# THE PATTERN AND REGULATION OF MAMMARY GLAND DEVELOPMENT DURING FETAL LIFE IN THE SHEEP

A thesis presented in partial fulfilment of the requirements

for the degree of Doctor of Philosophy in Animal Science

at Massey University, Palmerston North, New Zealand

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## SUPERVISOR'S DECLARATION

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# GENERAL ABSTRACT

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The production of colostrum and milk in sufficient amounts is essential for the survival of the neonate. Although there is limited data to indicate that the extent of fetal mammary development is essential to subsequent milk production, the secretory epithelial cells that proliferate during pregnancy do so on the epithelial ducts that have developed during prenatal life. Thus any reduction in duct development may ultimately impact on secretory cell mass and hence the capacity of the adult gland to produce milk.

A series of studies were carried out to establish patterns of fetal mammary gland development between male and female sheep and to identify factors that may be involved in the regulation/mediation of growth and differentiation, and that may contribute to the sexual dimorphism of the gland. In addition, mammary gland development was measured in fetuses from ewes in which the maternal environment was altered by hormones, nutrition or pre-lamb shearing.

The sequence of events in the development of the mammary gland of the fetal sheep was similar to that described for cattle. Sexual dimorphism in the ovine gland became pronounced during the formation of secondary ducts and was especially evident during the development of the fat pad where adipose tissue was far less abundant from the outset in the male. In terms of epithelial development, total duct area was similar in males and females up until day 120 of fetal age. Between days 120 and 140 of fetal age, total duct area doubled in females while the interval between day 140 and three weeks of postnatal age witnessed a four- to five-fold increase in the size of the duct system. Conversely, the male gland failed to progress beyond that observed at day 120. The sex differences observed in the histomorphogenesis of the gland were reflected in a relative growth analysis of mammary development. The growth of the mammary gland in the female followed the general development of the fetus, while in the male, mammary growth exhibited negative allometry from day 80 to 140 of fetal age.

Further experiments investigated factors that may be involved in the regulation of epithelial and mesenchymal growth within the fetal mammary gland. Receptors for androgen and oestrogen were localised in the mammary epithelial and mesenchymal cells of both sexes. An association between the localisation of androgen receptors (AR) and the divergence in the pattern of mammary development between males and females suggested the involvement of androgens in the sexual dimorphism of the gland. In support of this suggestion was the observation of a similar pattern of mammogenesis and AR immunoreactivity in the mammary glands of female fetuses whose dams were injected with testosterone during early gestation. Insulin-like growth factor-I receptor (IGF-IR) followed a similar pattern of immunoreactivity to AR in the mammary

tissue of the male indicating that the suppression of mammary growth may be mediated by IGFs. IGF-IR immunoreactivity tended to increase in both the epithelial and mesenchymal cells of the female mammary gland as gestation progressed. An abundance of IGF-IR in the developing fat pad of the female gland suggested a role for locally derived IGFs in stimulating adipose tissue growth and hence, the continued proliferation and morphogenesis of epithelial cells.

The final study demonstrated that a low plane of maternal nutrition throughout pregnancy was detrimental to development of the fetal mammary gland and hence, its future capacity to produce milk. In terms of total duct area, fetal mammary growth was more than two-fold greater in fetuses whose dams were exposed to a high plane of nutrition throughout pregnancy than in those fetuses whose dams remained at maintenance. This substantial difference in the amount of epithelial tissue present occurred without any significant effect on fetal or gland weights. Moreover, the increase in total duct area associated with a higher plane of maternal nutrition closely mirrored the increase in the intensity of IGF-IR immunostaining in the epithelial cells.

In conclusion, these results provide indirect evidence that inhibition of mammary gland growth in the fetal male sheep is dependent on its exposure to testosterone and may involve mediation by IGF-I. Oestrogens may act directly or indirectly, mediated by oestrogen-induced IGFs from the mesenchymal cells, to stimulate epithelial cell differentiation and proliferation in the mammary gland of the fetal female sheep. Furthermore, strong evidence indicates that the ewe is able to influence mammary development in her female offspring *in utero*, which may eventually affect their potential to produce milk.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AR | androgen receptor |
| BP | binding protein |
| CA | cyproterone acetate |
| cm | centimetre |
| CP | crude protein |
| CRL | crown-rump length |
| CT | computer-assisted tomography |
| DDE | dichlorodiphenyl dichloroethane |
| DDT | dichlorodiphenyl trichloroethane |
| DE | digestible energy |
| DES | diethylstilbestrol |
| DM | dry matter |
| DNA | deoxyribonucleic acid |
| 3-D | three-dimensional |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| FGF | fibroblastic growth factor |
| FGFR | fibroblastic growth factor receptor |
| GH | growth hormone |
| GHR | growth hormone receptor |
| h | hour(s) |
| IGFBP | insulin-like growth factor binding protein |
| IGF | insulin-like growth factor |
| IGF-IR | insulin-like growth factor-I receptor |
| iu | international unit |
| kg | kilogram(s) |
| KGF | keratinocyte growth factor |
| kPa | kilopascals |

| | |
|---|---|
| LEF-I | lymphoid enhancer factor-1 |
| Mcal | megacalorie |
| ME | metabolisable energy |
| mg | milligram(s) |
| MJ | megajoules |
| ml | millilitre(s) |
| mm | millimetre(s) |
| mRNA | messenger ribonucleic acid |
| ORα | oestrogen receptor alpha |
| ORβ | oestrogen receptor beta |
| ORKO | oestrogen receptor knockout mouse |
| pi | post injection |
| PTHrP | parathyroid hormone-related protein |
| PTHR1 | type 1 parathyroid hormone receptor |
| PUF | protected polyunsaturated fats |
| Tfm | testicular feminisation |
| TGF | transforming growth factor |
| µg | micrograms |
| µl | microlitres |

# CHAPTER ONE

# GROWTH AND DEVELOPMENT OF THE OVINE FETAL MAMMARY GLAND: A REVIEW

## 1.1 Introduction

New Zealand is the biggest exporter of sheep meat world-wide and supplies nearly 75 percent of the total amount of lamb traded internationally, earning lamb producers $1,635 million in the year to June 2001 (MAF statistics, 2002). In the next five years, it is expected that sheep meat production worldwide will decline. This will be due to a decline in the net-importing countries because of foot and mouth disease, and movement of sheep producers here and overseas to alternative land use or simply leaving the industry because of poor profitability. A fall in consumption is also expected, because of foot and mouth disease, but it will not be as great as the fall in production. To fill the international shortage in the supply of sheep meat, and benefit through higher prices, New Zealand farmers may gain by increasing lambing percentages.

The national average lambing percentage has been climbing gradually for a number of years, with a new record (116%) set in 2001. Meat New Zealand's strategic plan for 2001-2006 has focused on raising lambing percentages even further, from 116 to 125 percent over the next five years. However, the high fertility New Zealand crossbred ewe can only be regarded as a valuable asset if she produces viable lambs and, at the same time, sufficient milk to meet their energy requirements for rapid growth. At present, the milk requirements of twins are not always met by the ewes due to their limited capacity for milk production. This has a bearing on neonatal lamb mortality and growth rate to weaning.

The milk yield potential of a ewe is determined, to a large extent, by the growth of the mammary glands around puberty and during pregnancy. Consequently, most research has focused on the cellular changes during those two developmental phases with the implicit assumption that development of the mammary gland during fetal life is irrelevant to this later period. However, this developmental stage in life is of vital importance, in that initiation and differentiation of all cell types is completed and maturation of most is well underway before birth. Hence, early life is the time when the potential composition of an animal may be most vulnerable to modification.

The pattern and regulation of fetal mammary gland proliferation and morphogenesis has been most thoroughly studied in the mouse. Although there are significant differences in the growth and adult morphology of mammary glands in rodents and ruminants, there are few quantitative data on these fundamental processes during fetal life in ruminants. A study of the growth of the fetal mammary gland of the ewe and its regulation may reveal ways that the development of the gland could be manipulated to enhance milk production in the mature ewe so that she is better able to support the growth of multiple lambs.

## 1.2 Stages of fetal mammary growth and development

Various studies have been undertaken to describe the growth and development of the mammary glands during fetal life. The general features of development of the gland in the fetal

sheep were described briefly by Profé (1899). However, the first comprehensive description of the development of the mammary gland in cattle, sheep and goats was presented in the classic book *The Mammary Gland* written by Turner in 1952 and in which the terminology of Profé's description in sheep was changed to correspond with the description in cattle. Subsequently, Raynaud (1961) reviewed the more recent experimental studies on mammary gland morphogenesis in rodents and domestic animals and also provided an historical account of the literature dating from the 1860's. In her PhD thesis, Sonstegard (1972) described the normal histomorphogenesis of the developing bovine mammary gland from days 48 to 285 of fetal life as well as the glands' response to hormones *in vitro*. The literature was reviewed again by Anderson (1978) who incorporated various studies into a concise report outlining the sequential changes in the formation of the mammary glands of domestic ungulates (cattle, sheep, goats, pigs), rodents (rats, mice) and humans. In the context of this thesis it is noteworthy that quantitative data in sheep are limited to one study (Martinet, 1962).

Mammary gland development in the mouse is considered to be representative because it is now the most thoroughly studied gland of any species. It is questionable, however, whether the mouse is an appropriate model for the ruminant. In the next section the major stages of development will be compared with that of the rodent, with the objective of identifying any notable differences and similarities, and where further investigation is needed in the ewe.

### 1.2.1 The mammary epithelium

#### 1.2.1.1 Mammary band or 'milk' line

The epithelium of the mammary gland is derived from the epidermis of the embryo. In most species studied, the earliest morphological indication of its development is the formation of a band or crest of thickened epithelium termed the "mammary band (crest)" or "mammary line (milk line)". However, the mammary "band", "streak", "ridge", "line", "crest" and "hillock" are all terms that have been designated to define the earliest recognisable stages of fetal mammary gland development and represent serial changes in the thickened area of the ectoderm. Since each one of these developmental landmarks occupies a relatively short time period of several days, they are extremely difficult to differentiate. Furthermore, there are inconsistencies in the literature, within and between species, as to which structure appears first.

The differentiation of cells destined to develop into a functional mammary gland occurs very early in fetal development. The first outwardly visible sign of mammary gland development consists of a single layer of cuboidal cells that forms from the ectoderm and differentiates from the underlying mesenchyme tissue. This raised area of ectoderm appears on both sides of the midline in either the inguinal region (cattle, sheep and goats; Turner, 1952) or along the entire abdominal surface (rodents; Turner and Gomez, 1933a) and is referred to as the mammary band. The mammary streak is a slightly later development in which the area thickens, becoming more localised and forming a distinct line. These structures have been identified in goats

(Turner and Gomez, 1936) rats (Myers, 1917) and mice (Turner and Gomez, 1933a), but not in cattle or sheep (Turner, 1952) (Table 1.1).

**Table 1.1** Stages of development in the mammary glands of sheep, goats, cattle and mice (Anderson, 1978).

| Stage of development | Sheep | | Goats | | Cattle | | Mouse | |
|---|---|---|---|---|---|---|---|---|
| | CRL (mm) | Age (days) | CRL (mm) | Age (days) | CRL (mm) | Age (days) | CRL (mm) | Age (days) |
| Mammary band | 9 | - | 5 | 25 | 14 | 32 | 3.8 | 10 |
| Mammary line | 10 | - | 10 | - | 17 | 35 | 7.2 | 12 |
| Mammary crest | 15 | 30 | 28 | - | 19 | 37 | - | - |
| Mammary hillock | 20 | 33 | 29 | 37 | 21 | 40 | - | - |
| Mammary bud | 25 | 36 | 30 | 40 | 25 | 43 | 9.4 | 13 |
| Teat formation | 60 | 46 | - | - | 80 | 65 | 20 | 18 |
| Primary duct | 80 | 56 | 110 | 50 | 120 | 80 | 20 | 18 |
| Secondary duct | - | 59 | - | - | 160 | 90 | 27 | 20 |
| Canalisation of primary duct | - | - | 230 | 90 | 190 | 100 | 18 | 17 |
| Gland cistern | - | - | - | - | 230 | 110 | - | - |
| Teat cistern | - | - | - | - | 300 | 130 | - | - |
| Gestation length | | 147 | | 150 | | 280 | | 20 |

CRL = crown-rump length

The mammary line ("milk line") or ridge appears as a condensation of epithelial cells. In those animals that have inguinal glands only, such as cattle, sheep and goats, mammary lines are seen in the abdominal-inguinal area, posterior to the umbilicus. In the rat and mouse, which have mammary glands along the entire ventral surface of the body, the mammary line is evident from forelimb to hind limb. Hence, the location of the mammary lines corresponds with the position of the future normal and accessory glands and teats (Anderson, 1978). Mammary lines were readily discernible in 17, 10 and 10 mm long fetuses of cattle, sheep and goats, respectively (Turner, 1952) although Sonstegard (1972) found no indication of a pre-existing mammary line in cattle (Table 1.1). They were also reported to be present in rat (cited by Myers, 1917) and mouse (Turner and Gomez, 1933a) fetuses at days 13.5 and 12 of gestation, respectively, although Balinsky (1950a) and Hardy (1950) could not detect them in the mouse (Table 1.1). Individual glands were shown to make their appearance independently and not segment from a continuous longitudinal thickening (Balinsky, 1950a), which is the case in other litter-bearing species (e.g., pigs) (Raynaud, 1961). The relevance of "milk lines" in sheep, goat and human mammary gland development is questionable, particularly as only one pair of glands normally develops. However, the fact that about 20 percent of New Zealand Coopworth ewes form supernumerary glands (Maijala and Kyle, 1988), and, that supernumerary nipples can be

found from the groin to the armpit in humans (Howard and Gusterson, 2000) supports the concept of a milk line.

Differentiation of the mammary line into localised concentrations of epithelial cells that are spherical in shape takes place in three stages. The earliest is the mammary crest, in which it is proposed that cells of the germinal epithelium of the skin proliferate in several localised areas along the mammary line (Turner, 1952). This formation quickly changes to the mammary hillock, the rapid proliferation of cells that make up the mammary crest resulting in a hemisphere of epithelial cells. The hillock develops into a sphere, which penetrates the underlying mesenchyme. This sphere is called the mammary bud and is prominent between days 36 and 46 of gestation in the sheep (Table 1.1).

#### 1.2.1.2 Mammary bud

The bud is the main structure from which all mammary glands arise. The number of pairs of buds that form are characteristic of the species. In ruminants, one (sheep and goats) or two (cattle) pairs (one or two per side, respectively) of ectodermal buds develop in the inguinal area, while in mice and rats, five and six pairs, respectively, form along the milk line and invade the dermis. Two distinguishable compartments underlie these mammary buds. A dense mesenchyme, packed with fibroblast cells, is directly associated with the mammary epithelium while a second compartment comprises the precursor tissue for the mammary fat pad (Kimata et al., 1985).

Early studies of mammary gland morphogenesis presumed that the formation of the mammary bud involved increased local epithelial proliferation (Turner, 1930; Turner and Gomez, 1933a; Turner and Gomez, 1936; Turner, 1952). To the contrary, Balinsky (1950a) found that the mitotic index of early mammary epithelium in the mouse was lower than that of the surrounding epidermis. He therefore postulated that the epidermal thickening representing individual mammary glands and early growth of the bud was formed by the migration of epidermal cells. The characteristic shape of epidermal cells seen in scanning electron micrographs of the rabbit milk line was believed to support this theory (Propper, 1978). Cell migration and a change in cell shape explained the disappearance of the milk line, which occurs with mammary bud individualisation, and the rapidity of mammary bud morphogenesis. The continued centripetal migration of epithelial cells into positions corresponding with the glands of the mature animal causes the mammary bud to become spherical in shape and penetrate the mesenchyme (Propper, 1970). The mammary bud becomes fully enclosed in the mesenchyme except for a small indentation at the outer pole (Turner, 1952). Furthermore, there appears to be very little difference in the form and structure of mammary buds of ruminants (cattle, Turner, 1930; goats, Turner and Gomez, 1936; sheep, Martinet, 1962) and rodents (rat, Myers, 1917; mouse, Turner and Gomez, 1933a).

The relationship between the mammary bud and underlying mesenchyme was shown using mammary rudiments from rabbits (Propper, 1968) and mice (Kratochwil, 1969, 1975). In these

experiments cells from both the mammary rudiments and unrelated ectodermal and mesenchymal tissue of fetuses of different ages were separated and then cultured together *in vitro* in various combinations. When 12-day non-mammary epidermis (from the head or neck region) of rabbit fetuses is associated with 12-day mammary mesenchyme it gives rise to epithelial buds. On the other hand, if 12-day mammary epidermis is combined with mesenchyme from other areas the mammary epidermis fails to differentiate (Propper, 1968). The mesenchyme also determines the type of differentiation. When 12- to 14-day mouse mammary epithelium was combined with mesenchyme of the 13-day salivary gland, its morphogenesis became salivary-like with terminal adenomeres and a dichotomous branching pattern (Kratochwil, 1969).

It also appeared that the developmental fate of mammary epithelium was determined early during this inductive interaction since mammary bud morphology was maintained when 13- to 14-day embryonic rabbit mammary epithelium was grown in association with non-mammary mesenchyme (Propper, 1968). Likewise, Sakakura has shown mammary buds to be irreversibly committed to mammary development since 13-day-old fetal mouse mammary epithelium grown in association with salivary gland mesenchyme does not lose its ability to undergo glandular differentiation and produce milk protein (Sakakura et al., 1976).

Subsequently, Propper's interpretation, based solely on morphological criteria, was confirmed by examination of the induced buds for mammary-specific markers (Cunha et al., 1995). Mid-ventral or dorsal skin epithelium from 13-day rat or mouse embryos was associated with 13-day embryonic mouse mammary mesenchyme. These tissue recombinants were then grafted into virgin and lactating female athymic mouse hosts, and grown *in vivo*. Growing the tissue in athymic mice, which do not produce T-cells and hence do not develop an immune response, results in acceptance of the transplants, rather than rejection. After one month the tissue recombinants formed a keratinised skin with hair follicles and sebaceous glands. Associated with these epidermal structures were mammary ducts lined by tall columnar secretory epithelial cells underlain by myoepithelial cells expressing smooth-muscle $\alpha$-actin. Furthermore, tissue recombinants grown in lactating female hosts expressed milk proteins (Cunha et al., 1995).

Though these studies clearly show that the mammary mesenchyme is able to induce mammary development in the epidermis, they do not provide evidence that shows conclusively, which of the two tissues initiates mammary morphogenesis. There is fairly good evidence which suggests that the mammary mesenchyme form under the influence of the epithelial bud. Mammary epithelial gland buds were isolated and placed on mesenchyme that had not previously been in contact with a mammary epithelium (i.e., dorsal or ventral mesenchyme). They were grown in short-term organ culture then labelled with medium-containing $^{3}$H-glucosamine for 12 hours and processed for autoradiography. The epithelia became surrounded by a halo of mesenchymal cells, which exhibited responses characteristic of mammary mesenchyme. These responses included elevated production of tenascin-C (Chiquet-Ehrisman et al., 1986), elevated deposition of $^{3}$H-glucosamine in the extracellular matrix (Robinson et al.,

1999) and the synthesis of androgen and oestrogen receptors (Heuberger et al., 1982; Robinson et al., 1999). Moreover, the mammary mesenchyme did not respond to testosterone (i.e., it did not condense and cause the destruction of the male gland in the mouse) in the absence of mammary epithelium (Dürnberger and Kratochwil, 1980). Tissue recombination experiments in the mouse have demonstrated that a mesenchymal signal first determines mammary epithelial development. Once the early mammary bud is initiated, it induces the formation of the characteristic mammary mesenchyme, which surrounds the epithelial bud and governs the formation of a branched ductal system. Such experimental tissue recombinations have not been studied in ruminants, but it is expected that broadly similar epithelial-mesenchymal interactions would occur in all species.

Once the mammary buds are formed, they go through a period where they are morphologically inactive, the so-called "resting phase". This relative standstill in development occurs before the onset of ductal branching morphogenesis and seems to be a common feature between species. Turner (1930) found, in his investigation of mammary gland development in cattle, that after the formation of the spherical shaped bud there was no appreciable increase in its size for about three weeks. Similarly, for approximately two weeks in male and female sheep (Martinet, 1962) and 4 days in the female mouse (Balinsky, 1950a) the fetal mammary glands show no progressive differentiation and their growth is comparatively small (Table 1.1). This resting period has also been observed *in vitro* from explants of mammary epithelium from the ventral body wall of the fetal mouse (Hardy, 1950).

In the mouse, cellular proliferation of the bud, as measured by mitotic activity, decreases far below the level intrinsic for the epidermis from which the mammary gland has developed (Balinsky, 1950a). By measuring mammary gland volume from serial sections, Balinsky (1950a) also showed that, during the same period, growth of the mammary bud lags behind that of the body as a whole; the bud increasing only four times in size whereas the body weight increases 10-fold.

Many early studies described the "resting" bud as a spherical mass of cells attached to the epidermis by a narrow neck or stalk (Myers, 1917; Turner and Gomez, 1933a; Balinsky, 1950a) giving it a club-or bulb-shaped appearance. However, re-examination of mammary gland development during this stage using three dimensional reconstruction of 14-day fetal mouse mammary epithelia, showed that the gland had already begun to change shape and elongate (Cunha and Hom, 1996). This not only illustrated that the shape observed in histological section is totally dependent on the plane of section, but that elongation of the spherical mammary bud into a primary duct begins much earlier than previously thought. Observation of ruminant mammary bud in this plane of orientation may reveal similar morphological changes.

#### 1.2.1.3 Teat formation

Mammary buds determine the numbers of teats or nipples characteristic of an animal species. Sheep and goats typically have two mammary buds, which predetermine their two teats (Turner,

1952), whereas cattle have four (Turner, 1952) and mice and rats have ten and twelve, respectively (Turner and Gomez, 1933a; Anderson, 1978). Development of the teat (or nipple in rodents) and primary duct (or primary sprout) occur simultaneously, between days 48 and 50 of fetal life, or 33% of gestation length in the sheep (Martinet, 1962). This is very early in the development of the mammary gland compared to the mouse, where these stages don't begin until day 16 (Balinsky, 1950a; Sakakura et al., 1982), or approximately 80% of gestation length (Table 1.1). Teat development in cattle may be seen in the female fetus as early as 65 days of age (24% of gestation length), at least 15 days prior to the commencement of primary duct growth (Turner, 1930) (Table 1.1). A notable difference between cattle and goats during teat development is the prominence of hair follicles right up to the end of the teat in the latter. Hair on the teats remains a feature in the mature goat (Turner, 1952).

In ruminants, teat initiation consists of active proliferation of the mesenchymal tissue beneath and surrounding the mammary bud (Turner, 1952), hence its classification as "proliferation teat" (Turner and Gomez, 1933a). This proliferation forces the bud laterally and outwardly, causing it to rise very gradually until it protrudes from the outer surface of the body. The previously spherical bud opens at the epidermis forming a funnel-shaped crater at the tip of the teat composed of cornified cells, which are similar to the epidermal layer of the skin (Turner, 1930). This condition remains almost the same throughout fetal development. At the same time the primary duct begins to grow from the mammary bud into the newly forming teat (Turner, 1952).

In contrast, in rodents the "epithelial ingrowth teat", has evolved. Epithelial cells surround the bud and primary duct, which sink into the mesenchyme and a cone of cornified cells accumulate around the nipple. As a result, the nipple is deeply embedded rather than raised above the body surface (Myers, 1917; Turner and Gomez, 1933a) an obvious advantage in reducing injury while crawling on rough ground. The nipples only protrude above the body surface during puberty, and during the last stages of gestation in the pregnant mother, as a result of their increase in length to accommodate the suckling young (Turner and Gomez, 1933b).

#### 1.2.1.4 Primary duct (or primary sprout)

The formation of one or more primary ducts that develop as branches from the mammary bud determines the future number of galactophores that open onto the teat or nipple. It is at this stage in the progressive development of the gland that the greatest species differences appear. In ruminants and rodents, the number of primary ducts developing from each bud is one, resulting in one opening per teat (or nipple) (Turner, 1952; Myers, 1917; Turner and Gomez, 1933a). The number of openings per teat in the pig may vary from one to three, whereas humans have been reported to have 8-18 ducts traversing each nipple (Russo et al., 2001).

The development of the ruminant mammary gland during formation of the primary duct and subsequently has been largely neglected, particularly in sheep. Profé (1899) did not section sheep glands during the development of the primary duct and Turner (1952) assumed that further development of the gland up until birth quite likely followed the general development

observed in goats and cattle. Martinet's (1962) histological analysis of samples taken from mammary glands of 44- to 150-day-old fetuses provides the only descriptive account of the morphological progression of the sheep gland from mammary bud to secondary branching. Although 100 female and 50 male mammary glands were observed, Martinet's morphological description of the gland was very brief, confirming only those stages already described in sheep by Profé (1899) and in cattle by Turner (1930). Wallace (1953), in an experiment designed to compare mammary development in male and female lambs and calves, made histological observations from mammary tissue in fetuses at days 56, 84, 112 and 140 of gestation in sheep and days 84, 110, 167, 196, 224, 262 and 282 of gestation in cattle. In addition, the histological development of mammary gland tissue in fetal sheep at weeks 10, 15 and 20 of gestation was briefly described as part of a study investigating the role of insulin-like growth factors in mammary morphogenesis (Forsyth et al., 1999).

Taken together, these various studies in ruminants indicate that the primary duct expands as a result of rapid cell division, pushing the mesenchyme cells aside in its forward advance whilst following the path of least resistance. Its general course, however, is perpendicular to the body surface (Turner, 1952; Martinet, 1962). This invagination progresses up until day 55 of gestation in the sheep (Martinet, 1962) and day 90 in cattle (Turner, 1952; Sonstegard, 1972) or towards the end of the first trimester in both species.

Primary ducts in fetal mice were reported to begin their growth from the mammary bud relatively much later in pregnancy on day 18 (Turner and Gomez, 1933a). However, a later study revealed very clearly, in a photograph of the female mammary gland at day 18, canalisation of the primary duct (Elger and Neumann, 1966). Therefore, primary duct initiation had to occur prior to day 18. Sakakura et al. (1982) showed that the mammary bud elongates by rapid cellular proliferation to form the primary sprout late in the 16$^{th}$ day of fetal life in the female mouse. From observations of computer-generated three-dimensional reconstruction of the epithelial bud it is evident that elongation begins as early as day 14 (Cunha and Hom, 1996). Dense fibroblastic mammary mesenchyme surrounds the elongating mammary epithelium (Sakakura et al., 1982) and mitotic activity is increased approximately five-fold above the level measured during the resting phase (Balinsky, 1950a). Maturation of the elongating and widening primary duct coincides with the formation of a lumen. At approximately the same time that the primary duct begins to canalise, ductal branching morphogenesis commences.

#### 1.2.1.5 Secondary ducts (or secondary sprouts)

In ruminants, secondary ducts begin to grow from the basal germinal layer of cells of the primary duct when the primary duct reaches its greatest extension as a solid core of cells. Instead of continuing to grow perpendicularly inward, these ducts begin to grow at various angles to the direction of the primary duct, and into the surrounding mesenchyme (Turner, 1931). As they enlarge, the secondary ducts canalise and develop a two-layer epithelial lining (Turner, 1931; Wallace, 1953). These ducts develop into the major collecting ducts of the

mature gland. At a later stage, the secondary ducts give rise to tertiary branches. The latter are the primordial structures of the secondary milk ducts (Turner, 1952; Anderson, 1978, 1985). However, although the secondary ducts are developed at birth and some have canalised, a solid core of cells still persists at the growing ends (Turner, 1931). In relation to the total udder, the parenchymal tissue is limited to a small area of secondary ducts arising from the proximal end of the primary duct (Turner, 1931). The parenchyma is surrounded by a well-defined region of mammary mesenchyme, recognised histologically as fibroblastic connective tissue (Wallace, 1953; Forsyth et al., 1999). A photomicrograph presented by Forsyth et al. (1999) of the mammary gland of the ovine female at day 140 of fetal life also shows that the mesenchymal tissues comprise the greater proportion of the developing gland.

Wallace (1953) observed two or three secondary ducts already forming at the tip of the primary duct in sheep fetuses at 56 days of age. This corresponds well with Martinet's (1962) observation of secondary ducts emerging from the mammary cord at about day 59. By day 70, several secondary ducts, composed of three to four layers of cells, have formed (Martinet, 1962) and the epithelial tissue is restricted to an area of fibroblastic tissue immediately dorsal to the teat (Forsyth et al., 1999). At day 140 of gestation, the volume of epithelial tissue in female fetuses has increased approximately 100 times relative to the volume at day 70 of gestation (Martinet, 1962). Secondary ducts may be seen in 60-day goat fetuses (Anderson 1978) and in 90-day cattle fetuses (Turner 1931; Sonstegard, 1972). However, Turner and Gomez (1936) found it unnecessary to give all the details of the developing duct system in the goat because of its apparent similarity to that of cattle. In an 88-day goat fetus, the secondary ducts had already canalised (Turner and Gomez, 1936) (Table 1.1).

Secondary ducts were first observed in mouse fetuses at day 20, which is the day when parturition occurs in the mouse (Turner and Gomez, 1933a) (Table 1.1). It is now known that secondary branching is initiated in the mouse late on day 16 (Sakakura et al., 1982) and coincides with substantial DNA synthesis, although the distribution of this cell division is highly asymmetric in the mammary gland (Cunha and Hom, 1996). In 18-day fetal mammary gland, DNA synthesis is low in the epidermis and proximal mammary duct but very high throughout the distal branches (Cunha and Hom, 1996). Three to four secondary ducts are present at day 18 (Cunha and Hom, 1996) and by birth the mammary ductal tree system comprises 15 to 20 branches (Sakakura, 1987).

A notable morphological difference between species in the progression of the mammary epithelium occurs at this stage of development. When fat cell differentiation begins (at 16-17 days in mouse fetuses and 19-20 days in rat fetuses), the mammary epithelium breaks through the sheath of dense mammary mesenchyme and invades the precursor tissue of the fat pad (Sakakura, 1987; Cunha and Hom, 1996). In contrast, the mammary epithelium in day 140 sheep fetuses (parturition occurs at about day 147) remains surrounded by dense mesenchyme and has not started to penetrate the fat pad (Forsyth et al., 1999). DNA activity has not been measured during epithelial differentiation in the developing ruminant mammary gland.

#### 1.2.1.6 Canalisation of the primary duct

The manner in which the primary duct canalises, or forms a lumen, has been the subject of considerable controversy. Very early studies described the formation of the lumen in the human mammary gland as a process of cell degeneration, whereby the central cells of the solid primary duct degenerated, the debris being found in the developing lumen (cited by Myers, 1917). In the rat fetuses examined by Myers (1917) there were no traces of cell degeneration. Canalisation was observed at day 18.5 of fetal age when small irregular intercellular cavities appeared in the duct. The cavities were not confined to any particular part of the duct but usually appeared more developed at the proximal end. The lumen apparently formed from a rearrangement of the cells, thus producing numerous cavities, which later flowed together resulting in the formation of one lumen. Small cavities were also formed in the middle of the primary duct as a result of the rearrangement of cells in 20-day fetal mouse mammary gland (Turner and Gomez, 1933a). However, there appears to be some confusion in the terminology used by Turner and Gomez (1933a) to describe the process of canalisation. In the same study, the central cells of the duct were reported as having a tendency to separate as the duct lengthened and widened, forming a lumen (Turner and Gomez, 1933a). Sakakura (1987) reported that primary duct canalisation in the mouse began much earlier than day 20 of gestation. At day 16 of fetal age a funnel-shaped outline formed when the primary duct began to elongate. It was partly filled with cornified cells and directed towards the surface. Several intercellular vacuoles formed in the proximal end of the duct and later fused to make a canal opening through the mouth of the funnel. Turner and Gomez (1933a) also observed the formation of a funnel-shaped structure at the distal end of the duct.

The process of canalisation in ruminants is similar to that described by Turner and Gomez (1933a) in the mouse. As the primary duct widens, the cells in the centre of the duct begin to separate resulting in the formation of a lumen (Turner, 1931; 1952). The separation of cells begins at the proximal end (the area closest to the centre of the body) and gradually proceeds towards the distal end (Turner, 1931). The lumen associated with the proximal end is the primordial structure of the gland cistern while more distally, the opening later becomes the teat cistern. The duct narrows at its most distal end to form the streak canal, the last part of the primary duct to become canalised. In cattle, the primary duct begins to canalise when the fetus reaches 19 cm in length (Turner, 1931) or at approximately 100 days of gestation (Turner, 1952) (Table 1.1).

In contrast to earlier studies, Anderson (1985) likened the process of canalisation of the primary duct to the death of cells on the outside layer of skin. As the primary duct elongated and widened, the rapidly growing number of epithelial cells were unable to obtain nutrients once the structures reached more than six layers of cells in thickness. This resulted in cell death and the formation of a canal in the centre of the duct (Anderson, 1978, 1985). Recent examination of end buds using TUNEL assay revealed that apoptosis (or genetically programmed cell death) is involved in reducing the mass of cells in the end buds, possibly facilitating lumen formation

(Humphreys, 1999). Furthermore, apoptosis of various types of cells is now known to be part of normal development in many different tissues (Ganong, 1993).

#### 1.2.1.7 Development of gland and teat cisterns

In the ruminant, the gland cistern is formed by the continual growth of the lumen of the primary duct. The cells lining the primary duct push back the mesenchymal cells in all directions until eventually, the proximal end of the duct forms the cistern cavity (Turner, 1931, 1952). In cattle, the formation of the gland cistern is complete by day 130 of fetal age (Table 1.1). The teat cistern is formed by the progressive canalisation of the primary sprout towards the distal end. First evidence of a clearly defined teat cistern in cattle is at 130 days of gestation or when the fetus is approximately 30 cm in length (Turner, 1931, 1952). As the cells lining the cistern move in a horizontal direction the cavity of the teat cistern becomes larger (Turner, 1931) and is clearly delineated in the 48 cm bovine fetus, at which time the annular fold separates it from the gland cistern above (Anderson, 1978). The expansion of the gland and teat cisterns results in the lining being reduced to two or three layers of epithelial cells (Sonstegard, 1972). In contrast, the lining of the streak canal consists of a multi-layered epithelium similar to that of the skin covering the teat (Turner, 1931). The teat and gland cisterns in sheep and goats are presumed to develop similarly to cattle (Turner and Gomez, 1936; Turner, 1952).

In the 200-day bovine fetus the udder is fairly well developed with a highly differentiated median suspensory ligament readily defining the halves. Other features include the formation of blood vessels, which run perpendicular to the base of the udder, well-defined supramammary lymph nodes and a fat pad consisting of adipose cells (Turner, 1931). Thus, most of the development of new structures in the mammary gland in cattle is completed during this first 200 days of fetal growth (parturition occurs at about day 270). Beyond this time, further development is restricted to minor increases in size until the period immediately preceding puberty (Turner, 1931, 1952; Anderson, 1978).

Gland and teat cisterns are not a feature of the rodent mammary gland. Each individual gland at birth is represented by a ramifying system of some 15 to 20 ducts arising from the single duct that emanates from the nipple. It retains this morphology and, like the ruminant mammary gland, shows no progressive development until puberty (Myers, 1917; Turner and Gomez, 1933a; Balinsky, 1950a; Anderson, 1978; Sakakura, 1987). Large blood vessels and nerves have been observed in the mammary gland of 20-day fetal rats (Myers, 1917).

### 1.2.2 The mammary mesenchyme

The mammary gland in the fetal mouse comprises two distinct types of mesenchyme (Sakakura et al., 1982; Kimata et al., 1985). One is a dense mammary mesenchyme composed of several layers of fibroblastic cells surrounding the epithelium. The other consists of fat pad precursor cells, which appear separately, posterior to the mammary epithelium (Sakakura et al., 1982). Both the mammary mesenchyme and the fat pad precursor cells are derived from subcutaneous

mesenchymal cells. These cells contribute to the "stroma" of the mammary gland, which displays clearly distinct effects on the growth and morphogenesis of the fetal mammary epithelia.

#### 1.2.2.1 Fibroblastic mammary mesenchyme

In mammary gland development, the fibroblastic mammary mesenchyme assumes two different roles. One is an inductive function in which this specialised population of cells induces the epidermis to undergo ductal branching morphogenesis. The other is to impart sexual dimorphism on the gland, through its androgen responsiveness (Kratochwil and Schwartz, 1976; Sakakura, 1987). The mesenchyme underlying the mammary bud at the time of bud formation does not appear distinct from the rest of the dermis (Kratochwil, 1987). Very slowly, the mesenchymal cells bordering the mammary bud orient themselves around the epithelium. By day 43 in cattle fetuses (Turner, 1930) and day 13 in mouse fetuses (Sakakura, 1987) the epithelial buds are surrounded by a denser mesenchyme which is first recognised histologically as several layers of concentrically organised fibroblasts oriented around the mammary bud (Sonstegard, 1972; Sakakura, 1987). The mammary mesenchyme is distinguished from the dermal mesenchyme by elevated RNA synthesis (Robinson et al., 1999) and the expression of specific genes encoding for LEF-1 (refer to section 1.4.1) (van Genderen et al., 1994), and androgen (Heuberger et al., 1982) and oestrogen receptors (Robinson et al., 1999). LEF-1 is activated in day 11 mouse fetuses, during the formation of the mammary buds (van Genderen et al., 1994). Transcription of both steroid receptor genes is already elevated by day 12 of fetal age in the mouse (Robinson et al., 1999). These mesenchymal androgen receptors are responsible for setting in train the testosterone-induced destruction of the mammary gland in day 14 male fetuses by the mesenchyme (Kratochwil, 1971). The following are other factors that may also play a role in the regulation of mammary epithelial growth and development. Parathyroid hormone-related peptide (PTHrP) receptor expression has been localised to the mammary mesenchyme (Wysolmerski et al., 1998) and *in situ* hybridisation showed elevated levels of keratinocyte growth factor (KGF) transcripts (Cunha and Hom, 1996). Furthermore, the cells of the mammary mesenchyme are embedded in an extracellular matrix rich in tenascin, a protein that is not found in the adult mammary gland even during pregnancy (Chiquet-Ehrisman et al., 1986).

It is not known whether the short-lived association of fibroblastic mammary mesenchyme with the mammary bud is of importance in preparing the mammary bud for growth into the adjacent mammary fat pad. The destruction of the mammary bud in the male mouse under the influence of testosterone is the only established function of the fibroblastic mammary mesenchyme and this has no obvious biological significance. In the female, after the elongating epithelial bud breaks through the fibroblastic mammary mesenchyme, it enters the mesenchyme of the future fat pad while the population of the receptor-rich cells of the mesenchyme persists beyond the hormone-responsive stage and remains stationary, localised within the nipple (Wasner et al., 1983). The subsequent fate of the fibroblastic mammary mesenchyme is not clear. It may

penetrate the fat pad precursor tissue as it migrates with the mammary epithelium, or merely disappear (Sakakura, 1987). Wasner et al. (1983) reported that it may eventually become a part of the general subcutaneous tissue in the nipple area (where it is first recognised). While it is apparent that the fibroblastic mammary mesenchyme plays a key role in the development of the male murine mammary gland, the question remains as to whether or not this fibroblastic mesenchyme interacts with the newly formed mammary bud to allow its outgrowth from the epidermis in the female. Perhaps it is at this stage in development when oestrogen receptors in the female rodent and ruminant, and androgen receptors in the male ruminant, become relevant. (The roles of oestrogen and androgen receptors are discussed in sections 1.4.2.2 and 1.4.2.4).

#### 1.2.2.2 Fat pad precursor tissue

The fat pad differentiates independently of fibroblastic mammary mesenchyme, from more proximal mesenchymal tissue. On day 14 of fetal life in the mouse, these deeply placed subcutaneous mesenchymal cells appear as fat pad precursor tissue (Kimata et al., 1985). At this stage it is a relatively condensed tissue, becoming less compact on days 15 to 16 of gestation (Sakakura et al., 1982). Turner (1930) described this early mesenchyme tissue in the bovine mammary gland as loosely connected spindle shaped cells, which begin to differentiate into white fibrous tissue in fetuses of 8 to 12 cm in length (day 80 of gestation). Later congregating threads or bundles of cells appear, positioned perpendicular to the base of the udder (attachment to the abdomen). Proliferating preadipocytes form islets in the precursor tissue of the mammary fat pad at 16 to 17 days of gestation in the fetal mouse. These islets of preadipocytes increase in size and form into clusters of fat cells separated by connective tissue septa and are invested by a network of capillaries. The fat cells accumulate lipid gradually from day 16 and, two to three days after birth, a substantial depot of adipose tissue has formed (Sakakura et al., 1982; Sakakura, 1987). In the 12 to 13 cm long bovine fetus, whorls of cells are observed, the cells in the centre indicating their adipose nature by the presence of lipid in the cytoplasm. By the time the fetus reaches 60 cm in length, the larger part of the udder has been transformed into adipose tissue, which is seen histologically as aggregations of fat cells surrounded by connective tissue septa (Turner, 1931). The fat cells in fetal sheep at day 120 of gestation form distinct lobules (Wallace, 1953). In contrast to the late gestation mouse fetus, where the mammary epithelium has started to invade the fat pad (Sakakura 1987), bovine mammary epithelium does not grow in close association with adipose cells (Turner, 1952; Sheffield, 1988). Similarly, the mammary epithelium of 140-day fetal sheep is separated from the fat pad by multiple layers of fibroblastic tissue (Forsyth et al., 1999). Although formation of a definitive fat pad is seen at about day 80 of gestation in fetal cattle, very little is known of its subsequent development in ruminants (Sheffield, 1988).

Sakakura et al. (1982) demonstrated that the mammary fat pad precursor tissue is essential for the normal development and morphogenesis of the mammary gland. The combination of 17-day fetal mouse mammary epithelium with 14- or 17-day fat pad precursor tissue produced a typical

mammary gland pattern with elongated ducts and end bud formation. Dense 12- to 17-day fibroblastic mammary mesenchyme imposed an abnormal structure of frequent branching with many short ducts. The mechanisms whereby the fat pad influences mammary epithelial morphogenesis are not yet clear. There is strong evidence, mainly from studies of the development of the postnatal gland, that stroma-derived growth factors (e.g. KGF; Finch et al., 1995, Hovey et al., 2001) and regulatory molecules (e.g. *Wnt* genes; Weber-Hall et al., 1994) may regulate the growth and morphogenesis of the developing mammary gland. At present, there exists an expanding list of potential candidates, which may serve a morphogenic function.

### 1.2.3 Sexual dimorphism in mammary gland development

Most species show sexual dimorphism in mammary gland development during fetal life (Cowie et al., 1980). In sheep (Martinet, 1962) and mice (Jean et al., 1972) sexual differences in the volume of the mammary gland relative to body weight have been reported. Histologically, the mammary parenchyma of males is far less well developed from about day 70 of gestation in fetal sheep (Martinet, 1962). In the bovine fetus, Turner (1930, 1931) was able to detect sexual dimorphism from the late mammary bud stage. In some rodents (mice) androgen-induced regression of the mammary gland occurs in male fetuses (Kratochwil, 1971). In contrast to rodents and ruminants, male and female human mammary glands develop similarly *in utero* (Howard & Gusterson, 2000).

#### 1.2.3.1 Sexual differences in mammary gland growth and development

Sexual dimorphism of the development of the mammary glands of fetal mice was first described by Turner and Gomez (1933a). In 19-day male fetuses, according to Turner and Gomez, the primary duct detaches from the epidermis, but otherwise prenatal development of the bud and ducts is very similar to that of females. Nipples are not formed in male fetuses and the disconnected part of the gland may either disappear or undergo only a very poor development (Kratochwil, 1975). In this respect, considerable differences have been noted between mouse strains (Raynaud, 1971). This destruction of the male mammary gland is not typical for mammals, but represents a peculiarity of the family of some rodents (Kratochwil, 1977). Myers (1917) failed to find a sex difference in the morphology of the developing mammary gland in the albino rat.

In ruminants, marked differences between the sexes are apparent very early in fetal life. Turner (1930) reported that mammary buds of the bovine male were more spherically shaped and of greater volume than those of the female, which were more elongated on the axis perpendicular to the body surface. In males, buds invaginate well below the body surface whereas the buds of the female never invaginate a distance more than equal to their diameter (Turner, 1930). This is particularly evident during the later stages of bud development when the bovine fetus has a crown-rump length of approximately 11.5 cm (Sonstegard, 1972). No differences have been observed between male and female fetal mice in the shape of the mammary buds (Turner and

Gomez, 1933a). In a photomicrograph of the female fetal rat at 15.5 days, the bud appears ovoid in shape (Myers, 1917), similar to that of the female bud in cattle (Turner, 1930). Formation of the primary and secondary ducts in cattle is initiated earlier in females than in males. Furthermore, when sexes of similar crown-rump length were compared, the female primary ducts were significantly longer than those of the male although, in both sexes, the variation in length was huge (Sonstegard, 1972). Teat development in the bovine male is somewhat slower than that in the female and is characterised by a fairly flat tip. In contrast, the teat tip of the female is pointed (Turner, 1930). Furthermore, the teats in male fetuses are located either anterior to or upon the scrotal sac (Turner, 1952). The teat of the fetal male is also populated by hair follicles, which are present only in the dermis of the udder skin in females (Turner, 1952).

In addition to the sexual dimorphism in teat and duct morphogenesis, there are also differences in growth rates between the sexes. From day 44 to about day 70, mammary gland growth in female sheep fetuses is five times faster than body growth. By day 70, the teat cistern is evident, secondary ducts have developed and parenchymal tissue is well developed. Gland growth in the female then slows to 1.7 times body growth until term (Martinet, 1962). The male gland grows at a constant rate of 2.8 times that of body weight throughout gestation (Martinet, 1962). In male and female mice, the development of the mammary gland is influenced by stage of gestation and position, the inguinal glands growing more slowly than the thoracic (Jean et al., 1972). In the male mouse, the volume of the mammary gland increases slightly, but more slowly than body weight, between days 13 and 14 of fetal ages. There is then a period of relative inhibition between days 15 and 17 followed by positive allometric growth for the thoracic glands and isometric growth for the inguinal glands between days 17 and 19. In female mice, mammary gland volume increases more rapidly than body weight between days 14 and 15 of fetal age. Growth then slows between days 15 and 17. From days 17 to 19 positive allometric mammary growth occurs once more (Jean et al., 1972; Jean and Jean, 1973).

Very early in fetal life, the female develops a more extensive fat pad than the male. This is particularly true in ruminants, in which the mammary gland is in close proximity to the scrotum in the male. The fat pad has, therefore, no room to grow in the male, hence the early termination in udder development. As a consequence, the ducts are restricted to a much smaller area than that provided for the female (Turner, 1952; Anderson, 1985). A difference in the pattern of ductal growth has also been observed even during the stage when the degree of development is similar between the sexes. The primary duct of the male tends to show an open cistern with lateral branches, while that of the female appears longer and less dilated with a greater spread of mainly terminal branches (Wallace, 1953).

## 1.3 Methods of evaluating mammary gland growth

Growth of the mammary gland has been assessed in many ways using various techniques. Some of the methods used include: direct observation of the external appearance;

measurements of mammary volume or weight, area, DNA content or DNA synthesis; determination of mitotic and labelling indices; qualitative and quantitative histological and cytological methods; and the use of computer assisted image analysis to perform morphometric measurements.

### 1.3.1 Morphological measurements

Many of the early methods of determining mammary gland growth were morphological, such as whole mounts and histological examination (Munford, 1964). In rodents, where the gland develops as a sheet with ducts stretched in, what appears to be, two dimensions, the gland can be visualised as whole mounts. These were primarily qualitative assessments of development, although this technique allows planimetric measurements of the gland surface and examination of the ductal and alveolar structure. Turner and Gomez (1933b) studied the development of the mammary gland in mice from eight days of age, through pregnancy, lactation and involution by means of whole mount preparations. Morphological development of fetal mammary tissue was described using microscopic examination of serial sections and photomicrographs, but no attempt was made to quantify this development (Turner and Gomez, 1933b). Similarly, in studies of mammary gland development in the fetal albino rat, assessments of mammary development were primarily qualitative. Furthermore, the histological sections were hand drawn with the aid of a camera lucida. In fetal cattle, the only measurement recorded that quantified mammary development were the weights of the udders of larger fetuses (Turner, 1930, 1931). The use of whole mounts and histological sections gives both subjective and, more recently, objective values of mammary growth, and the normality of the growth can be evaluated.

Balinsky (1950a) investigated the prenatal development of the mammary gland in the mouse using quantitative methods. Serial sections of mammary tissue were drawn with the aid of a camera lucida and the surface area of each section measured with a planimeter. The sum of the surface areas gave an arbitrary volume, which was then compared with the growth of the whole fetus (determined by weight). As no attempt was made to exclude between-fetus variability in weight or to treat the results statistically, the figures obtained were a very crude approximation of relative growth rate. Histology, combined with mitotic arrest techniques, provided information on the rate of growth (Balinsky, 1950a).

### 1.3.2 Biochemical measurements

Mammary DNA has been used widely as an index of growth since the 1950's. Its use as a procedure to evaluate mammary gland growth is based on the assumption that the DNA content per nucleus remains constant during all physiological conditions. If this hypothesis is true then total DNA in the mammary gland gives an index of the number of epithelial cells (Munford, 1964). This measure of growth has been subject to controversy, with some researchers reporting that DNA per cell can vary among physiological states, particularly during lactation and involution (Simpson and Schmidt, 1969). In order to obtain a reliable picture of mammary gland

development, the best approach may be to combine DNA measurements with whole-mount observations and/or histological sections of mammary tissue (Munford, 1964).

Many studies of mammary gland development in the fetal mouse have measured cell proliferation by labelling mammary epithelium tissue cultures or serial sections of whole mounts with a radioactive tracer (e.g., $^{3}$H-thymidine) then processing them for autoradiography. DNA synthesis is detected as labelled nuclei, which is usually evidenced by the accumulation of silver grains over the nuclei of proliferating cells (Cunha and Hom, 1996; Robinson et al., 1999).

The distribution and changes with developmental stage of mRNA for various growth factors have been observed and measured in fetal mammary tissue using *in situ* hybridisation. In the fetal sheep, the expression of mRNA for insulin-like growth factor (IGF) in mammary intralobular stromal tissue and for IGF-I receptor in mammary epithelium was quantified by absorbance measurement using a microscope photometer (Forsyth et al., 1999) which measures the density of silver grains in the section and hence the amount of hybridisation.

### 1.3.3 Image analysis

Recent advances in computer assisted image analysis may improve the measurement of mammary gland development. Such measures as epithelial area or duct length can be determined but require the use of a digitiser pad to trace the areas or lengths to be measured, as computer programmes (e.g., Sigma Scan Scientific Measurement Programme) are not capable of recognising, for example, a mammary end bud. Measurement of the volume of different tissue compartments within the mammary gland can be made using computer-assisted tomography (CT). CT scans are able to distinguish between parenchyma and stroma, where stroma may be embedded within the parenchymal component. Sejrsen et al. (1986) estimated the volume of mammary tissue in heifers using CT. However, these measures are more reliable if complemented by estimation of DNA content and/or histological evaluation to obtain a measure of the composition of the parenchyma (Tucker, 1987).

The rapid progression of microscope and computer technology means it is now possible to create three-dimensional (3-D) images. A confocal scanning microscope creates multiple images at varying depths within, for example, a mammary bud that has been serially sectioned. The computer assembles each of the sectioned images creating a 3-D image that can be rotated and viewed from any orientation required. Three-dimensional reconstruction of 14-day fetal mouse mammary epithelium demonstrated that the mammary bud begins to elongate and change shape much earlier than previously thought (Cunha and Hom, 1996).

## 1.4 Factors influencing fetal mammary gland growth and development

Development of the mammary gland is initiated in the fetus, but unlike most other organs in the body the major part of its development occurs after birth, when, under hormonal stimuli, growth and differentiation take place. Growth of the fetal mammary gland appears to be independent of systemic hormone action although hormones that are known to influence the maturing postnatal mammary gland have been found in prenatal rodents and ruminants. Interactions between the mammary epithelium and the surrounding mesenchyme are critical to early morphogenesis, at least in the mouse, as demonstrated by the regression of male mammary glands in response to testosterone. Epithelial-stromal interactions lead to the establishment of a diffuse pattern of epithelial branching morphogenesis at birth, which is characteristic of mammary gland. Recently, some of the signalling molecules, which are known to govern these reciprocal tissue interactions during specific stages of mammary morphogenesis in the fetal mouse, have been identified through gene deletion experiments.

### 1.4.1 Transcription factors and Wnt genes

Recent data from gene deletion experiments have shown that the High Mobility Group (HMG)-box transcription factor, Lymphoid Enhancer Factor-1 (LEF-1) is necessary for fetal mammary gland development in the mouse (van Genderen et al., 1994). LEF-1 is activated at day 11 of fetal life in the mouse, before the mammary buds become morphologically distinct. The absence of this gene, which is activated early in all ectodermal rudiments, also affects the development of teeth, whiskers and hair. Both mammary and tooth development fail to progress past the bud stage and whisker development is not even initiated (van Genderen et al., 1994). Tissue combination experiments of LEF-1 deficient and wild-type epithelium and mesenchyme carried out in tooth and whisker buds have established that LEF-1 is necessary for epithelium-to-mesenchyme signalling (Kratochwil et al., 1996). This may also be true for mammary development. The mechanisms by which LEF-1 promotes bud formation remain unknown although there is considerable evidence that *Wnt* genes may be involved.

The *Wnt* gene family comprises at least 10 members sharing substantial amino acid identity with the secreted glycoprotein, the Wnt-1 proto-oncogene (normal genes that control growth). Five members of the Wnt gene family are expressed and differentially regulated in the normal mouse mammary gland during pregnancy and lactation (Gavin and McMahon, 1992). At least one *Wnt* gene, *Wnt-10b*, is expressed within the developing mammary bud (Christiansen et al., 1995), although whether it is essential in mammary gland development is not known. Three-dimensional reconstructions from serial sections of the mammary glands of *Wnt-1*-overexpressing transgenic mice showed slightly more ductal hyperplasia with fatter ducts than normal mammary glands at birth. By day 7 of postnatal life considerable ductal hyperplasia was evident. Moreover, male *Wnt-1* transgenic mice exhibit mammary gland ductal tissue even in

the presence of androgen levels adequate for normal development of the male genital tract. This suggests that some aspect of *Wnt-1* expression in the male mammary bud obstructs the normal cascade of cell-cell interactions in the fetal male mammary gland (unpublished from Cunha and Hom, 1996).

Other transcription factors thought to regulate tissue-to-tissue signalling during the early developmental stages are the homeobox genes, *Msx-1* and *Msx-2*, and their regulators, *Bmp-2* and *Bmp-4* (members of the TGF-beta superfamily). In the fetal mouse mammary gland at day 13.5 of gestation, *Msx-1 Msx-2* and *Bmp-4* were expressed in the epithelium, whilst *Bmp-2* was expressed in the mesenchyme (Phippard et al., 1996). Although analysis of transgenic mice that lack the functional *Msx-1* gene demonstrated the importance of *Msx-1* expression for normal tooth development (Satokata and Maas, 1994), no abnormal phenotype was detected in mammary glands from the same strain of mice (Phippard et al., 1996). Given that both *Msx-1* and *Msx-2* were expressed in the mammary epithelium, it was thought that *Msx-2* could functionally compensate for the loss of *Msx-1* at this stage. Support for this hypothesis came from observations in *Msx-2* and *Msx-1/Msx-2* double knockout mice in which defects in mammary development were observed (Phippard et al., 1996). These studies of the morphological changes and patterns of gene expression suggest that mammary development shares common features with other systems in which epithelial-mesenchymal interactions underlie morphogenesis. Although several regulatory genes have been implicated in the control of tissue interactions, the molecular mechanisms by which these processes function at the local level are poorly understood and require further investigation.

### 1.4.2 Steroid hormones

Oestrogens and androgens play a critical role in fetal development, influencing growth and differentiation in tissues such as the gonads, and mammary gland, in the case of the male rodent. In mammals, genetic sex is determined at fertilisation and this forms the basis for gonadal sex development. At this stage in embryonic development both sexes have the potential to develop a male or female phenotype (reviewed by Gray, 1992). Subsequently, a gene on the Y chromosome interacts with genes on other chromosomes to produce a signal that leads to the development of the testes in the male, irrespective of the number of X chromosomes (Bianchi, 1991). In the male, the gonads secrete testosterone and Müllerian inhibiting substance, which induce differentiation of the male sexual phenotype. The subsequent morphological and physiological development of males and females diverge, resulting in the formation of the male and female phenotypes (reviewed by Gray, 1992). It has generally been held that, in the absence of testosterone, the female phenotype is expressed independent of the presence of an ovary. Although the ovary secretes oestrogens during fetal development, the rodent model implies that the mesenchymal and epithelial cells of the female mammary gland are not under the influence of endogenous ovarian steroids during early development.

#### 1.4.2.1 The oestrogen receptors

Oestrogens are produced mainly in the ovaries and testes. While they freely diffuse across cell membranes, they are dependent upon interaction with specific receptor molecules in the cells to mediate their biological effects. The oestrogen receptor (OR) is a ligand-activated nuclear transcription factor that mediates the biological effects of oestrogens and anti-oestrogens, in both males and females (Nilsson and Gustafsson, 2002). Following the cloning of the OR-encoding cDNAs during 1986 there was general acceptance that only one OR gene existed (now referred to as oestrogen receptor alpha (ORα)). However, at the end of 1995 a novel OR (ORβ) was cloned from a rat prostate cDNA library (Kuiper et al., 1996). *In vitro* studies have shown that the ORβ protein binds the hormone 17-β oestradiol with an affinity similar to that of the classical OR and is able to mediate the effects of oestradiol in transfected mammalian cell lines (Couse et al., 1997).

Evidence of a role for ORα in postnatal mammary gland development was confirmed in studies in female mice from which the ORα gene had been deleted (ORα knockout mouse; αORKO). Deletion of the gene results in a primitive ductal tree devoid of terminal end buds, that does not grow out into the mammary fat pad (Korach, 1996). The final step in the biosynthesis of oestrogens from C-19 steroids is catalysed by the enzyme aromatase, the activity of which is eliminated in the aromatase knockout mouse. As a result, the mammary glands also fail to develop beyond the prepubertal stage in mice lacking aromatase (Fisher et al., 1998). In contrast, prepubertal ORβ knockout female mice (βORKO) appear to have a normal mammary histology but after puberty terminal differentiation of the mammary epithelium is incomplete (Förster et al., 1999).

It has generally been accepted that development of the fetal mammary gland does not require the presence of oestrogen or its receptor(s). Through the use of autoradiographic procedures, receptor sites for oestrogens have been identified in 14-day (Kratochwil, 1986) and 16-day (Narbaitz et al., 1980) fetal mouse mammary gland. Epithelial-mesenchymal combination experiments in mice have shown that the oestrogen receptor is induced by mammary epithelium. For example, oestrogen-binding sites can be induced in the mesenchyme of the lateral body wall by combining this mesenchyme with epithelium from mammary buds (Kratochwil, 1987). More recently the expression pattern of mRNA for both oestrogen receptors during mouse embryogenesis was examined and mRNA for both ORα- and ORβ- were detected using highly specific $^{35}$S-labeled riboprobes (Lemmen et al., 1999). On days 12.5 and 14.5 of fetal life, ORα and ORβ were expressed in the mesenchyme of the mouse mammary gland in both males and females (Lemmen et al., 1999). ORα does not play a role in fetal mammary development, at least in the mouse, as the mammary phenotype of female mice with a disrupted ORα gene is normal (Korach et al., 1996). Examination of the ORβ knockout mouse may provide new insights into the role of oestrogens during fetal development.

In the ruminant, oestrogen receptors have been identified in the prepubertal calf mammary gland by a ligand-binding assay (Rotondi and Auricchio, 1978), and by immunohistochemistry (Capuco et al., 2002). Whether or not the fetal phase of epithelial outgrowth occurs independently of oestrogen in ruminants remains to be elucidated.

#### 1.4.2.2 Oestrogens: their role in development of the fetal mammary gland

The importance of oestrogens in the stimulation of mammary growth during postnatal development in the rodent has been demonstrated in numerous studies (reviewed by Haslam, 1987). However, these hormones are assumed to play no physiological role in fetal mammary morphogenesis and function (at least not in the mouse). The destruction of the fetal ovaries, by localised irradiation, does not disturb the development of the mammary gland (Raynaud, 1961, 1971), which has been interpreted to mean that the mammary glands of the female do not require the stimulus of ovarian secretions during the fetal period.

Organ culture studies have also been undertaken to identify the potential effects of oestrogens on the developing mammary gland. Hardy (1950) cultured explants of ventral body wall fragments containing the presumptive mammary region from 10 to 13-day fetal mice in a medium of chick plasma and chick embryo extract for up to 25 days. Although no hormones were added to the medium differentiation and growth of the mammary buds proceeded to the point where secondary ducts had grown into the fat pad. Under similar *in vitro* conditions, Balinsky (1950b) obtained some differentiation of the mammary bud from lateral body wall epidermis as well as primary sprouts from explanted mammary buds, but observed no additional effects of adding oestrogens to the culture medium. Although Hardy and Balinsky concluded from these experiments that mammogenesis during prenatal life is independent of hormones, the likelihood of the occurrence of mammogenic growth factors in cultures comprising chick plasma and chick embryo extract medium, makes these experiments difficult to interpret. Mammary tissue from 10 to 15-day fetal mice differentiated to a point where the ducts had penetrated the fat pad when a biological medium was used but not to as great an extent when a synthetic medium, which is totally devoid of hormones, was used (Lasfargues and Murray, 1959). When oestradiol was added to the synthetic medium, growth of the epithelium was inhibited but, at the same time, development of adipose tissue was stimulated. Oestradiol concentrations in the media as low as 1 nM are sufficient to block outgrowth of the mammary bud and subsequent glandular morphogenesis *in vitro* (Kratochwil, 1985). When oestradiol was combined with progesterone in a ratio of 1:1000, an elementary duct system formed (Lasfargues and Murray, 1959), reflecting earlier results from *in vivo* studies (Elliott and Turner, 1953). The combined experimental results indicate that the fetal gland is capable of responding to oestrogen and its sensitivity to oestrogen may correlate with the ontological development of the oestrogen receptor. They would also suggest that endogenous oestrogens are not required for prenatal mammary development. However, the findings described below strongly suggest that the mammary gland is sensitive to oestrogens as early in development as formation of the epithelial bud.

Raynaud (1961, 1971) was the first to observe the teratogenic effect of oestrogens on the fetal mammary gland. In a series of experiments, Raynaud described the malformations resulting from injections of oestrogens (oestradiol dipropionate in olive oil) into pregnant mice or directly into fetuses at 12-15 days of gestation. High doses of the hormone (200 μg per pregnant female) produced premature development of the nipples in fetuses of both sexes and either complete or partial arrest of the developing mammary epithelium. The mammary buds were frequently replaced by cutaneous cavities lined with a thick keratinised epithelium or by cord-like invaginations of the basal and Maliphigian layers of the ectoderm. Similar malformations were observed in male and female rat fetuses after injection of oestradiol into the pregnant rat at day 14 of gestation. Furthermore, the mouse experiments established that the sensitivity of the mammary gland to oestrogen is determined by fetal age, with 95% of the glands arrested or inhibited when the hormone was applied between the 12$^{th}$ and 14$^{th}$ day of gestation. Only 5% were affected when oestradiol was given on day 15 or later. Moreover, the frequency of malformations rose with the dose of oestrogen administered to the pregnant mother (Raynaud 1961, 1971).

The developing mammary gland is clearly a hormone target but the normal mammary phenotype displayed in the ORα knockout mouse suggests that endogenous oestrogens are not required for prenatal development of the mammary gland. New models such as the ORβ knockout mouse may be useful in determining direct and indirect effects of oestrogen on the mammary gland during the fetal period.

#### 1.4.2.3 The androgen receptor

Studies in the fetal mouse show that androgen receptors form in the dense fibroblastic mammary mesenchyme surrounding the bud (Heuberger et al., 1982). The first receptor sites become detectable at the time of bud formation (day 12) but are only present in the estimated 3000 mesenchymal cells immediately surrounding the bud (Wasner et al., 1983). This finding suggested that the mammary epithelium induced the formation of androgen receptors in the adjacent mesenchyme and was demonstrated in experimental tissue combinations. Mesenchyme-free mammary epithelia were associated with mesenchyme from the mammary region. After culture, autoradiographs showed that each piece of epithelium was surrounded by a halo of receptor-positive mesenchymal cells. By contrast, epithelia from salivary gland or pancreas did not induce the formation of androgen receptors (Heuberger et al., 1982). By the stage of androgen responsiveness (day 14) each mesenchymal cell possesses approximately 30,000 binding sites for testosterone and receptors are present in both sexes (Wasner et al., 1983). There exists only a short (about 30 hours) androgen-responsive "window" during mammary development in the mouse. The gland becomes sensitive to the hormone from days 13 to 14 of fetal age, but is no longer affected on day 15 (Kratochwil, 1977). At day 14 of fetal life, androgen-induced regression of the male mammary gland occurs (Kratochwil, 1971). Interestingly, specific binding remains demonstrable at high levels after the end of the hormone-

sensitive phase and persists at least until birth in both sexes, as shown in [3H]DHT autoradiographs (Wasner et al., 1983).

To date, there have been no studies describing the ontogeny of androgen receptors in the mammary glands of fetal ruminants. In the sheep, the fetal testis contains more androgen (testosterone and dihydrotestosterone) than the fetal ovary during the first half of gestation and plasma concentrations in male fetuses are significantly higher than in females at this time (Pomerantz and Nalbandov, 1975). Testicular and plasma concentrations fall during the second half of pregnancy (Pomerantz and Nalbandov, 1975) and this corresponds well with the observations of Clarke et al. (1976) that the critical period during which testosterone causes masculinisation is the first half of gestation. Furthermore, androgen receptor is present in the primordial gonad and epididymis of the fetal ovine from day 40 of gestation (Sweeney et al., 1997). Hence, testosterone, which is produced by the fetal gonads as early as 30 days *in utero* (Attal, 1969), is not capable of regulating masculinisation before day 40 because there is no androgen receptor expressed in the fetal tissue before this stage (Sweeney et al., 1997).

Differences between the sexes in the morphological development of the ovine mammary gland become evident at about day 70 of gestation (Martinet, 1962). It is suggested that the subsequent "slowing down" of mammary growth and differentiation in the male ruminant is another example of hormone-induced and mesenchyme-mediated inactivation of the mammary gland.

#### 1.4.2.4 Androgens: their role in development of the fetal mammary gland

Mammary gland development in the fetal mouse is sexually dimorphic. Destruction of the gonads of 13-day male fetuses *in utero*, by localised X-ray resulted in female-type development (Raynaud 1961, 1971). Organ culture experiments confirmed that this response was the direct result of removal of androgenic hormones secreted by the fetal testis (Kratochwil, 1971). Combination experiments utilising wild-type and androgen-insensitive Tfm-mutant (testicular feminisation) tissues established that testosterone has only one target tissue in the gland and that its effect on the epithelium is mediated by the mesenchyme (Kratochwil and Schwartz, 1976). Moreover, critical to the regression of the epithelial bud in male mice is the expression of mesenchymal androgen receptors, the development of which correlates well with the development of androgen responsiveness.

Prenatal exposure of the male rodent to anti-androgens has demonstrated that the morphological development of the normal male mammary gland is dependent on androgens. In a series of experiments Neumann and his colleagues have shown that environmental anti-androgens are obviously detrimental to normal male sexual development *in utero*, altering growth and differentiation of the reproductive tract, including the mammary gland. Cyproterone acetate (CA) is a synthetic derivative of hydroxyprogesterone that exerts anti-androgenic and progestational activities (Neumann, 1994). It exerts its anti-androgenic activity by blocking androgen receptor binding. However, it is not considered a true anti-androgen as it exhibits both

agonist (at high concentrations) and antagonist (at lower concentrations) activity *in vivo*. At high concentrations, CA not only binds the androgen receptor, but it promotes nuclear transport, androgen receptor DNA binding and transcriptional activation (Kemppainen et al., 1992).

Treatment of pregnant rats with cyproterone acetate induces anti-androgenic effects in the male, such as the presence of a vagina and the inhibited development of accessory sexual organs (Neumann et al., 1970). Exposure during development also results in persistent abnormalities of the male urogenital tract in sheep and pigs (Neumann et al., 1970). The syndrome presented is known as "testicular feminisation" (Neumann, 1994). In male rat and mouse fetuses exposed to CA *in utero*, the mammary buds and teats grow as well as those in female fetuses. In the mouse, at day 15 of fetal age, the female pattern of development, whereby the continuity of the primary duct is preserved, is clearly seen in fetal males whose mothers have been treated with 3 mg CA/day from day 12 of pregnancy (Elger and Neumann, 1966). Injection of pregnant rats with 10 mg CA daily from the 13$^{th}$ day of pregnancy induced, in male fetuses, the development of nipples and of mammary gland tissue of the normal female type. Males carried by CA treated mothers did not develop the fibrous mesenchyme, which normally surrounds the mammary epithelium and leads to its ultimate destruction (Neumann and Elger, 1966). Moreover, feminised male rats orchidectomised as adults and treated with oestradiol benzoate + progesterone, developed a complete duct system capable of lactogenesis (Neumann et al., 1966). From these observations it can be concluded that expression of the male phenotype for mammary gland development is androgen-dependent, at least in the rodent.

In summary, the studies cited demonstrate that androgens such as testosterone are critical determinants of the male phenotype, but it is the steroid receptor that controls the timing of the fundamental events in fetal development and sex differentiation. Upon binding with the appropriate ligand, the receptor either activates or represses transcription of target genes. Interfering with the action of these hormones or their levels in the tissues results in masculinisation of the reproductive tract (in rodents and ruminants) and mammary gland (in rodents). Certain pasture species as well as widespread use of fungicides and pesticides in agriculture have the potential to alter male and female sex and development, in wildlife, domestic animals and humans by acting as environmental anti-androgens or anti-oestrogens.

### 1.4.3 Endocrine disruptors

Over the last 50 years, substantial evidence has surfaced on the hormone-like effects of environmental contaminants, such as pesticides, insecticides, fungicides and industrial chemicals, in wildlife and humans (Sonnenschein and Soto, 1998). These so-called environmental endocrine disruptors can interrupt reproductive development by mimicking or inhibiting the action of the gonadal steroid hormones, testosterone and oestradiol (Kelce and Wilson, 1997). Their toxicity is especially insidious during sex differentiation and fetal development due to the crucial role of gonadal steroid hormones in regulating these processes.

This raises the possibility that such chemicals could have an impact on mammary development and ultimately lactational performance in the adult ruminant.

#### 1.4.3.1 Effects of oestrogens on fetal mammary development

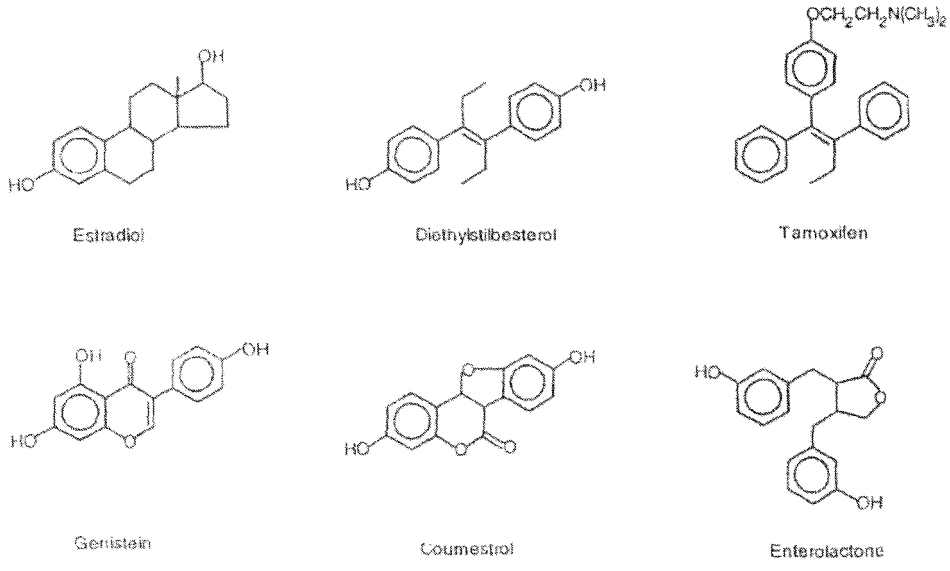
Oestrogens of varying potency are widespread in the environment and have the potential to alter reproductive tract and mammary gland development (Colburn et al., 1993; McLachlan, 1993). Studies regarding the effects of oestrogen exposure during the perinatal development of the mammary gland have focused primarily on oestradiol and diethylstilbestrol (DES). DES is a non-steroidal compound with properties similar to the natural female sex hormone, oestradiol. This potent, synthetic oestrogen, although not structurally similar to the natural oestrogens, can exhibit similar biological functions. However, DES also induces teratogenic and carcinogenic effects; effects which have been well documented in DES-exposed humans (reviewed by Newbold, 1999).

The potential toxic effects of oestrogens prompted development of a rodent model to study the adverse effects of developmental exposure to environmental oestrogens and other endocrine disruptors on genital tract and mammary gland development and differentiation (Bern et al., 1981). For example, exposure of newborn BALB/cCrgl female mice to daily injections of $10^{-3}$ µg DES or greater for the first 5 days of life resulted in hyperplastic alveolar nodules at 15 months of age. Significant abnormal secretory activity was observed at doses as little as $5 \times 10^{-4}$ µg DES (Bern et al., 1987). With regard to the histopathological effects of neonatal DES, the same study suggested that the mammary glands are 10- to 100-fold more sensitive than either the vagina or uterus to neonatal DES exposure. Furthermore, studies in rats have shown that prenatal and neonatal treatment with DES causes an increased incidence of mammary gland tumours (hyperplastic alveolar nodules, dysplasia, and neoplasia) (Rothschild et al., 1987) and increased sensitivity to hormones and carcinogens in later life (Bern et al., 1985). In the male ruminant, implants of stilboestrol into ram and castrated male lambs at birth caused great enlargement of the teats by 4 months of age, the effect being even more striking in bull and steer calves (Wallace, 1953).

Of particular relevance to domestic ruminants are the environmental oestrogens, which have the potential to alter sex differentiation and hence mammary gland development. One such class of environmental oestrogens are the phytoestrogens, which have been defined as any plant compound that is functionally or structurally similar to oestrogen or that produces oestrogenic effects (reviewed by Kurzer and Xia, 1997).

Broadly defined, phytoestrogens can be divided into three main classes: isoflavones, coumestans and lignans. All are diphenolic compounds with structural similarities to natural and synthetic oestrogens and anti-oestrogens (Figure 1.1). The isoflavones are the more intensely studied of the three classes.

**Figure 1.1** Structures of the phytoestrogens genistein (isoflavone), coumestrol (coumestan), and enterolactone (lignan) for comparison with oestradiol (natural oestrogen), diethylstibestrol (synthetic oestrogen), and tamoxifen (synthetic anti-oestrogen). (From Kurzer and Xu, 1997).




Mycoestrogens, or mycotoxins, which include zearalenone, are not intrinsic components of plants, but are a similar group of compounds that can have potent oestrogenic effects (reviewed by Patisaul and Whitten, 1999). They are secondary mould metabolites of the fungal genus *Fusarium*, which frequently infects pasture grasses and legumes (Kurzer and Xu, 1997). An oestrogenic mycotoxin that is commonly found in New Zealand pastures is zearalenone (di Menna et al., 1987). Ewes exposed to infected pasture grasses and clovers display markedly reduced reproductive performance through lowered ovulation rates, failure of fertilisation and an ovulation (Smith et al., 1990). Because of its oestrogenic properties, zearalenone has been used as an anabolic agent to enhance growth rate in lambs (Wiggins et al., 1979) and calves (Ralston, 1978).

Phytoestrogens have the ability to produce a range of hormonal effects in mammals due to their ability to mimic the actions of endogenous oestrogens. The severe reproductive abnormalities seen in sheep grazing clover-rich pasture in Australia first led to the discovery that phytoestrogens had physiological effects. Many of these animals became permanently sterile due to exposure to high levels of oestrogen for several months (Adams, 1995). Although phytoestrogen-induced sterility is rare in cattle (Adams, 1995) heifers grazing on phytoestrogen-rich red clover developed teats that were 35% longer than average (Nwannenna et al., 1994).

Because of their ability to mimic the actions of endogenous oestrogens, phytoestrogens and oestrogenic mycotoxins may influence fetal development and ultimately affect mammary physiology and lactation potential in the adult ruminant. *In utero* exposure to these compounds has been shown to alter mammary gland development in the fetal mouse (Hilakivi-Clarke et al., 1998). For example, the mammary glands of the female offspring of pregnant mice injected with 2 μg zearalenone from days 15 to 20 of gestation exhibited increased terminal end buds and

increased epithelial differentiation (Hilakivi-Clarke et al., 1998). The increase in terminal end buds is similar to the effect of oestrogen on the developing mammary gland, however oestrogen did not increase epithelial differentiation. It is possible that this latter effect may be caused by altered reproductive functions in the zearalenone-treated offspring, as *in utero* exposure to zearalenone induced persistent oestrus cycling differentiation (Hilakivi-Clarke et al., 1998).

Genistein, an isoflavone, is present in soybeans and soybean-based food products (Patisaul and Whitten, 1999). Dietary genistein is known to exert oestrogenic effects upon the uterus and mammary gland of adult rats. At a concentration of 750 μg/g of feed, genistein stimulated ductal and lobulo-alveolar development in the mammary gland of ovariectomised rats in a manner similar to that of 1 μg/g oestradiol (Santell et al., 1997). It is also suggested that genistein acts as an oestrogen in the fetal mammary gland as *in utero* exposure to genistein has been shown to alter mammary parenchymal patterns. The female offspring of pregnant mice injected daily with 20 μg genistein from days 15 to 20 of gestation exhibited more terminal end buds, and fewer differentiated epithelial structures, when compared to the glands from mice injected with the vehicle alone (Hilakivi-Clarke et al., 1998). The exact mechanisms by which genistein and zearalenone affect mammary gland development remain to be defined. However, the biological responses to these phytoestrogens are mediated through the oestrogen receptors α and β, which are expressed in the mammary bud from day 12.5 of fetal life (Lemmen et al., 1999) (refer to section 1.4.2. oestrogen receptors). Genistein interacts with both ORα and ORβ but has a higher binding affinity for ORβ (Kuiper et al., 1997). Thus, genistein is likely to be more biologically active through its interactions with ORβ. Zearalenone, on the other hand, does not exhibit a differential selectivity for ORβ and has a lower affinity for ORβ than genistein (Kuiper et al., 1997). The observations reported by Hilakivi-Clarke et al., (1998) indicate that both compounds are oestrogen receptor activators, however their biological responses are likely to depend on the relative levels of expression of each OR type. It will be interesting to know if the fetal ruminant mammary gland expresses oestrogen receptors and, if so, whether it is a target for hormonal disruption through exposure to exogenous oestrogens.

While their effect on the development of the ruminant mammary gland is still unclear, oestrogenic compounds can have profound effects on the development of the mammary gland in other species. Therefore it is suggested that in ruminants *in utero* exposure to these compounds may be detrimental to normal development, particularly of the mammary gland of the female fetus and hence to her future lactation potential.

#### 1.4.3.2 Effects of anti-oestrogens on fetal mammary development

The use of the anti-oestrogen, tamoxifen, in mammary cancer therapy is well established (Jordan, 1997). However, its biological effects on oestrogen target cells vary greatly depending on the species, organ, tissue, or cell type that is being examined. In the human mammary gland tamoxifen exerts anti-oestrogenic activities by acting as an oestradiol receptor blocker (Furr and Jordan, 1984). In the immature pig, tamoxifen alone acts as an oestrogen agonist in stimulating

mammary duct growth (Lin and Buttle, 1991). It is also known to have agonist activity in the uterus of the ovariectomised mouse (Campen et al., 1985) and in the vagina and uterus of the fetal mouse (Sato et al., 1966). Furthermore, the effect of tamoxifen also depends on the age of the animal examined. For example, tamoxifen completely antagonised the effect of oestradiol on mammary growth in mature rats whereas it induced mammary duct growth and did not antagonise oestradiol in immature postnatal rats (Nicholson et al., 1988). Observations by Hilakivi-Clarke et al., (1998) indicated that tamoxifen is an oestrogen receptor activator in the fetal mouse mammary gland. When administered to the pregnant mother (2 µg tamoxifen between days 15 and 20 of gestation), tamoxifen increased the density of terminal end buds in the fetal mammary gland when compared with the development in control mice. In contrast, administration of tamoxifen over a six-month period in intact adult mice completely blocked developmental growth of their mammary glands (Sourla et al., 1997). Slow release implants of ICI 163,438, a very specific oestrogen antagonist, into the mammary glands of pubertal mice also inhibited duct growth (Silberstein et al., 1994). These observations make it clear that any anti-oestrogen, whether environmental or manmade, that interferes with oestrogen action, has the potential of inhibiting mammary development and hence future milk production.

#### 1.4.3.3 Effects of androgens on fetal mammary development

Numerous studies have revealed that prenatal exposure to testosterone during a critical period for sexual differentiation can induce masculinisation of the external genitalia of the female (cattle: Jost et al., 1973; mouse: Manning and McGill, 1974; sheep: Clarke et al., 1976). Moreover, testosterone treatment of female mice or exposure to testosterone, as in bovine female fetuses of heterosexual twins (freemartins) during early gestation, masculinises the 'neutral' pattern of mammary development.

Injections of testosterone or testosterone propionate into pregnant mice or directly into the fetus, caused the mammary bud in the female offspring to become detached from the epidermis and isolated in the mesenchyme, as in the normal male (Raynaud 1961, 1971). The administration of testosterone propionate to pregnant mice also resulted in the inhibition of nipple formation in the female fetuses (Hoshino, 1965). As with masculinisation of the reproductive tract, androgen exposure was most detrimental to mammary gland development when administered during the 'critical period' or period of maximum sensitivity to androgen (day 12 of gestation) (Hoshino, 1965). This coincides with androgen receptor expression in the mammary mesenchyme of the female gland (Robinson et al., 1999) and the onset of gonadal testosterone secreting activity in the normal male mouse (Kratochwil, 1971).

The effect of androgens on mammary development in ruminants is not well documented. It was Lillie's (1917) classical study of the bovine freemartin that led to the suggestion that female fetuses might be masculinised by testicular hormones. In bovine freemartins, androgens secreted during uterine life by the male fetus may cause the female to be sterile. The mammary gland of the freemartin, although apparently normally developed at birth, cannot be stimulated

into normal milk production, producing only very small volumes with no colostrum (Anderson, 1974). Wallace (1953) described the udder of the freemartin from birth to 6 months as in effect, that of an ovariectomised heifer. Normal mammary duct proliferation and spread failed to occur either in the presence or absence of the abnormal gonads, but the gland was readily stimulated by oestrogen (stilboestrol implant), developing an extensive duct and gland system (Wallace, 1953). Turner (1959) also observed growth of the mammary gland in Guernsey freemartins after injection of oestradiol benzoate. Attempts to induce lactation in freemartins have been unsuccessful (Tervit et al., 1980) indicating that exposure of the gland to testosterone during fetal development has a permanent effect.

In the sheep, a further consequence of *in utero* exposure to androgens is the virilisation of the neuroendocrine axis, which leads to an inability of the female to respond to both the stimulatory and inhibitory effects of oestrogen in later life (Wood and Foster, 1998). Critical aspects of their female reproductive anatomy, physiology, and behaviour are permanently masculinised, and they are rendered infertile (Wood and Foster, 1998). The disruption of oestrogen-mediated stimulation of mammary gland development could potentially retard mammary growth in adult life and reduce milk yields.

#### 1.4.3.4 Effects of anti-androgens on fetal mammary development

It is only recently that environmental anti-androgens such as vinclozolin and Procymidone (fungicides) and the persistent DDT metabolite *p,p'* DDE (a pesticide) have been examined and their demasculinising effects reported. A multigenerational study showed that treatment of pregnant rats with *p,p'* DDE (dose rate of 100 mg/kg/day) from days 14 to 18 of gestation reduced anal-genital distance, and caused retention of thoracic nipples in male progeny (Kelce et al., 1995), both of which are indicative of prenatal anti-androgen exposure (Imperato-McGinley et al., 1992). Although DDT is an "oestrogenic" chemical, its metabolite *p,p'* DDE has little ability to bind the oestrogen receptor, but inhibits androgen binding to the androgen receptor, androgen-induced transcriptional activity, and androgen action in developing, pubertal and adult male rats (Kelce et al., 1995).

When administered to pregnant rats from day 14 of gestation until postnatal day 3, Procymidone, a fungicide similar in structure to vinclozolin (100 mg/kg/day) reduced anal-genital distance, and induced retained nipples and abnormal positioning of the urethral opening in the male offspring (hypospadias) (unpublished, Gray and Kelce, 1996). Similar malformations, including nipple retention, hypospadias and infertility, were observed in male rat offspring whose mothers were administered a high dose of vinclozolin (100 mg/kg/day). The female offspring were phenotypically normal (Gray et al., 1994). Treatment of pregnant rats treated with doses as low as 12 mg vinclozolin/kg/day will induce retained nipples, reduce anal-genital distance and permanently reduce ventral prostate weights in male progeny. In contrast, fertility is unaffected in adult male rats after prolonged exposure to 100 mg vinclozolin/kg/day. These results indicate that the developing male fetus is particularly sensitive to endocrine disruptors

such as vincolozin, which can produce deformities at doses that have little effect on reproductive processes in adult males. The extent to which domestic ruminants are exposed to environmental anti-androgens either directly through pasture intake or indirectly via processed feed is not known.

Of particular relevance to the dairy industry is the fact that DDE has a clearance half-life in the body and the environment of over 65 years. It is not unusual to see part per million levels of *p,p'* DDE (dichlorodiphenyl dichloroethane, the metabolite most stable in tissue) in animal and human tissues in contaminated areas (Kelce and Wilson, 1997). High concentrations of *p,p'* DDE (e.g., 2.7 ppm in heart, 3.5 ppm in kidney) were measured in tissues from stillborn infants in the mid-1960s when DDT was in use in the United States (USA), indicating exposure to the fetus during gestation (cited by Kelce and Wilson, 1997). Because *p,p'* DDE is stored in fat, it is not excreted under physiological circumstances, at least in humans, except during lactation (Adamovic et al., 1978). In a study (USA) comprising 868 women, the median level of DDE measured in breast milk at parturition was 2.4 ppm, the level declining substantially over the course of lactation (Rogan et al., 1986), suggesting a measure of exposure to the child. In a "pesticide area" of Mississippi, U.S.A., a milk study in lactating women found a mean of 14.7 ppm fat basis for *p,p'* DDE (Jensen, 1983). DDE contamination in ruminants, particularly dairy cattle, has an added significance in view of the important role of cow's milk in human nutrition.

In summary, much evidence has accumulated through the years to show that exposure to endocrine-disrupting substances during fetal development has adverse affects on the differentiating reproductive tract and mammary gland. Although the mature animal may experience adverse responses to these chemicals, it is the developing fetus that is particularly sensitive to perturbation and often experiences permanent, long-term consequences. Most of the studies cited measure the effect of injected substances whereas, in order for them to have these effects in (contaminated) "nature", they would have to pass the gastrointestinal and placental barriers of the dam. Although further investigation is required, the limited information available suggests that manmade and environmental contaminants have the potential to disrupt milk secretion. However, further research is needed to investigate the importance of these effects and the mechanisms involved. The impact of pollutants on mammary development and hence, milk secretion in the ruminant remain to be characterised.

### 1.4.4 The pituitary hormones

#### 1.4.4.1 Growth hormone

In all mammalian species studied, pituitary growth hormone (GH) has been shown to be an important regulator of postnatal growth and development (Isaksson et al., 1985). In addition to its well-established galactopoietic properties (reviewed by Burton et al., 1994), GH also plays a key role in postnatal mammary gland development (Kleinberg et al., 1990), but it does not act in isolation. In rodent models, in which pituitary glands, adrenal glands and ovaries or testes were

ablated, optimal mammary development occurred only when GH was administered in combination with oestrogen, prolactin, progesterone and corticosteroids (Lyons et al., 1958; Nandi, 1958). These findings were clarified and confirmed by Feldman et al. (1993) using recombinant DNA technology. The treatment of prepubertal and pubertal ewe lambs (Johnsson et al., 1986; McFadden et al., 1990) and heifers (Sejrsen et al., 1986; Sandles et al., 1987) with exogenous growth hormone resulted in substantial stimulation of the mammary parenchymal tissue, although the resultant morphogenesis of this enhanced development has not been described. Moreover, it does not translate into increased milk yield (Sandles et al., 1987).

Growth hormone actions require the presence of specific receptors on the surface of target cells. The GH receptor (GHR) is expressed in many tissues including fat, muscle and especially the liver (Gluckman and Breier, 1989). Although the systemic administration of GH markedly increases milk production in cattle (Knight et al., 1992) the mechanism of its action remains debatable. Controversy reigned for a long time over the existence of the GH receptor in mammary tissue as conventional binding assays failed to identify GH receptors in mammary tissue (Akers, 1990). However, GH receptors have been located in epithelial cells of the pregnant rat mammary gland by immunocytochemistry (Lincoln et al., 1990) and GH receptor mRNA has been found in mammary glands of cows (Hauser et al., 1990) rabbits, sheep and pigs (Jammes et al., 1991). Furthermore, the findings that IGF-I can substitute for pituitary hormones in mammary development (Ruan et al., 1992) and stimulate mammary cell growth *in vitro* (McGrath et al., 1991) suggest that growth hormone acts, at least in part, via local production of insulin-like growth factor (IGF-I) in the fat pad. Evidence that GH stimulates stromal cells to produce IGF-I was demonstrated by GH-stimulated, IGF-I mRNA synthesis in parenchyma-free fat pads of rats (Walden et al., 1998). GH also targets mammary stromal cells in ruminants, as adipose tissue from prepubertal ewe lambs binds GH (McFadden et al., 1990). Furthermore, expression of IGF-I mRNA in the ovine mammary fat pad is highest prior to puberty and in late pregnancy when serum GH concentrations are high (Hovey et al., 1998). Recent data indicate that the mammary fat pad mediates the mammogenic effects of systemic hormones through a local increase in the mammary ratio of IGF-I:IGFBP-3 (IGF binding protein). An increase in the proliferation of mammary parenchymal cells after treatment with oestrogen and GH was associated with increased IGF-I and reduced IGFBP-3 protein in the mammary tissue of 18 month-old heifers (Berry et al., 2001).

Whereas growth hormone is essential for normal linear growth after birth, the role of GH and its receptor (GHR) in fetal development remains controversial. Several studies have suggested that in the fetus, GH does not play a major role in somatic growth and IGF-I secretion appears to be largely independent of GH (Chard, 1989; Bassett et al., 1989). Initially, the only fetal tissue reported to possess GH receptors was the liver, and in the fetal sheep, binding of ovine GH to hepatic membranes is low and appears to be to lactogenic receptors (Gluckman, 1984). However, *in vitro* experiments have shown that several fetal tissues (e.g., human pancreas) respond to GH (Swenne et al., 1987), which suggests that functional GH receptors may be present in these tissues. Furthermore, GH receptor/binding protein (BP) immunoreactivity has been

demonstrated in all major organ systems of the rat fetus by day 18 of gestation (García-Aragón et al., 1992). In addition, total embryonic GH receptor/BP mRNA increases exponentially during fetal development (following the same pattern as the fetal growth curve), although concentrations are still much lower than in the adult (García-Aragón et al., 1992). Hill et al. (1992) localised the GHR protein in the germinal layer of the epidermis in skin of fetal and adult humans. *In situ* hybridisation studies have recently revealed high amounts of GHR mRNA in the epidermis during fetal development in rats and cattle (Edmondson et al., 1995; Knabel et al., 1998).

Published studies report conflicting results on the effects of GH administration to the mother on fetal development. Treatment of pregnant ewes with recombinant bovine GH (bGH) can stimulate fetal growth, but only after about day 100 of gestation (Jenkinson et al., 1999). Accelerated fetal growth was observed in pregnant sows treated with recombinant porcine GH (pGH) in early (days 30-43) (Sterle et al., 1995) and late (days 80-94) (Rehfeldt et al., 1993) gestation. In rodents, however, some studies report increased fetal weights (Clendinnen and Eayrs, 1961; Jörgensen et al., 1991) while others report no effect on fetal weights (Jean, 1968; Gargosky et al., 1991). It is reasonable to assume that the signal, which crossed the placental barrier and stimulated fetal growth after *in utero* exposure to GH, may also influence fetal mammary growth. This was demonstrated in mice where exogenous growth hormone was administered to the pregnant mother or directly into the fetus at day 14 of gestation. Fetal mammary gland development was measured by planimetry of serial sections at approximately day 20 of fetal life. Both methods of GH exposure induced hypertrophy of the mammary gland in male and female offspring. In addition, the mammary bud of the male gland remained attached to the epidermis (Jean, 1968). However, care must be taken in the interpretation of these results, as the source of GH is from pituitary extracts, thereby comprising numerous other hormones, which may have influenced the outcome.

To date, only one study has been undertaken to localise the GHR and its transcript in the mammary gland during fetal development and that was in the bovine (Knabel et al., 1998). Immunohistochemistry displayed the same distribution pattern of the GHR protein as *in situ* hybridisation location of the mRNA encoding for GHR. In 3-month-old bovine fetuses GHRs were found in the epithelial cells of the primary duct, in the endothelium of blood vessels and in the epidermis. By five months of gestational age the epithelium of the elongated and ramifying ducts displayed strong immunoreactivity for GHR, which was also expressed in the cytoplasm and nucleus of the mesenchymal cells surrounding the ducts and in the cytoplasm of adipocytes. Eight-month-old fetuses showed a distinct labelling in both the ductal epithelial cells and the adjacent mesenchymal cells of the mammary gland, while the more distant mesenchyme tissue was only weakly immunopositive. These results demonstrated that the amount of GHR and its mRNA in the glandular epithelial cells continuously increased as gestation progressed suggesting that GH may also be involved in prenatal proliferation and differentiation of mammary tissue. However, further investigation is required in order to evaluate the physiological role of GH in the fetal mammary gland.

### 1.4.5 Peptide growth factors

Recently, several growth factor families have been identified as having possible regulatory roles in normal postnatal mammary gland growth and development. These include members of the fibroblastic growth factor family (e.g., keratinocyte (KGF) and hepatocyte growth factors (HGF)), epidermal growth factor (EGF), transforming growth factors alpha and beta (TGF-$\alpha$ and TGF-$\beta$) parathyroid hormone-related protein (PTHrP) and the insulin-like growth factors (IGFs). Since mammary gland morphogenesis is dependent on epithelial-stromal interactions (Section 1.3.2) the expression patterns of the growth factors mentioned above and their location in the mesenchyme suggest that they may mediate short-range signals to influence mammary epithelial growth. Recent evidence has suggested roles for at least three locally produced growth factors in fetal mammary gland development; IGFs, KGF and PTHrP. Although no studies to date have examined the expression of EGF or TGF-$\alpha$ in the fetal mammary gland, it has become increasingly apparent that these growth factors play a significant role in regulating postnatal mammary growth and morphogenesis (Hovey et al., 2002).

#### 1.4.5.1 Parathyroid hormone-related protein (PTHrP)

Parathyroid hormone-related protein (PTHrP) is a peptide growth factor that is related to parathyroid hormone (PTH), a classic systemic calciotrophic peptide hormone produced by the parathyroid glands. In contrast to PTH, PTHrP is produced by a wide variety of fetal and adult tissues, does not circulate and exerts its actions locally (Broadus and Stewart, 1994). Both proteins retain the use of a common receptor, the PTH/PTHrP receptor or the Type 1 PTH receptor (PTHR1) (Jüppner et al., 1991).

PTHrP is expressed at various stages during mammary gland development and has been implicated as playing a role in epithelial-mesenchymal interactions. Over-expression of PTHrP in myoepithelial cells results in severe impairment of ductal branching morphogenesis and elongation during puberty and inhibits the formation of terminal ductules during early pregnancy (Wysolmerski et al., 1995).

In the mouse, PTHrP is expressed within the epithelial cells of the mammary bud late on day 11 of fetal age, after the mammary buds have already begun to form. PTHR1 expression is present on day 12 and is found throughout the ventral mesenchyme, both underlying the epidermis and surrounding the mammary buds (Wysolmerski et al., 1998; Dunbar et al., 1999). In the fetus, PTHrP, signalling through its receptor, is essential for the sexual dimorphism in normal mammary development in the mouse (Dunbar et al., 1999). Analysis of histological sections through wild-type male mammary buds at day 15 of fetal age showed the mesenchymal cells condensing around the degenerating epithelial stalk. This was accompanied by widespread TUNEL staining in the epithelial cells of the mammary stalk and the mesenchymal cells within the androgen-induced condensation (Dunbar et al., 1999). In contrast, in PTHrP and PTHR1 knockout embryos all mammary buds were present, well preserved and indistinguishable from

those observed in female fetuses (Dunbar et al., 1999). Furthermore, there was no apoptosis, as demonstrated by a lack of TUNEL staining, in PTHrP and PTHR1 knockout males, nor in the wild-type females. The mammary buds only persist in the knockout males until days 16 to 17 of fetal age, at which point they fail to undergo the initial round of branching morphogenesis observed in normal females and instead degenerate (Wysolmerski et al., 1998).

In the male, PTHrP appears to be the epithelial signal responsible for the induction of androgen receptor expression within the dense mammary mesenchyme, as the pattern of androgen receptor localisation is absent in PTHrP and PTHR1 knockout buds, but can be restored by expression of a PTHrP transgene (Dunbar et al., 1999). Moreover, androgen receptor staining is normal within the fetal testes (Wysolmerski et al., 1998) as is development of the Wolffian duct and descent of the fetal testes in the absence of either PTHrP or PTHR1 (Gilbert, 1994). This indicates that an intact androgen response is not sufficient to initiate destruction of the mammary bud in the normal male fetus.

A failure of PTHrP signalling also inhibits outgrowth of the primary duct in the female fetus. In the normal development of the mammary gland in female mice, the formation of the mammary buds occurs between days 10 and 12 of fetal age. From days 13 to 15 the gland remains relatively quiescent until day 16 when branching morphogenesis is initiated. By day 18, the primary duct has elongated and initiated the formation of secondary ducts, and makes contact with the fat pad. The nipple sheath has also formed (Sakakura, 1987). The mammary buds of PTHrP knockout females appear normal at days 12-13 and 15 of fetal age, but by day 18 there is a dramatic difference in the appearance of the mammary structures in the knockout as compared to normal females. In the PTHrP-knockout females, the mammary glands fail to elongate and the remaining bud becomes surrounded by an abnormally dense condensation of fibrous connective tissue. In addition, the mammary epithelial cells begin to degenerate, showing many pyknotic nuclei and indistinct cell borders. Furthermore, there was no evidence of nipple sheath development, and the developing fat pads, although present, appeared diminished in size (Wysolmerski et al., 1998). Ablation of the PTHrP receptor (PTHR1) leads to the same phenotype, a failure of the initial phase of branching morphogenesis during fetal mammary development (Wysolmerski et al., 1998). Further studies are required to explain why the loss of PTHrP-signalling renders the epithelial bud incapable of undergoing further morphogenesis in PTHrP and PTHR1 knockout females. It is unlikely to be due to the failure of the mammary mesenchyme of PTHrP and PTHR1 knockout females to express androgen receptor as the mammary epithelium of Tfm mice with inactivating mutations of the androgen receptor displays branching morphogenesis (Kratochwil and Schwartz, 1976). These observations raise the question as to the status of mesenchymal oestrogen receptors in the absence of PTHrP signalling. Although there is no evidence to date, other regulatory factors such as *Msx-2*, *Bmp-4*, FGF-7 and LEF-1 are expressed in the mammary mesenchyme (van Genderen et al., 1994; Cunha and Hom, 1996; Phippard et al., 1996; Robinson et al., 1999) and therefore, may be involved in regulating epithelial morphogenesis in the fetal mammary gland via a common genetic pathway.

#### 1.4.5.2 Insulin-like growth factors (IGFs)

The insulin-like growth factors, IGF-I and IGF-II are low molecular mass polypeptides that are structurally related to insulin (reviewed by Sara and Hall, 1990). The biological effect of the IGFs is influenced by association with IGF binding proteins (IGFBPs) (Jones and Clemmons, 1995). IGF-I plays a major role in promoting postnatal growth and in mediating the influence of growth hormone, thyroid hormones and nutrition on that growth (Humbel, 1990). While the liver is the major source of circulating IGFs and IGFBPs, numerous studies indicate that local IGF synthesis may be particularly important for the differentiation and growth of a variety of tissues (Jones and Clemmons, 1995). In the mammary glands of rodents (Kleinberg, 1998) and ruminants (Hovey et al., 1998) IGF-I is thought to be expressed in the stromal cells, indicating that it may act in a paracrine manner during mammogenesis. In primary cultures of luminal epithelial cells and myoepithelial cells prepared from the mammary glands of adult sheep, IGF-I stimulated DNA synthesis about five-fold (Forsyth, 1996).

Considerable experimental evidence exists to support the suggestion that IGF-I acts as a local mediator of growth hormone and oestradiol action in postnatal mammary growth and function (Walden et al., 1998). Expression of IGF-I mRNA within the mammary gland is upregulated by exogenous GH, but not prolactin and is further increased in the presence of oestrogen (Kleinberg, 1997). Locally implanted IGF-I and des(1-3)IGF-I, an amino-terminally shortened form of IGF-I, significantly increased the number of terminal end buds in mammary glands of hypophysectomised, castrated prepubertal male rats also treated with oestradiol (Ruan et al., 1992) and in female rats similarly treated (Ruan et al., 1995). Interestingly, des(1-3)IGF-I stimulates a greater (approximately four-fold) proliferative response by bovine mammary epithelial cells than its native IGF-I (Collier et al., 1989).

The precise function of IGF-II during postnatal mammogenesis is not well defined. IGF-II mRNA is expressed in the mammary fat pad of the mouse throughout postnatal life (Hovey et al., 1999) and *in situ* hybridisation has localised IGF-II to stromal cells in normal and neoplastic rat mammary gland (Manni et al., 1994). In cultures of ovine and bovine mammary epithelial cells, IGF-II was shown to stimulate the proliferation of ovine (Winder et al., 1989) bovine (McGrath et al., 1991) and mouse (Riss and Sirbasku, 1987) mammary epithelium, although to a lesser extent than IGF-I.

The IGFs exert their mitogenic effects by binding to specific cell-surface receptors. IGF-I has a greater affinity for the IGF-I receptor than does either des(1-3)IGF-I or IGF-II. In adult cattle, mammary epithelial cells express mRNA for the IGF-I receptor (Glimm et al., 1992) and possess high affinity ligand binding sites (Collier et al., 1989). In the prepubertal ovine mammary gland, IGF-I receptor mRNA localises to epithelial cells, its expression being greatest at 4 weeks of age (Morgan et al., 1996). The IGF-II receptor has a high affinity for IGF-II, a much lower affinity for IGF-I and does not bind insulin (Jones and Clemmons, 1995). The population of IGF-II receptors is elevated markedly during pregnancy within rat mammary tissue (Collier et al., 1989) and is greater than that for IGF-I in the pregnant bovine mammary gland (DeHoff et al., 1988).

However, as lactation progresses, the number of IGF-I receptors in lactating bovine mammary tissue declines (Disenhaus et al., 1988).

The expression of IGFBP-1, -2, -3 and –4 mRNA and their proteins in mammary tissue from prepubertal heifers suggest the involvement of the binding proteins in regulating the growth of mammary epithelial cells. Increased concentrations of IGFBP-3 are most often associated with blocking the mitogenic effects of IGF-I and IGF-II but not des(1-3) IGF-I, in heifers (Purup et al., 2000). However, the higher concentrations of IGFBP-3 in the mammary tissue of well-fed heifers together with reduced mammary tissue sensitivity to IGF-I are possible explanations for the negative effect of high feeding levels on mammary parenchymal growth (Weber et al., 2000).

While it is now well established that fetal IGF-I also affects fetal growth (Gluckman, 1997) its significance for fetal mammary development is still unclear. One study, to date, has reported the distribution of mRNA for IGF-I and IGF-II, and the IGF-I receptor in fetal mammary tissue using *in situ* hybridisation (Forsyth et al., 1999). In female fetal sheep, IGF-I and IGF-II mRNA were expressed in the cells of the intralobular connective tissue underlying the epithelial tissue at all stages examined (weeks 10, 15 and 20 of gestation). The abundance of IGF-II increased with gestational age. IGF-I receptor mRNA was localised to the epithelial cells in the fetal gland which is consistent with the results of Glimm et al. (1992) for the adult cow and Morgan et al. (1996) for the prepubertal lamb. Furthermore, the localisation of IGF-I and IGF-II mRNA to fetal stromal cells is consistent with the promotion of mammary epithelial proliferation in response to IGF-I and IGF-II in postnatal ruminants (Winder et al., 1989; McGrath et al., 1991) and rodents (Ruan et al., 1995). The distribution of mRNA for IGF-I and IGF-II, and the IGF-I receptor in the fetal mammary gland indicate a role for the IGF system in mediating epithelial-mesenchymal interactions in prenatal mammary development. Further investigation is required to determine the physiological role of the IGFs in the fetal mammary gland.

#### 1.4.5.3 Fibroblastic growth factor (FGF)

The fibroblastic growth factors are a large multigene family containing at least 22 identified polypeptides. Collectively, the FGFs are mitogens for cells of endodermal, ectodermal and mesenchymal origin. They bind and interact to various degrees with high affinity cytoplasmic tyrosine kinase receptors encoded by four separate genes. They also bind with a lower affinity to heparin sulphate receptors (reviewed by McKeehan et al., 1998; Ornitz, 2000). The FGFs that signal through the epithelial receptor FGFR2-IIIb are predominantly expressed in the adjacent mesenchyme, thereby playing a paracrine role, a process of major importance during organ morphogenesis (Finch et al., 1989).

Fibroblast growth factor receptor 2-IIIb (FGFR2-IIIb) signalling has been shown to have an important function in adult mammary gland development (Jackson et al., 1997). Examination of mice expressing a dominant negative FGFR2-IIIb receptor in the mammary epithelium caused a marked impairment of lobuloalveolar development during pregnancy. Although there was a

measurable lactational response, the low weight gain of the pups from these mice indicated that total milk production was insufficient to properly sustain the newborn pups (Jackson et al., 1997). This was attributable to the scarcity of alveoli in the mammary tissue.

To investigate a possible role for the IIIb isoform of fibroblastic growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during organogenesis in the mouse, mice lacking the IIIb form of FGFR2 were studied (DeMoerlooze et al., 2000). In FGFR2-IIIb null mice, the limbs, lungs and anterior pituitary gland undergo extensive apoptosis after initiation and developmental abnormalities arise in the salivary glands, inner ear, teeth, skin and skull. Many of these abnormalities can be explained by the fact that the mesenchyme-expressed ligand fails to initiate its normal response because of the absence of FGFR2-IIIb in the adjacent epithelium. Moreover, as the mammary gland is an organ that also develops by budding and branching morphogenesis it is likely to be affected in a similar manner. Analysis of FGFR2-IIIb gene expression in the fetal mouse has shown that it is present in the ectoderm and subsequently the basal layer of the skin epidermis, as well as the mammary bud (Spencer-Dene et al., 2001, Cunha and Hom, 1996). However, in mice that lack the FGFR2-IIIb gene, the mammary bud fails to form as shown in a photomicrograph presented by Spencer-Dene et al. (2001) of the fetal mammary gland at day 14.5 of gestation. A developmental oddity that occurs in the mutant mice is the slower differentiation of the epidermis, which takes one day longer than normal and is of a reduced thickness (DeMoerlooze et al., 2000). However, mammary buds have not been detected in these mice even after epidermal differentiation (Spencer-Dene et al., 2001).

Potential candidates for FGFR2-IIIb activation are FGF-10 and FGF-7 (keratinocyte growth factor) as both ligands accumulate in the mesenchymal tissue (Cunha and Hom, 1996; Ornitz, 2000). However, mice deficient for FGF7 appear to develop normal mammary glands (Guo et al., 1996), but mice deficient for FGF10 have been shown to have a complex phenotype similar to that of FGFR2-IIIb null mice (DeMoerlooze et al., 2000), suggesting that signalling by this ligand may be necessary for mammary development.

#### 1.4.5.4 Keratinocyte growth factor (KGF)

Keratinocyte growth factor (KGF) is a member of the heparin-binding fibroblast growth factor family (FGF-7) (Rubin et al., 1989). KGF is produced by fibroblasts and stimulates epithelial cell proliferation, leading to the hypothesis that it might function as a paracrine mediator of epithelial cell growth (Finch et al., 1989). *In vitro* studies have shown that KGF stimulates the growth of terminal end buds and epithelial organoids in the mouse mammary gland (Imagawa et al., 1994). Administration of KGF intravenously to virgin female rats stimulated ductal neogenesis and intraductal epithelial hyperplasia. KGF also induced acinar and ductal cell growth in pregnant rats, but the mammary epithelium of lactating rats was resistant, indicating a developmentally specific response to KGF (Ulich et al., 1994). The actual source of KGF in the rodent mammary gland is presumed to be cells of the mammary fat pad, although Ulich et al. (1994) did not detect any KGF mRNA in the fat pad itself. In the sheep, KGF is located within

the mammary fat pad, where expression of KGF mRNA has been detected *in vitro* and *in vivo* (Hovey et al., 2001).

In the mammary gland of the female fetal mouse, KGF is widely expressed in the cells of the mammary mesenchyme while the KGF receptor (KGFR) is expressed in the epithelium. Specifically, at day 14 of fetal age, KGFR mRNA expression in the mammary epithelial buds corresponded to the surrounding mesenchyme, which expressed KGF mRNA (Finch et al., 1995). The distribution of KGF and its receptor are the same as that reported by Forsyth et al. (1999) for IGF-I and IGF-II mRNA (in mesenchyme) and the IGF receptor (in epithelium) in ovine fetal mammary gland. The distribution of KGF and its receptor within the fetal mammary gland suggest that KGF might be a stromal factor that regulates prenatal mammary epithelial morphogenesis. Moreover, it may account for some of the differences observed postnatally between rodents and ruminants in parenchymal growth and development. In ruminants, the mammary parenchyma undergoes a lobulated morphogenesis in the presence of dense, fibroblastic connective tissue and a relatively high expression of KGF (Hovey et al., 2001). In contrast, the parenchyma of virgin mice and rats consists of a rudimentary ductal tree with minimal fibroblastic stroma (Sakakura, 1987). KGF expression, relative to the ruminant gland, is also reduced. Interestingly, the rodent gland can be stimulated to undergo lobular development in response to exogenous KGF (Cardiff and Wellings, 1999).

#### 1.4.5.5 Epidermal growth factor (EGF)/Transforming growth factor-α (TGF-α)

Epidermal growth factor and TGF-α belong to a family of at least 10 proteins with similar structures, including heparin-binding EGF, amphiregulin, and several heregulins. These proteins initiate their biological effects through the EGF (ErbB-1) receptor, which is a specific high-affinity receptor located on the plasma membrane (reviewed by Plaut, 1993). EGF stimulates vigorous growth of mammary epithelium *in vitro* as shown in studies utilising mouse and rat mammary epithelial cells in primary cell culture. In the mouse, EGF enhances the growth of ductal and alveolar cells (Imagawa et al., 1985) whereas, in the rat, EGF stimulates the proliferation of a myoepithelial cell type (McGrath et al., 1985). Indirect evidence for a stimulatory response to EGF *in vivo* was provided by sialoadenectomy, which removed the major source of EGF in the circulation, the submandibular gland. In sialoadenectomised female mice, mammary development is diminished during pregnancy and subsequent milk yields are lower. However, these effects can be reversed by the daily administration of exogenous EGF (Okamoto and Oka, 1984). More directly, when slow-release implants are introduced into the regressed mammary glands of ovariectomised mice, terminal end bud formation is stimulated around the region of the implant (Coleman et al., 1988). This observation led to the proposal that EGF may work in synergy with ovarian steroids in stimulating mammary gland development. Vonderhaar (1987) found that local ductal branching and lobuloalveolar development could be stimulated in intact adult mice treated with implants containing EGF and TGF-α in the mammary gland. TGF-

α was a more potent stimulator of lobuloalveolar development than EGF. In contrast to EGF, the effects of TGF-α occurred independently of oestrogen and progesterone supplementation.

Several studies have examined the expression of EGF and TGF-α within the mammary gland. Using polymerase chain reaction (PCR) with oligonucleotide primers specifying EGF or TGF-α, Snedeker et al. (1991) found mRNA for both EGF and TGF-α in mouse mammary glands during ductal growth and pregnancy, but only EGF mRNA was detected in lactating glands. In the same study, TGF-α mRNA was expressed by the cap cells of the ductal end bud, whereas EGF immunolocalised to the epithelial cells of the terminal end bud and the luminal cells of the ducts (Snedeker et al., 1991). *In situ* hybridisation indicates that TGF-α mRNA is also expressed by the mammary gland stroma of the adult rat (Liscia et al., 1990).

Little is known about the role of EGF, TGF-α and related growth factors in postnatal ruminant mammogenesis. A limited number of studies with ruminant cells have shown that EGF and TGF-α both stimulated DNA synthesis in mammary tissue explants from mid-pregnant heifers (Sheffield, 1998) in mammary epithelial cells from pregnant heifers (Collier et al., 1993) and in alveolar cells prepared from pregnant sheep (Forsyth et al., 1998). An effect of these growth factors *in vivo* was first suggested by the fact that infusion of EGF into the teat canal of sheep or cows stimulated mammary gland development (McGrath et al., 1988; Collier et al., 1993). However, Collier et al., (1993) showed that TGF-α is more effective than EGF at stimulating bovine epithelial cells in vitro. Similarly, the effect of EGF on the mammary alveolar epithelial cells of pregnant sheep was smaller and not clearly dose-related compared with TGF-α (Forsyth et al., 1998). DNA synthesis was also stimulated by amphiregulin in alveolar cells prepared from pregnant sheep (Forsyth et al. 1998). Furthermore, whereas TGF-α mRNA has been detected in bovine mammary tissue (Collier et al., 1993; Plath et al., 1997), expression of EGF mRNA is so far known only from a single study with ruminant tissue (Sheffield, 1997). These results, taken together, indicate the possible involvement of TGF-α and EGF in regulating ruminant mammary gland development. However, more information is needed about the suggested presence of these factors in mammary tissue.

In the mouse mammary gland, the EGF receptor (EGFR) is expressed in cap cells of end buds, in the stromal fibroblastic cells surrounding the terminal end buds, and in myoepithelial cells, luminal epithelial cells and adipocytes (Coleman et al., 1988; DiAugustine et al., 1997). EGFR is expressed in the mammary epithelial cells in sheep and cattle. Expression is increased during pregnancy and decreased during lactation (Moorby et al., 1995; Glimm et al., 1992). Recent studies where mammary glands of $EGFR^{-/-}$ neonatal mice (mice that lack the EGF receptor) were transplanted under the renal capsule of immuno-compromised female mice, indicated that EGFR plays a key role in stromal-epithelial interactions (Wiesen et al., 1999). Ductal growth and branching morphogenesis were impaired in tissue recombinants prepared with $EGFR^{-/-}$ stroma and wild-type epithelium or $EGFR^{-/-}$ stroma and $EGFR^{-/-}$ epithelium. In addition, the impaired ductal growth in the $EGFR^{-/-}$ mice was also associated with a marked reduction in the density of the fibroblasts that separate the ducts from the fat pad (Wiesen et al., 1999). Whether or not

EGF plays a role in fetal mammogenesis is yet to be determined. The fact that the EGF receptor is required for ductal development in the neonate, suggests a possible role for EGF in signalling prenatal ductal outgrowth, if not in the rodent, then perhaps in the ruminant, where the parenchyma advances as a much denser mass.

While EGF, TGF-α and the EGF receptors have not been examined in the fetal mammary gland, recent observations suggest that EGF may modulate androgen-dependent sexual differentiation of the fetus (Gupta et al., 1996). EGF, like androgen, stabilised the Wolffian duct of undifferentiated reproductive tracts of the thirteen-day-old female fetus that were grown in organ culture. EGF also induced cell proliferation of the fetal reproductive tract in a dose-dependent manner. In addition, a physiological level of EGF and androgen induced a synergistic effect on cell proliferation. Cyproterone acetate, which induces anti-androgenic effects in the male, not only blocked the EGF-induced masculinisation, but also blocked the increase in cell proliferation caused by testosterone and other androgens (Gupta et al., 1996). Using a quantitative competitive RNA PCR assay for EGF mRNA, Gupta and Singh (1996) showed that the level of EGF mRNA is higher in the male reproductive tract than in the female reproductive tract. Furthermore, testosterone-induced male sexual differentiation was accompanied by an increase in EGF gene expression. From these observations, it is reasonable to assume that androgens may act via mammary mesenchymal androgen receptors to elicit synthesis and secretion of EGF (or another autocrine/paracrine factor) that regulates mammary epithelial and stromal morphogenesis in the male ruminant.

### 1.4.6 Extracellular matrix proteins

Mammary mesenchyme is embedded in an extracellular matrix, comprised of proteins, four of which are present during embryogenesis in mice and rats (Kimata et al., 1985; Chiquet-Ehrisman et al., 1986). Fibronectin, laminin and proteoheparin sulfate are present throughout the subepithelial tissue, while tenascin C is present only in the dense mammary mesenchyme closely surrounding the mammary bud (Chiquet-Ehrisman et al., 1986; Sakakura, 1987; Dunbar et al., 1999). The expression of tenascin C, like androgen receptor, is directly dependent on PTHrP expression during formation of the mammary bud, as shown by the lack of tenascin C in PTHrP or PTHR1 knockout mice (Dunbar et al., 1999). The location of tenascin and its absence in adult mammary gland suggested a role for this protein in mediating interactions between the epithelium and mesenchyme during fetal mammary gland development. Surprisingly, in mice deficient for tenascin C, the mammary glands grow normally (Saga et al., 1992).

Epimorphin (EPM) is an extracellular matrix protein, originally characterised as a stromal cell surface molecule involved in embryonic epithelial morphogenesis of the lungs (Hirai et al., 1998). In the mouse mammary gland, EPM is expressed on the surface of myoepithelial and fibroblast cells, however, in culture, a subpopulation of mammary epithelial cells produce significant amounts of EPM. When EPM-expressing epithelial cell clones were cultured in collagen gels they displayed branching morphogenesis in the presence of KGF, HGF, EGF or

FGF, a process that was inhibited by anti-EPM, but not anti-HGF antibodies. Accordingly, EPM-negative cells simply grew in a cluster in response to the growth factors and failed to branch (Hirai et al., 1998), indicating that mammary gland branching morphogenesis is dependent, in part, on the extracellular matrix. Furthermore, the expression of EPM on the surface of mouse embryonic tissues suggests a central role in epithelial-mesenchymal interactions.

### 1.4.7 The maternal environment

There is very little evidence of an in utero effect on ovine fetal and subsequent mammary gland development, but a number models (e.g., hormones and growth factors, placental size, nutrition and temperature) have been shown to differentially influence fetal growth and development in general. For example, Mellor (1983) found that placental weight was the greatest single source of variation in the weight of the fetus at term. This was substantiated in studies in which uterine carunclectomies were performed in sheep prior to mating, thereby reducing subsequent placental surface implantation area and ultimate placental size (Owens et al., 1987). This resulted in reduced fetal weight and glucose transfer to the fetus. These studies suggested that it is possible to influence growth in the fetal sheep in general by influencing the maternal environment. However, it is not known whether the influence on fetal growth includes proportional effects on development of fetal mammary tissue.

#### 1.4.7.1 Nutrition

Throughout pregnancy the growth of the fetus is controlled by the maternal diet, both directly, through the supply of essential nutrients and indirectly, by altering the expression of key hormones and growth factors that regulate the uptake and utilisation of nutrients by the fetus. There is now increasing evidence that subtle modifications in nutrient supply during critical phases in fetal life can impart a legacy of developmental changes that affect body composition, postnatal growth and reproductive performance in farmed species (Robinson et al., 1999).

The most important nutritional factor influencing mammary growth in the postnatal animal is daily energy intake (feeding level). Numerous reports have shown that overfeeding during the commencement of positive allometric growth (from about two to three months of age until around the onset of puberty) impairs mammogenesis and may affect future milk production in ruminants. Feeding levels resulting in accelerated liveweight gain during prepuberty and puberty inhibited mammary parenchymal growth in heifers (Sejrsen et al., 1982) ewe lambs (Johnsson and Hart, 1985) and goats (Bowden et al., 1995) and reduced milk yields (Little and Kay, 1979). Moreover, the effect remained in subsequent lactations (Little and Kay, 1979). Unlike ruminants, high caloric intake in rodents enhances mammary development. Larger mammary fat pads and increased ductal development were observed in female mice fed high calorie diets to 8 and 18 weeks of age compared to mice fed low calorie diets (McFadden et al., 1988). It has been suggested that the critical aspect with respect to the effect of feeding level on mammary growth is the energy to protein ratio of the diet (Sejrsen et al., 2000). Mammary growth was inhibited in

prepubertal heifers reared at a high rate of body gain on a corn silage diet (3.4 Mcal DE/kg DM:16%CP), but mammary growth was not inhibited in heifers reared at a similar rate of body gain on an alfalfa silage diet (3.1 Mcal DE/kg DM:22%CP) (Capuco et al., 1995).

Mammary gland growth can also be affected by specific components of the diet. High levels of protected polyunsaturated fatty acids (PUFA) fed to prepubertal lambs resulted in enhanced mammary growth, especially in terms of parenchymal growth (McFadden et al., 1990). High levels of PUFAs also increased the amount of fat pad, and thus total gland size. Similar results have also been recorded in fetal rats, where exposure to a high fat maternal diet has the ability to alter the offspring's mammary gland morphology. A maternal diet high in *n*-6 polyunsaturated fats (the fat source was corn oil, which contains 59% PUFA, the majority being *n*-6 linoleic acid) significantly increased the number of terminal end buds in the mammary glands of the female offspring. The total mammary fat pad area was also larger in females exposed to a high fat diet *in utero* than in females exposed to a low fat diet (Hilakivi-Clarke et al., 1997).

For ruminants, there are many reports on the effects of maternal nutrition on fetal growth and size at birth (reviewed by Robinson et al., 1999). However, while no reports specifically studied mammary growth, the adverse effects of alterations in maternal nutrition on the subsequent growth and development of the fetus may also impact on specific organs and/or tissues. In ruminants, a low birth weight results in impaired postnatal performance (Bell, 1992), whilst in humans, studies reveal that low birth weight can have detrimental effects on disease aetiology (e.g., cardiovascular disease) which persist into adult life and even the next generation (Barker, 1998). It remains to be established whether modifications in maternal nutrition also influence fetal mammogenesis and ultimately lactational performance in adult life.

#### 1.4.7.2 Pre-lamb shearing

A number of studies, indoors *in* the U.K. (Austin and Young, 1977) and under New Zealand pastoral conditions (Kenyon et al., 1999), have shown that shearing ewes during pregnancy is associated with an increase in lamb birth weight. Shearing during mid-pregnancy increased the birth weight of twin lambs by more than 1 kg without having any effect on singleton birth weight (Revell et al., 2000). However, in another similar study enhanced birth weights were specific to single-born lambs (Morris et al., 2000). The birth weight response may reflect an increase in the delivery of IGF-I to the placenta by IGFBP-I as a result of reduced maternal concentrations of IGF-I and elevated maternal IGFBP-I (Revell et al., 2000). Although specific organs and tissues were not examined it is a reasonable assumption that if the factor influences fetal body growth, it may also influence fetal mammary gland growth.

## 1.5 Purpose and scope of the investigation

Development of the mammary gland occurs in distinct phases related to reproductive growth during fetal life, puberty, pregnancy and lactation. The basic structures of the mammary glands

are formed in fetal life and comprise, in the ruminant, a system of growing ducts that are confined to a very limited area around the cistern of the gland. The pubertal phase sees rapid growth of the fat pad and of the ducts that branch into it, but the main developments, quantitatively as well as qualitatively, occur during pregnancy. The mammary ducts during pregnancy proliferate and give rise to the lobulo-alveolar tissue, which synthesises and secretes milk to nourish the offspring.

The structural development of the mammary gland has been studied thoroughly in the fetal rodent and as a result, important advances have been made in our understanding of fetal mammogenesis and a large repertoire of experimental techniques has been developed. While this understanding is useful, extrapolating from the rodent model to the ruminant has to be done with caution, as there are significant differences between rodents and ruminants in the growth of the mammary gland, and its subsequent composition and parenchymal architecture in adult life. Fetal development of the mammary gland in farmed species was last described comprehensively fifty years ago. While observations of the sequential changes in the formation of the gland were meticulously described in cattle, the bovine model was considered representative of morphogenesis after primary duct elongation in sheep. Furthermore, there is a lack of quantitative data on these fundamental processes during fetal life in ruminants.

While prenatal development appears to progress autonomously (independently of systemic hormones), a peculiarity of some rodents is the androgen-induced destruction of the mammary rudiments. This sexual dimorphism in early morphogenesis of the mammary gland has provided insight into the interplay between classical systemic hormones and epithelial-mesenchymal interactions. However, the molecular mechanisms and signalling pathways through which these interactions take place are still poorly understood. Recent studies utilising transgenic and knockout mice have demonstrated that several genes are expressed in either the epithelium or mesenchyme of the developing mammary bud. However, to date, deletions of only three of these genes have resulted in a failure to form mammary glands.

Apart from the well-documented responsiveness to androgens, hormone responsiveness during fetal mammary gland development has received little attention. The adverse effects of environmental chemicals on the development of reproductive organs including the mammary gland, in laboratory species, demonstrate that these tissues are sensitive to oestrogens and androgens from the earliest stages. This raises the possibility that such chemicals could have an impact on mammary development in the ruminant and hence the lactation potential of the mother. An understanding of these processes is of fundamental importance in our quest to modify certain aspects of mammary gland function, which may result in enhanced milk production in the mature ewe.

The objectives of this research were to illustrate the ontological development of the normal mammary gland of the fetal sheep and identify the presence (or absence) of receptors for hormones and growth factors that have already been shown to be involved in mammary gland differentiation and development in the rodent.

# CHAPTER TWO

# A QUANTITATIVE MORPHOLOGICAL STUDY OF MAMMARY GLAND DEVELOPMENT IN THE FETAL SHEEP

## 2.1 Abstract

A study of the normal histomorphogenesis of the developing ovine mammary gland was made from 45 mammary glands, collected from a series of male and female fetuses at days 40, 60, 80, 100, 120 and 140 of gestational age (n=9 per age group) and from lambs at 21 days of age (n=1 per sex). Fetal ammary gland development was also evaluated in terms of relative growth.

The sequence of events in the prenatal development of the mammary gland of sheep was similar to that described for cattle. However, marked differences in mammary development between males and females, which were observed in the later stages of bud development in cattle, were not apparent in sheep until the formation of adipose tissue. Thereafter, development of the mammary gland became slower and ultimately less pronounced in males. In contrast, the female gland continued to increase in size (as measured by total duct area) and complexity, the epithelial tissue increasing some 24-fold from days 60 to 140 of fetal age.

Relative growth analysis of mammary development demonstrated that from days 40 to 80 of gestation, consistent positive allometric mammary epithelial growth was observed in both male and female fetuses. From days 80 to 140 of gestation there was little change in the magnitude of the allometric constant in the female mammary gland while that of the male grew somewhat slower than the body. This was also reflected in total duct area, whereby the epithelial tissue in the 21-day-old male lamb showed no advance on the condition at days 120 or 140 of fetal age.

These findings suggest that continuation of epithelial cell proliferation depends on the formation of adipose tissue and, subsequently, the mammary fat pad, the development of which is severely restricted in the male. Moreover, it is likely that the male sex hormones and local growth factors play an important role in regulating this development. It is intriguing to consider, given the substantial increase in epithelial growth from days 120 of fetal life to three weeks of postnatal age in the female, that mammary growth during fetal life may also be critical to future lactational performance.

## 2.2 Introduction

The most thoroughly studied mammary gland of any species is that of the mouse, hence its status as structurally representative. However, it is questionable as to whether it is an appropriate model for the ruminant as there are significant differences between rodents and ruminants in growth of the mammary gland, and its subsequent composition and parenchymal architecture in adult life. Furthermore, while development of the mammary gland in cattle has received attention since Turner's (1930, 1931) significant chronological account of mammary development from their earliest appearance in the embryo through gestation to birth, that of sheep has not previously been described in detail. Development beyond the formation of the primary duct in sheep was largely neglected, and assumed to quite likely follow the

morphological progression of that observed in cattle (Turner, 1952). Wallace (1953) examined mammary glands from two male and two female fetal sheep at each of days 56, 84, 112 and 140 of gestation. He described the normal course of development of the glands with the aid of histological sections and concluded that marked differences between the sexes were apparent from the outset of development. Martinet's (1962) histological analysis of male and female mammary glands from days 44 to 150 of fetal age provided a very brief account of the morphological progression of the sheep gland from mammary bud to secondary branching. However, the report confirmed only those stages already described in sheep by Profé (1899) and in cattle by Turner (1930, 1931). Martinet's study is, nonetheless, the only study to date that incorporates quantitative data. The histological development of mammary gland tissue in fetal sheep at weeks 10, 15 and 20 of gestation was also briefly described as part of a study of the role of insulin-like growth factors in mammary morphogenesis (Forsyth et al., 1999).

In all species, the basic structures of the mammary glands are formed in fetal life and comprise a system of growing ducts that are confined to a very limited area around the teat or cistern of the gland. The limited details of mammary gland morphogenesis described here relate to the ovine fetus. For a more comprehensive, comparative description of the stages of development in the ruminant and rodent the reader is referred to Chapter one. A mammary or "milk" line, which is a localised thickening of ectodermal cells closely adjoining a deeper layer of mesenchymal cells, precedes formation of the individual gland buds. The mammary lines appear on both sides of the midline in the inguinal region, where, subsequently, they fragment and regress, except where the single pair of mammary buds arise (Turner, 1952). The ectodermal cells proliferate and grow into the mesenchymal layer until, at day 44 of fetal age (Martinet, 1962) or in a 2.5 cm long fetus (Turner, 1952), a spherical cluster of cells gives rise to the mammary bud. Thereafter, differences in the patterns of development between males and females become apparent. From day 44 to about day 70 of fetal age the mammary gland of the male sheep grows at a constant rate of 2.8 times that of body weight while the female gland grows five times faster than body growth (Martinet, 1962). For approximately two weeks after the formation of the mammary bud there is no appreciable increase in its size (Martinet, 1962). Formation of the primary duct results from intensified cell proliferation at the proximal end of the bud (Turner, 1930) and occurs simultaneously with development of the teat, between days 48 and 50 of fetal life (Wallace, 1953; Martinet, 1962). Secondary ducts begin to form at the proximal end of the primary duct from 56 days of age (Wallace, 1953; Martinet, 1962). At day 70 the teat cistern is evident and the epithelial tissue is well developed. In female fetuses mammary growth then declines to 1.7 times the rate of body growth until term (Martinet, 1962). By day 84, mesenchymal precursor cells of a mammary fat pad appear posterior to the mammary epithelium (Wallace, 1953). These cells differentiate into a well-defined mammary fat pad, which consists of distinct lobules of mainly adipocytes, as well as blood and lymphatic vessels, nerves and connective tissue septa (Turner, 1931; Wallace 1953). Continued proliferation and branching leads to the formation of a rudimentary ductal system, which, in

contrast to that of the perinatal rodent, remains surrounded by dense fibrous connective tissue and has not started to penetrate the fat pad (Forsyth et al., 1999).

The histomorphological study to be described was undertaken for two main reasons:

1. To characterise the course of epithelial and mesenchymal morphogenesis of the mammary gland of both male and female fetal sheep, thereby confirming and extending those stages of development previously described by Turner (1930, 1931, 1952) and Martinet (1962);
2. To confirm and extend the previous observations of positive allometric growth in the female mammary gland from days 44 to 70 of fetal age (Martinet, 1962).

Quantification of the area occupied by epithelial tissue and the evaluation of mammary development in terms of relative growth will indicate periods of slow or rapid growth and hence, form a baseline for future studies on factors affecting fetal mammary development.

## 2.3 Materials and Methods

All procedures were approved by the Massey University Animal Ethics Committee.

### 2.3.1 Animals and Treatments

Thirty Coopworth ewes aged between four and six years were used in a 2 x 5 factorial design incorporating two fetal sexes (male versus female) and five dates of slaughter (days 40, 60, 100, 120 and 140 of gestation). They were selected in May 1997, 38 days after mating, from a commercial flock of one hundred and fifty ewes from Massey University's Keeble Farm, 9 km south of Palmerston North (latitude 40.23°S and longitude 175.37°E). Ewes were mated in March 1997 after oestrus synchronisation with progesterone–impregnated controlled internal drug releasers (CIDRs; Eazi-breed CIDR type G, Carter Holt Harvey Plastic Products, Hamilton, New Zealand). Ten Suffolk rams were introduced at CIDR removal and mating marks produced by the harnessed rams were recorded daily. Pregnancy status (dry versus pregnant) was determined by ultrasound 38 days after mating (and again at day 55 to confirm previous results). Nine single-bearing ewes pregnant to the first cycle, were assigned at random to each of the five slaughter dates.

Ewes were grazed on pasture as one mob under normal commercial conditions. Ewes were weighed on electronic scales (Tru-Test Distributors, Auckland) at CIDR removal and again 24 hours prior to slaughter.

### 2.3.2 Tissue collection

Ewes were euthanased by stunning with a captive bolt pistol and exsanguination. Euthanasia was conducted between 0900 h and 1400 h at each slaughter date, and the data on each ewe collected within 30 minutes of euthanasia. Immediately following exsanguination, the uterus was removed from the ewe and ligated at the utero-cervical junction and the vagina, cervix, ovaries

and excess tissue removed before the gravid uterus was weighed. The gravid uterus was carefully opened to remove the fetal fluids and expose the fetus. The umbilical cord was ligated near the point of attachment to the fetal abdomen and approximately four centimetres (cm) distal to that point, and the umbilical cord severed between the two ligatures. Fetuses still alive at this stage (n=9) were euthanased via intracardiac injection of sodium pentobarbitone (Pentobarb 500, Chemstock Animal Health, Christchurch, New Zealand). The fetus was then removed from the uterus and gently squeezed to remove amniotic fluid from the wool. The mammary glands of both sexes were then dissected, separated into left and right glands, weighed (except at days 40 and 60) and placed in Bouin's fixative (within 15 minutes of euthanasia). Fetal weight, curved crown-rump length (with the fetus lying in a 'relaxed' position) and girth measurements were recorded, as well as sex.

### 2.3.3 Histology Samples

At slaughter days 40 and 60 the mammary glands were too small to remove accurately so the whole fetus and the hindquarters (below the umbilical) respectively, were preserved. Mammary gland tissue from all fetuses was fixed in Bouin's fixative for up to 20 hours (due to its fibrous nature). After this time, excess fixative was washed out in two changes of 70% ethanol. The tissue was then stored in 70% ethanol before processing into paraffin wax. Sections six to seven microns thick were cut from the left gland of each animal, running through and parallel to the long axis of the teat in the anterior-posterior plane. Once the ducts became visible, every tenth section was observed under the microscope until the "complete duct system" (which included the bud at day 40 and a teat canal with ductal branching at days 60, 80, 100, 120 and 140) was located. If a complete system was not achievable (due to a bent teat or cracking of the tissue) the right gland was sectioned. Eight sections from each animal were mounted on individual PVA-treated (10% PVA glue) slides. Four to ten sections either side of the "complete duct system" were stored in dust-free boxes for future use.

The mounted sections were then oven dried overnight at 57°C to promote adherence to the slide. Four of the six slides were stained with haematoxylin and eosin (H&E), which stains the nuclei dark blue and the connective tissue pink. Sections, once stained, were mounted using DPX mountant (Product 360294H, Lot 84028841, BDH Laboratory Supplies, Poole, England), and stored at room temperature until analysis of duct and secretory cell area, and duct number. These sections were also used to compile a comprehensive pictorial overview of the normal histomorphogenesis of the developing ovine mammary glands. The remaining four sections were stored for immunohistochemical identification of androgen and oestrogen receptors (Chapter 3) and, epidermal growth factor and insulin-like growth factor-I receptors (Chapter 4).

### 2.3.4 Histomorphological observations

A histomorphological investigation of the developing ovine mammary glands was made from 45 rudiments collected from fetuses of both sexes at days 40, 60, 80, 100, 120 and 140 of

gestational age. Mammary glands from two, three-week-old lambs (1 male and 1 female) were also observed. Photomicrographs were used to identify the morphological and histological features of the mammary glands, as well as differences between males and females in the growth and development of epithelial and mesenchymal tissue. The photomicrographs were obtained using a digital camera (SPOT RT, Diagnostic Instruments inc., Sterling Heights, MI, U.S.A.) and a differential interference contrast (DIC) microscope (Axiophot, Zeiss, West Germany). Images were processed with the RT camera and Image-Pro PLUS, version 4.1 for windows (Media Cybernetics, Maryland, U.S.A.).

### 2.3.5 Morphological measurements

Total duct area ($\mu m^2$) for each gland was determined by viewing the H&E-stained section under a microscope and tracing the outline of individual ducts and their lumens (if present) using a digitizer pad attached to a computer and the Sigma Scan Scientific Measurement Programme (Version 3.90, Jandel Scientific, 1988). The area to be measured was calibrated using a micrometer slide under the 10x objective. Calibration was identical for all tissue measured. Total secretory cell area ($\mu m^2$) was calculated by subtracting the lumen areas from their respective duct areas. Individual duct and lumen areas for each gland were entered on a Microsoft excel spreadsheet, from which the total area and duct number (lateral branches from secondary ducts) were calculated. Total fat pad area was not measured due to the limited size of the paraffin-embedding moulds.

The development of the mammary gland was described in terms of relative growth as expressed by simple allometry $y = bx^{\alpha}$ (Huxley and Teissier, 1936). The variables associated with body weight and mammary gland growth were log transformed (natural $\log_e$). Linear regression was then used to calculate the allometric coefficient ($\alpha$), which related the growth rate of the mammary gland to that of the body as a whole. The independent variable ($x$) in the analyses was body weight$^{0.67}$ (to approximate surface area) while total duct area ($y$) served as an index of mammary gland growth. When $\alpha$=1, growth is said to be isometric. If $\alpha$>1, growth is said to be positively allometric (simple allometry) and if $\alpha$<1, then growth is negatively allometric (Huxley and Teissier, 1936).

### 2.3.6 Statistical analyses

Data were analysed using a general linear model procedure for analysis of variance to determine effects of stage of gestation, sex and their interactions. Data were log transformed (natural log) and adjusted to a common fetal body weight. Results are expressed as least square means and standard errors for male and female fetuses at each slaughter date. Statistical analyses were conducted using the computer package SAS for Windows (version 6.12, 1989-1996).

## 2.4 Results

### 2.4.1 Histomorphological observations

**Day 40 of fetal age**

The earliest recognisable stage of mammary gland development in the fetal sheep is the mammary band, which consists of a single layer of cuboidal cells that forms from the ectoderm and differentiates from the underlying mesenchyme tissue. This raised area of ectoderm, which appears as a condensation of epithelial cells, runs along both sides of the midline, posteriorly in the inguinal region and is referred to as the mammary or "milk lines" (Turner 1952). The location of the mammary lines corresponds with the position of the future normal and accessory glands and teats (Anderson, 1978). Two localised mammary buds, comprised of epithelial cells, arise separately along the mammary line and are prominent between days 36 and 46 of fetal age (CRL: 2.5 to 5.5 cm) (Turner, 1952; Martinet, 1962).

In the present study, the formation of the mammary buds of 40-day-old (CRL: 4.8 cm) male (Plate 2.1) and female (Plate 2.2) fetuses was completed and each was ovoid or egg-shaped. There was no indication of sexual dimorphism in the shape of the bud, the same ovoid form being characteristic of most of the buds at this stage of development. The buds of both sexes were completely invaginated into the underlying mammary mesenchyme and the overlying epidermis was pulled partially inward, forming an epidermal stalk with the bud at its proximal end (Plates 2.1 and 2.2).

As this stage of development of the mammary bud in the male and female were quite similar, the following histomorphological descriptions were based on a "generalised" bud. The bud was composed of a dense population of polyhedral epithelial cells, which were similar in appearance to the cells of the stratum germinativum layer of the developing epidermis. Irregular layers of larger, cylindrical-shaped cells were arranged in rows with the long axis of the cells pointing toward the centre of the bud. These cells rested on a very distinctive basement membrane, which separated the epithelial bud from the underlying mesenchyme. The cells in the central zone of the bud were not as closely packed together and their nuclei were frequently positioned near horizontally (Plates 2.1 and 2.2).

The cells of the mammary mesenchyme immediately beneath the basement membrane of the epithelial bud were closely packed together in several concentric layers oriented around the mammary bud and displayed deeply staining nuclei. Mitotic nuclei were readily observed in these cells and in the cells of the basal layers of the epidermis. Mitotic figures were also evident in the cells of the mammary bud (Plate 2.1). Further away from the bud, in the presumptive dermis, the mesenchymal cells were more loosely arranged and retained a more random orientation. A very fine fibrous network, from which collagen fibres would later develop, filled the mesenchymal background. Blood vessels were seen occupying the mesenchyme underlying the bud (Plates 2.1 and 2.2).

**Plate 2.1** Photomicrograph of the mammary bud of a male sheep at day 40 of fetal age. (Mag x400).

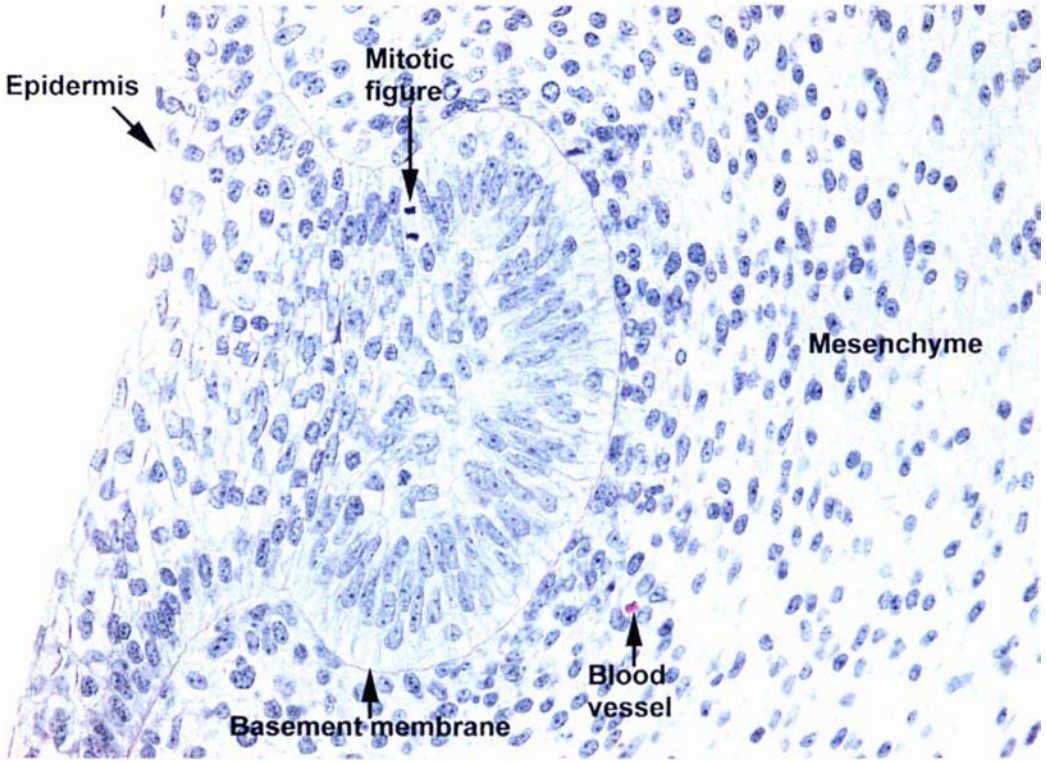
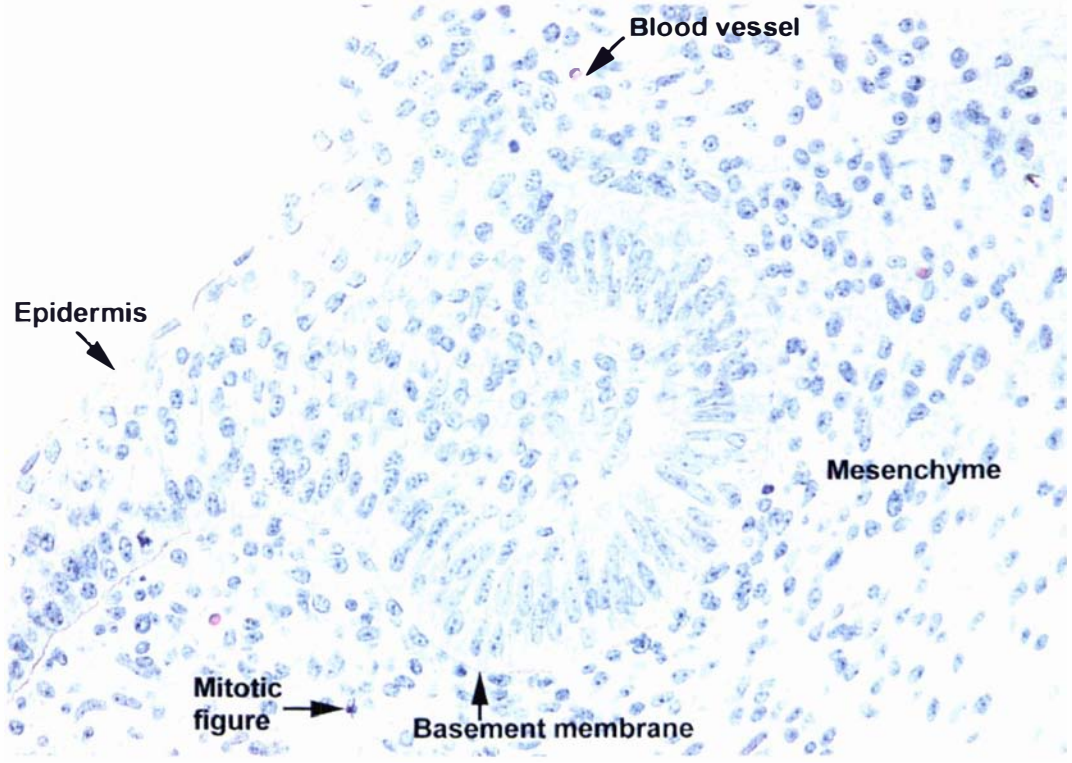



**Plate 2.2** Photomicrograph of the mammary bud of a female sheep at day 40 of fetal age. (Mag x400).



**Day 60 of fetal age**

A solid outgrowth of epithelial cells from the proximal end of the mammary bud had elongated and advanced through the underlying mesenchymal cells on a general course that was perpendicular to the epidermis. This structure was the forerunner of the primary duct, of which there was one developing from each bud, resulting in one opening per teat. In both sexes, this structure was composed of solid, homologous masses of polyhedral epithelial cells, which were arranged in irregular rows, and were similar in appearance to the basal stratum germinative cells of the epidermis. The superficial cells were becoming more flattened, appearing horizontal to the surface, as the structure elongated. At the same time the developing tissue had pushed outwards from the body to form a teat, its apex structurally similar in both sexes (Plates 2.3 and 2.4).

Mitotic figures were seen throughout the developing cell mass, but were more numerous at the proximal end. At a CRL of about 15.0 cm, this structure in the female had reached its full extension as a solid core of cells and was characterised by its club-shaped proximal end (Plate 2.4). Development of the primary duct in the male appeared to be somewhat slower compared to that of the female duct. While a small lumen could be seen forming at the proximal end of the solid core of cells in the male (Plate 2.3), canalisation had resulted in the formation of a lumen that extended almost the full length of the cell mass in the female (Plate 2.4). Upon canalisation, this solid mass of cells could now be termed a duct. Secondary structures, comprised of solid cores of cells, had begun to grow from the basal germinal layer of cells of the primary duct in all six of the female glands observed (Plate 2.4) while secondary initiation was evident in two of the three male glands (Plate 2.5).

The primary duct remained surrounded by a slightly denser mesenchyme, which will subsequently differentiate into fibroblastic connective tissue. At day 60 of fetal life, this developing connective tissue was largely undifferentiated and homogeneous in nature, consisting of mesenchymal cells, which were arranged in a swirling pattern around the growing ends of the ducts (Plates 2.4 and 2.5). The more fibrous nature of the mesenchymal background compared to that at day 40, was due to the early deposition of collagen. A network of fine blood vessels had formed in close proximity to the growing ducts (Plate 2.5).

The more deeply placed hypodermis remained largely undifferentiated, with many loosely arranged mesenchymal cells, containing round, dark-staining nuclei. Small bundles comprising Schwann cells with long, flattened nuclei (early innervation) and capillaries were appearing in an apparently random distribution (Plate 2.6).

**Plate 2.3** Photomicrograph of the partially canalised primary mammary duct of a male sheep at day 60 of fetal age. (Mag x100).

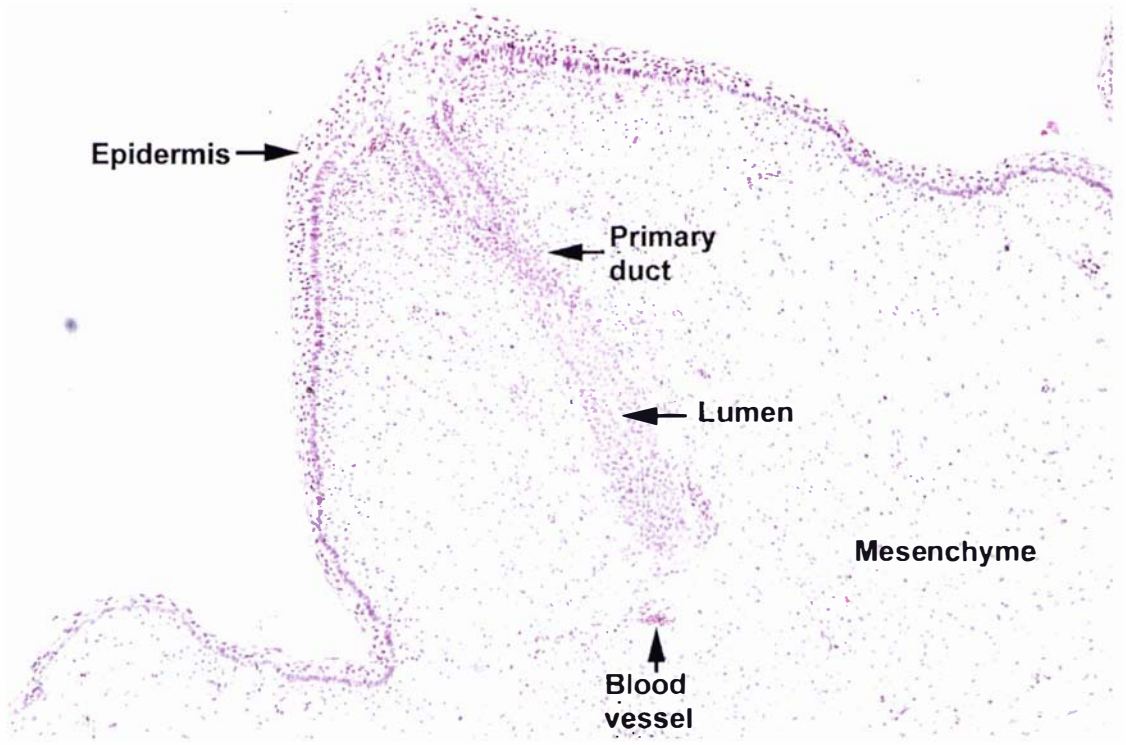



**Plate 2.4** Photomicrograph of the primary mammary duct of a female sheep at day 60 of fetal age. Canalisation of the solid mass of cells is almost complete. Several secondary structures, comprised of solid cores of cells, have formed. (Mag x100).

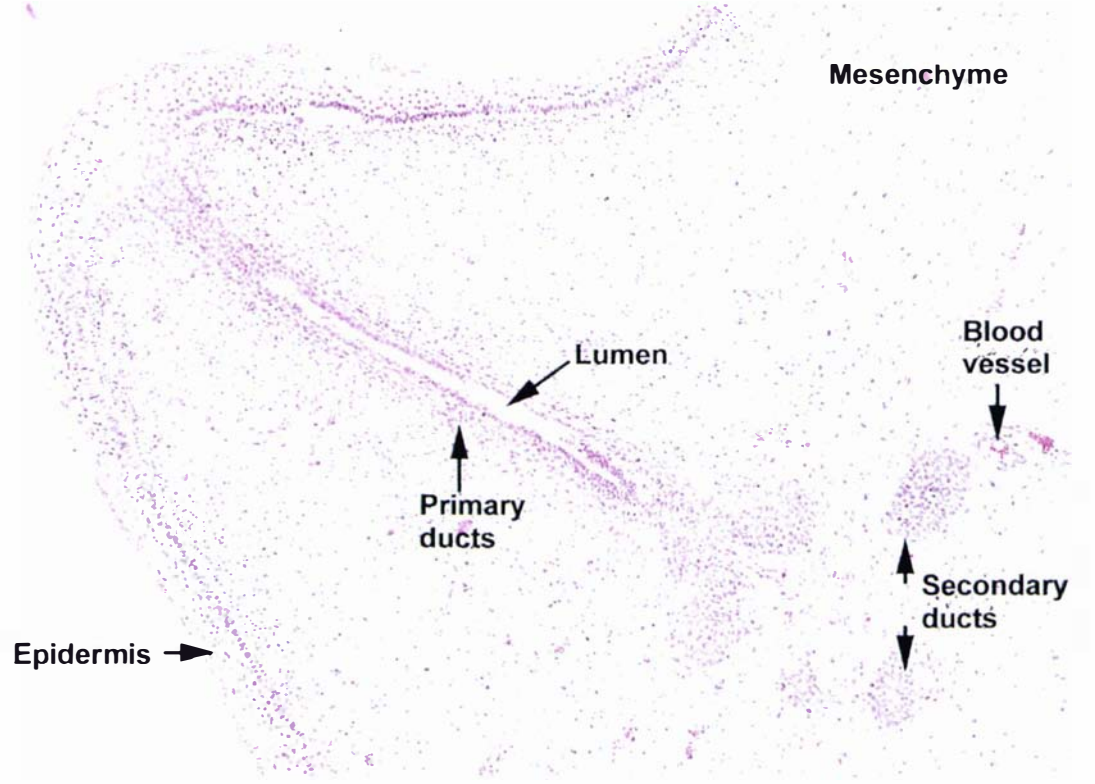


**Plate 2.5** Photomicrograph of the initiation of secondary structures from the proximal end of the primary mammary duct of a male sheep at day 60 of fetal age. The basement membrane surrounding the primary duct is well defined. (Mag x400).

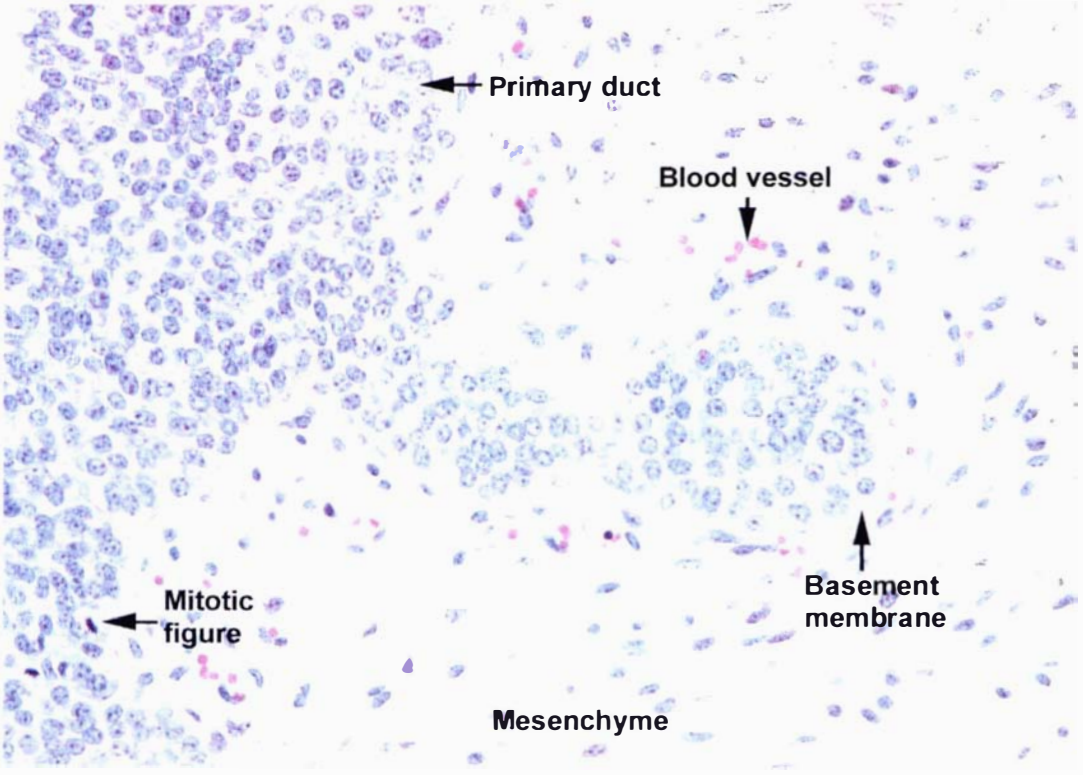
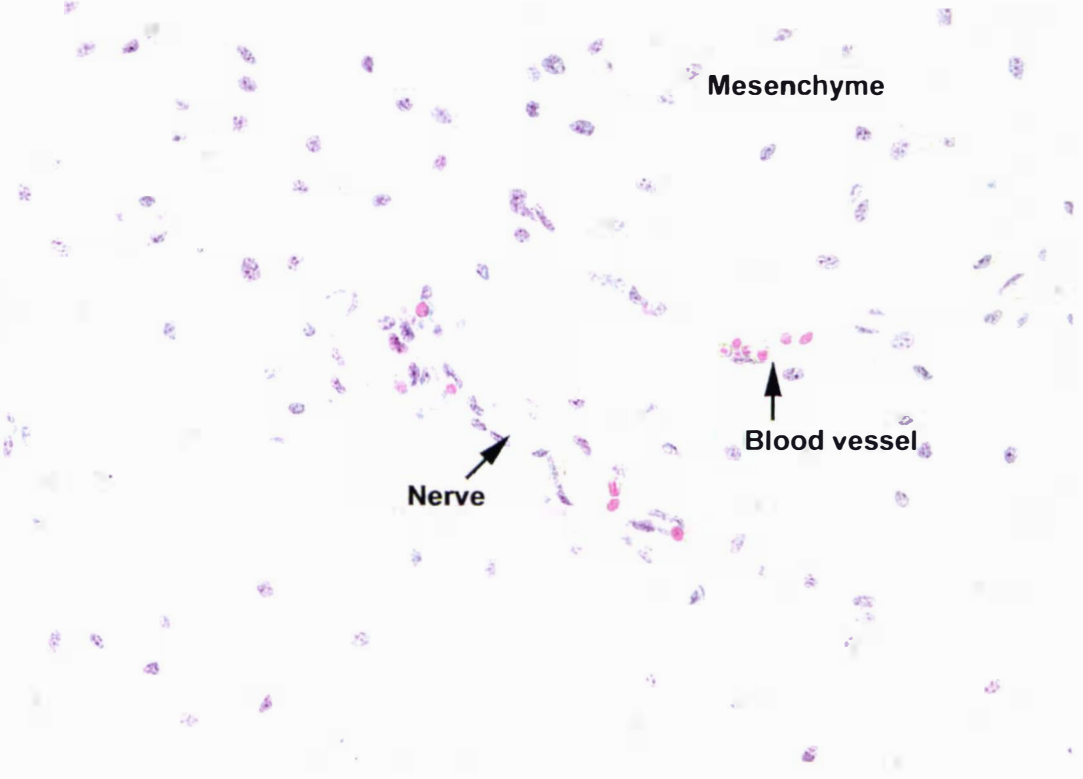



**Plate 2.6** Photomicrograph of the early development of the mammary fat pad (adipose tissue) of the female sheep at day 60 of fetal age. The process of angiogenesis is underway, with the formation of nerves and blood vessels. (Mag x400).



**Day 80 of fetal age**

The gland cistern had formed due to the continual growth of the lumen near the proximal end of the primary duct. The progressive canalisation of the primary duct towards the distal end had resulted in the formation of the teat cistern. The gland and teat cisterns were a structural feature in both males and females at day 80 of fetal age (Plates 2.7 and 2.8).

Numerous secondary ducts were present, appearing as irregular shaped hollow sacks; their lining composed of at least three layers of stratified cuboidal epithelial cells (Plate 2.9). These cells were proliferating quickly as evidenced by the presence of numerous mitotic figures on the growing ends of the ducts. Instead of continuing to grow towards the developing fad pad like that of the primary duct, these secondaries were growing at an angle. This change in direction was observed in both sexes, but to a lesser extent in males (Plates 2.7 and 2.8). The irregularly arranged connective tissue surrounding the ducts had become more fibrous in nature and was highly vascularised, with the appearance of large blood vessels.

In the male, skeletal muscle (i.e., twitch muscle (panniculus carnosa)) was developing in the hypodermis and comprised a mixture of myogenic and myoblastic cells. However, there was very little mesenchymal tissue present between the secondary ducts and the developing skeletal muscle, greatly restricting further epithelial development (Plate 2.8).

In the female, the fat pad was differentiating from a separate condensation of mesenchymal tissue that appeared distal to the mammary epithelium. The developing adipose tissue appeared as small, condensed bundles, consisting of a mixture of adipogenic cells, adipoblasts and immature adipocytes. Large, empty cytoplasms were a feature of the adipocytes while a granulated cytoplasm and an elongated nucleus were present in each of the adipoblasts (Plate 2.10). In intimate association with the developing adipose tissue were small blood vessels (capillaries) and nerves. Immediately surrounding each bundle of adipose tissue were swirls of fibrous connective tissue, the flattened, comet-shaped nuclei of collagen easily recognisable (Plate 2.10). Nerves and small blood vessels formed in close association with each bundle of developing adipose tissue. The less abundant mesenchymal tissue underlying the epithelial outgrowth in the male was beginning to differentiate into adipose tissue, appearing as small bundles of mainly adipogenic cells, with some adipoblasts. The nuclei were round as opposed to elongated, and the number of cells within each bundle was greatly reduced compared to that of the developing adipose tissue of the female. The formation of blood vessels and nerves were lagging behind that in the female (Plate 2.11). Lymph nodes were also developing in the loosely arranged connective tissue of the deep hypodermis. They appeared as a semicircle of densely packed cells with blue coloured nuclei, and little cytoplasm. At this stage of development, the periphery of the nodes was not very sharply defined (Plate 2.12).

**Plate 2.7** Photomicrograph of the formation of the gland and teat cisterns, and secondary ducts in the mammary gland of a female sheep at day 80 of fetal age. (Mag x25).

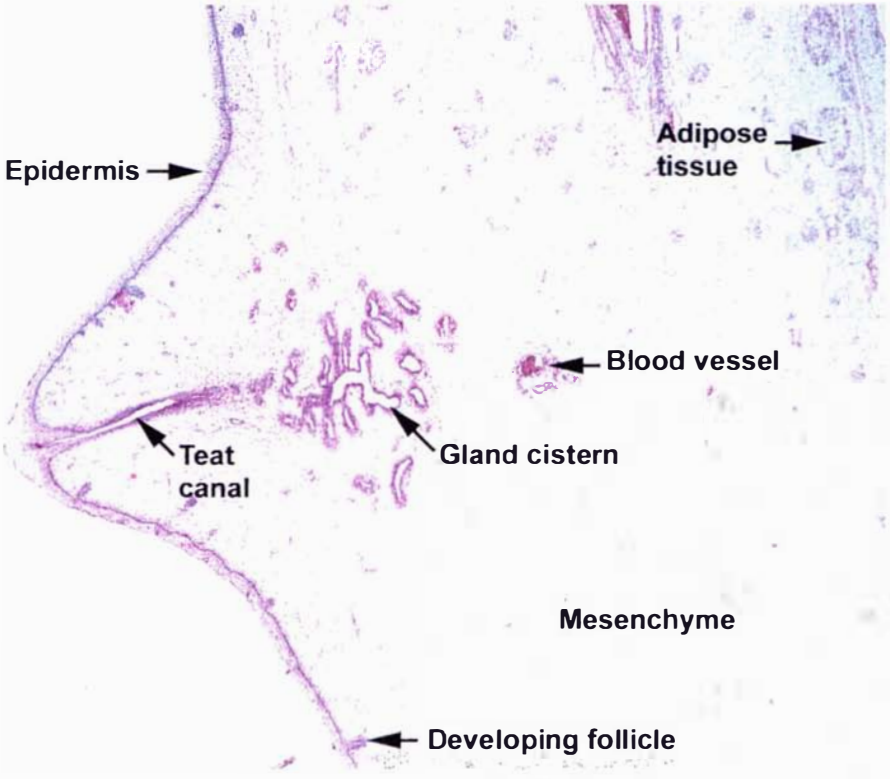



**Plate 2.8** Photomicrograph of the formation of the gland and teat cisterns, and secondary ducts in the mammary gland of a male sheep at day 80 of fetal age. (Mag x25).

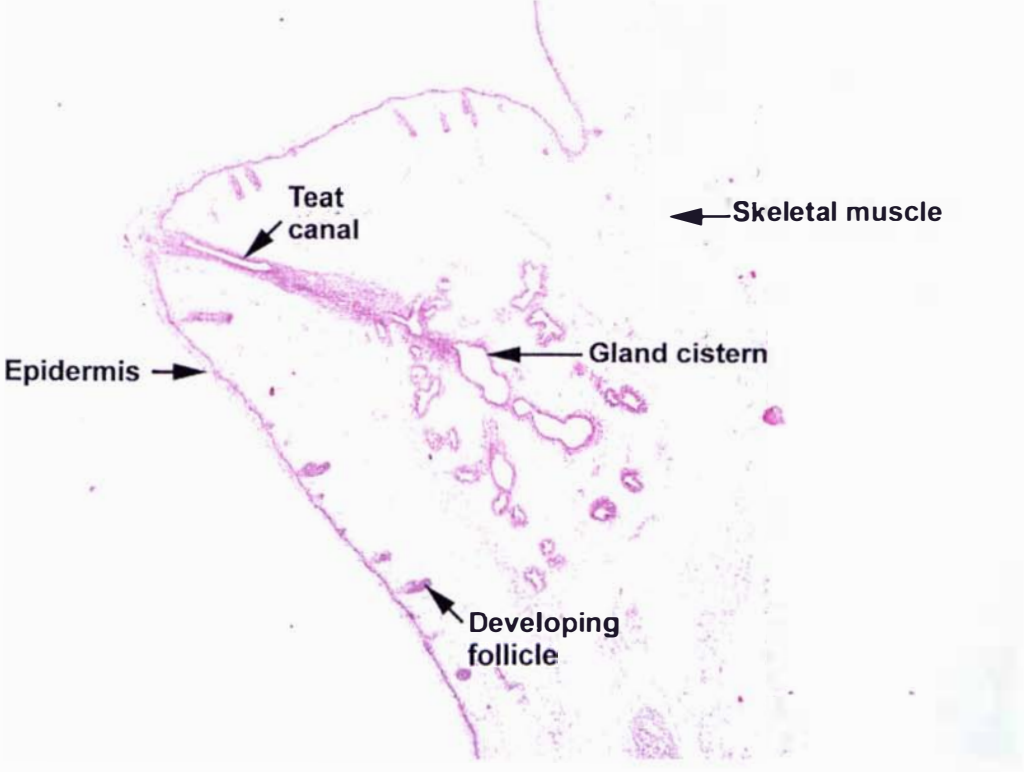


**Plate 2.9** Photomicrograph of the secondary ducts in the mammary gland of a female sheep at day 80 of fetal age. (Mag x400).

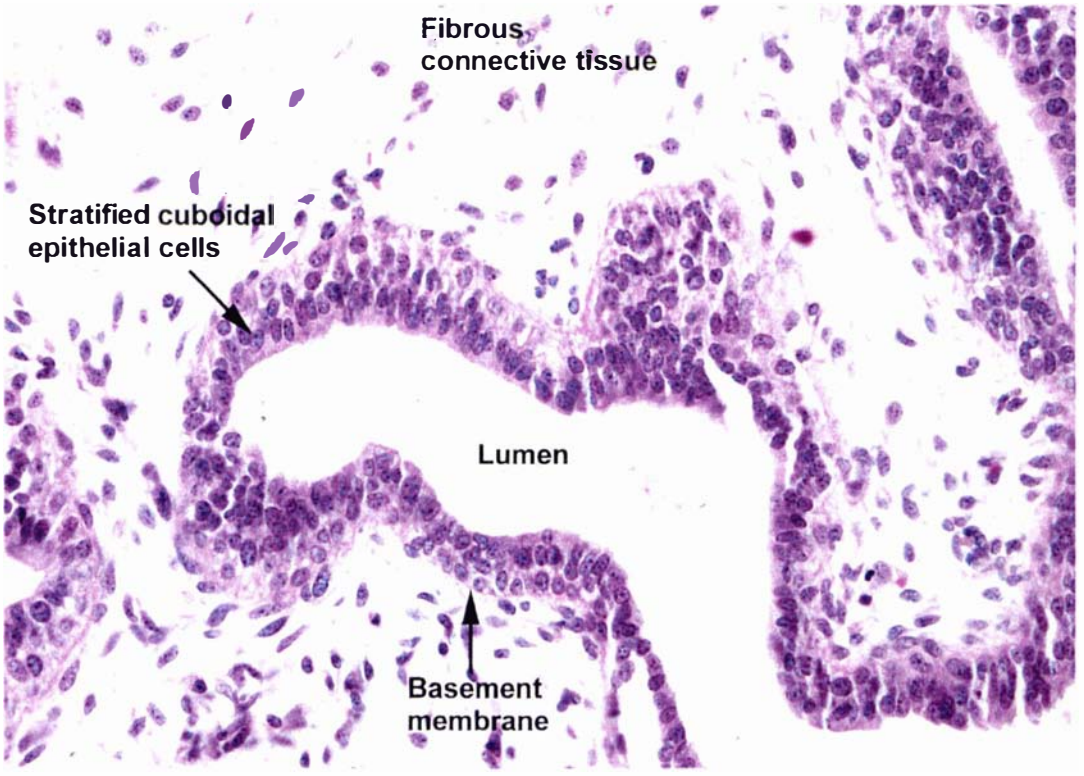



**Plate 2.10** Photomicrograph of the development of adipose tissue in the mammary gland of a female sheep at day 80 of fetal age. (Mag x400).

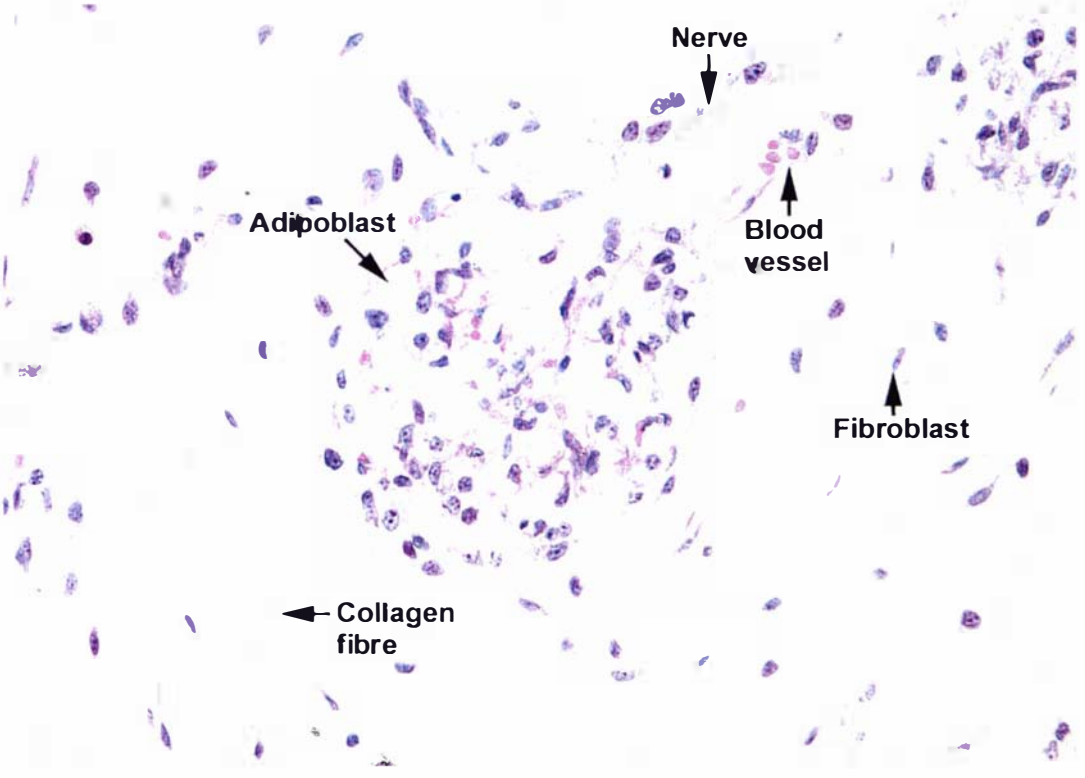


**Plate 2.11** Photomicrograph of the development of adipose tissue in the mammary gland of a male sheep at day 80 of fetal age. (Mag x400).

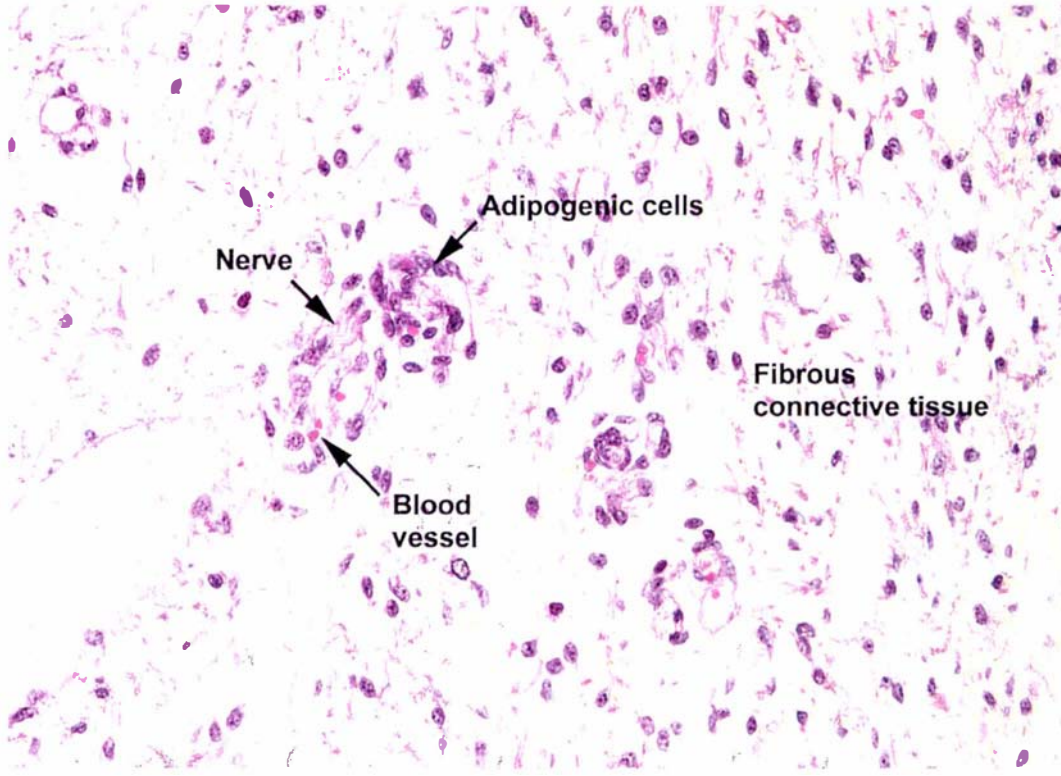
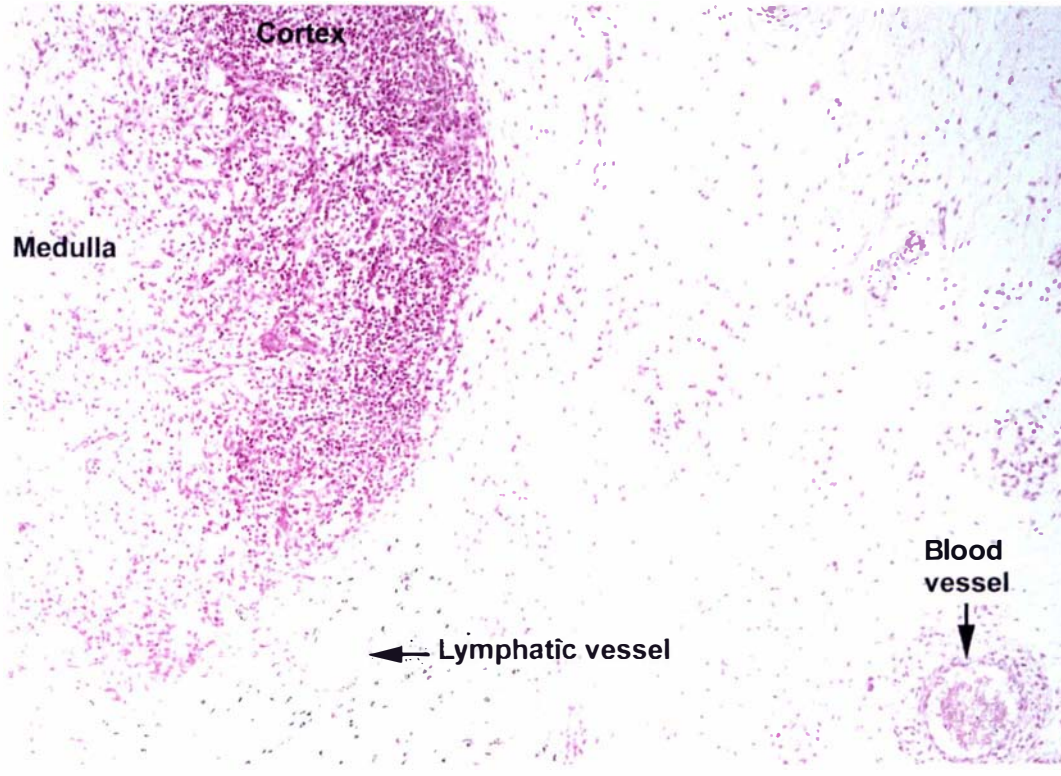


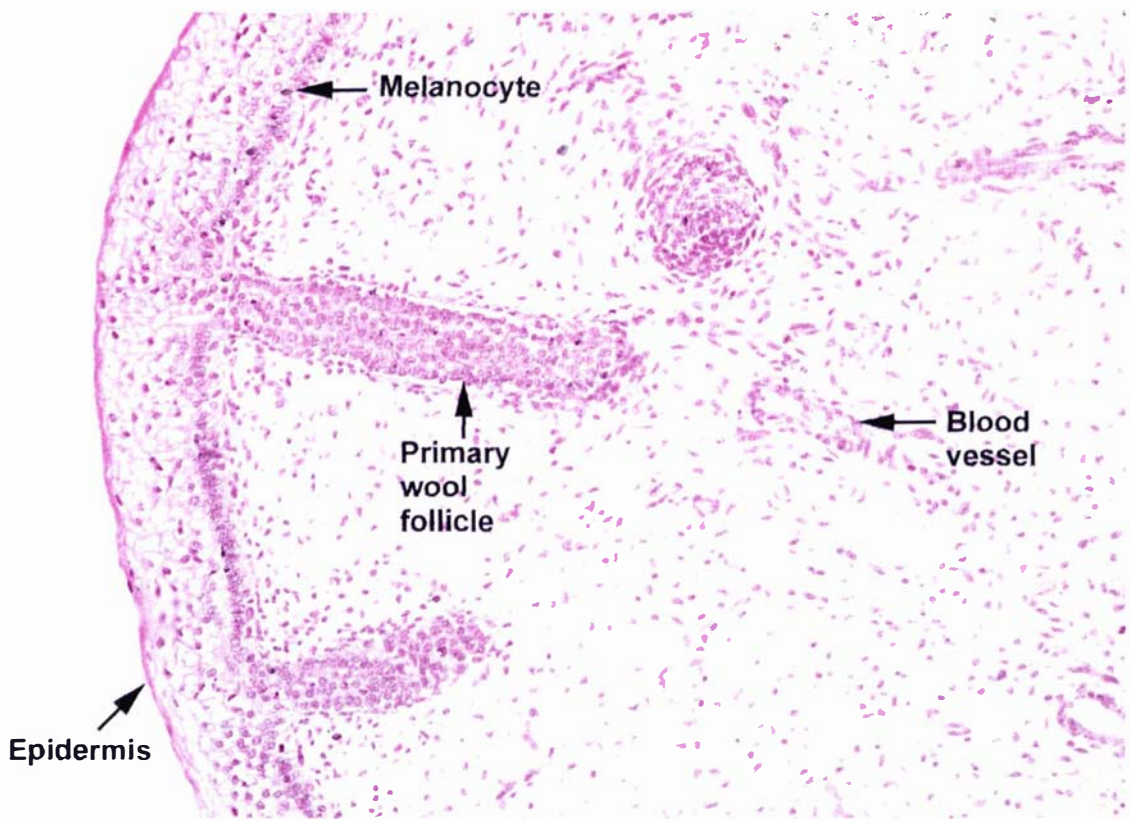

**Plate 2.12** Photomicrograph of the early development of a lymph node in the mammary gland of a female sheep at day 80 of fetal age. (Mag x100).



The epidermis comprised several layers of cells; an outer slightly pinkish layer (pre keratinising cells) underlaid by flattened cells, then enlarged cells then a basal layer of small rounded cells (Plate 2.13). In several glands, the lumen of the primary duct had opened to the exterior.

The first stage of wool follicle development was observed as a down growth of epidermal cells into the dermis, individual plugs of cells extending right up to the end of the teat (Plate 2.13). The sebaceous glands associated with the follicles were just starting to bud. Mitotic figures were present (metaphase and telophase) in the wool follicles, denoting the fast replication of cells growing down into the dermis. Melanocytes were observed in the hair follicle layer as well as in the deep layers of the epidermis (Plate 2.13).

**Plate 2.13** Photomicrograph of the formation of primary wool follicles. The down growth of a plug of epidermal cells from the teat surface into the dermis in a female sheep at day 80 of fetal age. (Mag x200).



**Day 100 of fetal age**

Numerous secondary ducts, of varying shapes and sizes, had sprouted from the growing end of the primary duct. In the female they continued to grow at various angles to the direction of the primary duct, and into the surrounding mesenchyme, whilst in the male, most of the secondaries grew in the same direction as the primary duct, towards the fat pad (Plates 2.14 and 2.15).

As they enlarged, the secondary ducts canalised and developed two layers of stratified cuboidal epithelial cells, although a solid core of cells still persisted at the growing ends (Plate 2.16). The nuclei remained randomly orientated. A new type of basal epithelial cell appeared at random, next to the basement membrane of the secondary ducts, their nuclei orientated at ninety degrees to the epithelial cells. These were assumed to be myoepithelial cells, based on their position within the ducts, and they were present in both males and females.

In relation to the total sectional area of the developing udder, the epithelial tissue was limited to a small area of secondary ducts arising from the proximal end of the primary duct. A well-defined region of mammary mesenchyme, recognised histologically as fibroblastic connective tissue, surrounded the ducts. This connective tissue was more fibrous than that observed at day 80 of fetal age with coarse collagenous fibres developing in close proximity to the primary duct. Fibroblasts and early collagen fibres aligned themselves along the deeper margins of the basement membrane of the secondary ducts while a more loosely arranged swirling connective tissue, rich in vascular channels, remained in intimate association with the entire ductal system (Plate 2.17).

The adipose tissue was far more developed and structured than that observed at day 80 of fetal age, although each bundle still comprised a mixture of adipogenic cells, adipoblasts and adipocytes. In the female, immature adipocyte cells with round nuclei were grouped together in distinct lobules, but individual lobules were not located in close proximity to one another. A single droplet of lipid resided in each cell. Each oval-shaped bundle of developing adipose tissue was enclosed by swirling fibrous connective tissue and remained deep in the hypodermis (Plate 2.18). Similar development was seen in the male, although the number of cells per unit area was reduced and the nuclei were not as numerous as those observed in the female (Plate 2.19). The lymph nodes were developing primary nodules.

**Plate 2.14** Photomicrograph of the developing duct system in the mammary gland of a female sheep at day 100 of fetal age. (Mag x25).

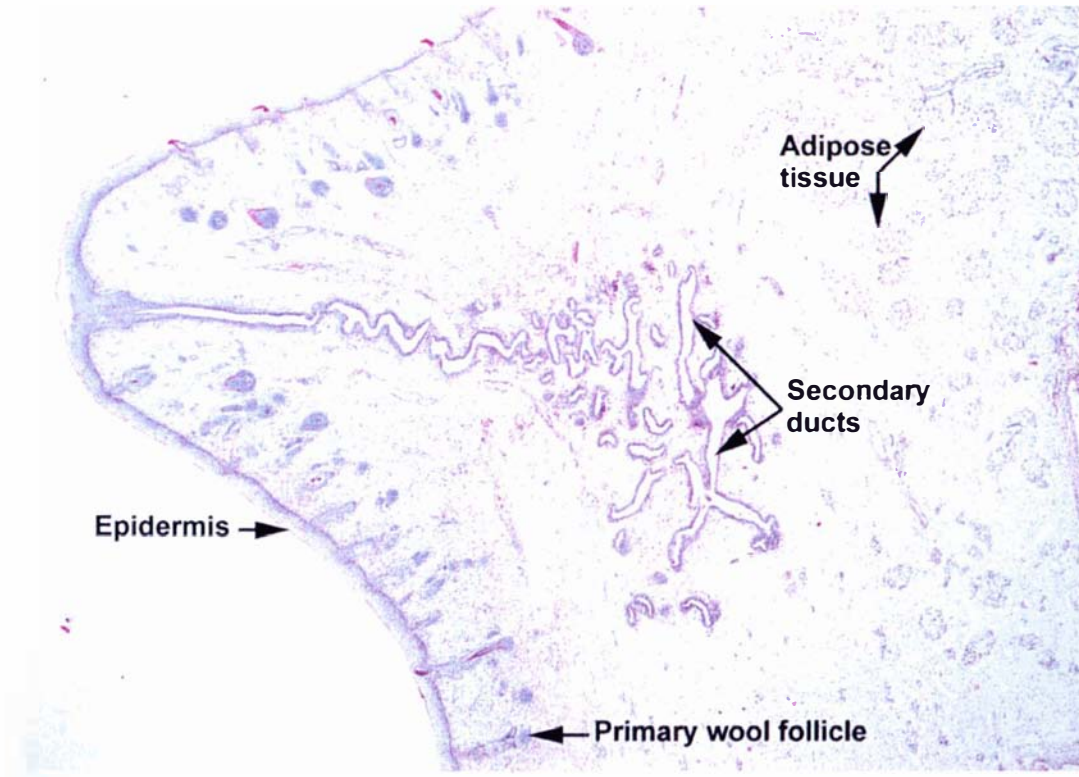



**Plate 2.15** Photomicrograph of the developing duct system of the mammary gland of a male sheep at day 100 of fetal age. (Mag x25).

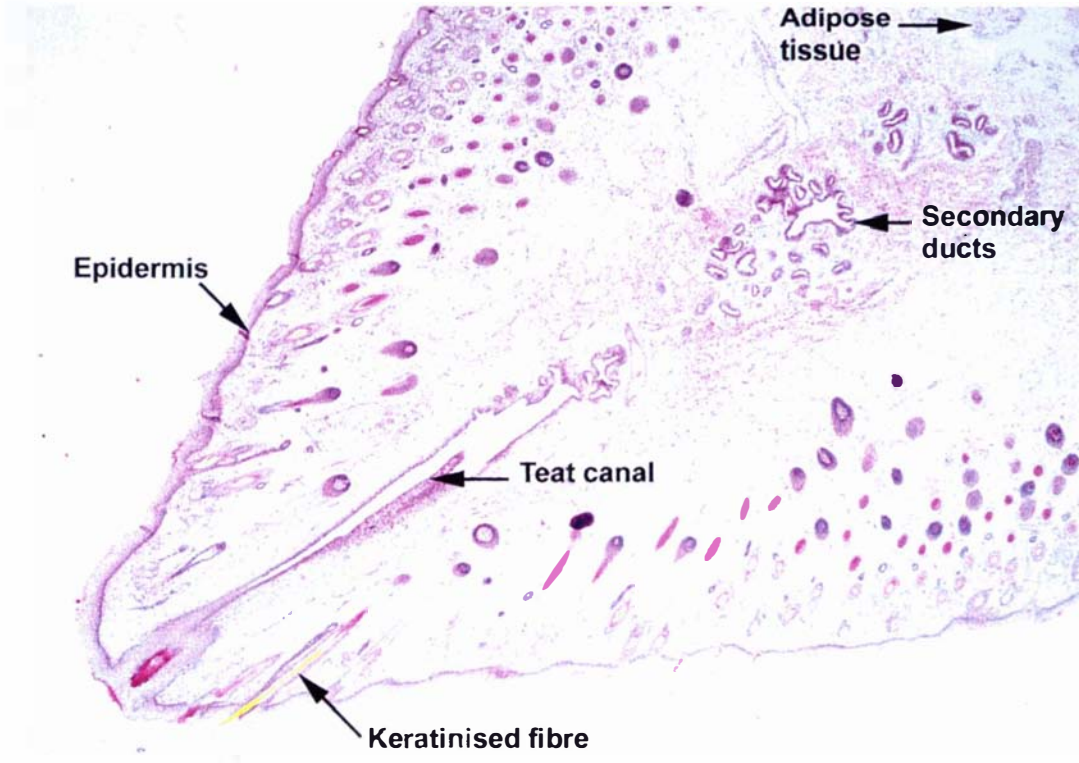


**Plate 2.16** Photomicrograph of the secondary mammary ducts of a male sheep at day 100 of fetal age. (Mag x400). Note the appearance of myoepithelial cells.

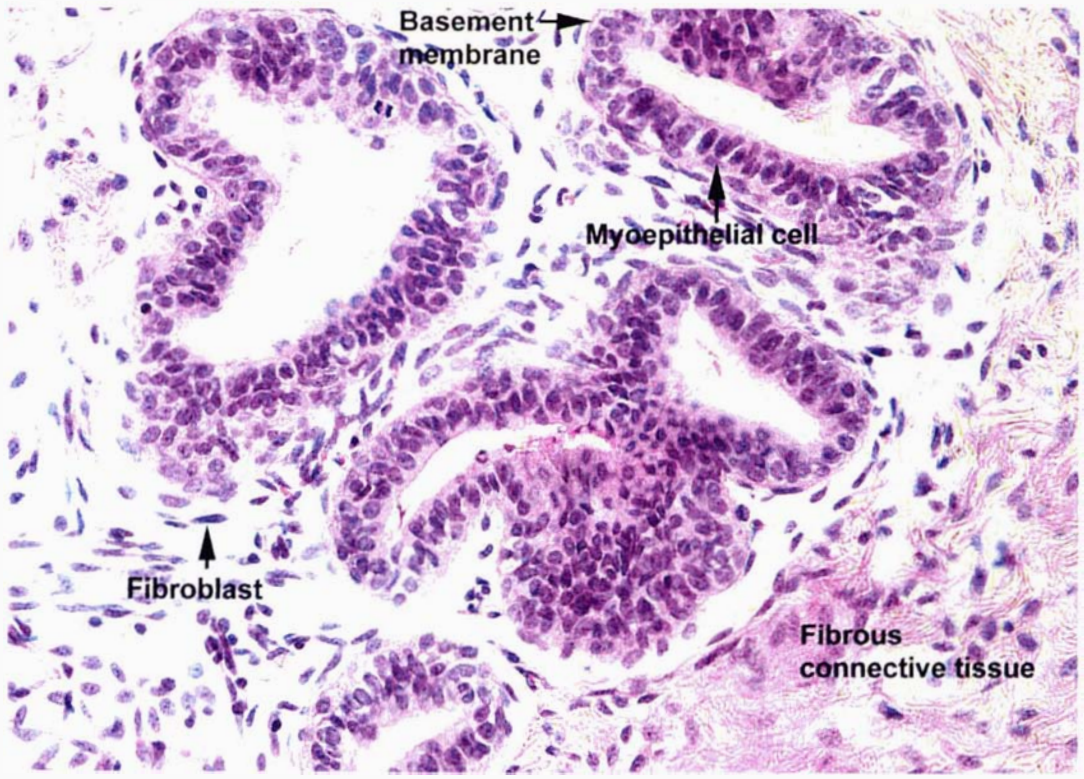



**Plate 2.17** Photomicrograph of the fibrous connective tissue surrounding the secondary mammary ducts of a female sheep at day 100 of fetal age. (Mag x400).

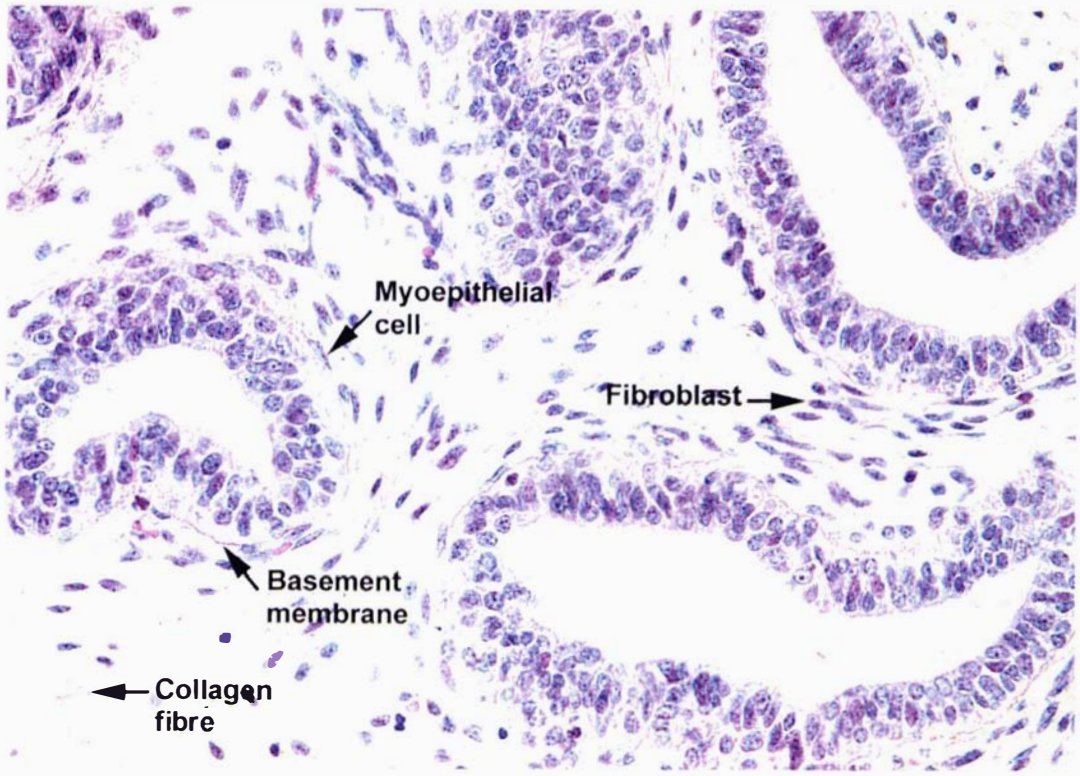


**Plate 2.18** Photomicrograph of the developing adipose tissue in the mammary gland of a female sheep at day 100 of fetal age. A lobule of adipose tissue contains mainly immature adipocytes (Mag x400).

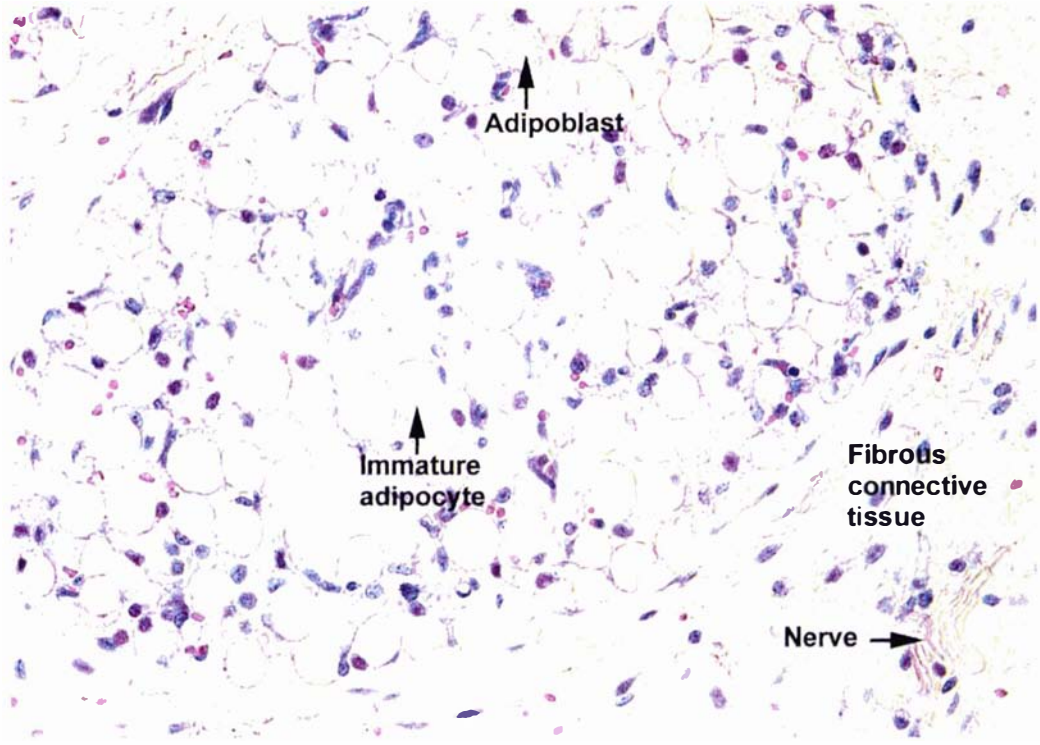
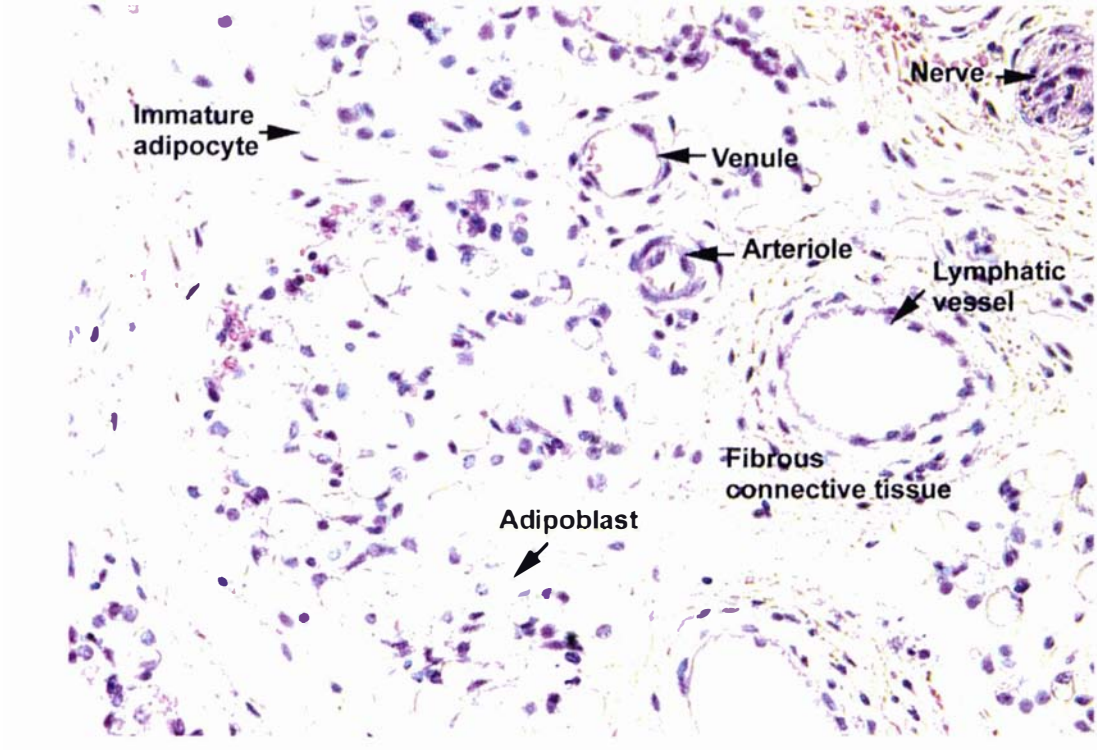



**Plate 2.19** Photomicrograph of the developing adipose tissue in the mammary gland of a male sheep at day 100 of fetal age. Each lobule contains a mixture of mainly adipogenic cells, adipoblasts and a few adipocytes. Note the triads comprising a venule, arteriole and lymphatic vessel. (Mag x400).



In comparison with the 80-day fetus, the epidermis was thicker and the outer layer was pinker in colour, indicating early keratinisation. The base of the wool follicle was invaginated to form a dome-like structure known as the papilla of the follicle bulb (Plate 2.20). Sebaceous glands and sweat glands were associated with each primary follicle and the sweat glands were partially canalised. In addition, arrector pili muscles were present in the dermis. At this stage of development there was a hair canal, which ran from the neck of the follicle to the upper part of the epidermis and the follicles were growing a keratinised fibre. Several fibres had ruptured the outer layer of the epidermis and emerged just above the surface of the skin (Plate 2.21). Dense capillary networks surrounded the primary follicles. The initiation of triads of primary follicles was complete (Plate 2.22) and the first secondary follicles were forming in close association with the triad groups.

**Plate 2.20** Photomicrograph of a developing primary wool follicle in the teat area of a female sheep at day 100 of fetal age. The dome-like structure is the papilla of the follicle bulb. (Mag x400).

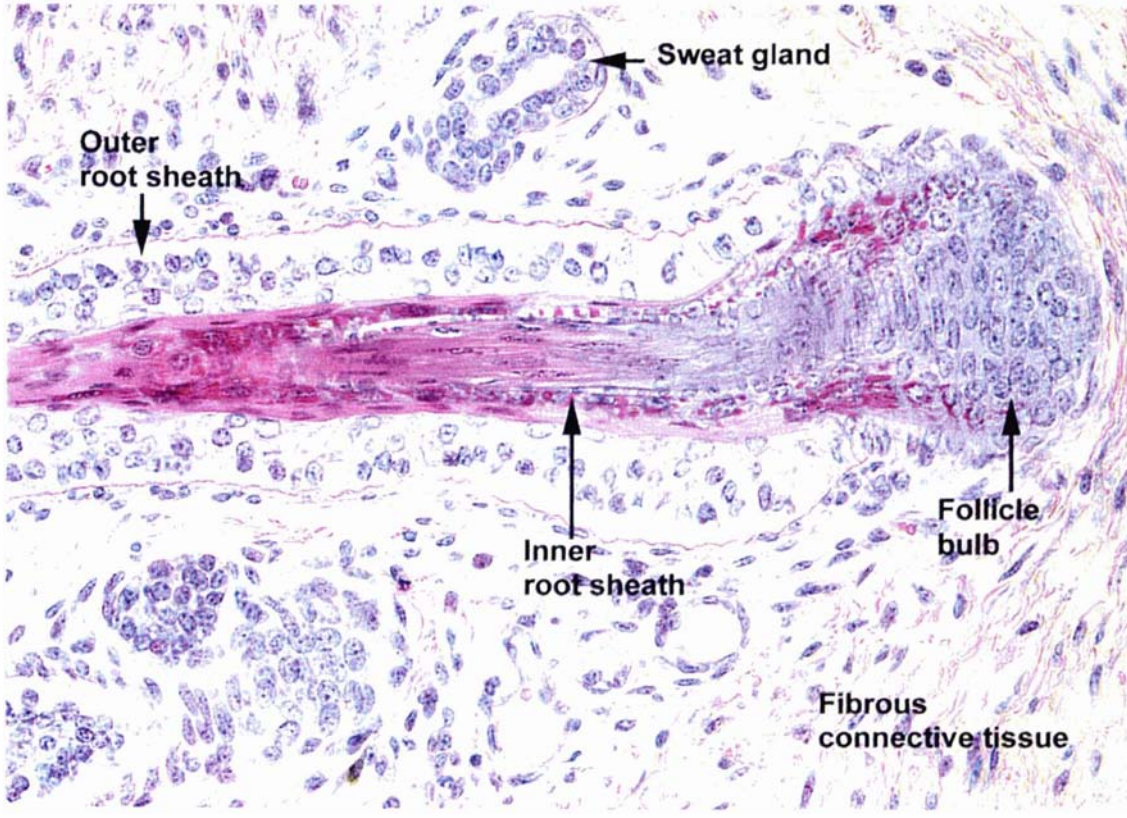


**Plate 2.21** Photomicrograph of the development of a primary wool follicle in a male sheep at day 100 of fetal age. The fibre has emerged above the surface of the skin of the teat. (Mag x400).

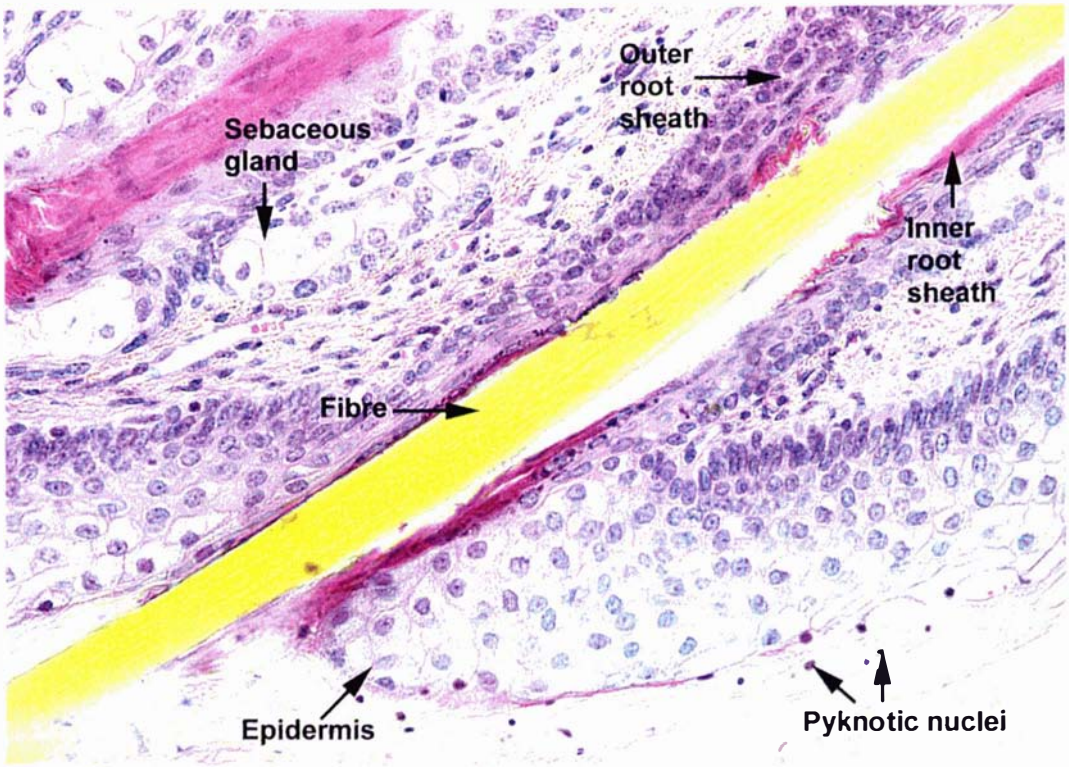
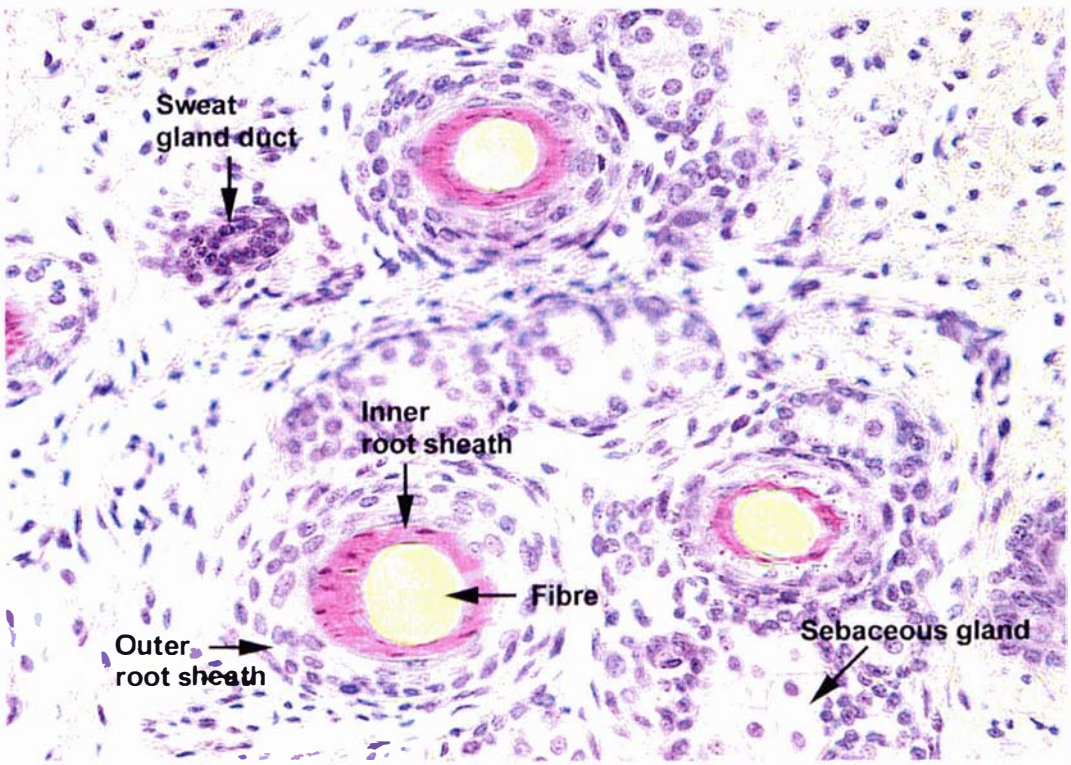



**Plate 2.22** Photomicrograph of the development of a primary wool follicle in the teat area of a male sheep at day 100 of fetal age. The initiation of triads of primary follicles is complete. (Mag x400 ).



**Day 120 of fetal age**

In the female, there was much variation in the size and shape of the secondary ducts. Large ducts were growing at varying angles towards the hypodermis and tertiary ducts were formed from lateral branching of the secondary ducts (Plate 2.23). The walls of the lateral branches comprised three to four irregular layers of polyhedral cells. Mitotic figures were present in the epithelial cells of the solid growing ends of the lateral branches indicating the rapid growth of these ducts. Two to three layers of epithelial cells lined the gland and teat cisterns. The superficial cells lining these regions were cuboidal to columnar. In contrast to the female, the lateral branches in the male appeared mainly terminal, with very few solid epithelial growing ends. Furthermore, there was less outward spread of the secondary ducts in the male, the developing epithelium continuing its growth towards the fat pad in a direction perpendicular to the tip of the teat (Plate 2.24). The secretory tissue in both sexes extended to the edge of the fat pad, where it was separated from the adipose tissue by multiple layers of dense fibrous connective tissue.

**Plate 2.23** Photomicrograph of the epithelial and mesenchymal compartments of the developing mammary gland in a female sheep at day 120 of fetal age. (Mag x25).

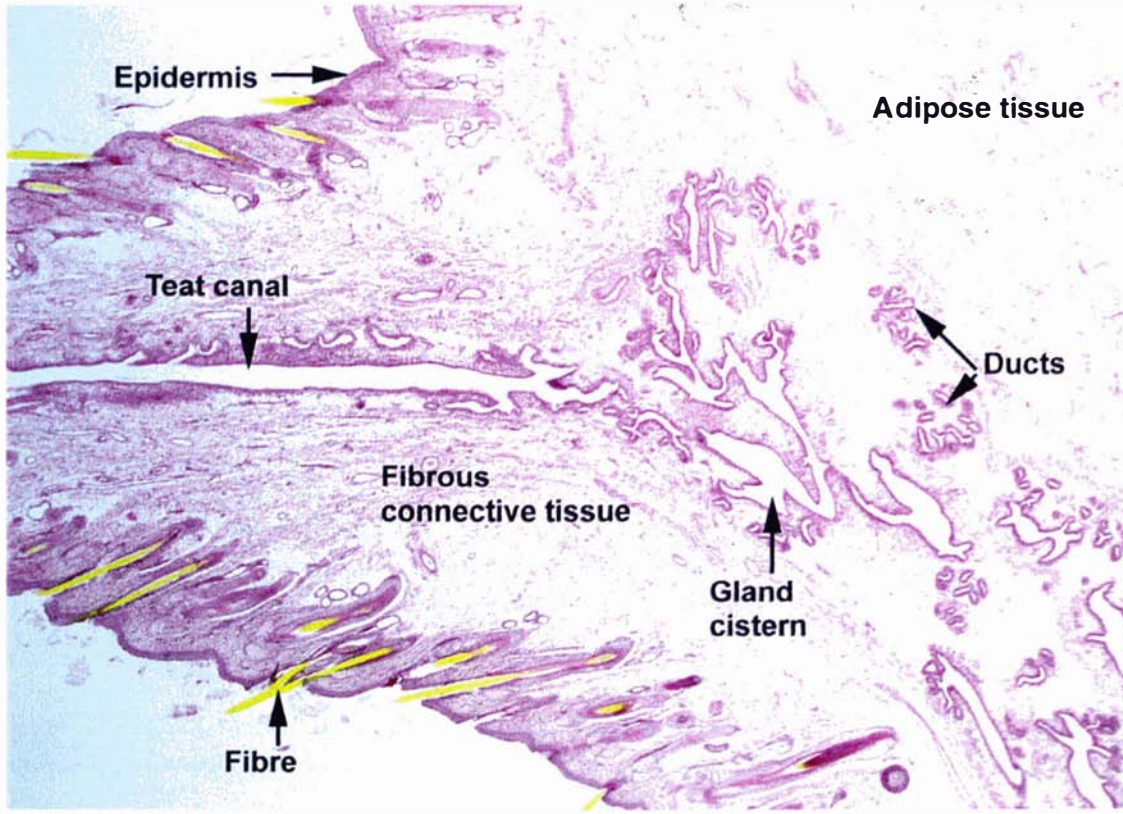


**Plate 2.24** Photomicrograph of epithelial and mesenchymal compartments of the developing mammary gland in a male sheep at day 120 of fetal age. (Mag x25).

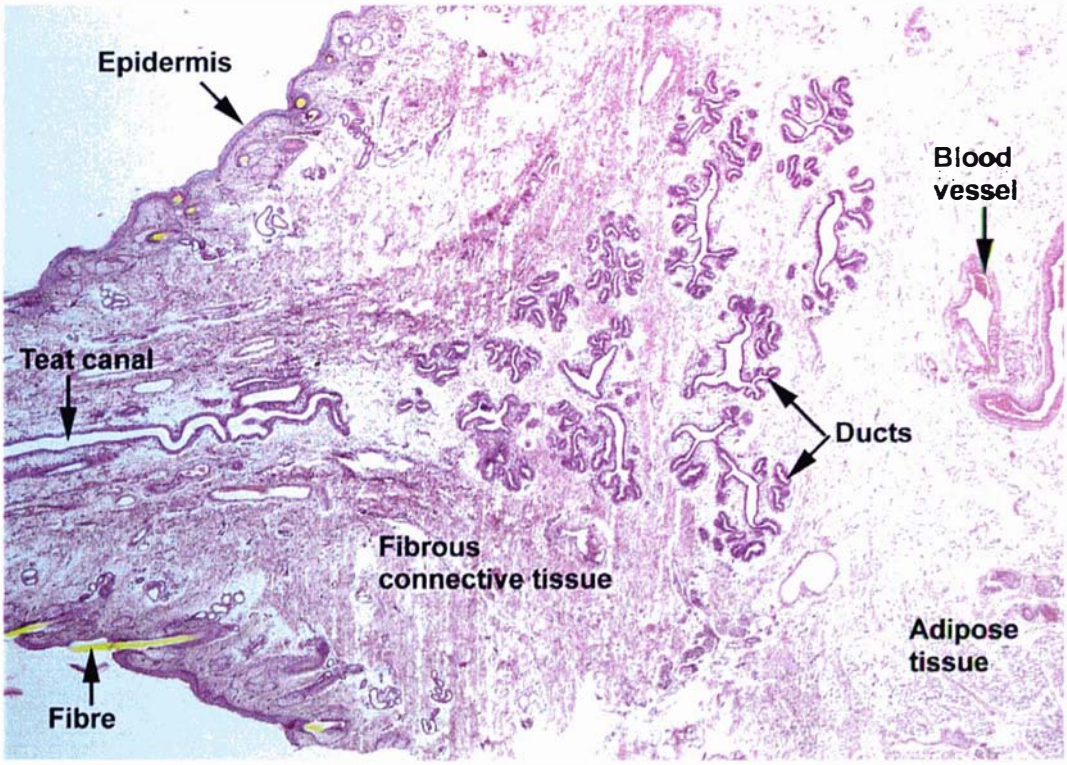
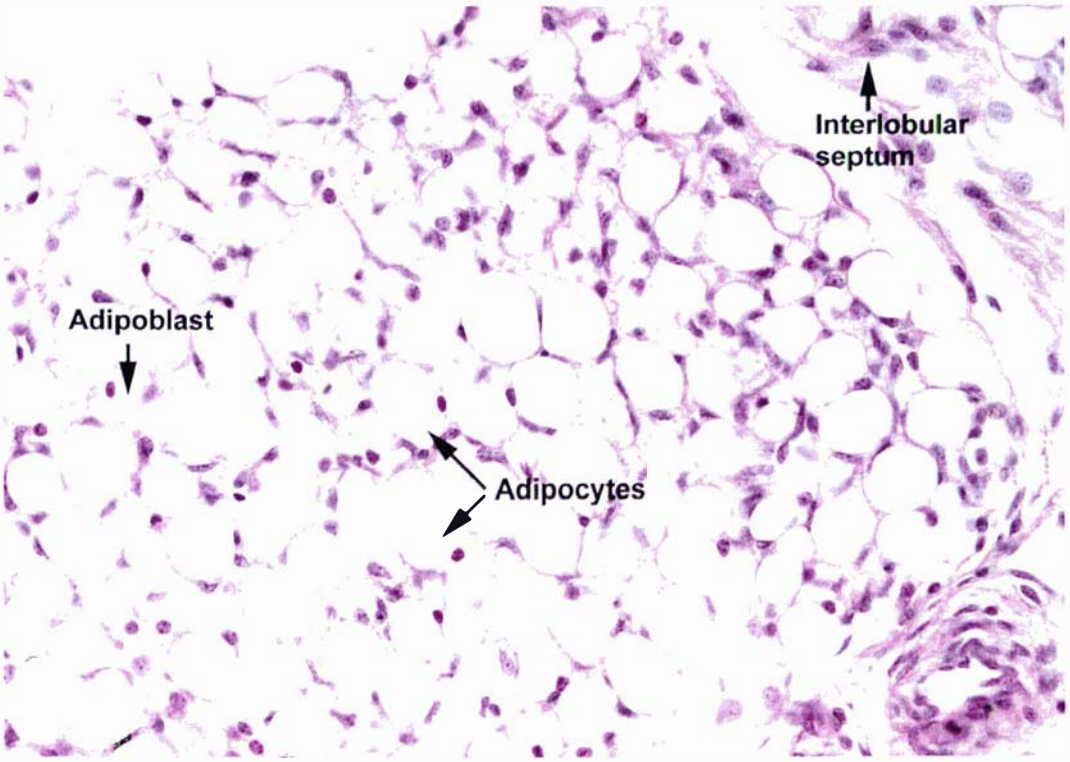


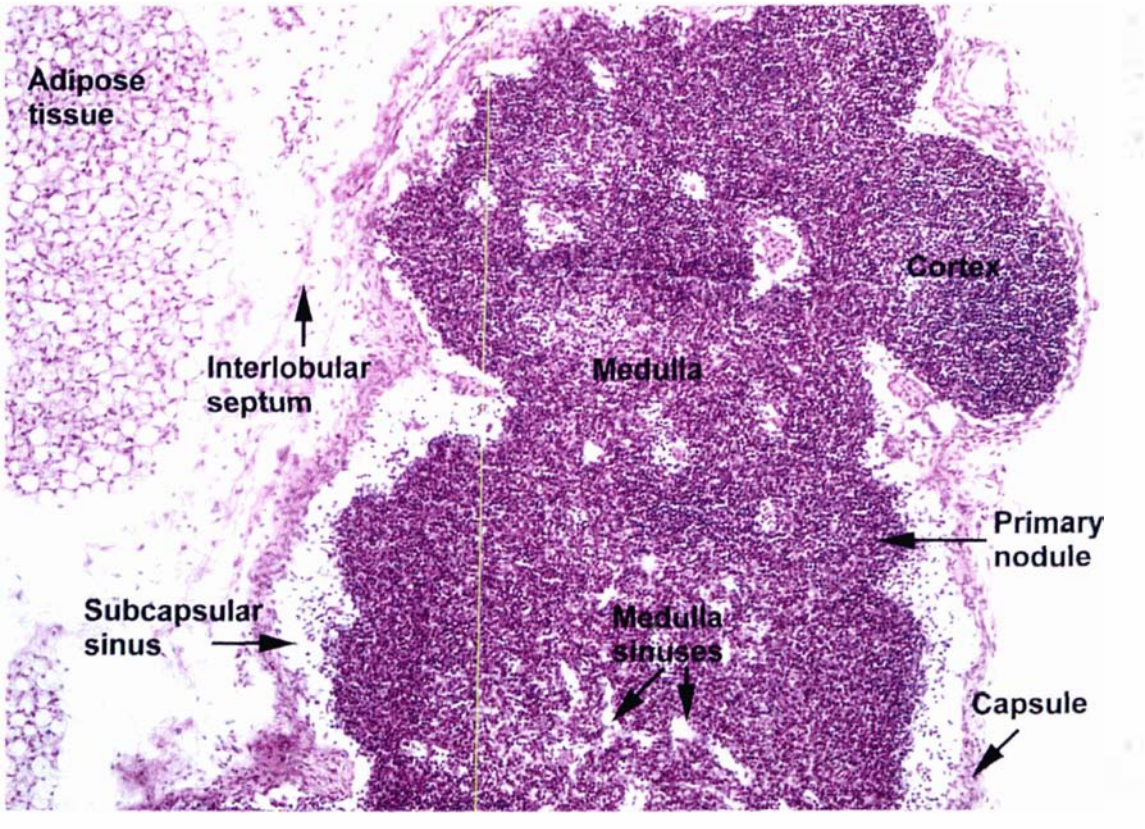

**Plate 2.25** Photomicrograph of the fat pad of a female mammary gland at day 120 of fetal age. Lobules of adipose tissue, comprising a mixture of adipoblasts and adipocytes, are separated from one another by fibrous connective tissue. (Mag x400).



A very dense, irregularly arranged fibrous connective tissue also surrounded the developing ducts, appearing to grow in every direction, giving a three-dimensional effect. The amount of collagen was increased from day 100 as evidenced by the deeper pink staining of interlacing strands of wavy fibres bordering the ducts.

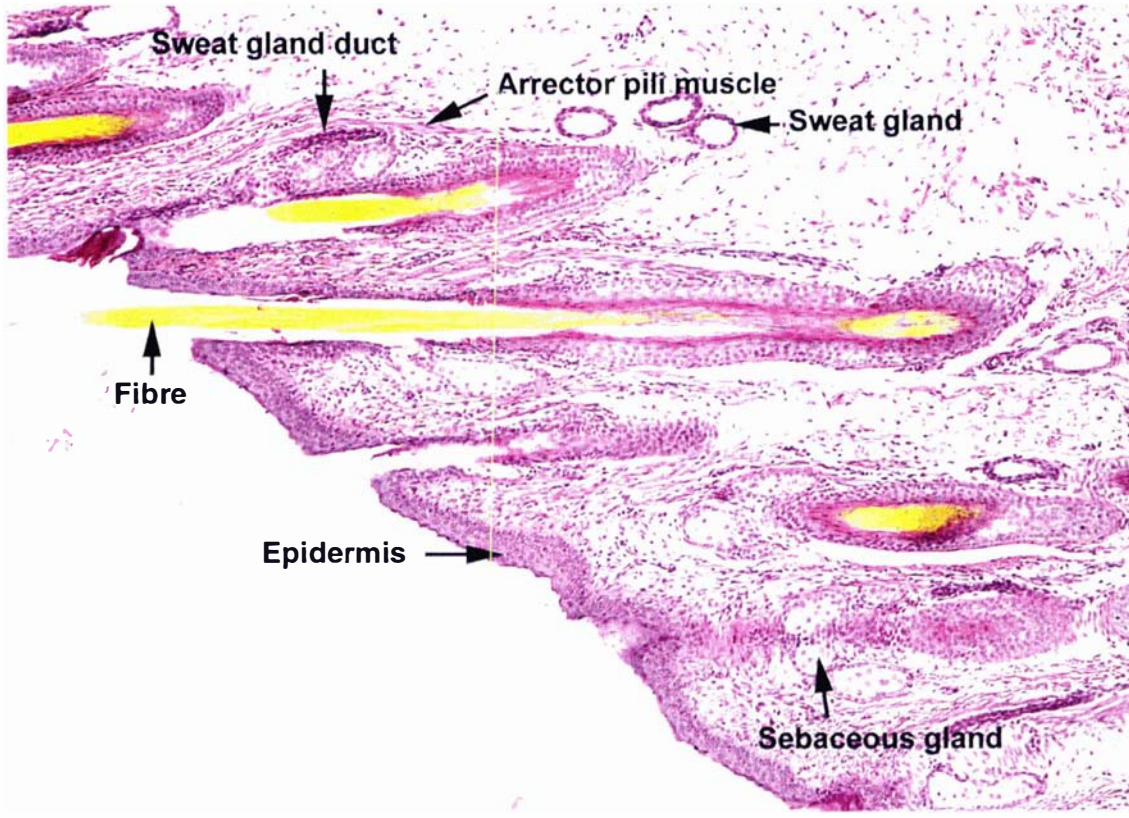
The fat cells (mainly adipocytes) of the adipose tissue were grouped into large lobules by partitions of interlobular connective tissue septa. Each adipocyte contained only one large droplet of fat while the less numerous adipoblasts each contained several. Because fat is dissolved during processing, the adipocytes in the fat pad appeared as empty spaces surrounded by thin layers of cytoplasm containing flattened nuclei (Plate 2.25). Although development was similar in both sexes, there was much less adipose tissue present in the male compared with that in the female. Large blood vessels were present in the developing gland. The margins of the lymph nodes were clearly defined and undulating in appearance due to the growth of primary nodules. A connective tissue capsule surrounded the entire node (Plate 2.26).

**Plate 2.26** Photomicrograph of a well-developed lymph node at day 120 of fetal age. (Mag x200).



The keratinised outer surface of the epidermis appeared undulated due to the eruption of keratinised fibres within the numerous primary wool follicles (Plates 2.23 and 2.27). Associated with each primary follicle were fully developed accessory glands, namely large and bilobed sebaceous glands, and sweat glands, which were coiling. Montgomery glands were present below the sweat glands.

**Plate 2.27** Photomicrograph of the eruption of keratinised fibres through the epidermis of the teat at day 120 of fetal age (female). (Mag x100).



**Day 140 of fetal age**

Sexual dimorphism in gland morphology was obvious at day 140 of fetal age (term = day 147). In the female, short quaternary branches formed as lateral branches from the tertiary ducts. The secondary and tertiary ducts were longer and the secondary ducts had large lumens. The entire gland was greatly increased in size due to the lengthening of the primary duct (teat and gland cisterns) as well as the continued expansion of the proximal branches (Plate 2.28). Similarly to day 120, the gland cistern and lateral branches of the developing ductal system in the 63-cm long female remained separated from adipocytes by fibrous connective tissue (Plate 2.29). Although there was great variation in the pattern of branching of the gland in both sexes, the size and shape of the female gland did not vary to the same extent as the male gland. Males often failed to produce extensive secondary ducts and lateral branching. Epithelial development was more or less restricted to the long axis of the teat area, due to the close proximity of the teats to the cranial surface of the scrotal sac (Plate 2.30). In both sexes, the epithelial ducts remained surrounded by a thick sheath of fibrous connective tissue.

**Plate 2.28** Photomicrograph of the development of epithelial and adipose tissue in the mammary gland of a female sheep at day 140 of fetal age. (Mag x25).

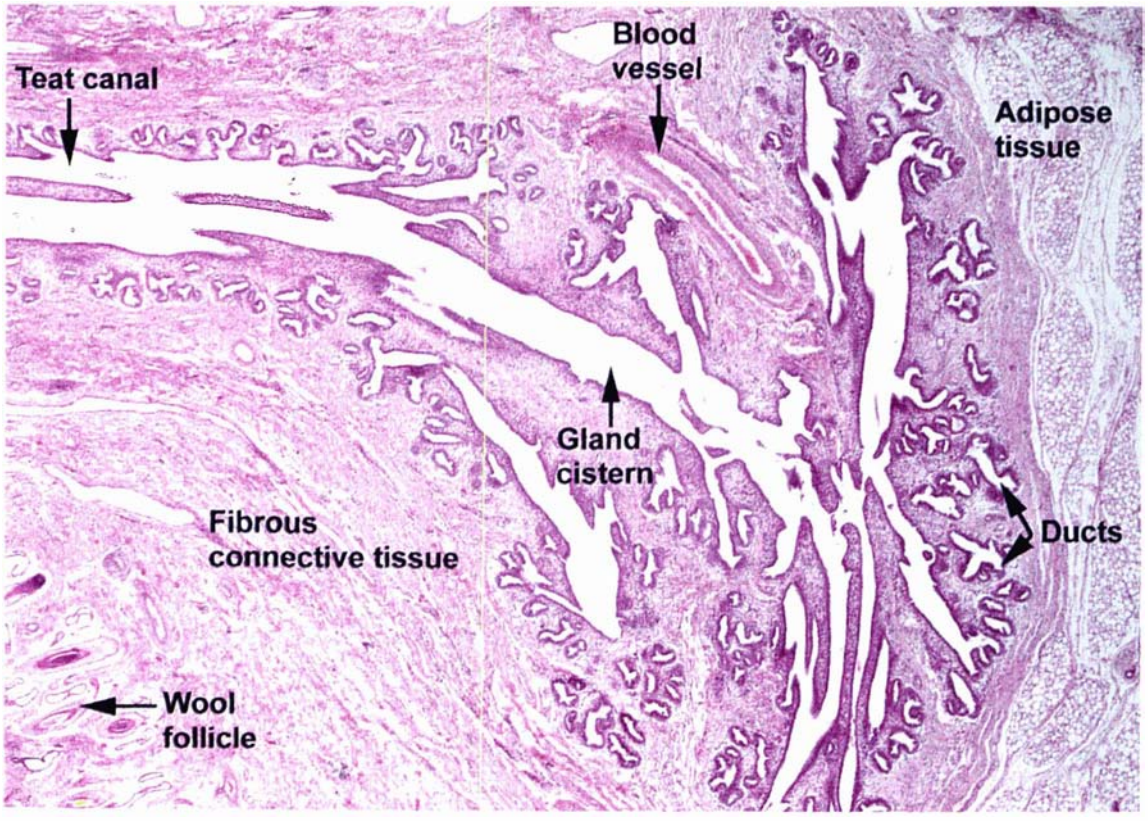


**Plate 2.29** Photomicrograph of the mammary gland of a female sheep at day 140 of fetal age. Dense fibrous connective tissue separates the epithelial and adipose tissue. (Mag x100).

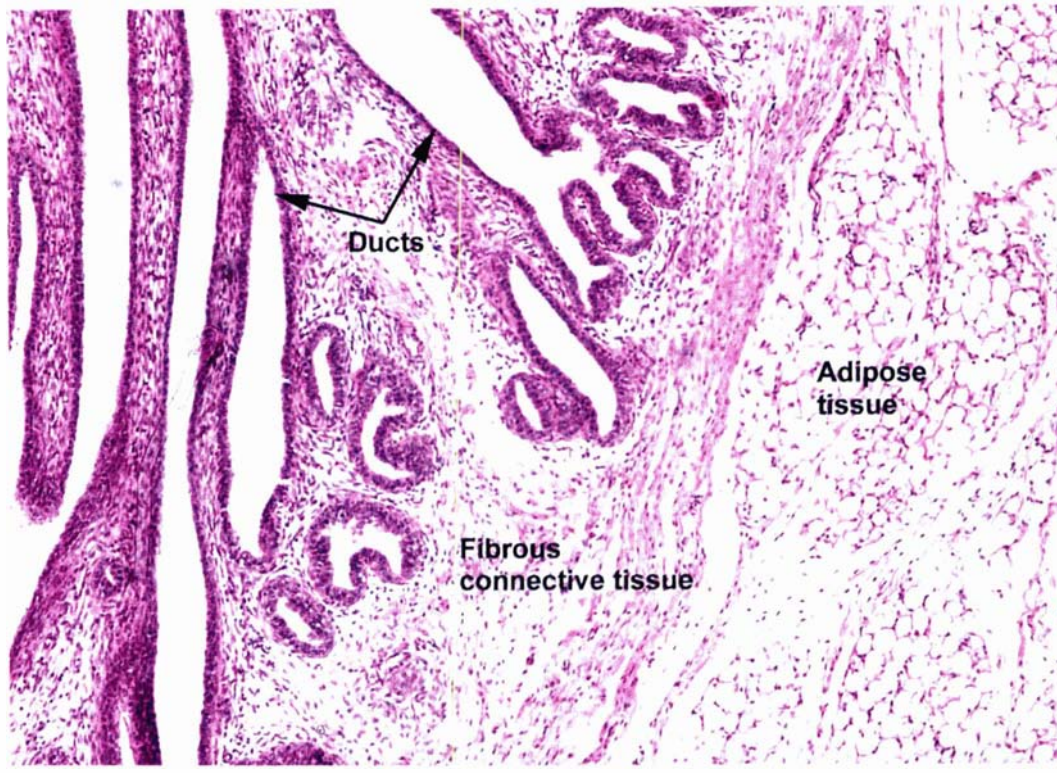
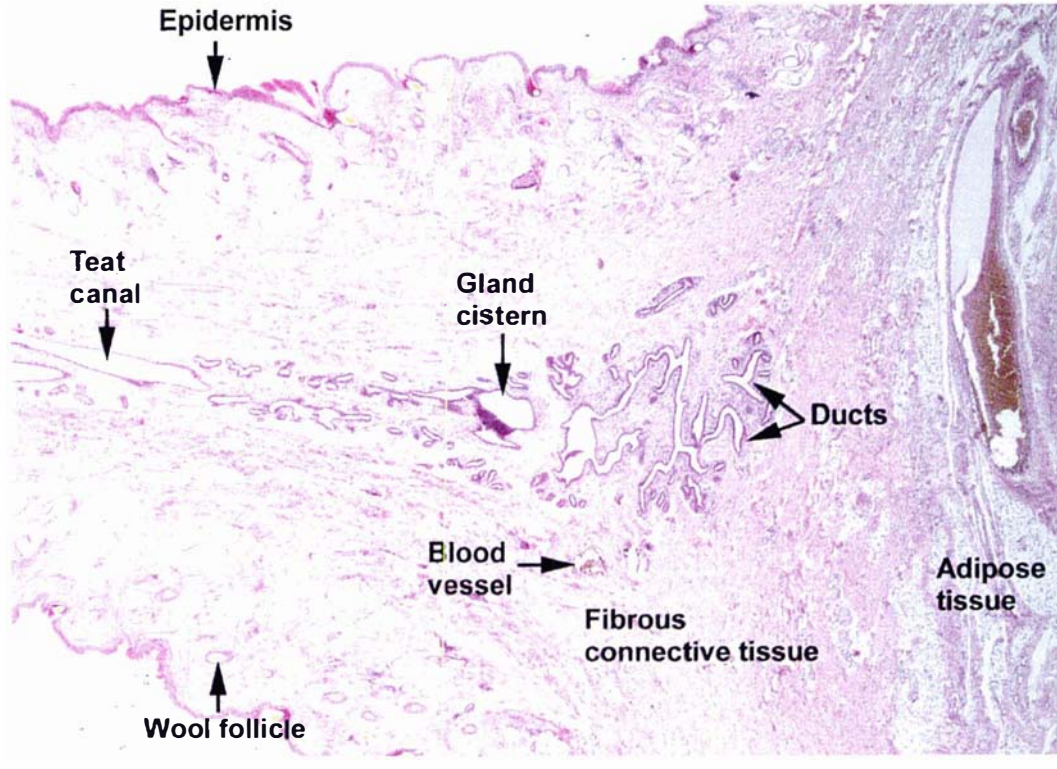



**Plate 2.30** Photomicrograph of the development of epithelial and adipose tissue in the mammary gland of a male sheep at day 140 of fetal age. (Mag x25).



The entire ductal system except for the teat duct was lined with a crowded cuboidal to columnar epithelial cell layer above a discrete, unevenly spread layer of basal cells. No secretory end-pieces (alveoli) formed in the prenatal stage of development.

An abundance of adipose tissue was a notable feature of the mesenchymal (or stromal) compartment of the female gland. The lobules of adipose tissue, which now contained quite large adipocytes as well as a few adipoblasts, were tightly packed but partitioned by narrow bands of connective tissue septa (collagenic and elastic fibres) (Plate 2.31). Substantially less adipose tissue was present in the male gland (Plate 2.32). Few distinct lobules formed and the long, shapeless bundles of mainly adipocytes were interspersed with fibrous connective tissue, their development closely resembling that observed at day 120 of fetal life.

The lymph nodes were easily visualised by eye in H&E-stained paraffin sections. They appeared as dark blue stained regions due to the density of the small, nucleated cells (lymphocytes) present. Blood and lymphatic vessels were present throughout the mammary gland and varied in shape, size and abundance. The vessels were lined by a simple squamous endothelium. The blood vessels were easily identified from the presence of red blood cells while the lymphatic vessels contained precipitated protein remnants of lymph.

The teat ducts routinely opened to the surface and were usually sealed from the external environment by a plug of keratin (Plate 2.33). The epithelium of the teat tip was continuous with the keratinised stratified squamous epithelium of the epidermis. Longitudinal sections displayed the well-developed keratinised fibres of the primary wool follicles emerging through the epidermis. Huge sebaceous glands were observed as clumps surrounding the primary follicles.

**Plate 2.31** Photomicrograph of the development of mammary fat pad in a female sheep at day 140 of fetal age. Lobules containing large adipocytes are tightly packed together, partitioned by collagenic and elastic connective tissue. (Mag x250).

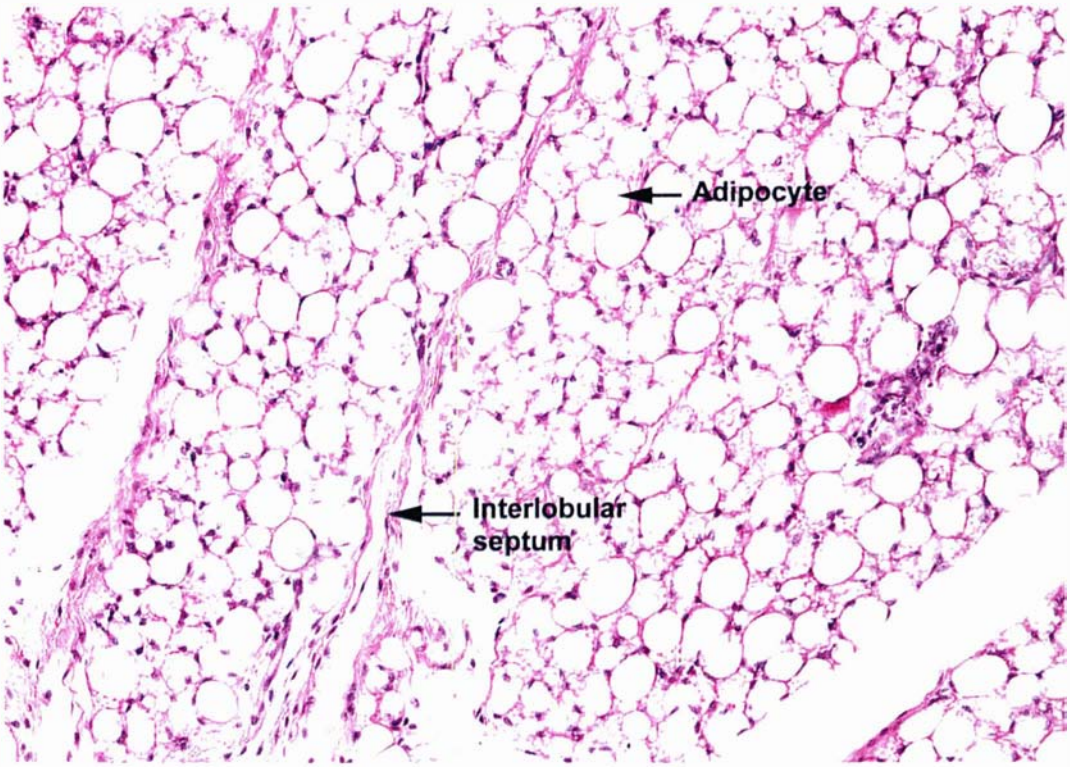



**Plate 2.32** Photomicrograph of the development of mammary fat pad in a male sheep at day 140 of fetal age. Long, narrow lobules containing large adipocytes are loosely arranged amongst fibrous connective tissue. (Mag x250).

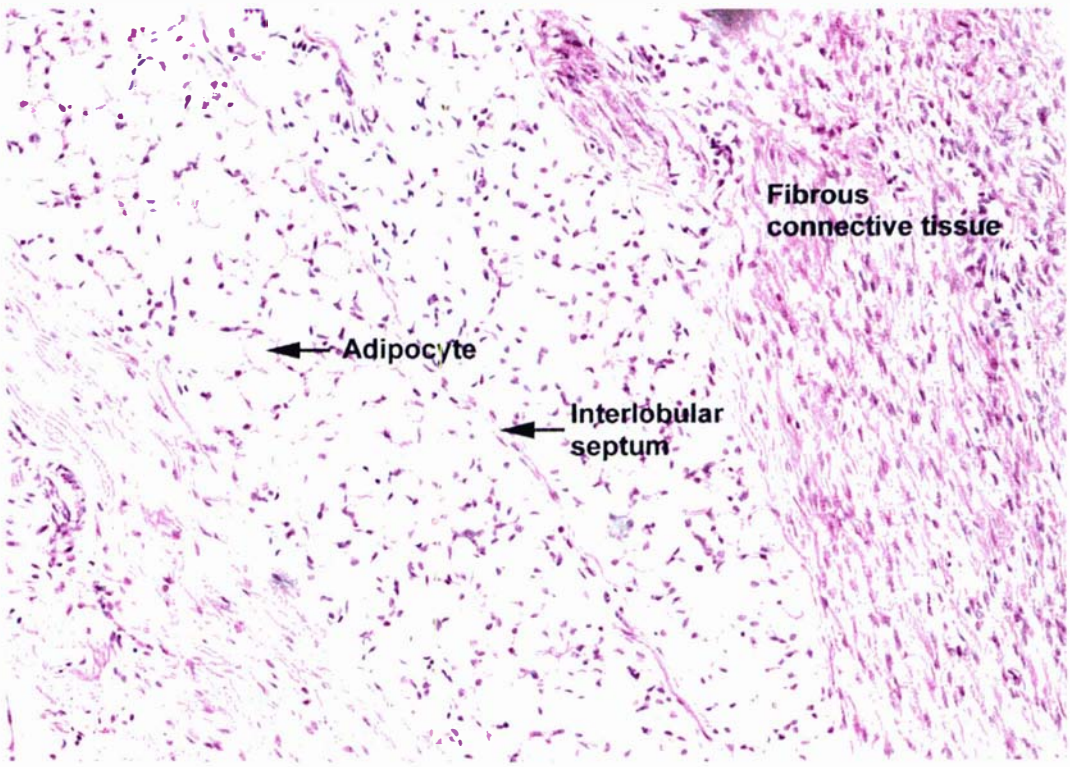
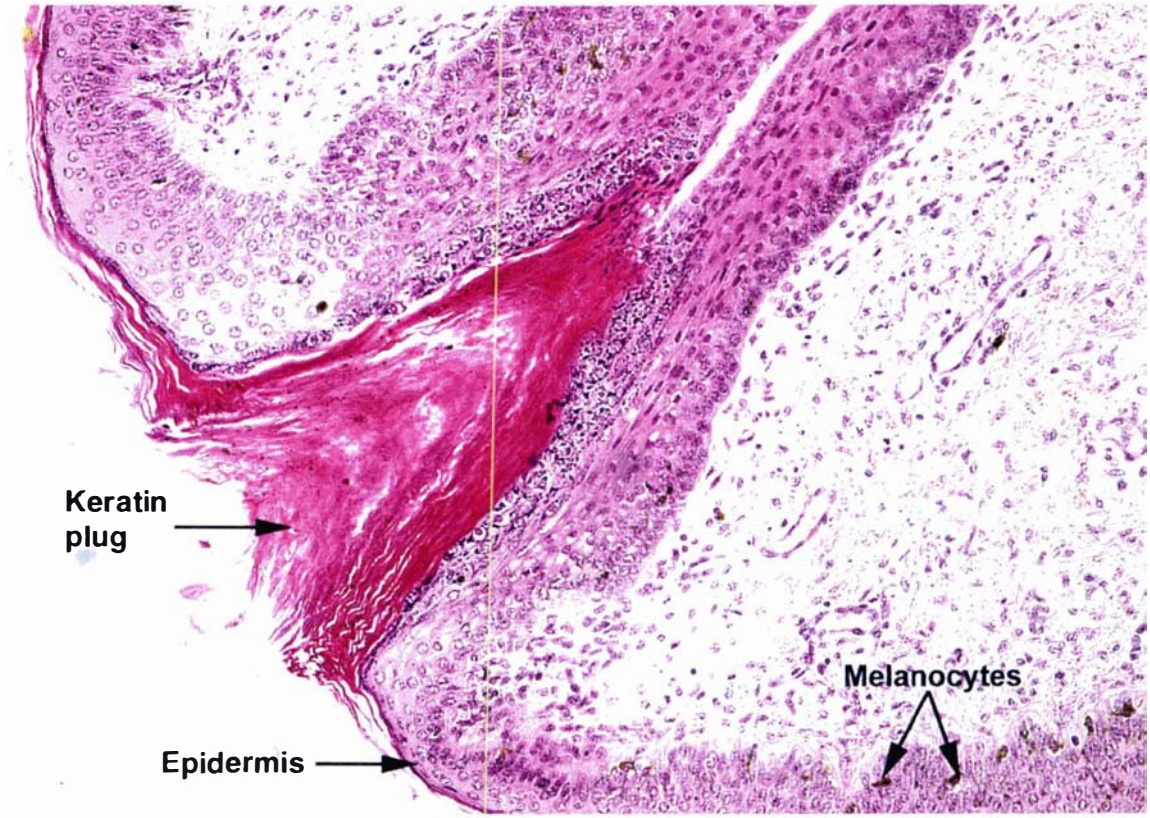


**Plate 2.33** Photomicrograph of the teat of a male sheep at day 140 of fetal age. A plug of keratin seals the entrance to the teat duct. The exterior surface of the epidermis has become highly keratinised and contains melanocytes (yellow/brown pigment). (Mag x250).



**Summary of the main features of mammary development during fetal life**

| Age | Female | Male |
|---|---|---|
| 40 | Mammary bud. An oval-shaped cluster of polyhedral epithelial cells, underlying, but still connected to, the epidermis, and surrounded by a concentric layer of undifferentiated mesenchymal cells. | Similar to the female bud. |
| 60 | Primary duct. Elongation of the mammary bud to its full extension. Canalisation almost complete. Development of several secondary ducts consisting of solid cores of cells. Mesenchymal cells remain largely undifferentiated. Angiogenesis initiated. | Elongation of the mammary bud to its full extension. Canalisation of primary duct initiated. Some secondary duct formation. Mesenchymal cells remain largely undifferentiated. |
| 80 | Teat and gland cisterns formed. Numerous secondary ducts with lumens, growing at various angles distal to the primary duct. Connective tissue more fibrous in nature and highly vascularised. Developing fat pad comprises small, condensed bundles, consisting of a mixture of adipogenic cells, adipoblasts and immature adipocytes. Pre-keratinising cells present in the epidermis. Initiation of primary wool follicle formation. | Mammary epithelial and connective tissue development similar to female. Growth of the secondary ducts continues mainly in the same direction as the primary duct. Less abundant mesenchymal tissue underlying ducts. Developing adipose tissue mainly adipogenic cells, with some adipoblasts. Blood vessel and nerve formation lagging behind female. Epidermal and wool follicle development similar to female. |
| 100 | Secondary ducts continue to grow at various angles to the direction of the primary duct. Appearance of myoepithelial cells. Collagenous fibres developing in close proximity to the primary duct. Early collagen fibres and fibroblasts aligned the basement membrane of the secondary ducts. Distinct lobules, comprising a mixture of adipogenic cells, adipoblasts and adipocytes located deep in hypodermis. Primary follicles growing a keratinised fibre. | Most of secondary ducts grow perpendicular to teat tip, towards fat pad. Appearance of myoepithelial cells. Fibrous connective tissue and adipose tissue development similar to female, but individual lobules of fat cells less numerous. Primary follicles growing a keratinised fibre. |
| 120 | Tertiary ducts formed from lateral branching of the secondary ducts. Secretory tissue had reached the edge of the fat pad. Amount of collagen was increased. Large lobules of adipose tissue comprising mainly adipocytes, with some adipoblasts. | Lateral branches mainly terminal, with very few solid epithelial growing ends. Much less adipose tissue present. |
| 140 | Extensive branching of secondary ducts and laterals. Secondary ducts formed large lumens. Ducts still lying outside the fat pad. Abundance of lobules of adipose tissue containing mainly adipocytes. Much denser fibrous connective tissue encloses ducts and adipose tissue lobules. | Duct development restricted to long axis of teat area. Few distinct lobules of adipose tissue comprised mainly of adipocytes. |

**Day 21 of postnatal age**

At three weeks of age marked differences were observed between the sexes in the pattern and extent of both ductal and adipose tissue development. In contrast to the relatively few growing ducts of the male, the female gland had large numbers of closely packed ducts radiating from the gland cistern in a broccoli-like fashion. The area of mammary epithelial tissue of the one female lamb was approximately five-fold greater than that of the fetuses at 140 days (Table 2.1). The area occupied by the epithelium increased relative to that of the surrounding fibrous connective tissue. Furthermore, the ducts had begun to invade the fat pad, while those of the male remained confined to the fibrous connective tissue beneath the teat structure (Plates 2.34 and 2.35). However, there were still large regions of the female fat pad devoid of ducts.

The epithelium of the gland cistern and ducts was uniformly composed of two crowded layers of cells, a luminal layer, which was cuboidal to columnar, and a basal layer. Contiguous with the six to eight layered teat duct was the stratified squamous keratinised epithelium of the epidermis.

**Plate 2.34** Photomicrograph of the mammary epithelial and adipose tissue of a 21-day-old female lamb. Fibrous connective tissue and bundles of adipocytes closely surround the developing ducts that have invaded the fat pad. (Mag x100).

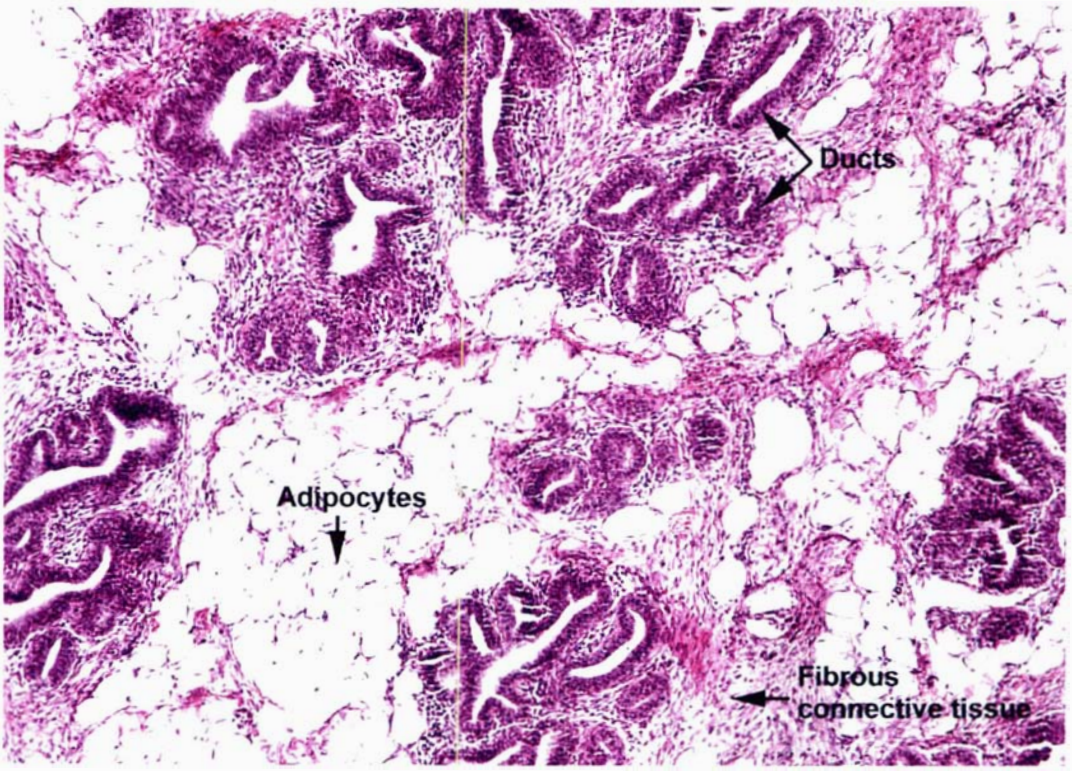
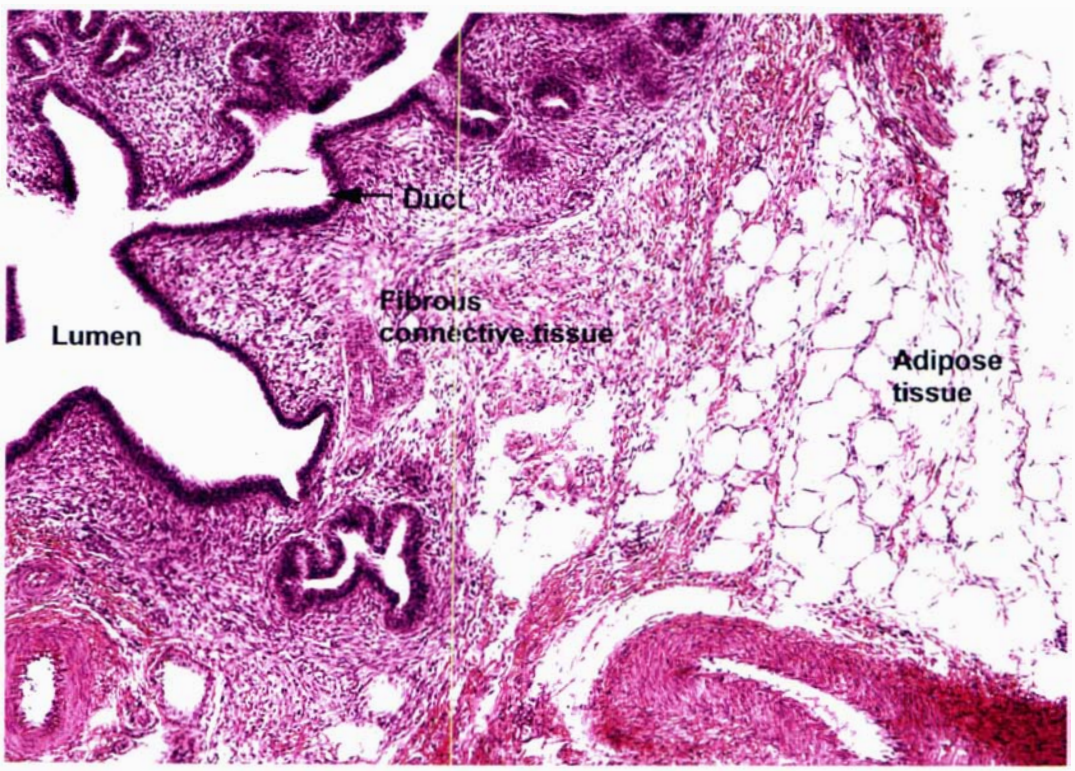



**Plate 2.35** Photomicrograph of the mammary epithelial and adipose tissue of a 21-day-old male lamb. The developing ducts remain separated from the fat pad by bands of fibrous connective tissue. (Mag x100).

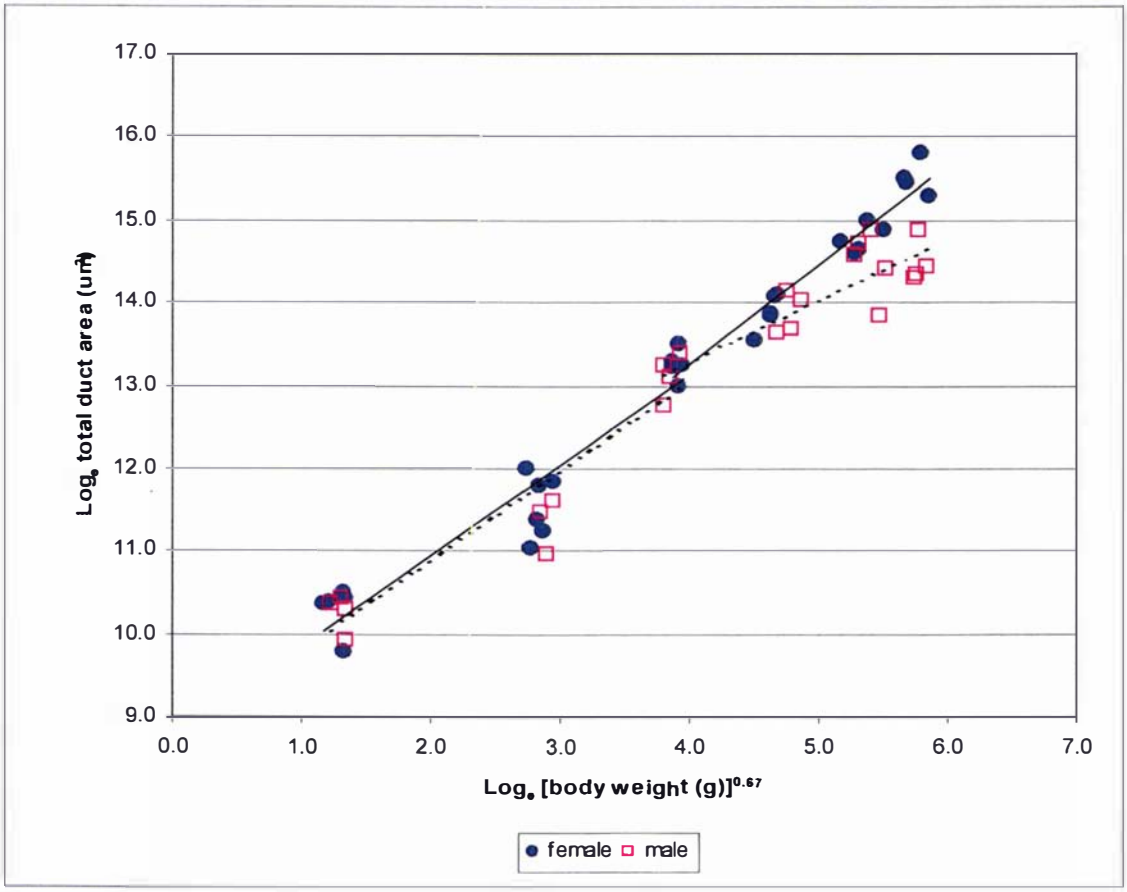


### 2.4.2 Morphological measurements

With the exception of the ovine data presented by Martinet (1962) I am not aware of any study that dealt with the growth rate of the mammary gland relative to that of the body in the fetal ruminant. It was therefore important to get an objective measure of growth from the data collected in this study.

The $\log_e$ of the data for mammary duct area ($um^2$) is plotted against (body weight)$^{0.67}$ (Figure 2.1). Relative growth analysis of the mammary glands revealed that the rate of increase in total duct area of both male and female fetuses was similar ($\alpha$=1.09) as the total increase in body weight from days 40 to 80 of gestation (i.e., growth was almost isometric). However, from days 80 to 140 of gestation total duct area increased 1.20 times as fast as body weight (i.e., positively allometric) in the female while in the male it accounted for approximately three-quarters ($\alpha$=0.76) of the total increase in body weight.

**Figure 2.1** The relation between total duct area ($um^2$) and (body weight)$^{0.67}$ in male and female fetal sheep from days 40 to 140 of gestation.



Quantitative data on the development of the mammary epithelial tissue in male and female fetal sheep at days 40, 60, 80, 100, 120 and 140 of gestation are presented in Tables 2.1 and 2.2 Three of the six ewes in each gestation group carried twins. However, preliminary analysis of the morphological data showed that age by rank effects were not significant, thus means were presented for age by sex cells (Table 2.2). Fetal weight increased significantly ($P<0.001$) with gestational age, but there were no differences in body weight over time between males and females. Mammary gland weights were significantly heavier ($P<0.001$) in females compared to those of males at each stage of gestation measured (days 100, 120 and 140). Within females, the weight of the mammary gland at day 120 of fetal age was heavier ($P<0.01$) than at day 100, but of a similar weight to that at day 140 ($P>0.05$). The mammary glands of fetal males were similar in weight at each stage of gestation measured.

Total duct area was measured as an indicator of mammary epithelial development (Table 2.2). There was no sexual dimorphism in the area of the ducts until day 140 of gestation, when total duct area of the female gland was some three-fold greater ($P<0.001$) than that of the male gland (see also Table 2.1). Females at days 120 and 140 of fetal age had significantly greater total duct areas ($P<0.001$) than females at all other stages recorded. Furthermore, there was a significant age by sex interaction ($P<0.01$) reflecting the gradual divergence in duct area (in favour of female fetuses) as gestation advanced. The same pattern held true for the total number of ducts and the number of canalised ducts (ducts with lumens), per gland. Total secretory cell area followed a similar pattern to total duct area, being approximately three-fold greater ($P<0.01$) in the female gland compared to the male gland at day 140 of fetal age. However, in contrast to duct area, only females at day 140 had secretory cell areas that were significantly ($P<0.001$) greater than females at all other stages of gestation. Total lumen area increased ($P<0.001$) with age in both sexes, but there was no significant difference between males and females at each stage of gestation. However, there was much variation in total lumen area between individual animals within each stage of gestation analysed.

**Table 2.1** Total duct area ($um^2 \times 10^3$) and body weight (grams) for male and female fetal sheep at days 40, 60, 80, 100, 120 and 140 of gestation and lambs at 21 days of age (unadjusted mean ± sem).

| | Males | | | Females | | |
|---|---|---|---|---|---|---|
| Fetal age[1] | Number | Duct area ($um^2 \times 10^3$) | Body weight (grams) | Number | Duct area ($um^2 \times 10^3$) | Body weight (grams) |
| 40 | 4 | 28.8 ± 1.5 | 7.2 ± 0.1 | 5 | 30.6 ± 1.5 | 6.9 ± 0.1 |
| 60 | 3 | 87.3 ± 8.9 | 76.0 ± 1.8 | 6 | 109.4 ± 6.7 | 68.9 ± 1.3 |
| 80 | 4 | 514.8 ± 31.8 | 313.8 ± 7.4 | 5 | 574.9 ± 21.3 | 342.2 ± 3.7 |
| 100 | 4 | 1078.2 ± 67.0 | 1255.3 ± 138.2 | 5 | 1092.1 ± 45.6 | 1002.6 ± 19.4 |
| 120 | 4 | 2338.9 ± 116.4 | 3135.5 ± 127.2 | 5 | 2640.7 ± 86.4 | 2901.2 ± 104.4 |
| 140 | 5 | 1826.8 ± 133.6 | 5170.4 ± 192.5 | 4 | 5532.8 ± 300.9 | 5397.5 ± 194.6 |
| Lamb[2] | 1 | 2595 | 8320 | 1 | 25631 | 11490 |

[1] Day of gestation; [2] 21 days old

**Table 2.2** Development of the mammary gland (lsmean±sem) of male and female fetal sheep at days 40, 60, 80, 100, 120 and 140 of gestation, and the significance of day of gestation, sex and the day of gestation by sex interaction. Data is log transformed ($log_e$).

| | | Day of gestation | | | | | |
|---|---|---|---|---|---|---|---|
| Parameter | Sex | 40 | 60 | 80 | 100 | 120 | 140 |
| Fetal number | M | 4 | 3 | 4 | 4 | 4 | 5 |
| | F | 5 | 6 | 5 | 5 | 5 | 4 |
| Fetal weight ($log_e$ g) | M | 1.96±0.06$^{a}$ | 4.20±0.07$^{b}$ | 5.79±0.06$^{c}$ | 7.11±0.06$^{d}$ | 8.02±0.06$^{f}$ | 8.52±0.05$^{h}$ |
| | F | 1.99±0.05$^{a}$ | 4.22±0.05$^{b}$ | 5.87±0.05$^{c}$ | 6.91±0.05$^{e}$ | 7.92±0.05$^{g}$ | 8.62±0.06$^{h}$ |
| Fetal mammary | M | na | na | na | 1.30±0.20$^{a}$ | 1.48±0.14$^{a}$ | 1.35±0.21$^{a}$ |
| gland weight ($log_e$ g) | F | na | na | na | 1.82±0.21$^{ab}$ | 2.27±0.11$^{b}$ | 1.98±0.23$^{b}$ |
| Total duct area | M | 10.50±0.21$^{a}$ | 11.61±0.22$^{b}$ | 13.33±0.18$^{d}$ | 13.91±0.14$^{e}$ | 14.42±0.20$^{f}$ | 13.83±0.36$^{g}$ |
| ($log_e$ $um^2$) | F | 10.55±0.20$^{a}$ | 11.78±0.19$^{c}$ | 13.46±0.17$^{d}$ | 13.98±0.13$^{e}$ | 14.58±0.17$^{f}$ | 14.93±0.39$^{h}$ |
| Total lumen area | M | na | 8.66±0.42$^{a}$ | 11.56±0.36$^{b}$ | 12.75±0.26$^{c}$ | 13.72±0.31$^{d}$ | 13.53±0.58$^{d}$ |
| ($log_e$ $um^2$) | F | na | 8.29±0.37$^{a}$ | 11.81±0.34$^{b}$ | 12.96±0.26$^{c}$ | 13.92±0.26$^{d}$ | 14.55±0.63$^{e}$ |
| Total secretory cell | M | na | 10.97±0.24$^{a}$ | 12.64±0.21$^{c}$ | 13.39±0.15$^{d}$ | 14.31±0.18$^{e}$ | 14.49±0.34$^{f}$ |
| area ($log_e$ $um^2$) | F | na | 11.20±0.21$^{b}$ | 12.76±0.20$^{c}$ | 13.27±0.15$^{d}$ | 14.31±0.15$^{e}$ | 15.43±0.36$^{g}$ |
| Number of ducts | M | na | 0.78±0.35$^{a}$ | 3.48±0.31b$^{c}$ | 4.17±0.22$^{ce}$ | 4.06±0.26$^{df}$ | 3.98±0.49$^{ef}$ |
| | F | na | 0.94±0.31$^{a}$ | 3.25±0.29$^{b}$ | 3.67±0.22$^{c}$ | 4.69±0.22$^{d}$ | 5.30±0.53$^{g}$ |
| Number of ducts with | M | na | 0.60±0.37$^{a}$ | 3.30±0.32$^{bc}$ | 3.96±0.23$^{cdf}$ | 3.68±0.27$^{de}$ | 3.59±0.52$^{df}$ |
| lumens | F | na | 0.70±0.33$^{a}$ | 3.12±0.31$^{b}$ | 3.46±0.23$^{cf}$ | 4.33±0.23$^{e}$ | 4.91±0.56$^{g}$ |

$^{abcdefgh}$ Means within rows with different superscripts are significantly different (P<0.05).

## 2.5 Discussion

The foundation for the large and dramatic alterations in mammogenesis during adult life begins when the animal is a fetus. However, the majority of studies have usually focused on the changes in the mammary gland that occur around puberty and during pregnancy with the implicit assumption that development of the mammary gland during fetal life is irrelevant to this later period. In order to demonstrate that critical aspects of mammary differentiation are established during fetal life, an important first step was to obtain quantitative data during the prenatal period.

The structural aspects of mammary gland growth and development have been well described in the fetal rodent, and, except for the timing related to gestation length, the sequence of events are generally similar in all species. For ruminants, the few studies to date have been at a descriptive level, giving details of mammary gland morphogenesis in cattle (Turner, 1930, 1931; Sonstegard, 1972) and to a lesser extent in goats (Turner and Gomez, 1936), and have assumed that the sheep gland follows a similar developmental pattern (Turner, 1952). Wallace's (1953) and Martinet's (1962) accounts of mammary development in the fetal sheep were brief with Martinet's study failing to provide any observations on development of the mesenchymal or adipose tissues. Moreover, quantitative information on mammary development in the fetal ruminant is limited to one study on the sheep (Martinet, 1962). Therefore, the objectives of this study were to expand, firstly, on those stages of mammary gland morphogenesis in the fetal sheep previously described by Turner (1952), Wallace (1953) and Martinet (1962), and secondly, on the very limited quantitative data pertaining to ruminants.

Turner's (1930, 1931 and 1952) bovine studies relied on animal tissue obtained from abattoirs, hence the use of crown-rump length (CRL) as an estimate of fetal age. The ages of embryos were also estimated from CRL in Turner's (1952) very brief description of the general features of ovine mammary development, which were based on Profés (1899) account (refer to Chapter one for details). Moreover, the fetuses were not of the same breed. Wallace (1953) and Martinet (1962), on the other hand, acquired fetuses of known age and of the same breed. The ages of the fetuses used in the present study were also known and the animals were of the same breed. Day 40 of fetal age was the earliest stage at which development of the mammary gland was described and quantified, due to the difficulty in determining pregnancy status in the ewe and sex of the fetus prior to this time.

In the present study, there was no indication of sexual dimorphism in the size, shape or depth of invagination of the mammary bud at day 40 of fetal age (Plates 2.1 and 2.2). This observation of morphologically similar buds during the more advanced (day 40) stage of bud development (bud present from days 36 to 46) supports the observations of Martinet (1962) who also failed to record sexual dimorphism at a similar stage of development (day 44). In contrast, it is possible to distinguish differences in mammary development between bovine males and females at the

mammary bud stage (Turner, 1930). Although there are slight variations in regard to the form of the buds, female bovine buds generally have a smaller volume and are more oval in shape compared with males, particularly in the later stages of bud development. The buds of females also appear to invaginate a shorter distance from the surface than in males, seen again in the more mature bud, when the bovine fetus is approximately 11.5 cm in length (Sonstegard, 1972).

The observation of concentrically-arranged, closely packed, mesenchymal cells with deep staining nuclei, orientated around the distinct basement membrane of the epithelial bud is exactly as that described by Turner (1930) and Sonstegard (1972) for the relatively undifferentiated mesenchymal tissue underlying the bovine bud. Martinet (1962) however, failed to describe the developmental progress of the mesenchymal tissue in sheep, focusing primarily on epithelial development.

At day 60 of fetal age (approximately 15.0 cm CRL) the partially canalised primary duct in the female had reached its full extension as a mass of epithelial cells and the formation of three or four secondary ducts, comprised of solid cores of cells, had begun (Plates 2.3 and 2.4). Canalisation of the primary duct and the initiation of secondary duct formation are consistent with the observations of Wallace (1953) and Martinet (1962) who found that these processes occur sometime around days 56 to 59 of fetal age. The absence of secondary ducts in one of the three male glands in the present study may have been due to the plane of sectioning. As no intermediate stages (between days 40 and 60) were available any delays in development attributed to the male could not be confirmed. However, it was noted that canalisation of the male primary duct lagged behind that of the female. This is consistent with observations in cattle where developmental progress in primary duct (Turner, 1930) and secondary duct (Sonstegard, 1972) formation in the female were initiated before that in the male.

Teat development occurred simultaneously with the invasion of the mammary bud into the underlying mesenchyme (Plates 2.3 and 2.4). Turner (1930) reported that early teat formation produced a fairly pointed teat end in females compared with a more flattened teat in males. No such structural differences in the apex of the teat were observed in the ovine gland. In fact, the teat ends of individual males and females varied enormously in their shape. The mesenchymal cells were still largely undifferentiated, although some fibroblastic cells were identified aligning the growing ends of the bud. The appearance of blood vessels in close proximity to the growing ducts was probably an indication of the mesenchyme's future differentiation into fibrous connective tissue. Furthermore, early differentiation of the more deeply placed hypodermis was marked by the appearance of small bundles comprising Schwann cells (early innervation) and small blood vessels (angiogenesis). Martinet (1962) did not describe the development of adipose tissue while Wallace (1953) mentioned the appearance of "future fat cells" at day 84 of fetal age.

By day 80 of fetal age the primary duct had given rise to numerous secondary ducts and the gland and teat cisterns were a structural feature in both males and females (Plates 2.7 and 2.8). This is consistent with the observations of Martinet (1962) and Forsyth et al. (1999), where, by

day 70 of fetal age, several secondary ducts had formed from the primary duct and the teat cistern was evident. Sexual diversity in the direction of secondary duct growth was also noted in the present study. As compared with the direction of growth of the primary duct the secondary ducts were beginning to grow at an angle. Although similar developmental progress was observed in the male at day 80, growth of the secondary ducts continued mainly towards the developing fat pad, like that of the primary duct. However, there was much variation between individual males. Similar progress by the epithelial tissue was recorded by Martinet (1962), at day 76, and Wallace (1953), at day 84.

Another characteristic of the male gland at day 80, which was also reported by Martinet, (1962) for fetuses on day 76, was the relatively small amount of mesenchymal tissue present between the secondary ducts and the developing skeletal muscle, greatly restricting further epithelial development. This is a prominent feature of the ruminant male udder from secondary duct formation through to birth (cattle: Turner, 1931, Sonstegard, 1972; goats: Turner and Gomez, 1936).

At day 80 the first sign of a developing fat pad became evident, with the differentiation of hypodermal mesenchymal cells into mainly adipogenic cells, with some adipoblasts (Plates 2.10 and 2.11). This was seen in males and females although the numbers of cells within each bundle (or whorl) of developing adipose tissue were greatly reduced in the male compared to the female. A similar pattern of adipose tissue development was observed by Turner (1931), who described the characteristic "whorls of cells" during the early stages of adipose tissue formation in fetal cattle (around day 80 of gestation, term = 270 days). However, little is known about its subsequent development in ruminants (Sheffield, 1988).

The presence of hair follicles along the entire length of the teat is also a feature in sheep. At day 80 of gestation the down growth of plugs of epidermal cells into the dermis marked the initiation of primary wool follicle growth in the fetal sheep (Plate 2.13). Although not mentioned by Martinet (1962) the formation of wool follicles was obvious in photomicrographs of the male teat at day 76 and the female teat at day 97 of fetal age. A notable difference between female cattle and goats of both sexes is the prominence of hair follicles right up to the end of the teat in the latter (Turner, 1952).

The development of the mammary epithelium at day 100 was exactly as that portrayed in a photomicrograph presented by Martinet (1962) of a female fetus at day 97 of gestation. Myoepithelial cells were first noticed at day 100, lying below the luminal epithelial cells, and were present in males and females (Plate 2.16). Forsyth et al. (1999) was able to localise these basal epithelial cells from week ten of gestation (approximately day 70 of fetal age) in the female gland using an antibody to α-smooth muscle actin. The progress in development of the fat pad from adipogenic cells towards more-mature adipose tissue was marked visually by the accumulation of stored lipids in some adipose cells.

The amount of collagen in this very dense, irregularly arranged fibrous connective tissue had increased substantially by day 120 of fetal age and was easily visualised as deep pink staining

on examination of the H&E-stained paraffin sections. The secretory tissue in both sexes lay in an extensive body of intralobular connective tissue and the ducts were separated from adipose tissue by multiple layers of fibrous connective tissue (Plates 2.23 and 2.24). At day 112, Wallace (1953) described the differentiation of a well-defined region of connective tissue surrounding a duct system of great complexity. In terms of area, the epithelial ducts of the male gland failed to show anything but the slightest progress, while lateral branching of the ducts of the female gland continued to grow in a direction perpendicular to that of the primary duct.

A feature of adipose tissue development at day 120 was the arrangement of adipose cells into large, distinct lobules (Plate 2.25). These lobules comprised mainly adipocytes, identified by their flattened nuclei, which appeared on the periphery of the large unoccupied spherical cells. A similar pattern of development was seen in the 36 cm long (about 150 days) bovine fetus, in which the gradual storage of fat was indicated by the large empty spherical adipose cells (Turner, 1931).

Consistent with the observations of Wallace (1953), the male glands in the present study at 140 days showed little, if any further development from that present at 120 days. Unlike the male, the extension of the ducts continued in the female, hence the calculated increase in total duct area and secretory cell area. However, the epithelial tissue remained confined to the edge of the fat pad (Plate 2.29 and Forsyth et al., 1999). There was substantially less adipose tissue present in the male compared with that in the female, development of the fat pad in the male closely resembling that observed at day 120 of fetal life. Turner (1931) concluded that the formation of the fat pad appeared to be complete in the 60 cm long (about 200 days) female bovine fetus. This equates to about 74% of gestation length and is similar to that observed in sheep, where the formation of a distinctive fat pad is seen at day 120 of fetal life, or at 82% of gestation length.

The most dramatic changes in mammary development were observed in the three-week-old female lamb, whereupon examination of the gland revealed the advancement of the epithelial ducts into the fat pad (Plate 2.34). The extent of the duct system in relation to the size of the udder had increased substantially, total duct area was now almost five-fold greater than at day 140 of fetal age. In contrast, the epithelial tissue of the male gland remained restricted to an area of fibrous connective tissue immediately dorsal to the teat, having progressed no further than that observed at day 120. These observations confirmed those cited previously by Wallace (1953) in the one-month old lamb.

The observations of mammary glands of female ovine fetuses suggest that the epithelial (or future parenchymal) tissue per gland increases approximately 24-fold from the initiation of the secondary ducts until just prior to birth; and a further four- to five-fold in the three weeks after birth. Although the absolute amount of mammary growth during the fetal period is only a small percentage of the mature mammary gland growth during pregnancy and lactation it is intriguing to consider that growth during the prenatal period may have a major effect on lactation potential in later life.

Relative amounts of growth during fetal life in the male and females' development have been determined in several species. Data from rodents demonstrated that development of the mammary gland is influenced by stage of gestation and position of individual glands, with mice exhibiting sexual dimorphism in growth rate of the mammary gland relative to growth of the body as a whole (Jean et al., 1972; Jean and Jean, 1973). Considering the differences in the number of mammary glands, their position on the body and the length of gestation, it is perhaps not wise to assume that the rodent is an appropriate model for relative growth analysis of mammary development in the ruminant. As demonstrated for fetal sheep, mammary gland growth in the female is five times faster ($\alpha$ = 5.01) than body growth from day 48 to day 69 of gestation. It then slows to 1.70 times body growth until term (Martinet, 1962). Although data from the present study also indicated positive allometric growth in female fetuses, it was not as dramatic as that observed by Martinet (1962). The allometric constant ($\alpha$) averaged 1.09 from days 40 to 80, and 1.20 from days 80 to 140. Values for relative growth analysis in the present study were derived from 30 female fetuses at six stages of gestation whereas Martinet (1962) relied on values for 24 female fetuses of various ages between days 48 and 150 of gestation. Furthermore, the techniques differed. Martinet (1962) obtained the volumetric growth of the mammary gland by planimetric analysis. Similar discrepancies were seen in the male data. Martinet (1962) found that the mammary gland of the male grew at a constant rate of 2.8 times body weight and did not show the initial accelerated rate of relative growth in the female. Mammary glands of males in this study grew at the same rate as the females from days 40 to 80 ($\alpha$ = 1.09), then slowed to 0.76 times that of body growth from days 80 to 140, exhibiting negative allometry. In the male, development of adipose tissue lags behind that of the female from the outset and this is reflected in the decline in relative growth of the mammary gland from day 80. This is consistent with studies in female sheep that indicate the importance of the mammary fat pad in stimulating prepubertal allometric mammary growth (Hovey, 1996). Furthermore, transplantation studies in female mice demonstrated that the amount of adipose tissue in the fat pad dictated the ultimate number of epithelial cells within the mammary gland (Hoshino, 1964). Given that milk yield is proportional to epithelial cell number (Akers, 1990) these findings suggest that the fat pad is the limiting factor in terms of the amount of tissue that is grown during fetal development and, hence, the amount of secretory tissue present at lactation in the adult.

In conclusion, these results showed that the ovine mammary gland does not undergo sex-specific destruction in males, as in widely studied mice. There was a gradual divergence in the extent of development of the mammary gland epithelial and mesenchymal structures of male and female fetal sheep. Unlike the mammary gland of fetal cattle, where it is possible to distinguish differences early in gestation (about 20% of gestation length) sexual dimorphism in the fetal sheep does not become pronounced until the formation of secondary ducts (about 54% of gestation length). These differences were especially evident during development of the fat pad where adipose tissue was far less abundant in the male from the outset. In terms of epithelial development, female fetuses at days 120 and 140 of gestation had substantially greater duct areas as compared to female fetuses at earlier stages of gestation. Moreover, the

interval between day 140 and three weeks of postnatal age witnessed an extensive increase in the size of the duct system and the invasion of the epithelial tissue into a well-developed fat pad.

The significance of the mammary fat pad's contribution to the morphological development of the epithelial tissue has become increasingly apparent. Hence, it would appear that the prevention of the complete differentiation of the epithelial cells into a mammary gland capable of synthesising milk in the male ruminant could be simplistically explained by the lack of adipose tissue into which the ducts may penetrate. On the other hand, the rodent model has provided overwhelming evidence that testosterone produced from the fetal male testis is involved in the sexual dimorphism of the mammary gland, which modifies the neutral (or female) pattern of growth. Whether such sex-dependent development occurs in sheep remains to be explored. Therefore, it was proposed, in the following study, to identify the presence (or absence) of sex steroid receptors in the ovine gland, which have already been shown to be involved in differentiation and development of the rodent mammary gland.

# CHAPTER THREE

# SEX DIFFERENCES IN THE REGULATION OF DEVELOPMENT IN OVINE FETAL MAMMARY GLAND – ROLE OF ANDROGEN AND OESTROGEN RECEPTORS

## 3.1 Abstract

Androgens and oestrogens are steroid hormones that regulate male and female reproductive organ development and functioning upon binding to specific receptors. In the mammary gland of the fetal mouse, androgen and oestrogen receptors are formed in the mesenchymal cells surrounding the epithelial bud. However, although androgen receptors are responsible for the testosterone-induced destruction of the epithelial bud in the murine male, oestrogens and their receptors are assumed to play no role in regulating prenatal mammary gland growth and development.

An immunohistochemical assay was used to locate the presence or absence of androgen and oestrogen receptors in the mammary tissue of male and female fetal sheep at days 40, 60, 80, 100, 120 and 140 of gestation. At day 40, both androgen and oestrogen receptors were localised in the mesenchymal cells immediately surrounding the epithelial bud. Receptors for both steroids were present in the epithelial cells of mammary ducts from day 80 of gestation onwards. Immunostaining was also observed in the nuclei of the fibroblasts that comprised the connective tissue matrix.

Immunohistochemistry confirmed an association between the gradual developmental divergence in epithelial, connective and adipose tissue differentiation in males and females and the intensity of immunostaining for both androgen and oestrogen receptors.

The location of androgen and oestrogen receptors after the initial bud stage in the ovine mammary gland is clearly different from that observed in the mouse. This would infer that there might well be differences in the hormonal regulation of mammary morphogenesis in rodents and ruminants.

## 3.2 Introduction

It is well accepted that mammary development during puberty and pregnancy is dependent on regulation by reproductive hormones (Daniel and Silberstein, 1987; Haslam, 1987; Fendrick et al., 1998). In contrast, prenatal development of the mammary glands appears to progress independently of systemic hormonal cues and instead is governed by reciprocal signalling between the epithelium and the mesenchyme (Kratochwil, 1987; Sakakura, 1987). Tissue separation and recombination studies in rabbits (Propper, 1968) and mice (Kratochwil, 1969; Cunha, 1995) demonstrated that mammary epithelial buds emerge from the embryonic epidermis as a result of mesenchymal induction. Once the early mammary bud is initiated, its development depends on the presence of mesenchymal cells (Kratochwil, 1987) the formation of which are governed by signals emanating from the mammary bud (Heuberger et al., 1982; Kratochwil, 1982). The characteristic "primary mammary mesenchyme" is formed from adjacent mesenchymal cells that are morphologically distinct from the more distant dermal mesenchyme

and express receptors for androgen (Heuberger et al., 1982, Wasner et al., 1983) and oestrogen (Kratochwil, 1987; Lemmen et al., 1999). However, the capacity of the mammary epithelial bud to induce these mesenchymal markers is transient and is lost at later stages (Heuberger et al., 1982).

The mouse model has demonstrated that androgens play a role in the determination of sex differences in the ontogenesis of the prenatal mammary gland. It also suggests that oestrogens play no physiological role at this stage. The model has, however, established that androgen and oestrogen binding in the mammary gland is due to the expression of specific steroid receptors and that the fetal gland shows endocrine sensitivity.

The only recognised function ascribed to mesenchymal androgen receptors is the destruction of the mammary bud in the male under the influence of testicular androgens. This sexual dimorphism in fetal mammary development is not typical for mammals but represents a peculiarity of the family of some rodents (e.g., mouse and rat) (Kratochwil, 1971). The mammary bud either becomes separated from the epidermis, in which case no nipple development takes place and the gland remains rudimentary, or the gland disappears. In the absence of influences from the fetal testes or testosterone, explanted mammary tissue from male and female fetal mice, irrespective of their genetic sex, follows a female-type pattern of development (Kratochwil, 1971). This female pattern of development, whereby the continuity of the primary duct is preserved, is also clearly seen in male rat and mouse fetuses exposed to anti-androgens, especially cyproterone acetate, *in utero* (Elgar and Neumann, 1966).

The formation of mesenchymal androgen receptors in the mouse starts at day 12 of fetal age. By day 14, the mammary bud in males and females is surrounded by approximately 3000 mesenchymal cells, each cell possessing about 30,000 binding sites for testosterone (Wasner et al., 1983). At day 14 androgens from the gonads of male fetuses cause this mesenchyme to condense around the mammary bud, which is subsequently destroyed. The target tissue for testosterone is the mesenchyme since the epithelial cells of the mammary bud do not possess receptors (Wasner et al., 1983) and, experimentally, combinations of wild type epithelium with androgen-insensitive (Tfm) mesenchyme fails to react (Kratochwil and Schwartz, 1976). Moreover, the mammary mesenchyme does not respond to testosterone in the absence of mammary epithelium (Dürnberger and Kratochwil, 1980) indicating that processes within the epithelial bud must therefore control the mesenchymal response to testosterone.

Development of the fetal mammary gland is not only affected by androgenic steroids, but also by oestrogens. Although oestrogenic hormones are assumed to play no physiological role in fetal mammary morphogenesis and function (at least not in the mouse) their effects have been demonstrated experimentally. In a series of experiments, Raynaud (1961, 1971) showed that injections of high doses of oestrogen into pregnant mothers or directly into the fetus produced varying degrees of malformation and mammary gland inhibition in the offspring, while mammary development was normal following destruction of the foetal ovaries. The latter has been interpreted to mean that the mammary glands of the female do not require the stimulus of

ovarian secretions during the fetal period. Nevertheless, it is possible that oestrogens of fetal or maternal origin may play a role in normal mammary development. Nipples developed prematurely in the offspring of mice and rats when oestrogens were injected into the mother during gestation (Raynaud, 1961, 1971) while administration of an anti-oestrogen (MER-25) suppressed nipple development and reduced the volume of the mammary epithelial tissue (Jean-Faucher et al., 1977).

A prerequisite for oestrogen action is the presence of oestrogen receptors. Steroid autoradiography showed that oestrogen receptors could be detected in the murine mammary gland at 14 to 16 days of fetal age (Narbaitz et al., 1980). The cloning of oestrogen receptor β (ORβ) (Kuiper et al., 1996) combined with riboprobe technology demonstrated the expression of both ORβ and the classical oestrogen receptor (ORα) in the mesenchyme of male and female fetal mammary gland as early as day 12.5 (Lemmen et al., 1999). Oestrogen receptor alpha has also been located in the mammary epithelial cells of human fetuses from the 30$^{th}$ week of gestation (term = 40 weeks) onwards (Keeling et al., 2000). In the rodent, oestrogen receptors are not present in the epithelial cells until day three of postnatal age (Haslam and Nummy, 1992). The lack of any gross abnormalities in the mammary gland of the ORα knockout mouse strengthens the case for endogenous oestrogens playing no role in prenatal development of the mammary gland. Moreover, female mice lacking ORβ have normal mammary gland histology and function (Krege et al., 1998). However, the question arises as to what role oestrogens and their receptors play in mammary tissue during prenatal life.

To date, there are no reports on the expression of androgen or oestrogen receptors in the mammary gland of the fetal ruminant, and the effect of steroid hormones on mammary development in ruminants is not well documented. Hence, the involvement of sex steroids and their receptors in the sexual dimorphism of the ruminant mammary gland remain to be explored. In the fetal sheep, differences in the morphological development of mammary tissue between males and females become evident at about day 70 of gestation, when, histologically, the mammary epithelial tissue is far less well developed in the male (Martinet, 1962). Furthermore, sexual dimorphism in the growth and development of adipose tissue is apparent at day 80 of gestation. Thereafter the formation and abundance of adipose cells in the male lags behind that of the female (Chapter two).

Sexual differentiation in the sheep occurs around days 30 to 32 of fetal age (McNatty et al., 1995). While fetal androgen biosynthesis is required for expression of male phenotype, oestrogen synthesis does not appear to be essential for normal development of female phenotype. However, the expression of genes at all levels of the steroidogenic pathway has been observed in the gonads of males and females between days 32 and 35 of fetal age, around the time of sexual differentiation (Quirke et al., 2001). In the male sheep, androstenedione and testosterone are present in the fetal testis from about day 30 (Attal, 1969; Mauleon, 1977; Lun et al., 1998). Some studies detected very low concentrations of oestrogens (oestradiol-17β) in the fetal testis at day 40 (Lun et al., 1998; Quirke et al., 2001) and after day

70 (Attal, 1969) of fetal age while other studies did not (Mauleon et al., 1977). Oestrogen (oestradiol-17β) secretion was detected in the ovary of the female sheep at day 31 until day 62 of fetal life but not beyond (Mauleon et al., 1977). This corresponds well with the observations of Lun et al., (1998) who showed the presence of oestradiol-17β in the female ovary at 35 days, and Quirke et al. (2001) who detected significant amounts of oestradiol-17β from day 35 onwards. Androstenedione and testosterone were present in the fetal ovaries at days 32 and 40, respectively (Lun et al., 1998).

In the female sheep, the total area occupied by the mammary ducts doubled from day 120 to day 140 of gestation. Duct area then increased almost five-fold from day 140 of fetal age to day 21 of postnatal age (Chapter two). Furthermore, a significant amount of adipose tissue developed in the female gland before birth, which was not present in the male (sheep: Chapter two; cattle: Turner, 1931). It could be hypothesised that oestrogen receptor activation within the fetal mammary gland is required to stimulate branching morphogenesis of the mammary ducts and development of the fat pad. Hence, the development of the female mammary gland would come under the influence of ovarian or maternal oestrogens, or oestrogen in combination with other hormones, relatively early in fetal life. The developing conceptus of sheep and cattle is exposed to significant concentrations of oestrogens at various times during fetal life (Challis et al., 1974; Findlay and Cox, 1969). The principal site of oestrogen biosynthesis is the placenta, which appears to produce the range of oestrogens (mainly oestradiol-17α) found in the fetal plasma and maternal urine of the sheep (Findlay and Seamark, 1973). Placental production of oestrogens commences at about day 70 and increases rapidly between days 90 and 120 of gestation and may indicate increasing oestrogen availability to the fetus (Challis and Patrick, 1981) as a similar pattern of increasing plasma concentrations was observed in the fetus (Findlay and Cox, 1969). This corresponds to the stage of gestation when sexual dimorphism becomes evident in the fetal mammary gland (Martinet, 1962; Chapter two). These oestrogens may exert biological effects within the mammary tissue of the developing fetus. Certainly near the end of gestation the fetal mammary gland is hormone responsive as demonstrated by the short period of precocious secretion just prior to or after birth, which has been described in numerous species including female lambs and calves (Wallace, 1953).

It is also probable that androgens, acting via the androgen receptor, are responsible for the sex differences observed in mammary gland morphogenesis during prenatal development in the sheep. Mammary duct development was more extensive and the depth of the fat pad was greater in castrated (at birth) male lambs than in ram lambs by two months of age. This led Wallace (1953) to suggest that the inherent capacity for development in the male gland is partially inhibited by the testis. However, by six months of age the morphological development of the mammary gland of castrated lambs was indistinguishable from that of the intact males. Wallace (1953) also reported no differences in the mammary development between freemartins and normal heifers from five months of fetal age to about one month of postnatal age. However, while some attempts to induce lactation in freemartins have been unsuccessful (Tervit et al.,

1980) others have met with a more favourable outcome, with two Holstein freemartins producing 7.5 and 12.5kg/day of milk, respectively, in the third month after treatment (Nouri and Kohli, 1999). This volume of milk is, however, substantially less than the 25kg/day produced by a typical lactating Holstein cow (Grossman et al., 1999). The differences between studies may be due to the degree of masculinisation of the reproductive tract, and hence the pattern of steroidogenesis. Abnormally high concentrations of testosterone and an absence of normally occurring oestrogen have been measured in freemartin cattle as early as day 47 of gestation (Shore and Shemesh, 1981). Altered steroidogenesis coupled with minimal lactational performance would indicate that exposure of the gland to testosterone during fetal development has a permanent effect.

The normal progression of mammary morphogenesis in female mice lacking a functional oestrogen receptor suggests that oestrogens serve no role during prenatal development. However, numerous studies have demonstrated that the fetal gland shows endocrine sensitivity; the classic example being that of androgen-mediated mammary destruction in the male mouse. In the ruminant, the histomorphological studies conducted to date have reported differences between patterns of mammary development in males and females as early as the mammary bud stage in cattle (Turner, 1930; Sonstegard, 1972) and the formation of secondary ducts and adipose tissue in sheep (Wallace, 1953; Martinet, 1962; Chapter two). Whether these sexual differences are mediated via mesenchymal steroid receptors remains to be determined. It was therefore proposed to identify the presence (or absence) of steroid receptors in the ovine gland, which, in the case of androgen receptors, have already been shown to be involved in differentiation and development of the rodent mammary gland.

## 3.3 Materials and Methods

All procedures were approved by the Massey University Animal Ethics Committee.

### 3.3.1 Animals and Treatments

Mammary gland samples used in this study were derived from animals used in an experiment described previously (Chapter two).

### 3.3.2 Histology Samples

The method used for mammary gland sample collection, processing and storage was the same as that described in Chapter two.

### 3.3.3 Immunohistochemistry

Immunohistochemistry was used to locate and assess the relative distribution of androgen and oestrogen receptors in the mammary tissue of male and female sheep during prenatal

development. The advantages of immunohistochemistry are that only a small piece of tissue is required and the technique permits localisation of receptors to a specific tissue or cell type. One disadvantage of this procedure is the inability to objectively quantify the results in that the intensity of immunohistochemical staining can only be estimated subjectively.

Androgen receptors were located histochemically in the sections using a high temperature antigen unmasking technique. The primary antibody for the androgen receptor analysis was a rabbit polyclonal raised against the first 17 amino acids of the N-terminal region of the androgen receptor (Batch 400103, Novocastra, Newcastle, U.K.). Rat prostate, which is known to contain the antigen, acted as the positive control.

Sections were dewaxed in xylene, rehydrated in graded ethanol solutions, then placed in tap water followed by distilled water for one minute. Slides with sections were placed horizontally in a metal staining rack, submerged in 0.01 M sodium citrate buffer (pH 6.0) and heated to 100°C in a pressure cooker for one minute and then left to stand in distilled water for a minute. Slides were transferred to slide mould trays. Three to four drops of 0.01 M phosphate buffered saline (PBS: pH 7.2) were gently flooded onto each section with a pipette and left for five minutes. It was found in preliminary trials that flooding the slides with the various solutions, as opposed to washing the slides, had to be done to avoid damaging the tissue.

All sections were "blocked" for five minutes with bovine serum albumin (BSA, Lot 86H1367, Sigma, St. Louis, MO, USA) diluted 1:100 with PBS to reduce non-specific binding. All slides except the negative control were then rinsed three times by flooding with PBS and leaving for one minute. This was the standard rinsing procedure. Sections were then incubated overnight at 4°C with the primary antibody diluted 1:20 in 1% BSA in PBS. Control sections (adult rat prostate and fetal mammary gland) were processed by omitting the primary antibody.

Next day the sections were rinsed with PBS and incubated for 30 minutes in a humid atmosphere with biotinylated donkey anti-rabbit immunoglobulin (Batch 93825, Amersham Life Science) diluted 1:200 in 1% BSA in PBS. Sections were rinsed with PBS and then incubated with streptavidin biotinylated horseradish peroxidase complex (Batch 122645, Amersham, Buckinghamshire, UK) for 15 minutes. Sections were rinsed again and incubated in a solution containing 4 mg 3,3' Diaminobenzidine (DAB Lot 60K0820, Sigma, St. Louis, MO.), 10 µl hydrogen peroxide activator and one drop of heavy metal intensification (HMI) in 10 ml PBS until the colour reaction product developed (a brown colour, after 20-60 seconds). The reaction was halted by rinsing the slides in PBS, then in tap water. The sections were dehydrated in 70%, then absolute alcohol, cleared in xylene, and mounted using DPX mountant and a glass cover-slip.

Oestrogen receptor was also located histochemically in the sections using the high temperature antigen unmasking technique. Rabbit uterus, which is known to contain the antigen, and fetal mammary gland, acted as the controls. The primary antibody for the oestrogen receptor analysis was a mouse monoclonal (NCL-ER-6F11) raised to the full-length alpha form of the oestrogen receptor molecule (Batch 115140, Novocastra, Newcastle, UK). This antibody was

diluted 1:80 in 1% BSA in PBS. The secondary antibody was biotinylated species-specific anti-mouse immunoglobulin (Amersham Life Science) diluted 1:200 in 1% BSA in PBS. The methodology was similar to that described for androgen receptor.

Slides for androgen and oestrogen receptors were examined for intensity of immunohistochemical staining using a differential interference contrast (DIC) microscope (Axiophot, Zeiss, West Germany). Staining was subjectively estimated and expressed as negative (-), weakly positive (+), moderately positive (++), or strongly positive (+++). Photomicrographs of the slides were obtained using a digital camera (SPOT RT, Diagnostic Instruments inc., Sterling Heights, MI U.S.A.) and a differential interference contrast (DIC) microscope (Axiophot, Zeiss, West Germany). Images were processed with the RT camera and Image-Pro PLUS, version 4.1 for windows (Media Cybernetics, Maryland, U.S.A.).

## 3.4 Results

To localise androgen and oestrogen receptors to specific cell types, immunohistochemistry was performed on paraffin-embedded sections of mammary gland tissue from the same fetal sheep that were used in the ontogeny trial (Chapter two). Due to the small amount of mammary tissue present at days 40 and 60 of fetal age, and the angle of the teat at later stages, the cells were sometimes sectioned more obliquely, as opposed to square-on, hence the discontinuous pattern of immunoreactivity exhibited by some ducts. Positive immunoreactivity was confirmed when staining was present beyond the actual surface of the cells, and the cells stained darker than the surrounding tissue. The skin and sweat glands of the mammary gland acted as a control for edge artefact. Three reaction patterns were recognised:

1. linear cell surface – intense staining of the cell membrane (apical plasmalemma) and adjacent cytoplasm. Usually discontinuous (stop/start) around the luminal surface of the duct.
2. cytoplasmic – staining of the apical cytoplasm with or without obvious reactivity of the cell membrane or nuclear membrane. Usually continuous around the luminal surface of the duct.
3. nuclear – staining of the cell nucleus.

The distribution of androgen and oestrogen receptors in the mammary gland at days 40, 60, 80, 100, 120 and 140 of gestation are presented in the Appendices (Tables A.1 and A.2).

### 3.4.1 Ontogeny of androgen receptor within the developing mammary gland

Rat prostate was used as a control tissue for immunoreactivity to androgen receptor antibody as it is known to contain the antigen. No immunoreactivity was visible in the negative control (Plate 3.1). Omission of the primary antibody in fetal mammary tissue also resulted in no visible

immunoreactivity. Addition of the primary antibody to the rat prostate resulted in strong nuclear staining of the stromal cells. Some nuclear staining was observed in the secretory epithelial cells (no photomicrograph shown).

At day 40 of fetal age, the mesenchymal cells of the developing mammary gland displayed a well-defined envelope of immunoreactivity to androgen receptor antibody around the mammary bud and its stalk (Plate 3.2). However, one female exhibited immunoreactivity beyond the well-defined sub-population of cells surrounding each bud, in the more distant dermal mesenchyme. Although the distribution of receptor-positive mesenchymal cells close to the mammary bud was similar in males and females, the intensity of staining varied amongst individual animals within and between the sexes.

The epithelial and mesenchymal cells of the developing 60-day-old fetal mammary gland showed no reaction to androgen receptor antibody in either sex (data not shown).

Immunoreactivity to androgen receptor antibody was present in the epithelial cells of the teat canal and secondary ducts at day 80 of fetal age. In the male, staining extended from the lumen surface to deep in the apical cytoplasm. In the secondary ducts reactivity was observed as a dark brown halo around the lumen edge (Plate 3.3). For most of the tissue from female fetuses analysed at this age, staining was less intense compared with that in the male and the line of reactivity was not always continuous around the lumen edge of the duct (Plate 3.4). The pattern and intensity of staining in the female gland varied from light staining of the cell surface to intense staining of the apical cytoplasm. A feature of the male gland was the extensive nuclear staining in the cells of the fibroblasts that surrounded the ducts (Plate 3.3).

The pattern of immunoreactivity in the male at day 100 was very similar to that observed at day 80. However, one male exhibited an intense immunoreactivity to androgen receptor antibody that was localised to the nucleus of the ductal epithelial cells (Plate 3.5). In general, the female ducts continued to stain less strongly than the male ducts, although the intensity of staining varied greatly between individuals. Some females displayed very patchy linear cell surface reactivity (Plate 3.6), while others showed a similar intensity of staining to that seen in the male. Nuclear staining in the cells of the fibroblasts, which resided in close contact with the ducts, was commonly seen in both sexes, although the intensity of staining observed in the females was much weaker than that seen in the males.

At day 120 the nuclei of the fibroblasts in the connective tissue that surrounded the growing ducts of the male gland continued to stain strongly for androgen receptor, while negative reactivity was observed in the females. All four males displayed deep cytoplasmic staining of the epithelial cells and, although the intensity of staining varied, reactivity occurred almost continuously around the lumen edge of the ducts. The nuclei of the ductal epithelial cells were slightly stained in two of the males (Plate 3.7). All females exhibited patchy cell surface immunoreactivity to androgen receptor antibody, but there was no nuclear staining of the epithelial cells (Plate 3.8).

For most of the sections of mammary gland tissue from male fetuses analysed at day 140 (term = 147 days) immunoreactivity to androgen receptor antibody was weaker in the epithelial cells and in the fibroblasts of the connective tissue compared to that exhibited at days 80, 100 and 120 (no photomicrographs shown). Reactivity in the epithelial cells was variable in the females. Of the four female glands examined, two showed no staining of the epithelial cells, one showed patchy cell surface immunoreactivity and one exhibited continuous staining around the duct with reactivity localised in the cytoplasm and nucleus of the epithelial cells (no photomicrograph shown).

In summary, androgen receptors were found in the fetal mammary tissue at all stages examined, except day 60. The pattern of immunostaining in the mesenchymal cells was similar between the sexes, always appearing in the cell nuclei, although the intensity of staining was greater in the male than in the female gland. Males also tended to exhibit immunoreactivity in the cytoplasm of the epithelial cells rather than along the cell surface, while linear cell surface staining was a common reaction pattern amongst the females. Whereas the pattern of immunoreactivity was similar between individual males and females, the intensity of immunostaining varied greatly, particularly in the epithelial cells.

Androgen receptor immunoreactivity was strongest in the mesenchymal and epithelial tissues of the male mammary gland at days 80, 100 and 120 of fetal age, and in the female at days 80 and 100. However, the cells of the female mammary gland frequently stained less intensely than those of the male at each stage of gestation where androgen receptors were present.

**Plate 3.1** Photomicrograph of the negative control (rat prostate) for the localisation of androgen receptor in fetal sheep mammary gland tissue. Fetal mammary gland tissue was incubated in the absence of primary antibody to androgen receptor. Note the lack of immunoreactivity. (Mag x400).

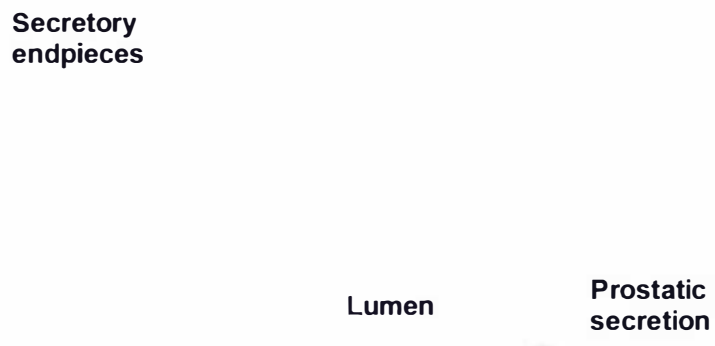



**Plate 3.2** Photomicrograph of the localisation of androgen receptors in the mammary bud of an ovine female at day 40 of fetal age. Only the few mesenchymal cells around the basement membrane show immunoreactivity to androgen receptor antibody. (Mag x400).

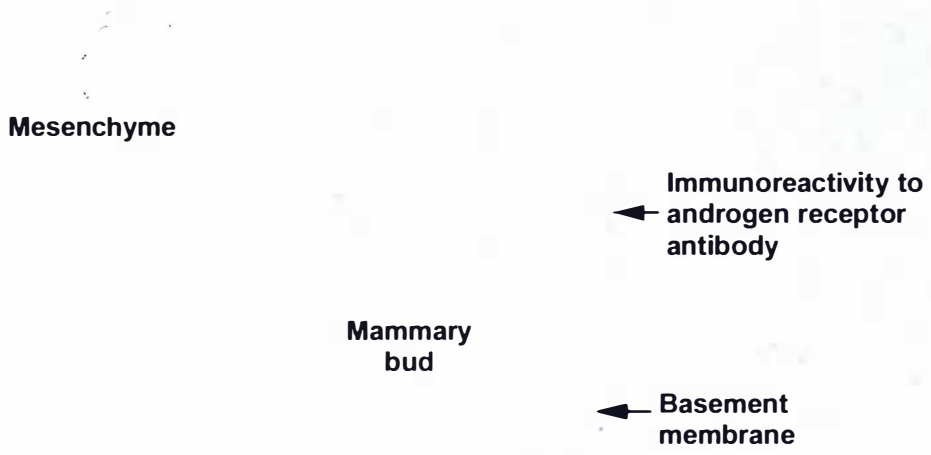


**Plate 3.3** Photomicrograph of the localisation of androgen receptors in the ducts and the dermal mesenchyme of the mammary gland of an ovine male at day 80 of fetal age. Staining is restricted to the apical surface of the luminal epithelial cells and the nuclei of the fibroblasts. (Mag x400).

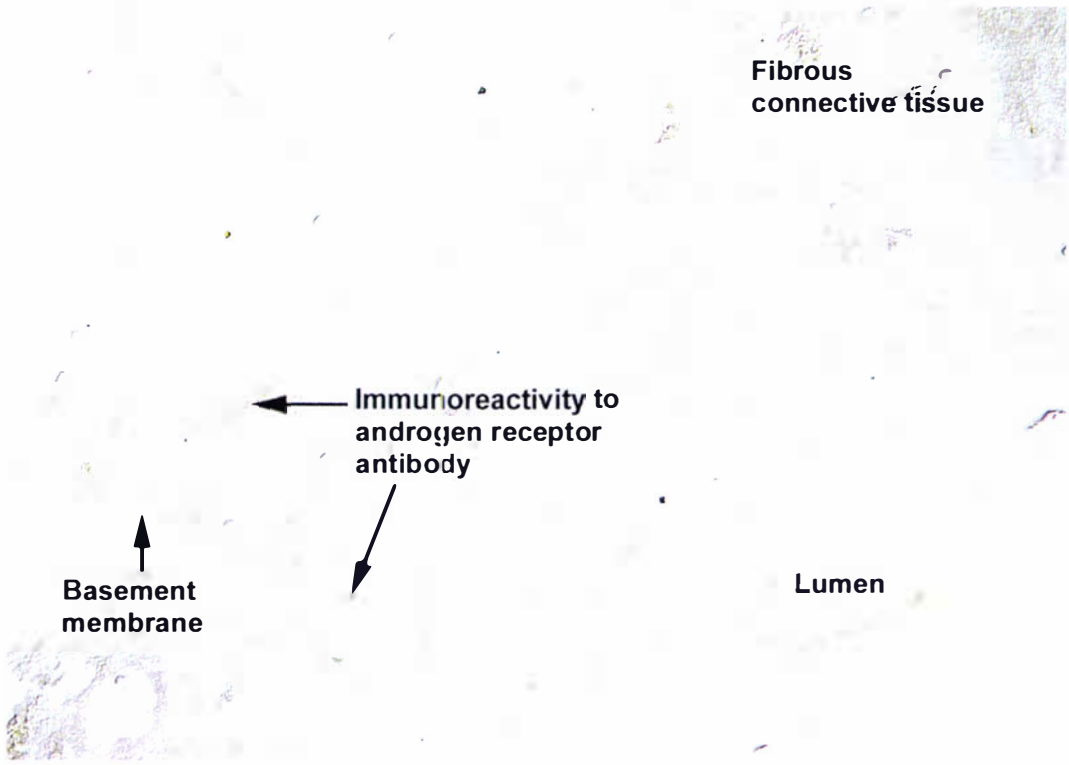



**Plate 3.4** Photomicrograph of the localisation of androgen receptor in the mammary gland of an ovine female at day 80 of fetal age. (Mag x400).

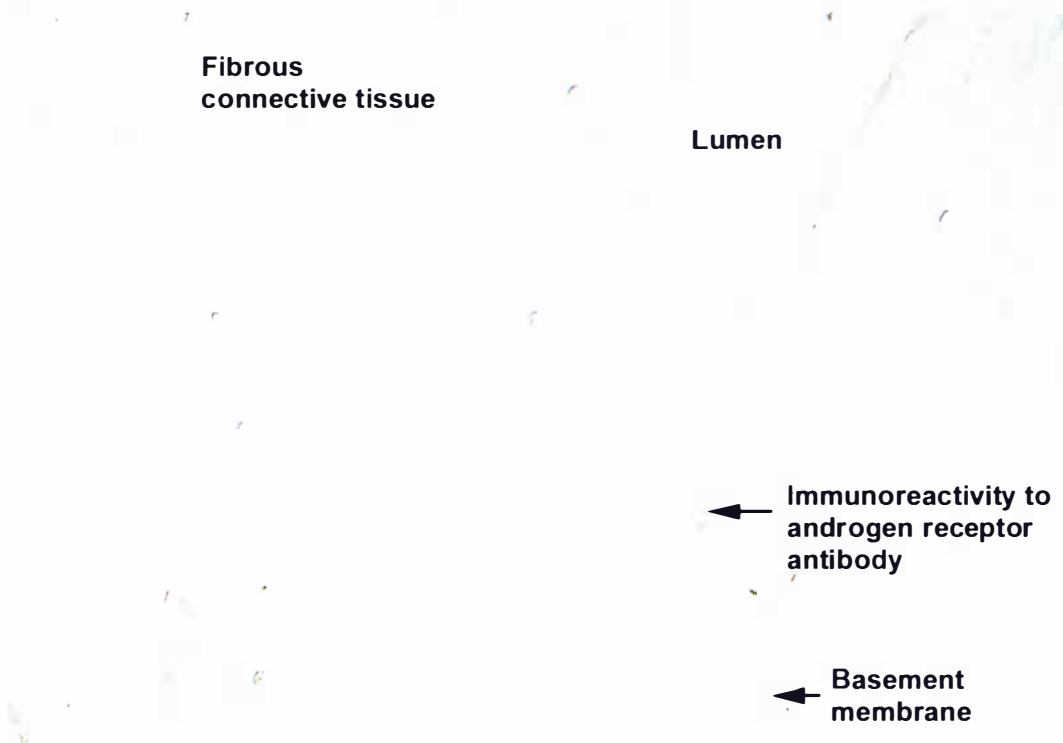


**Plate 3.5** Photomicrograph of the localisation of androgen receptors in the ducts of one ovine male mammary gland at day 100 of fetal age. Staining in the nuclei of the epithelial cells. (Mag x400).

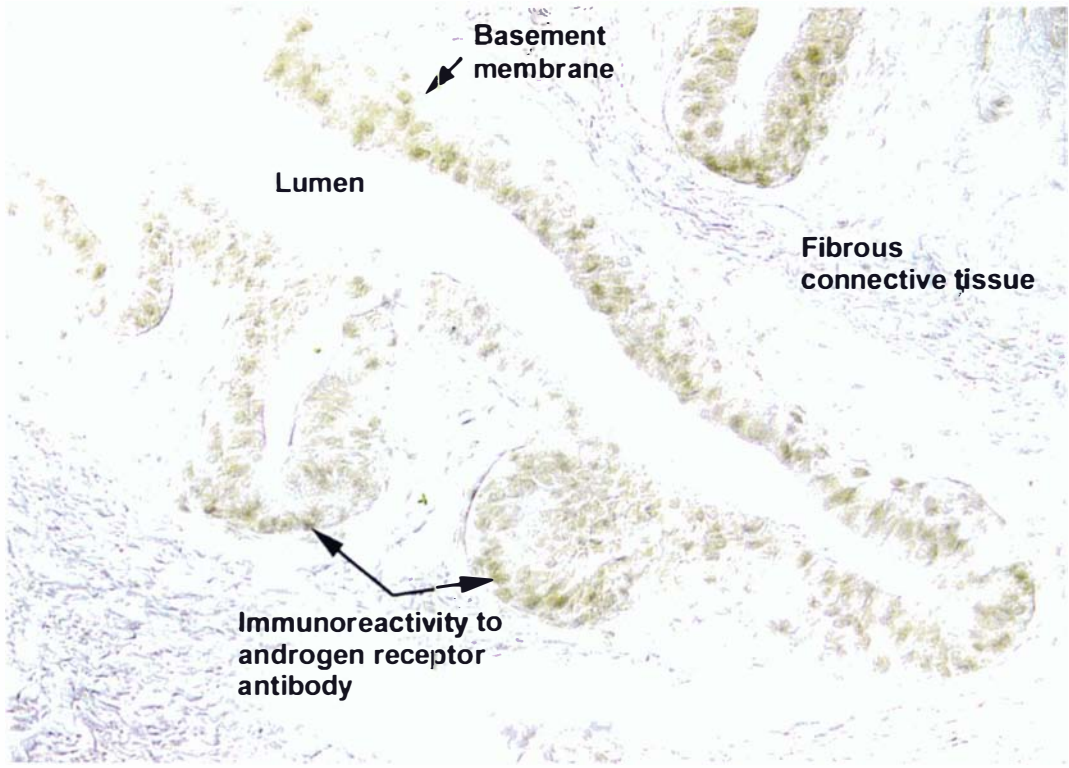



**Plate 3.6** Photomicrograph of the localisation of androgen receptors in the ducts of an ovine female mammary gland at day 100 of fetal age. Staining is restricted to the apical surface of the luminal epithelial cells. (Mag x400).

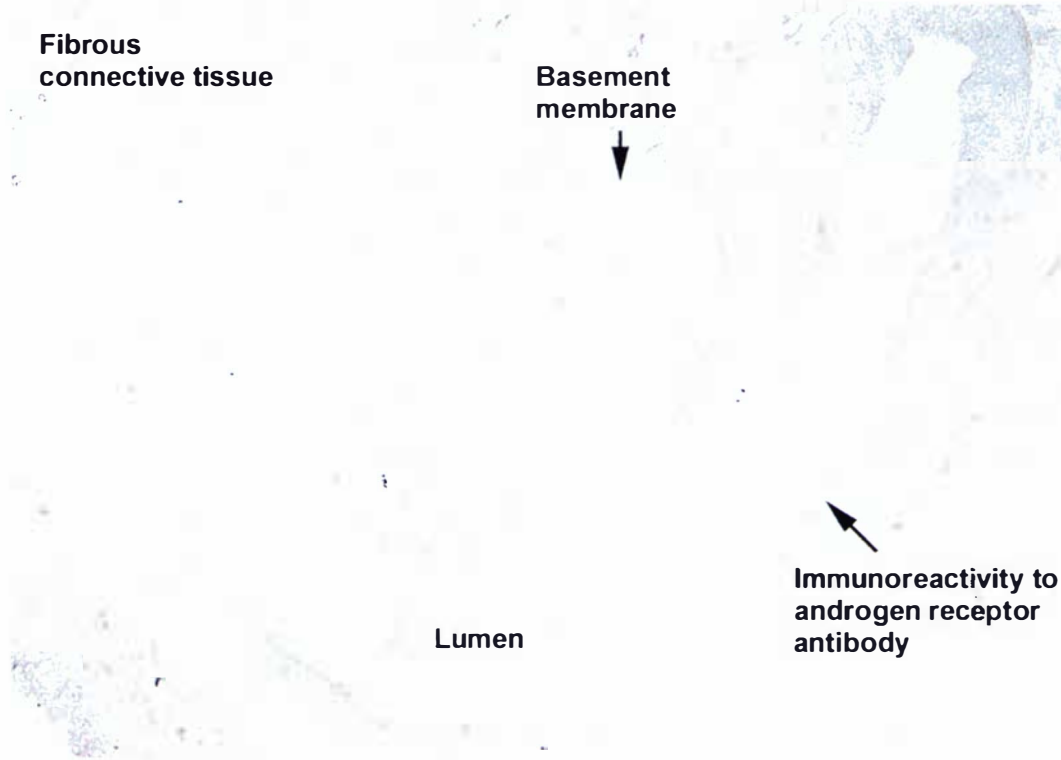


**Plate 3.7** Photomicrograph of the localisation of androgen receptors in the ducts of an ovine male mammary gland at day 120 of fetal age. Staining occurs beyond the cell surface, moving into the nuclei. The fibroblasts in the surrounding mesenchyme exhibit nuclear staining (Mag x400).

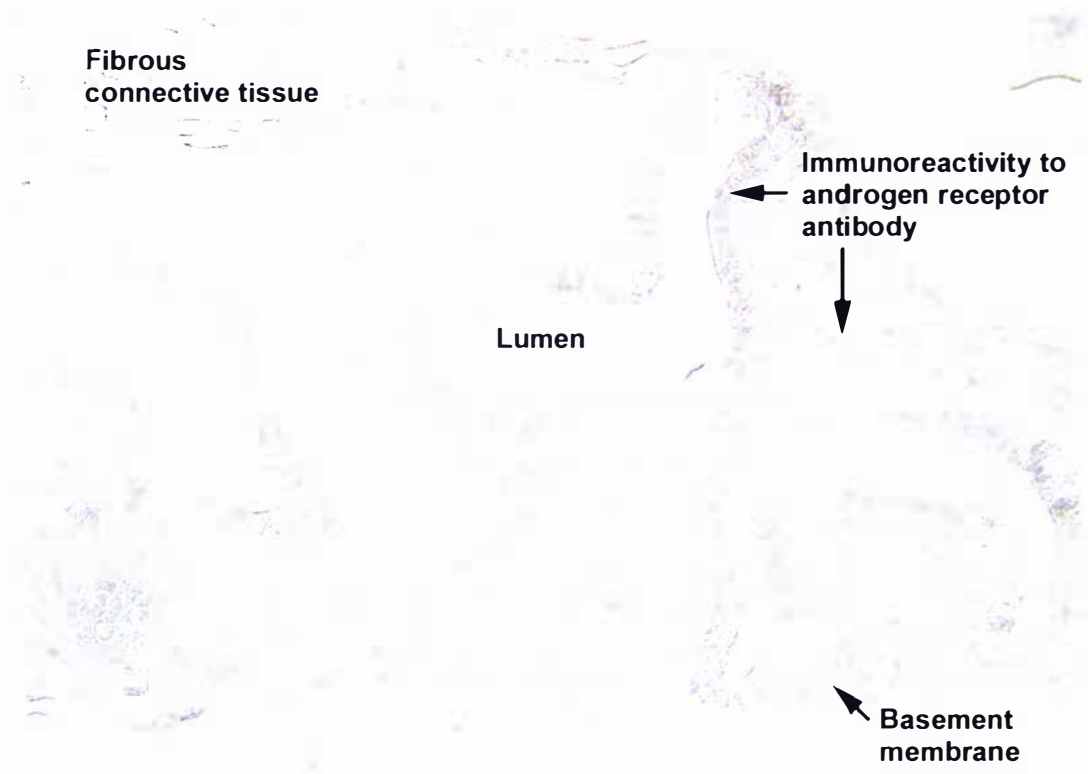
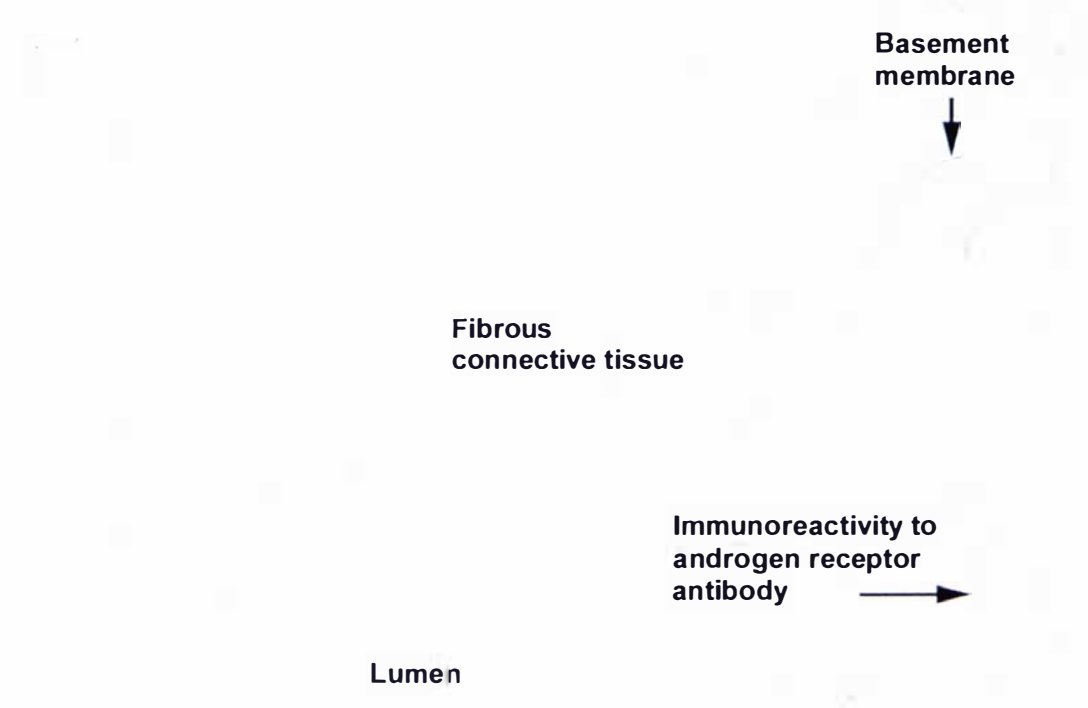



**Plate 3.8** Photomicrograph of the localisation of androgen receptors in the ducts of an ovine female mammary gland at day 120 of fetal age. (Mag x400).



### 3.4.2 Ontogeny of oestrogen receptor within the developing mammary gland

The rabbit cervix, which acted as a control, did not exhibit immunoreactivity to oestrogen receptor antibody when the primary antibody was omitted from the immunohistochemical procedure (Plate 3.9). Addition of the primary antibody revealed staining of the fibroblastic nuclei, the apical plasmalemma of the luminal epithelial cells and the ciliated columnus cells.

In male and female fetal sheep, at day 40 of gestation, a very distinctive line of immunoreactivity to oestrogen receptor antibody was seen around the basement membrane of the mammary bud and its stalk (Plate 3.10). Only the few mesenchymal cells that were concentrated around the mammary bud possessed oestrogen receptor. None of the samples exhibited immunoreactivity beyond this well-defined sub-population of mesenchymal cells.

Extensive nuclear staining was observed in the fibroblasts at day 60 of fetal age (Plate 3.11). In both sexes, oestrogen receptor positive cells in the largely undifferentiated connective tissue were distributed evenly throughout the mesenchyme without any specific pattern of distribution or association with the epithelial cells of the developing secondary ducts.

In contrast to the strong immunoreactivity of the mesenchymal cells, the epithelial cells were non-reactive in the mammary glands of both sexes. (Plate 3.12).

One out of five female mammary glands showed immunoreactivity to oestrogen receptor antibody in the epithelial cells of the ducts at day 80 of fetal age. Staining was confined to the apical cytoplasm of the cells and formed a continuous colour band around the luminal perimeter of the duct (Plate 3.13). The epithelial cells remained non-reactive in the other females and in all four males. Similarly to day 60, nuclear staining was observed in the fibroblasts throughout the differentiating fibrous connective tissue (Plate 3.13). Furthermore, the interlobular connective tissue of the developing adipose tissue of the female who showed staining of the ductal epithelial cells also exhibited weak, yet positive, immunoreactivity (no photomicrograph shown).

Immunoreactivity to oestrogen receptor antibody was observed in the epithelial cells of the mammary ducts in males and females at day 100 of fetal age. The pattern of reactivity in the ducts from females ranged from linear cell surface through apical cytoplasmic and nuclear staining (Plate 3.14). A similar pattern appeared in the male although the linear epithelial cell surface staining was weaker than that seen in the female. The nuclei of the epithelial cells of one male were very clearly immunoreactive (Plate 3.15).

The nuclei of the fibroblasts continued to show reactivity in both sexes although staining was less intense in the male gland. In the females, the fibroblasts that displayed reactivity were generally found in close association with the epithelial cells of the lateral ducts. However, the differentiating mesenchymal cells of the deep hypodermis of one female, in particular, exhibited immunoreactivity to oestrogen receptor antibody (Plate 3.16). It was difficult to establish whether the cells that were staining were fibroblasts or adipoblasts.

At day 120 of fetal life the female mammary gland showed the same range in immunoreactivity to oestrogen receptor antibody as observed at day 100. In two females, reactivity was confined to the cell surface but was patchy and formed a discontinuous line around the lumen edge. An example of this pattern of reactivity is portrayed in Plate 3.17. Reactivity in the male ducts was limited to the luminal surface of the epithelial cells. Both sexes continued to exhibit nuclear staining of the fibroblasts that enveloped the mammary ducts although the intensity of staining was much weaker in the male. Similarly to day 100, one of the five female glands showed reactivity in the differentiating cells of the developing adipose tissue. Once again, positive identification of those cells could not be established.

Immunoreactivity to oestrogen receptor antibody was observed through the whole depth of the cytoplasm and around the nuclear membrane of the epithelial cells in two of the four female glands at day 140 of fetal age (Plate 3.18). However, as reported at previous stages, there was variability in the distribution and intensity of staining between individual animals. One female exhibited reactivity in the apical cytoplasm of the epithelial cells while patchy epithelial cell surface staining was seen in another female. Variability also occurred in the male, where immunoreactivity was either negative or observed as patchy cell surface staining. Furthermore, while the nuclei of the adjacent fibroblasts continued to stain in the female, they were non-reactive in the male.

Immunoreactivity was not observed in the cells of the adipose tissue or in the interlobular connective tissue of the fat pad at day 140.

In summary, oestrogen receptors were present in the mesenchymal cells of the mammary tissue at all stages examined, except in the male, where they were non-reactive at day 140. Immunoreactivity in the epithelial cells was first observed at day 80, but only in one female. At days 100, 120 and 140 both males and females exhibited oestrogen receptor immunostaining in the epithelial cells, although there was variability in the distribution and intensity of staining between individual animals. However, there was a tendency for females to show a greater intensity of staining in the epithelial and mesenchymal cells relative to that seen in the male. Similarly to the pattern of immunostaining for androgen receptor in the mesenchymal cells, oestrogen receptor staining was restricted to the cell nuclei. Immunoreactivity was occasionally observed in the cells of the fibrous connective tissue that enveloped the developing adipose tissue, but only in the female gland.

**Plate 3.9** Photomicrograph of the negative control (rabbit cervix) for the localisation of oestrogen receptor in fetal sheep mammary gland tissue. Fetal mammary gland tissue was incubated in the absence of primary antibody to oestrogen receptor. Note the lack of immunoreactivity. (Mag x400).

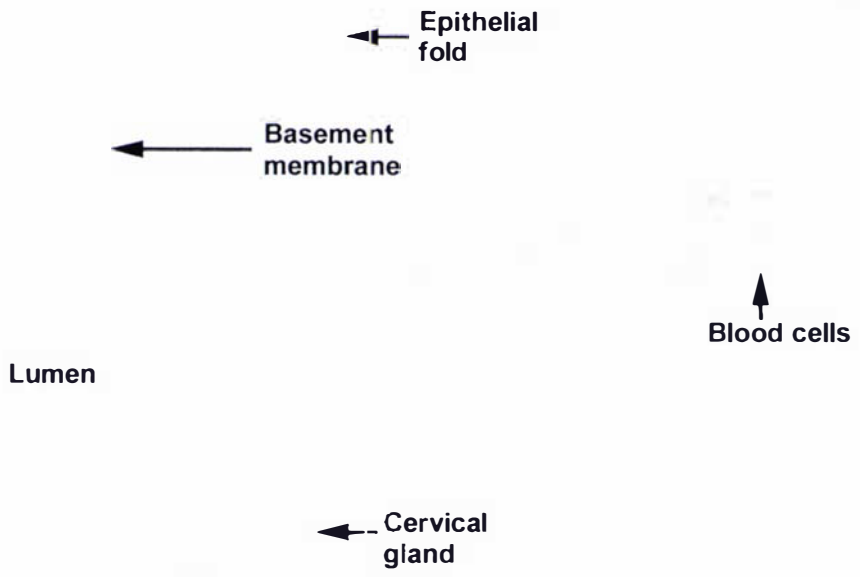



**Plate 3.10** Photomicrograph of the localisation of oestrogen receptors in the mammary bud of an ovine female at day 40 of fetal age. Only the few mesenchymal cells around the basement membrane show immunoreactivity to oestrogen receptor antibody. (Mag x400).

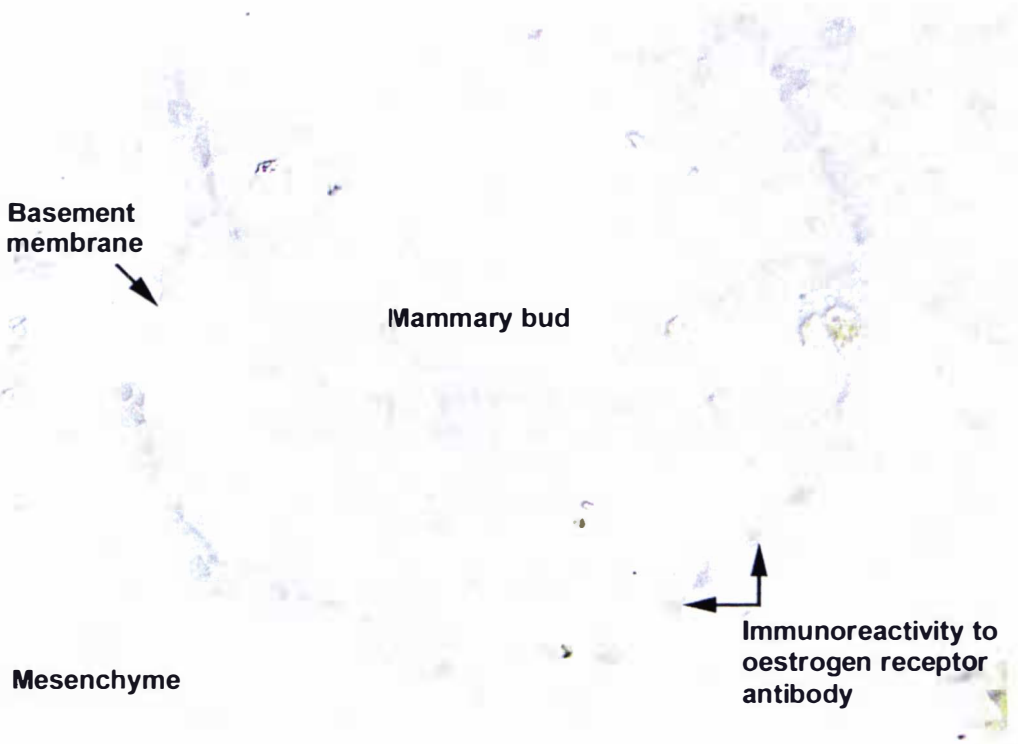


**Plate 3.11** Photomicrograph of the localisation of oestrogen receptor in the dermal mesenchyme of the mammary gland of an ovine female at day 60 of fetal age. (Mag x400).

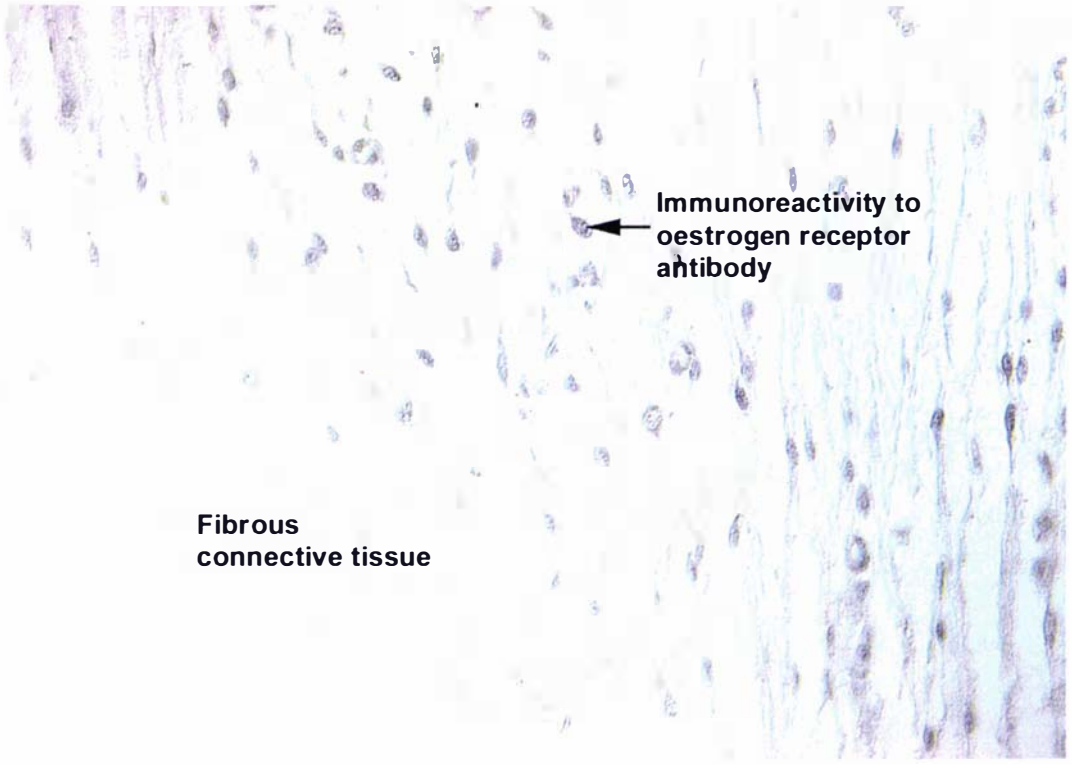



**Plate 3.12** Photomicrograph of the localisation of oestrogen receptors in secondary ducts of the mammary gland of an ovine female at day 60 of fetal age. Note the absence of immunostaining in the epithelial cells. (Mag x400).

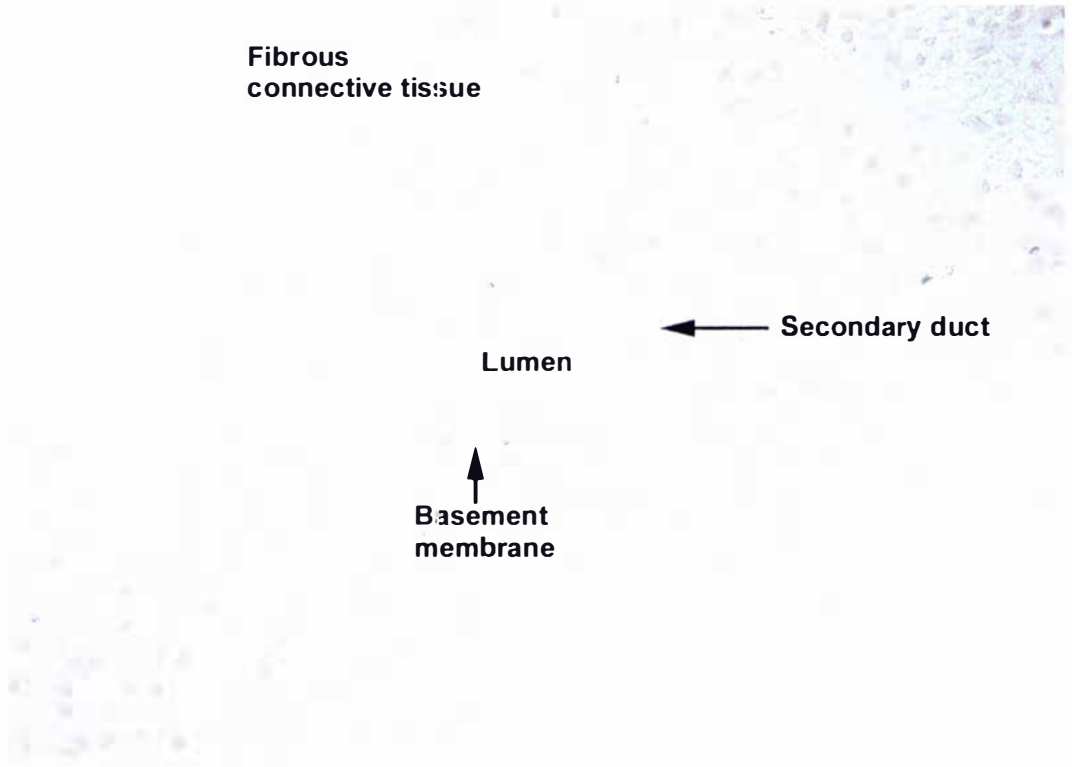


**Plate 3.13** Photomicrograph of the loca isation of oestrogen receptors in secondary ducts of the mammary gland of an ovine female at day 80 of fetal age. Staining is localised in the apical cytoplasm of the luminal epithelial cells. The fibroblastic nuclei are also immunoreactive. (Mag x400).

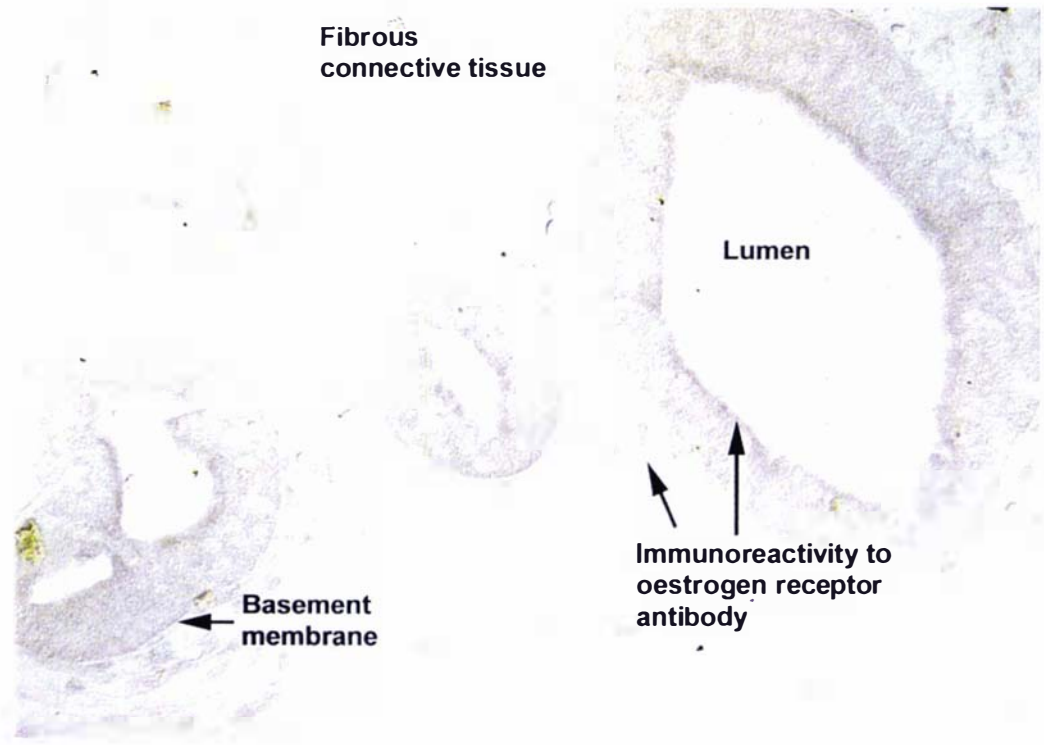



**Plate 3.14** Photomicrograph of the localisation of oestrogen receptors in the ducts of the mammary gland of an ovine female at day 100 of fetal age. Staining is localised in the apical cytoplasm of the luminal epithelial cells. (Mag x400).

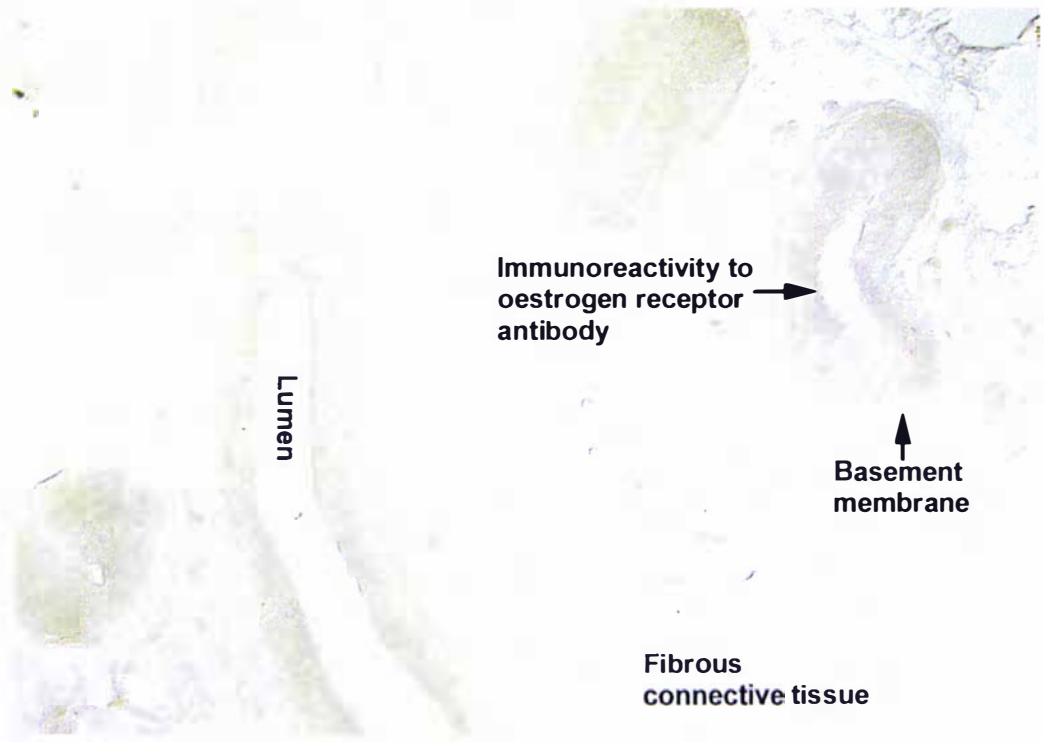


**Plate 3.15** Photomicrograph of the localisation of oestrogen receptors in the ducts of the mammary gland of one ovine male at day 100 of fetal age. Staining is localised in the epithelial cell nuclei. (Mag x400).

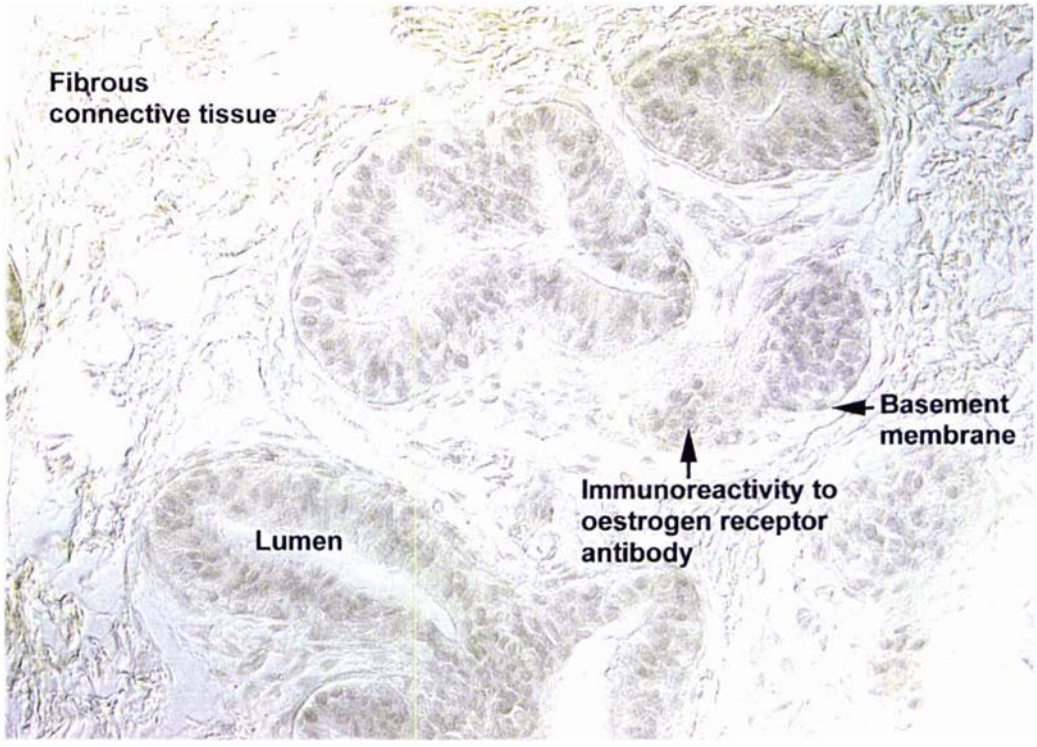



**Plate 3.16** Photomicrograph of the localisation of oestrogen receptors within the developing adipose tissue of the mammary gland of one ovine female at day 100 of fetal age. Note the nuclear staining of the fibroblasts that surround the growing ducts and the staining of the differentiating mesenchymal cells within the developing adipose tissue. (Mag x400).

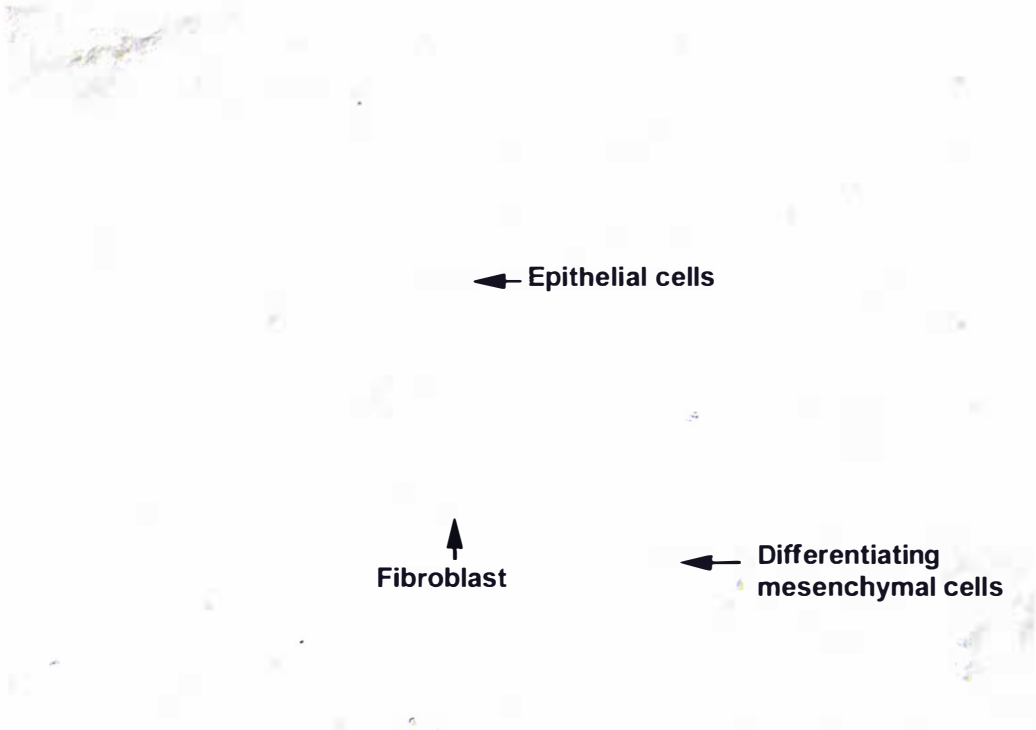


**Plate 3.17** Photomicrograph of the localisation of oestrogen receptors in the ducts of the mammary gland of an ovine female at day 120 of fetal age. Note the intense staining along the luminal surface of the epithelial cells. (Mag x400).

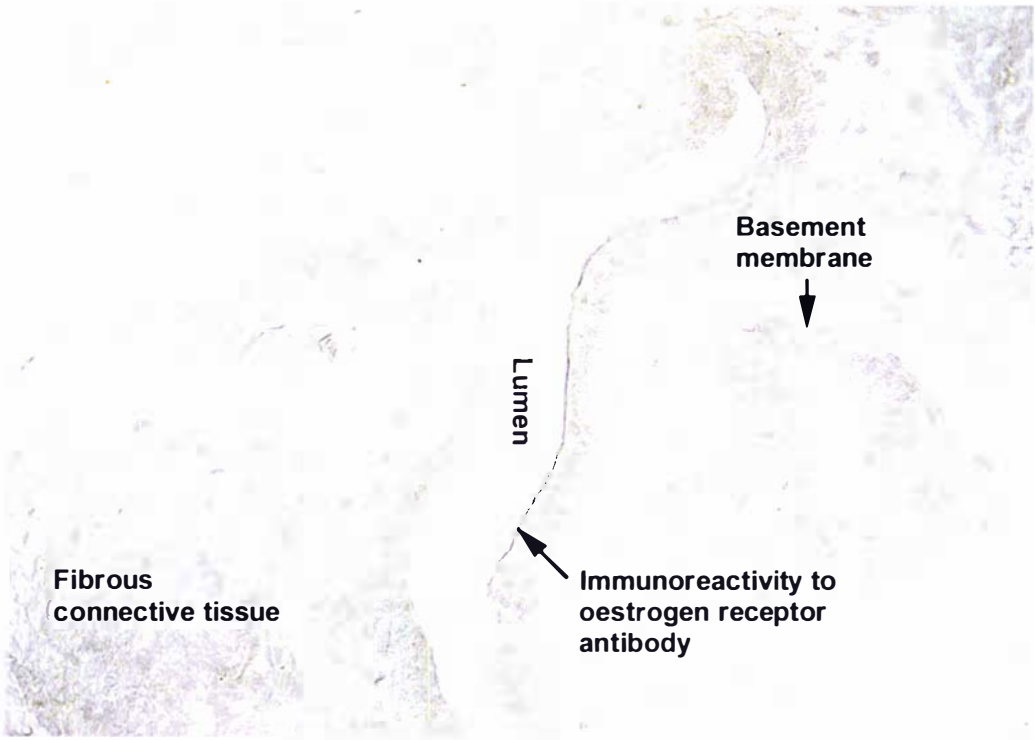
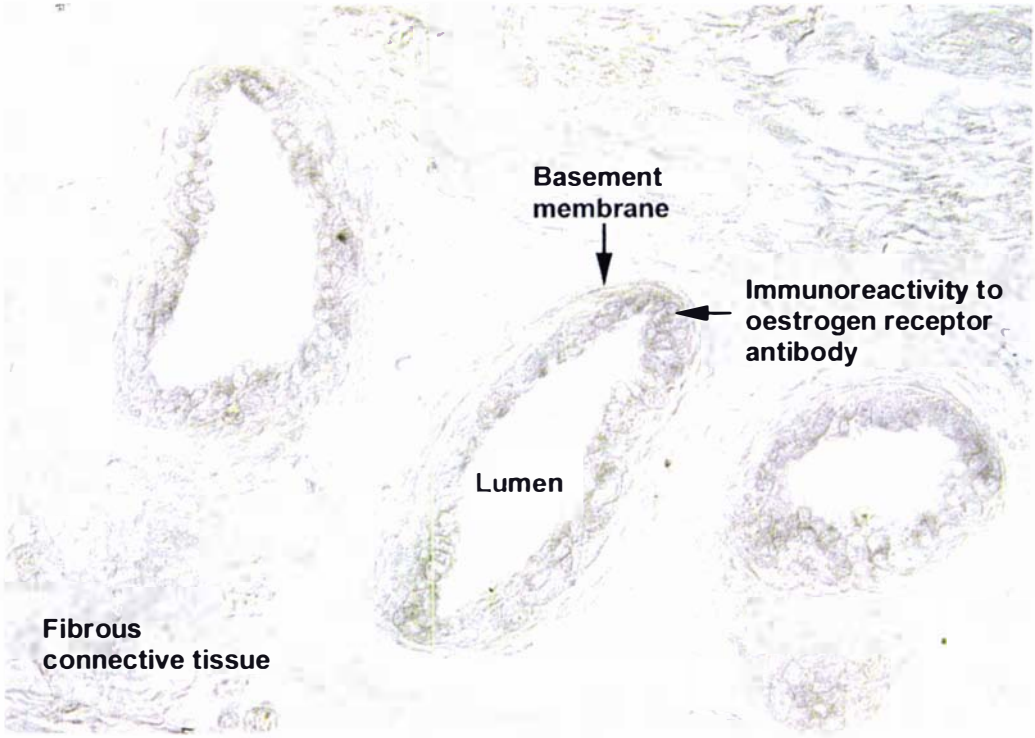



**Plate 3.18** Photomicrograph of the localisation of oestrogen receptors in the ducts of the mammary gland of an ovine female at day 140 of fetal age. Perinuclear cytoplasmic staining of the epithelial cells. (Mag x400).



## 3.5 Discussion

Studies in rodent models have shown that androgen and oestrogen receptors are present in the mammary gland during prenatal development. This study is the first report, to my knowledge, outlining the ontogeny of these steroid hormone receptors in the developing ovine mammary gland.

**Presence and location of androgen and oestrogen receptors**

Androgen and oestrogen receptors were present in the mesenchymal cells from day 40 and in the epithelial cells from day 80, of fetal age. In the mesenchymal cells, immunoreactivity was restricted to the nucleus whereas the epithelial cells displayed mainly cytoplasmic staining. Androgen receptors were present in the nuclei of the epithelial cells in one of four males at day 100 and two of four males at day 120 of fetal age. Oestrogen receptors were present in the nuclei of the epithelial cells in one of four males at day 100 and one of five females at each of days 100 and 120 of fetal age. In an extensive review of the literature, Yamashita (1998) concluded that "steroid hormone receptors (SHR) are basically localised in the nucleus, regardless of hormonal status, and that considerable amounts of unliganded SHR may be present in the cytoplasm of target cells in exceptional cases". Nevertheless, there are still divergent views on the intracellular distribution of steroid hormone receptors. According to Hager (2000) steroid hormones come in three varieties, nuclear, cytoplasmic or both, based on where they reside in the cell when unbound by hormone. Oestrogen receptors are nuclear proteins, whereas androgen receptors localise in the cytoplasm. However, it is well known that SHR shuttle continuously between the nucleus and cytoplasm independent of hormonal status (Perrot-Applanat et al., 1992). Nucleocytoplasmic shuttling suggests that nuclear proteins such as the oestrogen receptor may exert specific biological activities within the cytoplasm, and hence, may play a role in the growth and development of the ovine mammary gland. On the other hand, it may be that the mainly cytoplasmic immunostaining of the epithelial cells of the fetal ovine gland is one of those "exceptional" cases.

At day 60 of fetal age, the epithelial cells of the primary duct and the few secondary ducts present did not exhibit immunoreactivity to androgen and oestrogen receptor antibodies. However, as demonstrated by Korach et al. (1988) and Greco et al. (1991), the absence of immunohistochemical and autoradiographic reactions of sex steroid hormone receptors in the fetal and early neonatal period does not necessarily mean the complete absence of receptors in the cells. It may indicate that their concentration is below the detection limit of these techniques. Alternatively, the lack of immunoreactivity in the epithelial cells early in gestation may reflect a developmental phenomenon. Receptor appearance and disappearance may coincide with increasing and decreasing circulating concentrations of steroid hormones within the fetus (discussed below with respect to the ontological development of each steroid hormone receptor examined).

**Ontogeny of androgen receptor in the fetal mammary gland**

The results of this study demonstrated that the mesenchymal cells of the developing mammary glands of male and female fetuses showed immunoreactivity to androgen receptor antibody at day 40 of gestation – the earliest stage examined. Receptors were detectable at mammary bud formation and were, with the exception of one female, exclusively localised to the few layers of mesenchymal cells surrounding each epithelial bud and its stalk (Plate 3.2). This is similar to the distribution observed in rodents (Heuberger et al., 1982; Kratochwil, 1985). The close proximity of mesenchymal cells containing receptors to the bud would suggest, as demonstrated in the mouse with epithelium-mesenchyme recombination cultures, that the mammary epithelium had induced the binding sites in the mesenchyme (Kratochwil and Schwartz, 1976; Dürnberger and Kratochwil, 1980; Heuberger et al., 1982).

In the male mouse, receptors decide the fate of the gland and sexual development in response to circulating hormones. At day 14.5 of fetal life (76% of gestation length), testosterone, upon binding to the mesenchymal androgen receptors that surround the bud, causes the complete or partial destruction of the epithelial bud. This degree of sexual dimorphism in the prenatal development of the mammary gland does not occur in the ruminant. The mammary bud of the male sheep continues to differentiate and penetrate the deeper layers of mesenchyme during the first two trimesters of gestation although, morphologically, its development lags behind that of the female gland (Chapter two). However, there is little further development of mammary epithelium, connective tissue or adipose tissue from day 120 of gestation (82% of gestation length) through to day 21 of postnatal age while the female gland continues to increase in size and complexity with advancing age (Chapter two). Whether the differential patterns in mammary growth and development seen in male and female fetal sheep are caused by androgens secreted by the male testes is not known.

Sexual differentiation in lambs occurs within a broad critical period of approximately 30 to 100 days of the 147-day gestation (Short, 1974; Clarke et al., 1976). However, each sexually dimorphic trait, such as reproductive anatomy, physiology, behaviour, and neuroendocrine function, is presumed to have an individual period of sensitivity to steroid hormones (Clarke et al., 1976; Wood and Foster, 1998). Testosterone is produced by the gonads of the ovine male from about day 30 of fetal age (Attal, 1969; Mauleon, 1977; Lun et al., 1998) and gonadal concentrations are highest during the first half of gestation (Pomerantz and Nalbandov, 1975). Hence, testosterone is capable of regulating masculinisation of the mammary gland at day 40 because androgen receptor is expressed in the mammary tissue at this stage. However, the mammary gland must be sensitive to testosterone for developmental changes to occur. In the male mouse, there exists only a short androgen-responsive "window" during mammary development. Once the bud is severed from the epidermis, the mammary gland loses its responsiveness to testosterone (Kratochwil, 1977). Nevertheless, as shown in autoradiographs of glands from 16-day fetuses (Heuberger et al., 1982), and new born mice (Wasner et al.,

1983), the population of receptor-rich mesenchymal cells persists beyond the hormone-responsive stage and remain stationary, located predominantly within the nipple area.

In males and females at day 60 of fetal age the epithelial and mesenchymal cells of the developing mammary gland showed no reaction to androgen receptor antibody. Between days 35 and 48 of gestation, the ovine mammary bud undergoes a resting phase where growth is slow and there is little or no morphogenetic activity (Martinet, 1962). This developmental phase is apparently intrinsic to the epithelium as demonstrated in the mouse by separating mammary epithelium from mesenchyme and recombining epithelia of different ages (Kratochwil, 1969). It is possible that following the resting phase (about day 50), the ovine mammary bud induces expression of androgen receptors in the surrounding mesenchymal cells, which are initially undetectable but sharply increase in their distribution and intensity as gestation progresses. The appearance of mesenchymal androgen receptors at day 80 and their location in the dermis suggest a role for the androgen receptor in the suppression of further differentiation of the fibrous connective and adipose tissues in the male.

In the ovine male, at day 80 of gestation, the epithelial cells of the teat canal and growing ducts, and the fibroblasts that surrounded the ducts, exhibited moderately to strongly positive intensity of immunohistochemical staining for androgen receptor antibody. This is compatible with the observations of Martinet (1962) and the present study that sexual dimorphism in mammary gland development became apparent at days 70 and 80, respectively. Interestingly, the rete testis stains intensely for androgen receptor at day 70 of gestation and continues to stain at a similar intensity in 100- and 130-day-gestation fetuses (Sweeney et al., 1997). The mammary gland of the male follows a similar trend, with the epithelial and mesenchymal cells continuing to exhibit strong immunoreactivity to androgen receptor antibody at days 100 and 120 of fetal age. The peak in androgen receptor intensity during this time is consistent with the observations that the allometric coefficient for mammary epithelial growth from days 80 to 120 of fetal life is less than one, and that it is close to zero from day 120, both of which would suggest the involvement of androgens. Ovine fetal testicular testosterone is measurable from day 30 (Attal, 1969), increases steadily from days 35 to 75 of gestation (Quirke et al., 2001) then tends to fall in the last 80 days of gestation (Pomerantz and Nalbandov, 1975). Circulating plasma concentrations of testosterone become progressively greater as gestation proceeds in both sexes, although the rise in concentration is of a greater magnitude in the male compared to the female (Pomerantz and Nalbandov, 1975).

Although immunoreactivity for the androgen receptor was seen in the epithelial cells of the female gland, staining was always weaker than that in the male at each stage of gestation examined. By day 140 of gestation, the epithelial cells of two of the four females were non-reactive. Significantly lower plasma testosterone concentrations in the female relative to those in the male in late gestation (Pomerantz and Nalbandov, 1975) may contribute to the decline in the intensity of AR immunostaining seen in the female gland.

These observations would suggest that in the male, testosterone, presumably from the testes, acts directly on:

1. epithelial androgen receptors to prevent further differentiation of the mammary epithelium.
2. mesenchymal androgen receptors to prevent further differentiation of adipose tissue and fibrous connective tissue.

However, it is also possible that growth factors may be involved. Recent reports suggest that epidermal growth factor (EGF) may modulate androgen-dependent sexual differentiation of the fetus (Gupta et al., 1996) as EGF, like androgen, stabilised the Wolffian duct of undifferentiated reproductive tracts of thirteen-day-old female murine fetuses that were grown in organ culture. From these observations, it may be postulated that androgens act via mammary mesenchymal androgen receptors to elicit synthesis and secretion of EGF (or another autocrine/paracrine factor) that regulates mammary epithelial and mesenchymal morphogenesis in the male ruminant. Localisation of EGF receptors in the tissues would support this hypothesis.

**Ontogeny of oestrogen receptor in the fetal mammary gland**

Oestrogen receptors (ORα) were detected in the ovine mammary gland by immunohistochemistry from days 40 to 140 of fetal age. At day 40 (27% of gestation length), immunoreactivity to ORα antibody was confined to the mesenchymal cells in the immediate vicinity of the mammary bud, and the stalk that connects the gland epithelium to the epidermis (Plate 3.16). Receptors were present in males and females. The same situation was found to prevail for the appearance of oestrogen receptors in the rodent gland, whereby both ORα and ORβ were expressed in the mesenchyme surrounding the bud at day 12.5 of fetal life (65% of gestation length) (Lemmen et al., 1999). ORβ was not examined in the present study.

By day 60 of fetal age the fibroblasts of the differentiating dermal mesenchyme displayed strong positive nuclear staining for ORα (Plate 3.17). This was evident in the mammary glands of both sexes. However, while the presence of ORα in the nuclei of the fibroblasts persisted at a similar intensity in the female glands at each stage examined, intensity in the male gland became progressively weaker and by day 140 no immunoreactivity could be detected. In male and female mice, oestrogen receptors have been shown to persist at similar concentrations in the densely populated mesenchymal cells that surround the epithelial cord until at least day 16 of fetal age (Narbaitz et al., 1980). This equates to 84% of gestation length in the mouse, which is consistent with the last recorded appearance of mesenchymal oestrogen receptors in the ovine male at day 120, or 82% of gestation length.

Although restricted to one female, oestrogen receptors were first detected in the epithelial cells of the mammary ducts at day 80 of fetal age. At day 100 (68% of gestation length), all male and female mammary tissue exhibited immunoreactivity to ORα antibody in the epithelial cells. Similarly, in the human mammary gland, Keeling et al. (2000) reported the appearance of ORα in the epithelial cells from the 30$^{th}$ week of gestation (75% of gestation length) onwards. This

phenomenon is not seen in the mouse until day three of postnatal age (Haslam and Nummy, 1992). In cattle, immunohistochemical localisation of oestrogen receptor has only been performed postnatally (Capuco et al., 2000; Capuco et al., 2002).

The lack of appearance of ORα in the epithelial cells of all the tissue examined until day 100 may involve regulation by oestrogens. Notable increases in fetal circulating concentrations of plasma oestrogens commence at about day 70 of gestation and levels rise rapidly between days 90 and 120 of gestation (Findlay and Cox, 1970; Findlay and Seamark, 1973) coincident with the appearance of epithelial ORα in the present study. From a peak at approximately day 120, fetal plasma oestrogen concentrations fall gradually, so by term (day 147) they are approximately the same as those found at 90 days. During the same period, ORα is down regulated in the mammary gland of the male, and, at day 140 very little or no immunoreactivity is observed in the epithelial or mesenchymal cells. In contrast, immunostaining for epithelial and mesenchymal ORα in the female mammary gland remains at a similar intensity to that seen at day 120. Furthermore, while the total area occupied by the mammary ducts in the female gland doubles from day 120 to day 140, very little further development takes place in the male.

Fetal and neonatal development of the mammary gland appears to be oestrogen independent in so far as these early stages of development occur in the oestrogen receptor knockout mouse (ORKOα - transgenic mice that lack expression of ORα) (Korach, 1996). Certainly, the similarity in the pattern of circulating plasma oestrogen concentrations in males and females from mid-gestation until term and the developmental divergence in total duct area suggest that oestrogens do not play a role in ovine fetal mammary gland development. However, the mammary gland of the fetal sheep exhibits both epithelial and mesenchymal oestrogen receptors, which suggests that the proliferating epithelial cells could respond directly to oestrogen. But, in the absence of oestrogen stimulation, or endocrine influences, the stimulation of mammary epithelial cells in the fetus may be mediated by locally produced growth factors. Between days 60 and 80 of gestation, epithelial cells do not exhibit oestrogen receptors, and plasma oestrogen concentrations (Findlay and Seamark, 1973) and gonadal oestradiol-17β content (Quirke et al., 2001) in the fetus are relatively low. However, fetal mammary tissue continues to differentiate and proliferate. In the absence of oestrogen receptors, the rudimentary ductal structure of the ORKO mouse is believed to develop in response to the action of maternal growth hormone (GH) during fetal development (Korach et al., 1996). However, it has been reported that GH is not transported across the placenta from the maternal circulation to the fetus, at least in the rat (Fhölenhag et al., 1994).

In the fetal sheep, the concentration of GH is considerably higher than that in the maternal plasma, from 60 days of gestation (Gluckman et al., 1979). Furthermore, fetal plasma concentrations of GH are triphasic, with high concentrations at 60 to 70 days, falling to a nadir at 100 to110 days and a second increase to a peak at 130 to 140 days (Gluckman et al., 1979). Growth hormone receptors are present in the epithelial and mesenchymal tissues of the bovine mammary gland from as early as 3 months of fetal age (Knabel et al., 1998). Therefore, it could

be speculated that GH receptors in ovine fetal mammary tissue from day 60 of gestation act either directly, or indirectly to stimulate epithelial proliferation.

Based on the early pubertal rodent model, evidence is emerging that GH, via GH receptors in the mammary gland, stimulates local IGF-I production within the stroma (mesenchyme) of the mammary gland. Thereafter, IGF-I synergises with oestrogen to induce epithelial growth (Kleinberg, 1997). A paracrine effect with oestrogen acting on the mesenchyme could also be hypothesised as a pathway to induction of mammary growth in the fetal sheep. At weeks 10, 15 and 20 of gestation, *in situ* expression data showed mRNA for insulin-like growth factor I and II (IGF-I and IGF-II) in the interlobular connective tissue underlying the epithelial tissue in the fetal ovine mammary gland (Forsyth et al., 1999). This would strongly indicate that the stromal cells are critical for IGF-I production. Furthermore, mRNA for IGF-I receptor was located in the epithelial cells of the growing ducts. Although limited evidence exists regarding a possible role for EGF in ovine mammogenesis, transplantation of neonatal mammary glands from EGFR null mice demonstrated that EGFR is essential for mammary ductal growth and branching morphogenesis, but not for mammary lobulo-alveolar development (Wiesen et al., 1999). Whether EGF is important in mammary ductal morphogenesis in the fetus is yet to be determined. These findings would support a paracrine model, in which the mesenchymal cells underlying the epithelium serve as a local source of IGF that may then stimulate the growth of the ducts through binding to IGF-I receptors present in the epithelial cells.

ORα were occasionally observed in the differentiating cells of the developing adipose tissue of the female ovine gland at days 80, 100 and 120 of gestation. As plasma oestrogen concentrations are high during this period it could be postulated that oestrogen, via oestrogen receptors in the hypodermal mesenchymal cells, plays a role in priming the fat pad for growth factor-mediated epithelial penetration during postnatal life. This is supported by numerous studies, which have provided convincing evidence that adipose stroma is not only absolutely required for postnatal ductal elongation, but that its constituents are integral mediators of oestrogen-stimulated growth (Cunha et al., 1997; Woodward et al., 1998).

**Summary**

The sexual divergence of mammary growth, in terms of morphological differentiation and relative growth, was evident at day 80 of fetal age. Thereafter, development of the mammary gland in the male became slower and less pronounced than that of the female gland; the epithelial, connective and adipose tissues of the 21-day-old lamb showed no advance on the structure at day 120 of fetal age. These observations were compatible with the intensity of immunohistochemical staining for androgen receptor antibody, which increased in both the fibroblasts and epithelial cells of the growing ducts from day 80, reaching a peak between days 100 and 120 of gestation. Hence an association between "masculinisation" of the mammary gland and the intensity of androgen receptor immunostaining exists. Alternatively, the inhibition of mammary growth in the male may involve mediation by mesenchymal factors such as EGF.

In terms of total duct area, the male and female mammary glands develop similarly until day 120, when, in the female, there is a change in the signal, either from the fetus or the dam that stimulates branching morphogenesis of the epithelium. The presence of oestrogen receptors in the fetal mammary gland of the sheep agrees with the rodent model (Narbaitz et al., 1980) indicating that these glands can respond to oestrogen stimulation (Hilakivi-Clarke et al., 1998). Oestrogens of fetal or maternal origin may act directly or indirectly, mediated by oestrogen-induced growth factors from the underlying mesenchymal cells, to stimulate mammary ductal elongation. Or, is it solely the absence of testosterone that allows the female mammary gland to proceed in its development? Hence, it was considered necessary to further investigate the role of testosterone and growth factors in the sexual dimorphism of the mammary gland.

Two experimental strategies were proposed:

First, to determine the presence or absence of receptors for two growth factors (IGF-I and EGF) that may be involved in growth and differentiation of the fetal mammary gland (Chapter four).

Second, *in utero* exposure of female fetuses to androgens, which may give indirect evidence that inhibition of mammary gland growth in the male is dependent on its exposure to testosterone (Chapter five).

# CHAPTER FOUR

# ROLE OF GROWTH FACTORS IN THE DEVELOPMENT OF THE MAMMARY GLAND IN FETAL SHEEP

## 4.1 Abstract

Locally produced insulin-like growth factors (IGF-I and IGF-II) have been implicated in mediating mesenchymal-epithelial interactions in the female mammary gland during fetal development. In the present study, an immunohistochemical assay was used to determine, in the mammary gland of male and female ovine fetuses, the presence or absence of IGF-I receptor (IGF-IR), which is thought to mediate the mitogenic response of epithelial cells to IGF-I and IGF-II.

IGF-IR was located in the epithelial and mesenchymal cells of the mammary gland at days 60, 80, 100, 120 and 140 of fetal age in males and females. It was not present at day 40. All three reaction patterns were recognised: linear cell surface, cytoplasmic and nuclear. In the epithelium of the female gland immunostaining increased in intensity as gestation progressed, reaching a peak at days 120 to 140. The intensity of staining reached a peak in the epithelial cells of the male gland at day 100, and declined thereafter.

IGF-IR was also found in the adipoblasts of the developing adipose tissue of both sexes, from day 80 of fetal life onwards. Immunoreactivity was seen mainly in the nucleus although cytoplasmic staining was occasionally observed. The dermal and hypodermal mesenchymal cells (fibroblasts) frequently showed both nuclear and cytoplasmic staining. Although the intensity of staining in the mesenchymal cells was greater in females relative to males, it did not vary much as gestational age increased.

EGFR were localised in the cytoplasm of the epithelial cells of both sexes, but only at day 120 of gestation.

These findings indicate a role for IGF-IR in the differentiation and proliferation of the epithelial cells in the female mammary gland during fetal development. Moreover, they suggest that suppression of mammary growth in the male may involve mediation by IGF-IR. Finally, they demonstrate that the presence of IGF-IR is regulated by the stage of development, ovarian and testicular hormones, and mesenchymal-epithelial interactions.

## 4.2 Introduction

Mammary development in the fetus is apparently autonomous, however, the pattern of oestrogen receptor expression in the fetal mammary gland observed in Chapter three supports the possibility of a role for oestrogen in prenatal mammogenesis. Moreover, the data and the literature indicate that the action of oestrogen is not direct, but may be mediated by growth factors, such as IGF-I, in the mammary gland. Since growth hormone (GH) stimulates the paracrine release of IGF-I in a variety of tissues, there is interest in the effect of GH on the development of the prenatal mammary gland.

The similarity in gland structure at birth between ORKO and "wild-type" female mice has led to the suggestion that early growth of the gland develops in response to the action of maternal growth hormone (Korach et al., 1996). The increase in mammary gland volume of fetal mice after the administration of exogenous GH to the pregnant mother, or directly into the fetus (Jean, 1968) support the idea that the GH receptor (GHR) has a direct role in mediating the effects of GH on the fetal mammary gland. *In situ* hybridisation and immunohistochemistry have demonstrated the presence of GHR in the epithelial and mesenchymal cells of bovine mammary glands during fetal development (Knabel et al., 1998). Furthermore, the amount of GHR in the epithelial cells continued to increase with gestational age, inferring the involvement of GHR in cell proliferation and cell differentiation of the mammary ducts.

Oestrogen and growth hormone continue to be of primary importance in postnatal mammary growth, but there are sufficient data to suggest that IGF-I also plays a significant role in this process. Tissue recombination studies in the mouse in which various elements of the IGF-I axis have been deleted confirmed that IGF-I and/or IGF-I receptor are/is essential for normal mammary development (Kleinberg et al., 2000). Neither oestradiol nor GH alone or in combination had any affect on prepubertal mammary development in these knockout mice. These findings and several other studies have led to the proposal that the action of GH in postnatal mammary development is achieved by local IGF-I synthesis. These include demonstrations that GH up-regulates the synthesis of local IGF-I mRNA (Ruan et al., 1995), that locally released IGF-I can mimic the mammogenic effect of GH (Ruan et al., 1992), and that targeted deletion of IGF-I results in complete retardation of ductal growth that can be rescued by exogenous IGF-I, but not GH (Ruan and Kleinberg, 1999).

Within the ovine mammary gland IGF-I mRNA expression is markedly higher in the mammary fat pad than in the parenchymal tissue and increases during the prepubertal period of allometric growth (Hovey et al., 1998). Strong support that the stromal cells comprising the fat pad are critical for local IGF-I production comes from *in situ* expression data in the ovine fetus. At 10, 15 and 20 weeks of gestation, mRNA for IGF-I and IGF-II were expressed in the cells of the intralobular connective tissue (mesenchyme) while the IGF-I receptor was expressed in the epithelium (Forsyth et al., 1999). Quantification by absorbance measurements showed that mRNA expression increased with gestational age for IGF-I and IGF-II, and that IGF-II was more highly expressed than IGF-I (Forsyth et al., 1999). Furthermore, this correlates well with circulating plasma concentrations of IGFs in the fetal lamb. IGF-I concentrations are relatively low in the fetus and rise soon after birth (Bassett et al. 1989) while IGF-II concentrations are high in the fetus and decrease at birth to adult levels (Gluckman et al. 1987). Finally, arguing against the idea that IGF-I is essential for mammary development, transgenic mice that lack expression of IGF-I and of the IGF-I receptor both show normal fetal development of the mammary gland (Hadsell and Bonnette, 2000). However, there is no published data pertaining to mammary development in the IGF-II knockout mouse.

With respect to a possible role for IGFs in male mammogenesis, male animals and testosterone-treated castrates have higher plasma IGF-I concentrations than castrates or females (Bass and Gluckman, 1990). This would indicate that testosterone can also stimulate the somatotrophic axis, implying that the GH axis may play some part in androgen-induced control of mammary morphogenesis in the male ruminant.

In addition to the role of IGFs, gene deletion experiments have demonstrated that signalling through the EGF receptor (EGFR) is essential for mammary ductal branching morphogenesis in the mouse (Wiesen et al., 1999). Using recombinations of tissues derived from animals that lack the EGF receptor it was found that the stromal receptor was required for ductal development but its absence did not affect lobulo-alveolar development. However, because there are multiple ligands for the EGFR it was difficult to deduce which of these proteins was involved in the phenotype expressed by the EGFR null mouse. Recent experiments in mice lacking various combinations of EGF, transforming growth factor alpha (TGFα) and amphiregulin, demonstrated that amphiregulin is essential for ductal morphogenesis of the mammary gland during puberty, and suggested that EGF and TGFα act in a collaborative fashion to regulate lactogenesis (Luetteke et al., 1999).

Only limited evidence exists regarding a possible mitogenic role for epidermal growth factor (EGF) in ruminant mammogenesis (Plaut, 1993). EGFRs are present on ovine and bovine mammary epithelial cells, the expression of which is elevated during pregnancy, but greater during mid- than during late pregnancy or lactation (Moorby et al., 1995; Koff and Plaut, 1995) and down-regulated by GH treatment (Glimm et al., 1992). No studies have been undertaken to localise the EGFR and its transcript in the mammary gland of the fetal ruminant, hence its involvement in growth and differentiation of the fetal mammary gland remains unknown.

Several studies have shown that EGF modulates androgen-dependent male sexual differentiation in the mouse (Gupta and Singh, 1995; Gupta et al., 1996). Quantitative competitive RT PCR showed that the expression of mRNA for EGF was higher in the male than in the female reproductive tract, with increased levels of EGF mRNA in the male corresponding to periods of cellular growth and differentiation. Furthermore, exposure of fetuses to testosterone during days 13 to 17 of gestation not only induced the Wolffian duct in the females, but also resulted in a significant increase in the expression of mRNA for EGF. These results imply a role for EGF in male sexual differentiation, and hence, the possibility of a role in the sexual dimorphism of the ruminant mammary gland.

Given the preliminary state of our knowledge of the role of IGF/IGFR and EGF/EGFR in the fetal ruminant mammary gland, the following questions emerged:

1. Does immunohistochemistry display the same distribution pattern of the IGF-IR protein as its mRNA (confirm Forsyth et al., 1999).
2. Are EGF receptor proteins present, and if so, are they localised on epithelial and/or mesenchymal cells?

3. Does the period of marked mammary epithelial growth (days 120 to 140 of gestation) in the female correspond with increased intensity of IGF-IR and/or EGFR immunostaining?
4. If present, does EGFR and/or IGF-IR immunostaining in the male mammary gland coincide with the period of increased intensity of androgen receptor immunostaining?

The following study was proposed to find answers to those questions.

## 4.3 Materials and Methods

All procedures were approved by the Massey University Animal Ethics Committee.

### 4.3.1 Animals and Treatments

Mammary gland samples used in this study were derived from animals used in an experiment described previously (Chapter two).

### 4.3.2 Histology Samples

The method used for mammary gland sample collection, processing and storage is the same as that described in Chapter two.

### 4.3.3 Immunohistochemistry

Insulin-like growth factor-I receptors (IGF-IR) were located histochemically in the sections using a high temperature antigen unmasking technique. The primary antibody for the IGF-I receptor analysis was produced in goats immunised with purified insect cell line *Sf* 21-derived, recombinant human insulin-like growth factor one soluble receptor (Lot VL018101, R&D Systems, Minneapolis, M.N., U.S.A.). Skin, from mammary gland tissue, which is known to contain the antigen, acted as the positive control. This antibody was diluted 1:15 in 1% BSA in PBS. The secondary antibody was anti-goat immunoglobulin (Batch 113401, Dako Corporation, California, USA) diluted 1:200 in 1% BSA in PBS. The methodology was similar to that described for androgen receptor (Chapter three).

Receptors for epidermal growth factor (EGFR) were also located histochemically in the sections using a high temperature antigen unmasking technique. The primary antibody for the EGFR analysis was a mouse monoclonal antibody (NCL-ER-6F11) produced using recombinant fusion protein to the external domain (Batch 114403, Novocastra, Newcastle, U.K.). Normal skin from the fetal sheep, which is known to contain the antigen, acted as the positive control. This antibody was diluted 1:20 in 1% BSA in PBS. The secondary antibody was anti-mouse immunoglobulin (Batch no. illegible, Amersham Life Science) diluted 1:200 in 1% BSA in PBS. The methodology was similar to that described for androgen receptor (Chapter three).

Slides for detection of EGF and IGF-I receptors were examined for intensity of immunohistochemical staining using a differential interference contrast microscope. Staining was subjectively estimated and expressed as negative (-), weakly positive (+), moderately positive (++), or strongly positive (+++). Photomicrographs of the slides were obtained using a digital camera and a differential interference contrast microscope. Images were processed with the RT camera and Image-Pro PLUS, version 4.1 for windows.

## 4.4 Results

The reaction patterns described hereafter, are exactly the same as those described in Chapter three: linear cell surface, cytoplasmic and nuclear. The distribution of IGF-I receptors in the mammary gland at days 40, 60, 80, 100, 120 and 140 of gestation is presented in the Appendices (Table A.3).

### 4.4.1 Ontogeny of insulin-like growth factor-I receptor (IGF-IR) within the developing mammary gland

The ovine skin (at day 100 of fetal age), which acted as a control, did not exhibit immunoreactivity to IGF-IR antibody when the primary antibody was omitted from the immunohistochemical procedure (no photomicrograph shown). Addition of the primary antibody revealed staining of the skin epidermis, the hair follicle and the inner root sheath enclosing the follicle.

At day 40 of fetal age, immunoreactivity to IGF-IR antibody was not observed in the cells comprising the mammary bud or in the surrounding mesenchyme of the developing mammary gland in either sex. Although a dark ring enveloped the basement membrane, reactivity was questionable as staining was only slightly above background levels. However, faint immunostaining was seen in the epidermis (no photomicrograph shown).

Males and females exhibited immunoreactivity to IGF-IR antibody in the epithelial cells of the mammary ducts at day 60 of fetal age. Staining was intense along the luminal surface of the epithelial cells, incorporating the cell membrane and adjacent cytoplasm (Plate 4.1) (male data not shown). Two female fetuses showed intense nuclear staining in the cells of the fibroblasts of the differentiating mesenchyme that surrounded the ducts (Plate 4.1) and of the more distant mesenchyme. The remaining three females and all males displayed weak immunoreactivity in the fibrous connective tissue. The outer edge of the epidermis (stratum corneum) stained in both sexes.

At day 80 of fetal age, immunoreactivity to IGF-IR antibody was frequently seen in the epithelial cells of the secondary ducts. In males and females, immunostaining in the larger ducts was continuous around the luminal surface and extended into the apical cytoplasm. In two females, epithelial staining extended to the nuclear membrane. Although the pattern of immunoreactivity was similar between the sexes, the intensity of staining varied. Females displayed an intense

granular cytoplasmic reactivity (Plate 4.2) whereas males exhibited finely granular apical and cell surface reactivity (Plate 4.3). Nuclear staining of the fibroblasts that surrounded the ducts was observed in both sexes, although occasionally, fine granular cytoplasmic reactivity was seen. In the connective tissue of the deep hypodermis, the fibroblasts exhibited granular staining of their nuclei. Moreover, a considerable number of the adipoblasts of the developing fat pad showed nuclear staining.

Compared to 60-day-old fetuses, immunostaining of the stratum corneum of the skin epidermis was more intense at day 80. The first stage of wool follicle development, observed as a down growth of epidermal cells into the dermis, coincided with the noticeable presence of IGF-IR in the inner root sheath.

In the mammary gland at 100 days, staining extended from the lumen surface to deep in the apical cytoplasm and nuclei in two of the four males (Plate 4.5). For most of the female glands analysed at this age, staining was mainly restricted to the cell surface, but was by no means superficial. No nuclear staining was observed in the epithelial cells of the female ducts (Plate 4.6).

Cytoplasmic staining of the fibroblasts surrounding the ducts was frequently observed whereas the fibroblasts of the deep hypodermis displayed nuclear staining. Similarly to day 80, the nuclei of the adipoblasts continued to show immunoreactivity to IGF-IR antibody in males and females, although staining of the cytoplasm was occasionally observed. The intensity of staining was also similar to that observed at day 80.

In the skin of the mammary gland, the stratum basale, stratum spinosum, and stratum superficiale were moderately stained while the germinal layer showed strong positive immunoreactivity. Although this pattern of staining was observed in most of the tissue, the epidermis of some fetal glands only showed immunoreactivity in the stratum basale. The components of the wool follicles, including the inner and outer root sheaths, the erector pili muscle and the sweat glands were intensely immunostained (Plate 4.7).

In contrast to the pattern of immunoreactivity displayed at day 100, the ducts of the male gland at day 120 displayed finely granular apical and linear cell surface reactivity, which was generally discontinuous around the luminal perimeter (photomicrograph not shown). The epithelial cells of the female ducts now exhibited reactivity in the apical cytoplasm and around the nuclear membrane (Plate 4.8). The intensity of staining was greater in females at day 120 relative to that at day 100, but weaker in the males.

The fibroblasts surrounding the ducts continued to show a similar pattern and intensity of staining to that observed at day 100. More nuclear, as opposed to cytoplasmic, staining was observed in the fibroblasts that enveloped the developing lobules of adipose tissue. Well-defined nuclear staining of the adipoblasts was seen in the female fat pad, although cytoplasmic staining was occasionally seen in both sexes. Immunoreactivity in the adipose tissue of the male was weaker than that in the female.

Reactivity in the epidermis was quite superficial and confined mainly to the stratum corneum and stratum granulosa. Staining of the wool follicles and their accessory glands was much less intense than that observed at day 100.

At day 140 of fetal age, the intensity and pattern of immunostaining in the epithelial cells was very similar to that observed at day 120 (no photomicrograph shown). The fibroblasts of the dermis and hypodermis displayed a similar pattern of reactivity to that seen at day 120 with staining essentially confined to the nucleus. Nuclear and cytoplasmic reactivity was observed in the adipose tissue but the amount and intensity of staining was greater in the female than in the male gland.

While all layers of the epidermis stained intensely for IGF-IR, the wool follicles were essentially non-reactive.

**Plate 4.1** Photomicrograph of the localisation of IGF-I receptors in the ducts and dermal mesenchyme of the mammary gland of an ovine female at day 60 of fetal age. The luminal surface of the epithelial cells and the nuclei of the surrounding fibroblasts show strong immunoreactivity to IGF-IR antibody. (Mag x400).

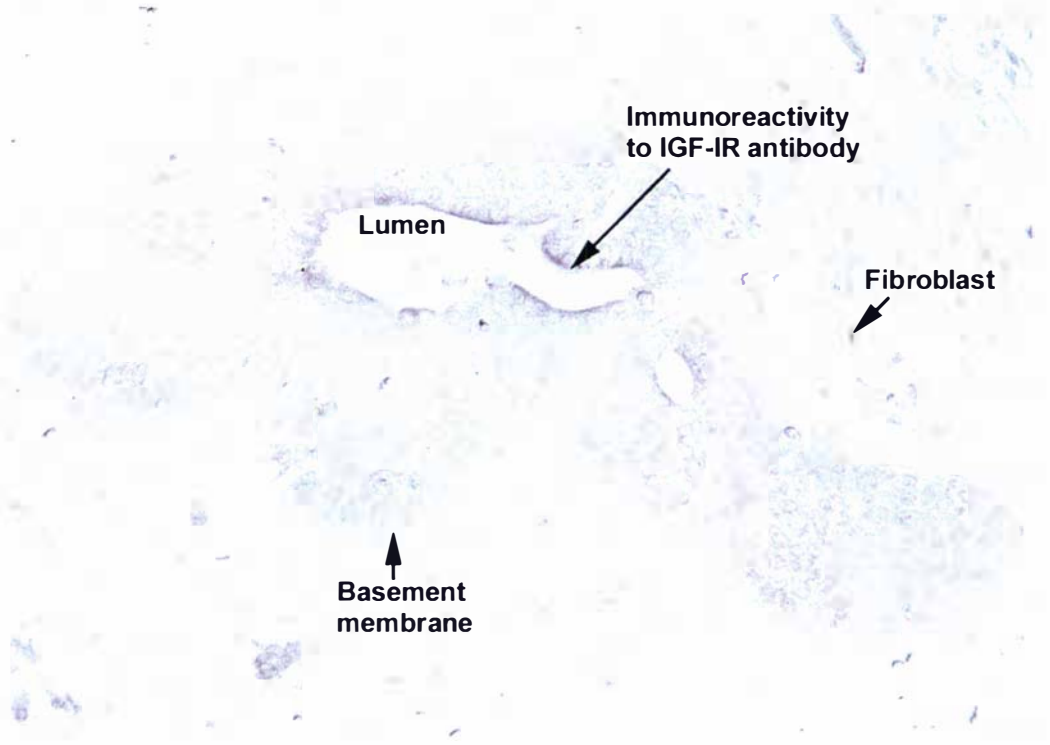



**Plate 4.2** Photomicrograph of the localisation of IGF-I receptors in ducts of the mammary gland of an ovine female at day 80 of fetal age. The epithelial cells exhibited an intense, coarse granular cytoplasmic reactivity. (Mag x400).

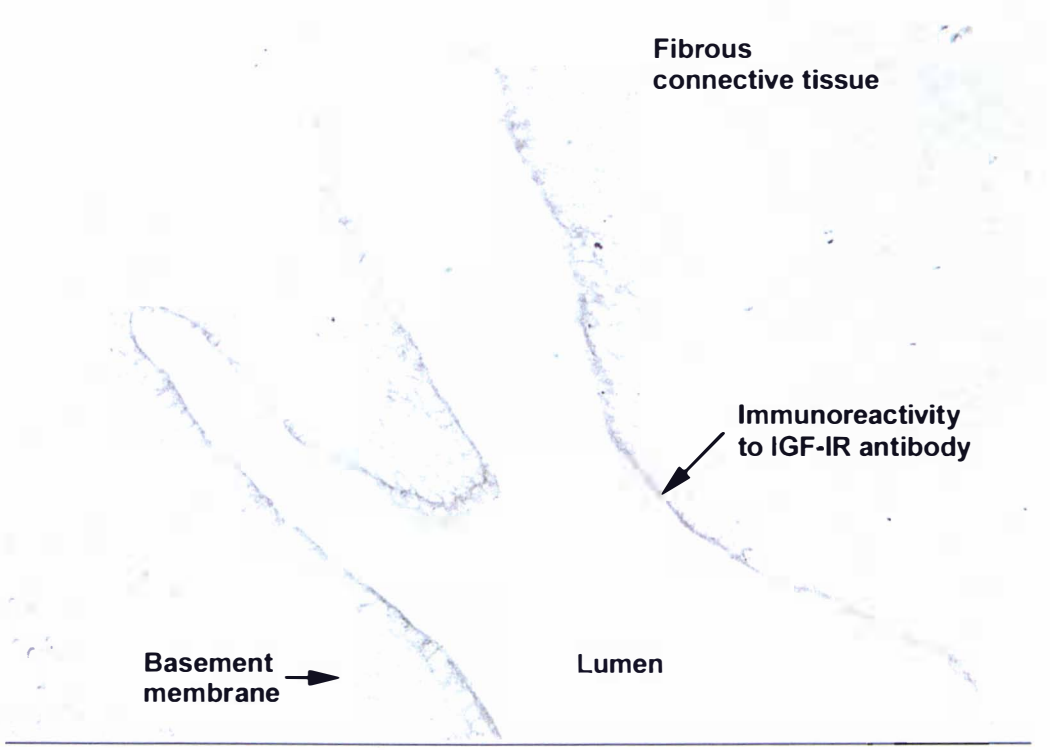


**Plate 4.3** Photomicrograph of the localisation of IGF-I receptors in ducts of the mammary gland of an ovine male at day 80 of fetal age. The epithelial cells displayed a finely granular cell surface and apical reactivity. (Mag x400).

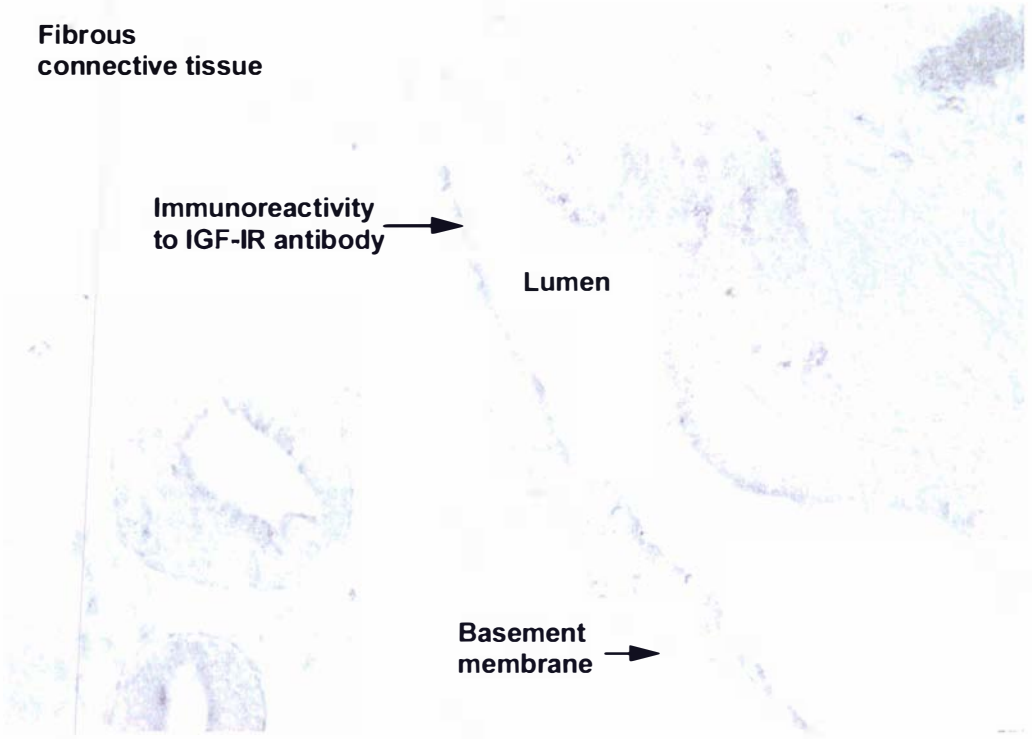



**Plate 4.4** Photomicrograph of the localisation of IGF-I receptors in the differentiating cells of the developing fat pad of an ovine female at day 80 of fetal age. Note the nuclear staining of the adipoblasts and surrounding fibroblasts. (Mag x400).

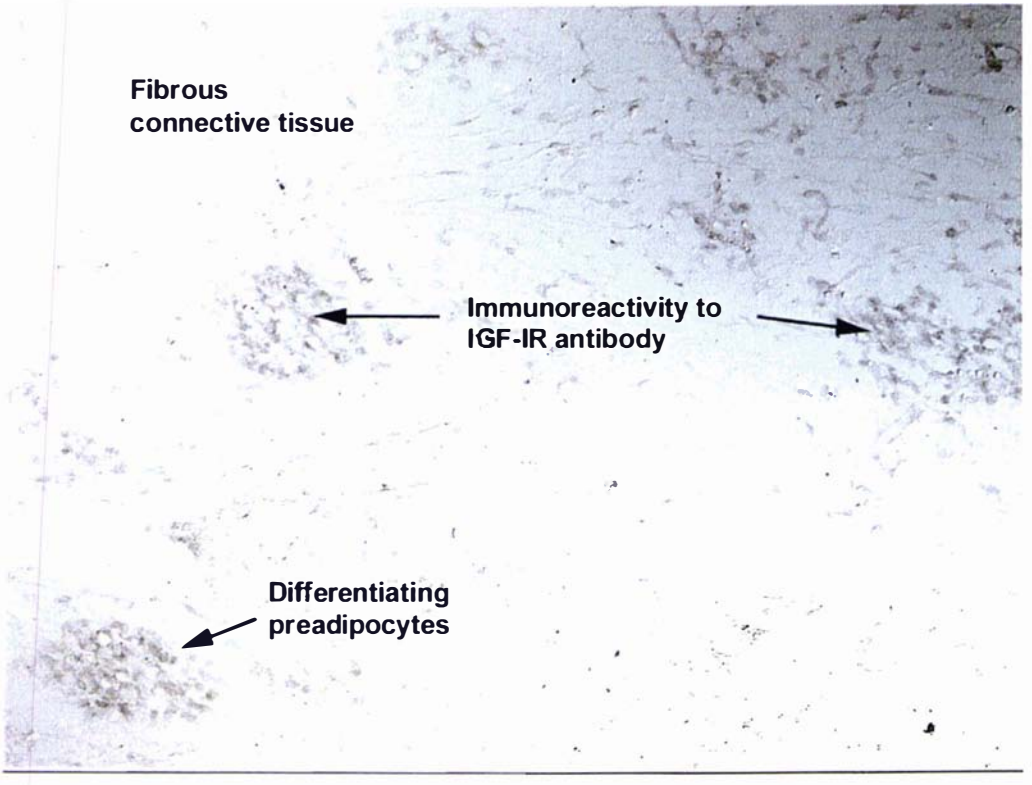


**Plate 4.5** Photomicrograph of the localisation of IGF-I receptors in ducts of the mammary gland of an ovine male at day 100 of fetal age. The IGF-I receptor is localised in the nucleus and cytoplasm of the epithelial cells. (Mag x400).

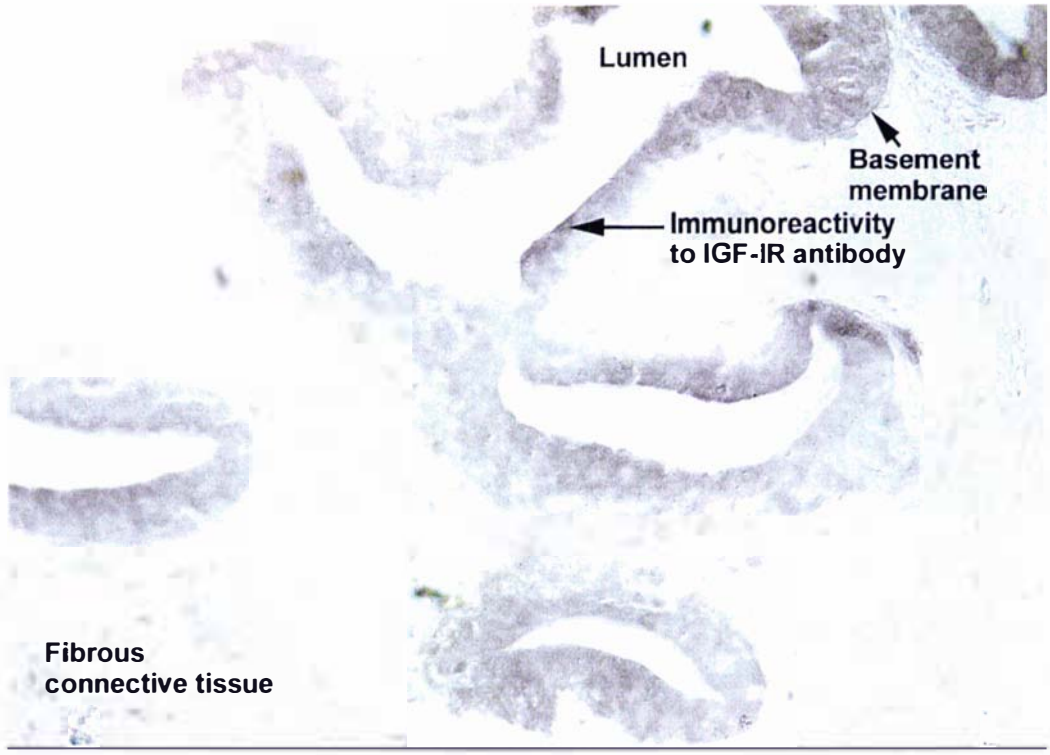



**Plate 4.6** Photomicrograph of the localisation of IGF-I receptors in ducts of the mammary gland of an ovine female at day 100 of fetal age. The epithelial cells display patchy granular cell surface reactivity. (Mag x400).

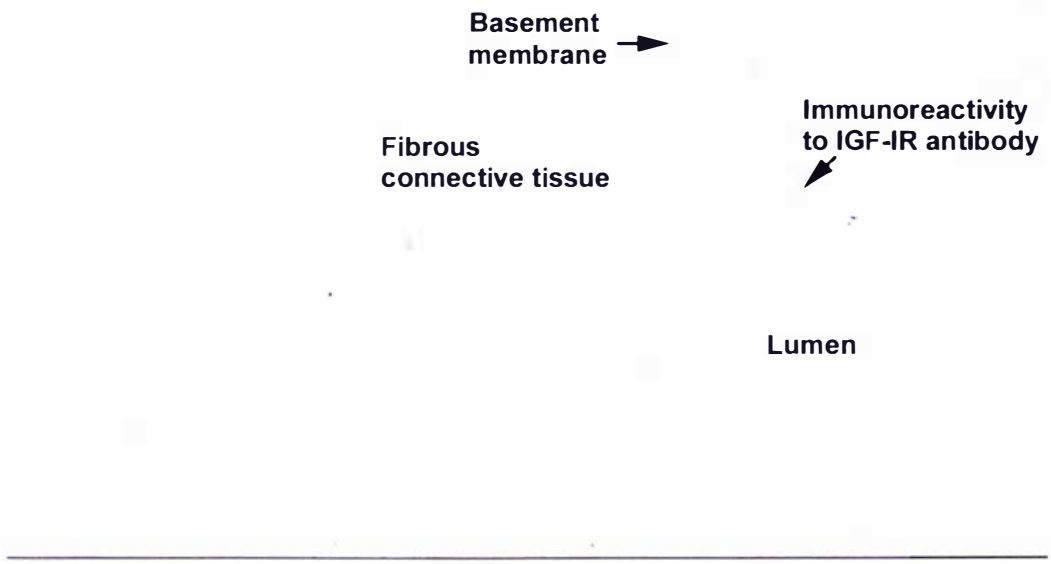


**Plate 4.7** Photomicrograph of the localisation of IGF-I receptors in the skin of the mammary gland of an ovine female at day 100 of fetal age. The inner root sheath of the primary wool follicles shows distinctive immunoreactivity to IGF-IR antibody. (Mag x400).

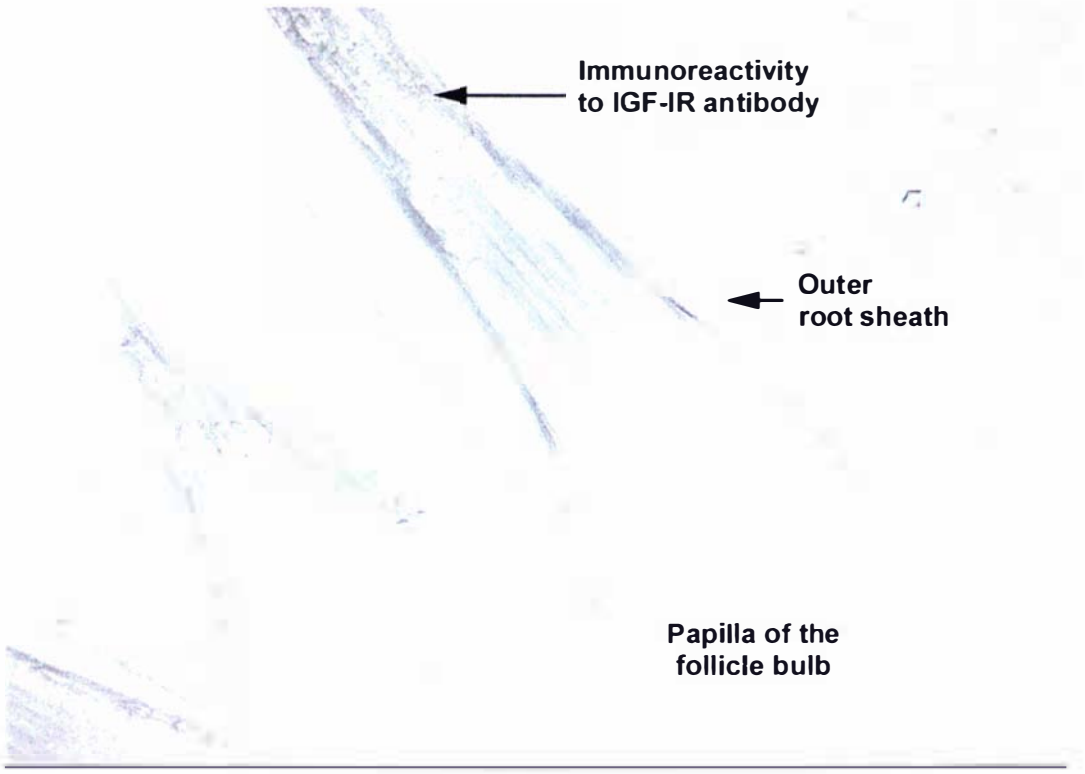
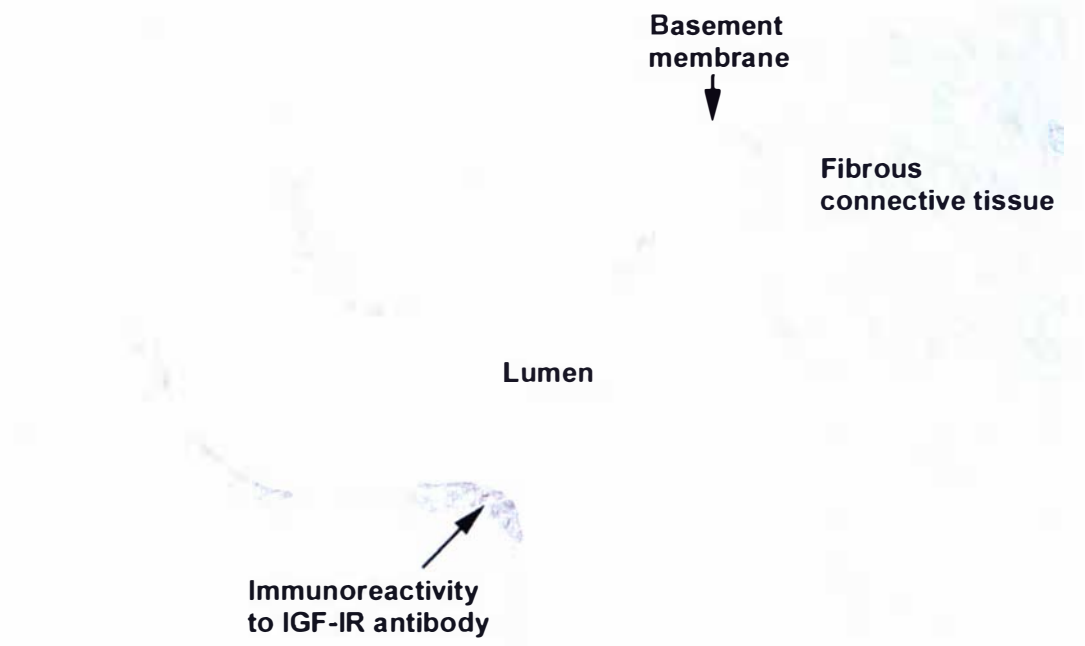



**Plate 4.8** Photomicrograph of the localisation of IGF-I receptors in ducts of the mammary gland of an ovine female at day 120 of fetal age. (Mag x400).



In summary, IGF-IR was present in the epithelial and mesenchymal cells of male and female mammary gland from day 60 of fetal age. Immunoreactivity was localised to the epithelial cytoplasm with intense immunostaining along the luminal surface. In contrast, immunoreactivity in the surrounding mesenchymal cells was variable, and ranged from weak to strongly positive. Females exhibited coarse cytoplasmic immunostaining of the epithelial cells with some reactivity of the nuclear membrane at days 80, 120 and 140, and cell surface staining at day 100. In contrast, fine granular staining of the cytoplasm and cell membrane was a feature of the male epithelial cells at days 80, 120 and 140, while at day 100, immunoreactivity was observed in the cytoplasm and nucleus. Nuclear staining of the adipoblasts of the developing adipose tissue was evident at day 80 in both sexes. However, there was a tendency for females to show a greater intensity and amount of staining relative to that seen in the male. The mesenchymal cells (fibroblasts) frequently showed both nuclear and cytoplasmic staining, and remained at a similar intensity from days 80 to 140.

### 4.4.2 Ontogeny of epidermal growth factor receptor (EGFR) within the developing mammary gland

The ovine fetal skin, which is known to express the EGFR at the cell surface (M.J. Birtles, personal communication) acted as a control. The skin of the ovine fetus at day 120 of gestation did not exhibit immunoreactivity to EGFR antibody when the primary antibody was omitted from the immunohistochemical procedure (Plate 4.9). Addition of the primary antibody revealed staining of the skin epidermis (stratum corneum), the deep follicle bulb, hair follicle and the sweat gland duct.

Similarly to IGF-IR, immunoreactivity to EGFR antibody was not observed in the cells comprising the mammary bud or in the surrounding mesenchyme of the developing mammary gland in either sex at day 40 of gestation. In fact, the only stage at which EGFR was reactive in the fetal mammary gland was at day 120. Three of the five females displayed patchy staining of the cell membrane, while two females showed staining of the apical cytoplasm (Plate 4.10). Immunoreactivity ranged from weak to moderately positive. A discontinuous dark line was observed along the lumen edge of the epithelial ducts in the males, but it did not extend beyond the cell surface, indicating edge artefact. The mesenchymal cells surrounding the branched ducts, as well as those of the deep hypodermis, showed no immunoreactivity to EGFR antibody.

Distinct staining of the presumptive stratum corneum of the epidermis, the upper one third of the wool follicles and the sweat gland ducts were seen in the day 120 female glands. Only the ducts of the sweat glands exhibited positive immunoreactivity in the mammary gland of the male at day 120.

Immunoreactivity was not seen in the mammary glands of either sex at day 140 of fetal age. The epidermis and the wool follicles were faintly immunostained.

**Plate 4.9** Photomicrograph of the negative control for the localisation of EGF receptors in fetal sheep mammary gland tissue. Fetal mammary gland tissue was incubated in the absence of primary antibody to EGFR. Note the lack of immunoreactivity in the epithelial and mesenchymal tissues. (Mag x400).

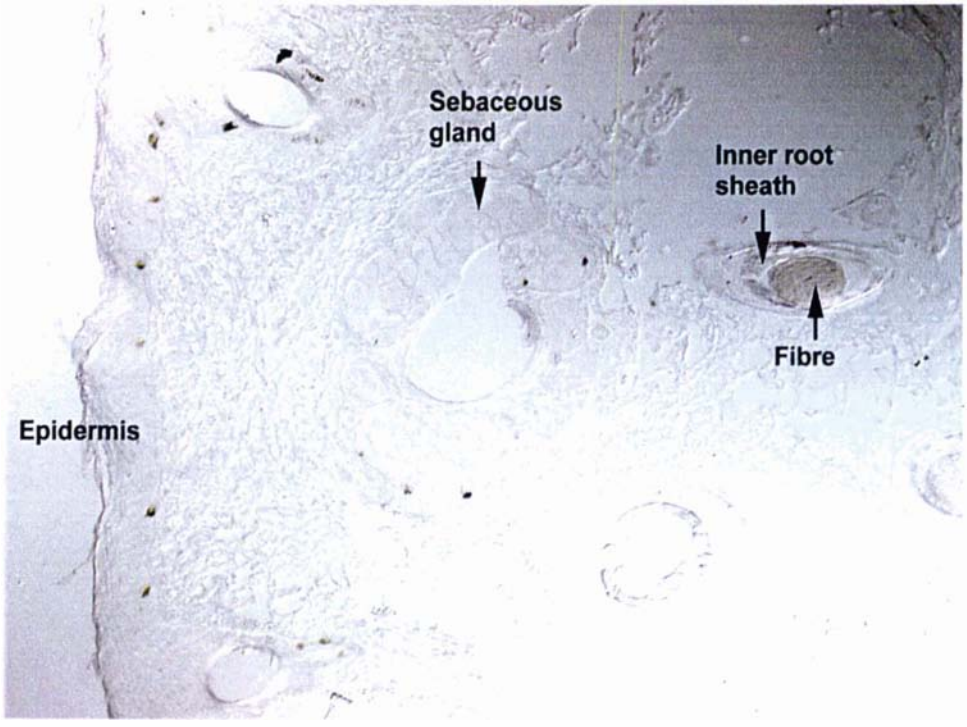
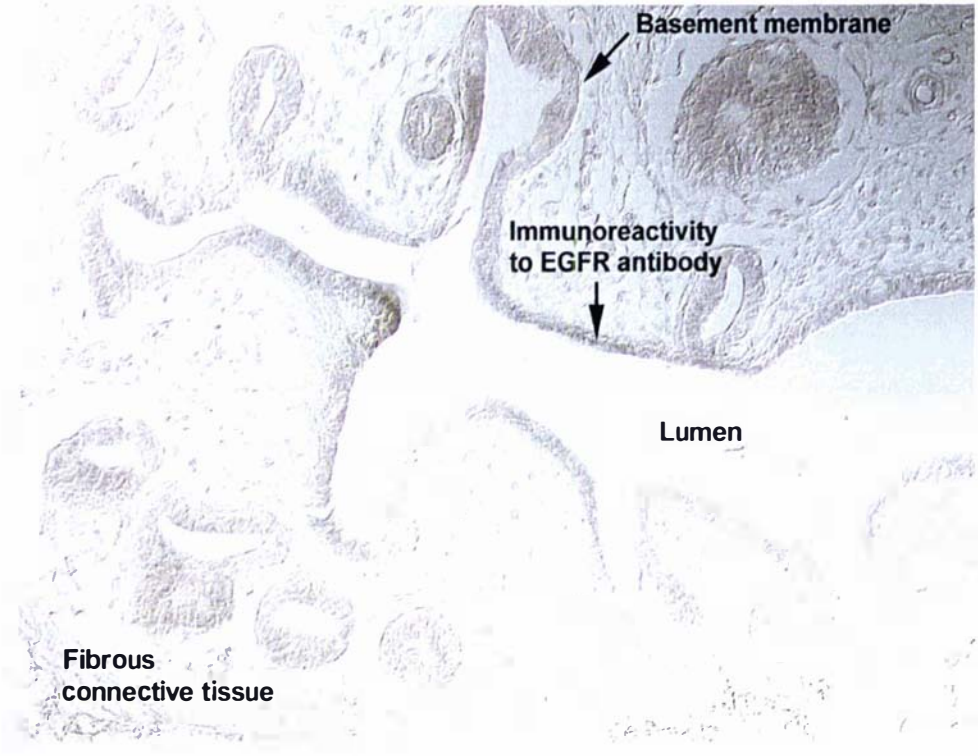



**Plate 4.10** Photomicrograph of the localisation of EGF receptors in the ducts of the mammary gland of an ovine female at day 120 of fetal age. The EGF receptor is localised in the cytoplasm of the luminal epithelial cells. (Mag x400).



## 4.5 Discussion

The characteristic morphology of the mammary gland appears to be programmed and maintained by the interactions that occur between the epithelial and mesenchymal tissues during fetal development, independent of endocrine influences. In the previous study I demonstrated the presence of oestrogen receptors in the mesenchymal and epithelial cells of the fetal mammary gland, which suggests that the fetal gland is capable of responding to oestrogenic stimuli. However, the absence of oestrogen receptors in the epithelial cells until at least day 100 of fetal life lends strong support to suggestions that epithelial-mesenchymal interactions are mediated by polypeptide growth factors.

**Ontogeny of IGF-I receptor (IGF-IR) in the fetal mammary gland**

The insulin-like growth factors are potent paracrine mitogens for epithelial cells in the postnatal ovine mammary gland (Hovey et al., 1998) although their precise role during fetal mammary gland development has not been defined. The finding that insulin-like growth factor-I receptor protein (IGF-IR) is present in the mammary gland of the prenatal sheep not only confirms the *in situ* expression data of Forsyth et al. (1999), but upholds their suggestion of a role for the IGF system in mediating epithelial-mesenchymal interactions in fetal mammary development.

As early as day 60 of fetal age, immunohistochemistry demonstrated immunoreactivity to IGF-IR antibody in the epithelial cells of the mammary ducts and in the adjacent mesenchymal cells, in male and female sheep. Previous studies have shown that IGF-I receptor mRNA localises to epithelial, and not mesenchymal, cells in fetal and prepubertal ovine mammary gland, its expression being greatest at four weeks of postnatal age (Morgan et al., 1996; Forsyth et al., 1999). In the female fetal mouse a similar distribution exists for keratinocyte growth factor (KGF), whereby the receptor is localised exclusively in the mammary epithelium (Finch et al., 1995). However, similarly to the present study, growth hormone receptor protein and its mRNA were found in both the ductal epithelial cells and the adjacent mesenchymal cells of the bovine mammary gland from three months of fetal age (Knabel et al., 1998).

Measurement of absorbance showed a tendency for increased expression of IGF-I receptor mRNA in the mammary epithelial cells of the female ovine fetus with stage of pregnancy, although the correlation was not significant (Forsyth et al., 1999). In the present study, the intensity of immunostaining in the epithelial cells of the female ovine gland increased gradually from day 60 of fetal age, reached a peak at day 120 and remained elevated at day 140. A similar pattern emerged in the expression of GHR in the mammary epithelial cells of fetal cattle, where immunoreactivity increased from 3 to 6 months of gestation and remained elevated thereafter (Knabel et al., 1998).

The normal development of the mammary gland of the ORKO mouse has led to the suggestion that the fetal mammary gland develops in response to the action of maternal growth hormone (Korach et al., 1996). In support of this idea, Jean et al. (1977) demonstrated that administration of exogenous GH into either the pregnant mouse, or directly into the fetus, increased the

volume of the fetal mammary gland. However, it has been reported that exogenous GH is not transported across the placenta from the maternal circulation to the fetus (Fhölenhag et al., 1994). Furthermore, suggestions of an indirect action of GH via systemic IGF-I are countered by the fact that IGF-I does not cross the placenta (D'Ercole and Underwood, 1981). Therefore, the effects of maternal GH and IGF-I on fetal development are not mediated through direct effects on the fetus (i.e., placental transfer of the hormones to interact with fetal mammary tissue). Rather, they must act through secondary mechanisms by influencing maternal or placental metabolism in a manner, which then leads to enhanced fetal growth, and hence mammary growth.

GHR localise to the epithelial and mesenchymal cells of the prenatal bovine mammary gland (Knabel et al., 1998), which supports the idea that the GH receptor (GHR) has a direct role in mediating the effects of fetal GH on the developing mammary gland. However, two lines of evidence suggest that the effect is indirect. First, ovine fetal mammary growth, in terms of total duct area, over time is exponential (personal observation). Second, the pattern of GH concentration in the ovine fetal plasma is triphasic with high concentrations in mid-gestation, followed by a subsequent fall and then a second peak in late gestation (Gluckman et al., 1979). Furthermore, ovine mammary epithelium does not bind GH, at least in the prepubertal lamb (McFadden et al., 1995). Therefore, at least part of the effect of GH on fetal mammary growth may be mediated by IGF-I through the IGF-IR.

The liver is the major source of circulating IGF-I, but suggestions of an indirect action of GH via systemic IGF-I are countered by the fact that circulating plasma concentrations of IGF-I are relatively low in the fetal sheep throughout gestation (Bassett et al. 1989). However, a role for locally synthesised IGF-I in the ruminant mammary gland is likely. Support for this idea came from the finding that expression of mRNA for both IGF-I and IGF-II was higher in intact rather than in cleared fat pad of postnatal ovine mammary gland (Hovey, 1996). Furthermore, *in situ* expression data for IGF-I and IGF-II in the fetal ovine mammary gland confirmed that the mesenchymal (stromal) cells are critical for local IGF-I production (Forsyth et al., 1999). Given the finding that IGF-IR localises to epithelial cells from day 60 and oestrogen receptors (OR) are not immunodetectable in the epithelial cells until day 100 of fetal age (Chapter three), it is tempting to speculate that paracrine IGF-I stimulates epithelial proliferation prior to day 100. The proliferating ductal epithelial cells then induce the upregulation of IGF-I in the surrounding mesenchyme, which, in turn, stimulates further differentiation and proliferation of the epithelium.

Very little is known about the influence of IGF-II on the developing mammary gland. Forsyth et al., (1999) reported a greater abundance of IGF-II mRNA compared to IGF-I mRNA in the mesenchymal cells underlying the epithelial ducts of the fetal ovine mammary gland from days 70 to 140 of gestation. The abundance of IGF-II mRNA increased with gestational age and was reduced in 4-week old postnatal and adult tissue (Morgan et al., 1996; Forsyth et al., 1999). This correlates well with circulating plasma concentrations of fetal IGF-II, which are high in the fetal lamb and decrease at birth to adult levels (Gluckman et al., 1987). The primary mitogenic

effects of IGF-II are likely initiated through the IGF-I receptor, as IGF-II is able to bind to IGF-I receptors with the same affinity as IGF-I (Lacroix et al., 1995). Taken together, these findings support the possibility of a role for IGF-II in mediating mesenchymal-epithelial interactions.

IGF-IR was elevated within the fetal adipose tissue from day 80 of fetal age, the time of which also corresponds with the onset of rapid growth of the female fat pad (Chapter two). IGF-IR also localised to the mesenchymal (fibroblast) cells of the interlobular connective tissue that envelop the developing adipose tissue. Hence, the possibility of two modes for IGF-I regulation: a paracrine model in which the mesenchymal cells adjacent to the developing adipose tissue serve as a local source of IGF-I and an autocrine model in which the mesenchymal cells of the deep hypodermis stimulate their own proliferation. In refutation of this proposal, Forsyth et al., (1999) did not observe a strong signal for expression of IGF-I or IGF-II mRNA in the more distant interlobular connective tissue. However, in lambs IGF-I mRNA expression was significantly higher in the mammary fat pad relative to the epithelium and during the course of postnatal development, highest expression was observed during the prepubertal period of allometric growth (Hovey, 1996). Interestingly, IGF-IR immunoreactivity in fetal mammary tissue was greatest in late gestation when mammary growth of the female was positively allometric (Chapter two).

The high concentration of circulating plasma oestrogens in late gestation (Findlay and Seamark, 1973) combined with the presence of oestrogen receptors in the mammary epithelial cells from day 100 of gestation (Chapter three) suggest a direct effect of oestrogen on epithelial development of the ovine fetus. On the other hand, the increase in the concentration of fetal plasma oestrogen in late gestation (Findlay and Seamark, 1973) could act as a stimulus for fetal GH release, which in turn may regulate IGF-I levels within the mammary gland. Alternatively, oestrogen may upregulate IGF-I and/or IGF-IR as exogenously added oestrogen increased mRNA expression of IGF-IR by two to three-fold in transplanted normal human breast tissue (Clarke et al., 1997). The presence of IGF-I and IGF-II mRNA in the mesenchymal cells that reside in close contact with the epithelium, and of IGF-IR in the epithelial cells, suggest paracrine regulation, whereby oestrogen acts on the mesenchyme to induce mammary growth in the fetal sheep.

In the fetal male, the intensity of immunohistochemical staining for IGF-IR increased in both the fibroblasts and epithelial cells of the growing ducts from day 60, reaching a peak between days 100 and 120 of gestation. This was very similar to the pattern of immunoreactivity observed for androgen receptor (Chapter three), suggesting that inhibition of mammary growth in the male may involve mediation by IGF-I. As the mesenchyme is well known to play a dominant role in morphogenetic tissue interaction, at least in the mouse (Kratochwil, 1969) it is suggested that androgens control morphogenesis of the male mammary gland during this phase of sexual dimorphism by acting on mesenchymal IGF production.

## Ontogeny of EGF receptor (EGFR) in the fetal mammary gland

At day 120 of fetal age the epithelial cells of the female ovine mammary gland expressed immunohistochemically detectable EGFR. Receptors were located exclusively at the cell surface. This is in agreement with the linear cell surface distribution of EGFR in the ductal cells of the normal adult human breast (Damjanov et al., 1986). EGFR were not present at any of the other stages examined, nor were they positively identified in the mammary tissues of the ovine male.

Interestingly, the infusion of EGF into the fetal sheep from days 110 to 125 of gestation resulted in marked hypertrophy of the skin and wool follicles, together with a reduced ratio of primary to secondary follicles and shedding of the fibres (Thorburn et al., 1981). This is consistent with the presence of EGFR in the epidermis, wool follicles and accessory glands seen in the present study. EGFR is also widely distributed in the epidermis of human fetal skin (Damjanov et al., 1986). In a discussion section, Thorburn et al. (1981) commented that no changes were observed in the fetal mammary glands after EGF infusion. However, they did not report as to whether the evaluation of the mammary glands was based on gross measures or histological indices.

These results indicate that mammary epithelial cells are most probably dependent on other external/internal factors, e.g., IGFs, for growth regulation during fetal development. The participation of EGFR in fetal mammary growth remains uncertain.

## Conclusions

These findings suggest that IGF-IR expressed by the epithelial and mesenchymal tissues of the female mammary gland may be a putative mediator of mesenchymal-epithelial interactions in ovine mammary development prior to day 100 of fetal age. Thereafter, it is proposed that oestrogens of placental origin act directly or indirectly, mediated by oestrogen-induced IGF-I and/or IGF-II from the underlying mesenchymal cells, to stimulate cell differentiation and cell proliferation in the prenatal mammary gland.

The similarity in the ontogeny of AR and IGF-IR expression in the mammary gland of the ovine male supports a paracrine model. It is proposed that androgens act via mammary mesenchymal androgen receptors to regulate the pattern of synthesis and secretion of IGF-I and hence mammary morphogenesis, in such a way that little further development takes place after day 120 of fetal age.

Further studies need to be performed to clarify the precise relationship between steroid hormones and growth factors in the fetal mammary gland.

# CHAPTER FIVE

# EFFECTS OF ANDROGEN, ANTI-ANDROGEN AND ANTI-OESTROGEN ON DEVELOPMENT OF THE MAMMARY GLAND IN FETAL SHEEP AND NEWBORN LAMBS

## 5.1 Abstract

The presence of androgen and oestrogen receptors in the epithelial and mesenchymal tissue of the developing ovine mammary gland suggests not only a role for steroid hormones in fetal mammogenesis, but also potential sensitivity to androgenic and oestrogenic stimuli from the environment. Therefore, it was proposed to inject pregnant ewes with substances that were representative of the compounds that are likely to cause disruption of mammary gland development and assess their effects on both the ewes and their fetuses.

The effects of androgen, anti-androgen and anti-oestrogen treatment of pregnant ewes on development of the fetal mammary gland were examined in two studies. In a preliminary study (experiment one), twin-bearing ewes (n=5) were treated by once-weekly intramuscular injection of testosterone 17β-cypionate (200 mg/ml) at days 36, 43, 50 and 57 of gestation. Ewes and fetuses were euthanased at day 140 of gestation.

In experiment two, 36 twin-bearing ewes were randomly assigned to one of four treatments (n=9 ewes per treatment group). Testosterone (representative androgen) and cyproterone acetate (Androcur, representative anti-androgen) treatments were administered in commercially available slow release formulations by twice weekly intramuscular injection (100mg or 1ml) at days 36, 40, 43, 47, 50, 54 and 57 of gestation. Tamoxifen (representative anti-oestrogen) (0.085g in 1.7ml cottonseed oil) and cottonseed oil treatments were also administered by twice-weekly intramuscular injection on the same days. Three ewes, and their fetuses, from each treatment group were euthanased at days 61 or 117 of gestation. The remaining 12 ewes were allowed to proceed to term and their milk production recorded for the first seven days of lactation. All lambs were euthanased at birth. Histomorphological and immunohistochemical analyses determined the extent of mammary development and immunoreactivity to androgen receptor antibody, respectively.

In both experiments, prenatal testosterone exposure partially masculinised the external genitalia of female fetuses producing a penis and empty scrotum with no external vaginal opening. These females also possessed visually normal ovaries and all the derivatives of the Müllerian ducts (fallopian tubes, uterus, cervix, anterior vagina) and Wolffian ducts (epididymides, vas deferens, seminal vesicles). Administration of Androcur to pregnant ewes did not cause feminisation of the male offspring.

In experiment two, mammary gland development of males and masculinised females at birth did not differ ($P<0.06$), whilst females from the other three treatment groups had significantly ($P<0.01$) greater total duct and secretory cell areas than their male counterparts. In contrast, total duct area at day 140 of gestation, in experiment one, was significantly ($P<0.05$) greater in masculinised females relative to males, but severely suppressed relative to phenotypically normal females (Chapter two).

Where immunoreactivity to androgen receptor antibody was positive, the sites of reactivity were predominantly localised in the apical cytoplasm of the ductal glandular epithelium and the fibroblasts of the dermal mesenchyme. At each stage of development examined, the intensity of immunostaining was greater in males and testosterone-treated females relative to control females and females exposed to Androcur.

The milk yield of Tamoxifen-treated ewes was significantly ($P<0.05$) lower than that of all other treatment groups at each milking, but there was little discernible effect on milk composition. The effect on lactational performance was reflected in significantly reduced maternal plasma progesterone concentrations relative to control ewes at days 120 and 140 of gestation.

These findings indicate a role for testosterone in the sexual dimorphism of the ovine mammary gland. Moreover, they emphasise the sensitivity of the developing fetus to the adverse effects of exposure to environmental androgens and oestrogens during the period of sexual differentiation. Furthermore, there is cause for concern with respect to the secretory capacity of the maternal mammary gland after exposure to environmental 'endocrine disruptors' given the current focus on increased lambing percentages.

## 5.2 Introduction

Earlier observations (Chapter two) and those of others (Wallace, 1953; Martinet, 1962) demonstrated a gradual divergence in the extent of development of the mammary gland epithelial and mesenchymal structures of male and female fetal sheep. While the epithelial and adipose tissue of the female gland continued to proliferate within an extensive network of fibrous connective tissue with increasing fetal age (Chapter two; Forsyth et al., 1999), complete differentiation of those cellular structures in the male gland was prevented. By three weeks of postnatal age the rapidly proliferating dichotomously branched epithelium of the female gland had penetrated an extensive fat pad (Chapter two; Akers, 2002). In contrast, the epithelium of the male was restricted to an area of fibrous connective tissue immediately dorsal to the teat, having failed to penetrate the fat pad, which was present in a much lower abundance within the male mammary gland (Chapter two).

The rodent model has provided overwhelming evidence that androgens, specifically testosterone, produced from the fetal testis are directly responsible for the prevention of the complete differentiation of the epithelial cells into a mammary gland capable of synthesising milk in the male. This model also established that androgen binding in the mammary gland is due to the expression of androgen receptors and that the fetal gland shows endocrine sensitivity. Androgen-induced regression of the mammary gland can also be elicited in females with exogenous androgen. Testosterone propionate administered to pregnant mice during the period of androgen responsiveness caused separation of the mammary bud in female fetuses as well as males (Hoshino, 1965). Cyproterone acetate, an anti-androgen, prevented regression of the mammary bud in male fetuses resulting in nipples being present on all rats born to mothers

treated from day 12 of gestation with this compound (Elger and Neumann, 1966; Neumann and Elger, 1966).

Whether differences between the patterns of mammary development in male and female sheep are a result of the action of androgen receptors in the male under the influence of testicular androgens is not known. The mammary gland of the ovine fetus does possess androgen receptors (Chapter three) but there are two distinct differences between mammary development in the mouse and the sheep that discourage an extrapolation of results. First, whereas the mouse mammary gland possesses androgen receptors exclusively in the mesenchymal cells (Heuberger et al., 1982; Wasner et al., 1983), the ovine mammary gland possesses androgen receptors in both the mesenchymal cells and the epithelial cells of the ducts (Chapter three). Second, while sexual dimorphism of the ovine mammary gland appears to be a gradual process (Chapter two), in the mouse there exists only a short (about 30 hours) androgen-responsive "window" when androgen-induced regression of the male mammary gland occurs (Kratochwil, 1971).

As initially determined by Short (1974), and later refined by Clarke et al. (1976), sexual differentiation in the fetal sheep occurs from approximately 30 to 100 days of the 147 day gestation. However, only a short period of sensitivity to androgens exists (between day 40 and 50 of gestation), which results in complete masculinisation of the external genitalia of the female offspring. Treatment of pregnant sheep with testosterone, either as an implant (Short 1974; Clarke et al. 1976; Tarttelin 1986) or as repeated intramuscular injections of testosterone cypionate, a long-acting androgen (Wilson and Tarttelin 1978; Tarttelin 1986; Wood et al. 1991; Wood et al. 1995), from days 20, 30 or 40 of gestation caused complete masculinisation of the external genitalia in the female offspring. Later treatments were ineffective. These findings suggest that *in utero* exposure of female fetuses to androgens during the period of sensitivity to masculinisation by gonadal steroids may give indirect evidence that inhibition of mammary gland growth in the ovine male is dependent on its exposure to testosterone.

Like that of the fetal rodent, the mammary gland of the fetal sheep also possesses oestrogen receptors (Chapter three). However, in contrast to the rodent, oestrogen receptors in the mammary gland of the sheep are present in both the mesenchymal and epithelial compartments during prenatal life. The normal progression of mammary morphogenesis in oestrogen receptor knockout mice suggests that prenatal development of the mammary gland is autonomous (Bocchinfuso and Korach, 1997). In view of this, one would expect to observe, through maternal treatment with an oestrogen or anti-oestrogen, the mammary gland of the female offspring to develop as normal. However, the mammary mesenchyme of the fetal rodent displays oestrogen sensitivity. For example, epithelial cell density was significantly increased at four weeks of age in the female offspring of rats treated with 20 ng oestradiol per day between days 14 and 20 of gestation. But, the effect was not permanent: growth in the controls was equivalent by seven weeks of age (Hilakivi-Clarke et al., 1997). In contrast, the prevalence of terminal end buds was

increased by oestrogen at both ages, although it was not known whether the effect persisted into maturity.

Given that fetal mammary tissue is responsive to stimuli from exogenous steroid hormones, the gland is potentially at risk from exposure of the mother to endocrine-disrupting chemicals, such as pesticides and industrial chemicals (Sonnenschein and Soto 1998). Of particular relevance to domestic ruminants are the environmental oestrogens, which have the potential to alter sex differentiation and hence, mammary gland development. One such class of environmental oestrogens are the phytoestrogens, which are plant compounds with structural similarities to natural and synthetic oestrogens and anti-oestrogens (reviewed by Kurzer and Xia, 1997). A similar group of compounds are the oestrogenic mycotoxins, one of which, zearalenone, is commonly found in New Zealand pastures (di Menna et al., 1987). Ewes grazing pasture grasses and clovers infected with mycotoxins may display markedly reduced reproductive performance through lowered ovulation rates, failure of fertilisation and anovulation (Smith et al., 1990).

Rodent models in which the pregnant mother was treated with oestrogens or oestrogenic compounds have provided evidence that the mammary gland is sensitive to the effects of exogenous steroids during prenatal development. The mammary glands of the female offspring of pregnant mice injected with 2 µg zearalenone from days 15 to 20 of gestation exhibited increased terminal end buds and increased epithelial differentiation (Hilakivi-Clarke et al., 1998). On the other hand, *in utero* exposure to 20 µg genistein from days 15 to 20 of gestation resulted in more terminal end buds, and fewer differentiated epithelial structures, when compared to the glands from offspring exposed to the vehicle alone (Hilakivi-Clarke et al., 1998).

While their effect on the development of the ruminant mammary gland is still unclear, the possibility exists that compounds that interfere with oestrogen action have the potential to alter mammary development and hence, impede the capacity to produce milk in later life. Therefore, it was hypothesised that the expression of oestrogen receptor in the fetal ruminant mammary gland makes it a target for hormonal disruption through exposure to exogenous oestrogens. Furthermore, it was proposed that the pattern of mammary morphogenesis in the male was, in part, dependent on the secretion of androgens from the male testes. The experiments herein have compared:

1. Morphological measurements and immunoreactivity to androgen receptor antibody in normal males and females, and in males and females whose dams were treated with testosterone cypionate or Androcur from days 36 to 56 of gestation, to provide indirect evidence of a role for testosterone in suppression of mammary growth in the male.
2. Morphological measurements in normal females and in females whose dams were treated with tamoxifen (an anti-oestrogen) from days 36 to 56 of gestation to provide evidence that the mammary gland is/is not sensitive to the effects of exogenous steroids during prenatal development.

The effects of androgen, anti-androgen and anti-oestrogen treatment upon mammary development at parturition and subsequent milk yield in the ewes were also investigated.

## 5.3 Materials and Methods

All procedures were approved by the Massey University Animal Ethics Committee.

## Experiment one

### 5.3.1 Animals and Treatments

Five twin-bearing Romney ewes, aged 3 or 4 years, were used in this preliminary study. They were selected in May, 35 days after mating, from a commercial flock of 100 ewes on the Sheep and Beef Cattle Research Unit (SBCRU), Massey University. Ewes were mated in March (autumn) after oestrus synchronisation using progesterone-impregnated controlled internal drug releasers (Eazi-breed CIDR Type G, Carter Holt Harvey Plastic Products, Hamilton, New Zealand). The CIDRs were inserted for 12 days. Six Suffolk rams were introduced at CIDR withdrawal.

Rams were harnessed with Sire-Sine® mating crayons (Radford et al. 1960), and crayon marks were recorded on a daily basis for the first 7 days of the first cycle (between 1600 and 1800 hours (h)). Crayon colour was changed on day 7 and 17 of the first cycle and returns to service were recorded at the end of the second cycle. Multiple pregnancy was identified at day 35 of gestation (and confirmed at day 47 to verify previous results) using real-time ultrasound scanning (Carter 1987).

Once selected, the five twin-bearing ewes were transferred to Massey University's Terrace Block, 5 kilometres south of Palmerston North (latitude 40.23° S and longitude 175.37° E).

Ewes grazed pasture (mainly ryegrass *(Lolium perenne)* and white clover *(Trifolium repens)*), under commercial conditions, at the Terrace Block for the duration of the trial. Ewes were weighed on electronic scales (Tru-Test Distributors, Auckland) at CIDR insertion (mean weight of 58.7±4.6 kg) and again 24 hours prior to slaughter (day 139) (mean weight of 79.1±2.5 kg).

To induce masculinisation of female lambs *in utero*, 2 ml Depo® -Testosterone (each ml of the 100 mg/ml solution containing 100mg testosterone 17β-cypionate, 0.1 ml benzyl benzoate, 736 mg cottonseed oil and 9.45 mg benzyl alcohol (Pharmacia & Upjohn Company, Kalamazoo, MI 49001, USA)) was administered to the pregnant ewes by intramuscular injection at days 36, 43, 50 and 57 of gestation (term is ~147 days).

### 5.3.2 Blood Sampling and Assays

To monitor the concentrations of testosterone in maternal circulation, blood samples were collected from each ewe by jugular venipuncture at day 35 (24 h prior to the first injection) days 37, 44, 51 and 58 (24 h post-injection) and day 140 of gestation. Samples (7 ml) were withdrawn into vacutainers (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA) containing sodium heparin as the anticoagulant and immediately placed on ice. Within one hour, the samples were centrifuged at 4000 x g and 4°C for 10 minutes. Plasma was pipetted into duplicate vials and stored at -20°C until assayed. In addition, blood samples were obtained from both male and female fetuses by cardiac puncture at day 140 of gestation (day of slaughter).

Circulating plasma concentrations of maternal testosterone were measured in duplicate using a commercial solid-phase Testosterone RIA kit (TESTO-CTK (P3093), DiaSorin s.r.l., 13040 Saluggia (VC) Italy). Assay sensitivity was below 0.05 ng/ml at 95% confidence limit. Within and between assay coefficients of variation for testosterone were 9.0% and 12% respectively (based on the manufacturer's sample pools).

### 5.3.3 Tissue Collection

At day 140 of gestation, ewes were euthanased by stunning with a captive bolt pistol and exsanguination. Slaughter was conducted between 0900 h and 1400 h, and the data on each ewe collected within 45 minutes of slaughter. Immediately following exsanguination, the mammary gland was dissected off and trimmed of skin, fat and connective tissue before being weighed. Consistency and colour of the mammary secretions were also noted. Following removal of the mammary gland the abdominal cavity was opened and the uterus removed. The utero-cervical junction was ligated and the cervix, vagina, ovaries and excess tissue removed before the gravid uterus was weighed. An incision was made along the greater curvature of each pregnant horn and the fetal fluids removed. The umbilical cord was ligated near the point of attachment to the fetal abdomen and approximately 4 cm distal to that point, and the umbilical cord severed between the two ligatures. Fetus(es) were then blood sampled and any still alive at this stage (n=9) euthanased via intracardiac injection of sodium pentobarbitone (1.5 ml) (Pentobarb 500, Chemstock Animal Health, Christchurch, New Zealand). The fetus was then removed from the uterus. Amniotic fluid was removed from the fetal wool by gentle squeezing. Each fetus was given an identity number. The length of the penis and scrotum, and the distance between the penis and anus were measured using digital callipers. The external genitalia were photographed and a description of external features was made. The mammary glands of both sexes were then dissected, separated into left and right glands and placed in Bouin's fixative (within 15 minutes of euthanasia). Fetal weight, curved crown-rump length (with the fetus lying in a "relaxed" position) and girth measurements were recorded, as well as rank and sex.

The entire urogenital tract was removed from the body then photographed. The presence of normally developed ovaries and all the derivatives of the Müllerian ducts that distinguished

female fetuses from their male counterparts was recorded. The patency of the urethra was determined by injecting coloured water into the urinary bladder with a syringe after ligation of the ureters.

### 5.3.4 Histology samples

Fetal mammary gland tissue was fixed in Bouin's fixative for up to 20 hours (due to its fibrous nature). The tissue was then stored in 70% ethanol before embedding in paraffin wax. Sections 7 µm thick were cut from the left gland of each animal, running through and parallel to the long axis of the teat in the anterior-posterior plane. Once the ducts became visible, every tenth section was observed under the microscope until the "complete duct system" (which included the teat canal and ductal branching) was located. If a complete system was not achievable (due to a bent teat or cracking of the tissue) the right gland was sectioned. Six sections from each animal were mounted on individual silane-treated slides. Four to ten sections either side of the "complete duct system" were stored in dust-free boxes for future use.

The mounted sections were then oven dried overnight at 57°C to promote adherence to the slide. Four of the six slides were stained with haematoxylin and eosin (H&E), which stains the nuclei dark blue and the connective tissue pink. Sections, once stained, were mounted using DPX mountant (Product 360294H, Lot 84028841, BDH Laboratory Supplies, Poole, England), and stored at room temperature until analysis of duct and secretory cell area, and duct number. The remaining two sections were stored for immunohistochemical identification of androgen receptor.

The protocol for treating the slides with silane was as follows:

Slides were washed in chromic acid over night, then rinsed for 2 hours in warm (60°C) running water. Slides were oven dried at 57°C, allowed to cool, then immersed (individually) in a fresh solution of 2% 3-aminopropyltriethoxysilane in dry acetone (dried with $MgSO_4$). Slides received two washes in dry acetone followed by a further two washes in distilled water. A dust-free box was used to store the slides until required.

### 5.3.5 Morphological measurements

Total duct area ($\mu m^2$) for each gland was determined by viewing the section under a microscope and tracing the outline of individual ducts and their lumens (if present) using a digitizer pad attached to a computer and the Sigma Scan Scientific Measurement Programme (Version 3.90, Jandel Scientific, 1988). Total secretory cell area ($\mu m^2$) was calculated by subtracting the lumen areas from their respective duct areas.

### 5.3.6 Immunohistochemistry

The primary antibody for the androgen receptor (AR) analysis and the methodology used to identify AR in the fetal mammary gland are explained in Chapter three.

Slides were examined for intensity of immunohistochemical staining using a differential interference contrast (DIC) microscope. Staining was subjectively estimated and expressed as negative (-), weakly positive (+), moderately positive (++), or strongly positive (+++). Photomicrographs of the slides were produced using an image analysis programme.

### 5.3.7 Statistical analyses

Data were analysed using a general linear model procedure for analysis of variance to determine the effects of maternal testosterone treatment on fetal mammary gland development and measures of the external genitalia. Data are expressed as least square means and standard errors for male and female fetuses. Fetal data were adjusted to a common fetal weight. Statistical analyses were conducted using the 'SAS' system for windows, version 6.12. (1996).

## Experiment two

### 5.3.8 Animals and Treatment

A 4 x 3 x 2 factorial design incorporating four treatments (cottonseed oil, testosterone cypionate, Androcur and Tamoxifen) three stages of gestation (days 61, 117 and birth) and two fetal sexes (male and female) was used. One hundred and fifty, mature Romney ewes were mated naturally in March following treatment with progesterone-impregnated CIDRs to synchronise oestrus. Suffolk rams fitted with harnesses and crayons were used to identify mated ewes. Pregnancy status was determined using ultrasound at day 35 of gestation. Only those ewes mated within the first 3 days of the first oestrous cycle and carrying twins were used (n=36).

At day 35 of gestation, ewes (n=9 per group) were assigned at random to one of the four treatments: (a) cottonseed oil (control group), (b) testosterone 17β-cypionate (Depo-Testosterone, representative androgen), (c) Tamoxifen (representative anti-oestrogen) or (d) cyproterone acetate (Androcur Depot, representative anti-androgen).

Testosterone (Lot #20DJT, Pharmacia & Upjohn Company, Kalamazoo, MI 49001, USA) and Androcur (Lot #92134, Schering (Pty) Ltd/ (Edms) Bpk) treatments were administered in commercially available slow release formulations by twice-weekly intramuscular injection (100 mg in 1 ml) from days 36 to 57 of gestation (i.e., inject at days 36, 40, 43, 47, 50, 54 and 57). Tamoxifen (85.7 mg in 1.7 ml cottonseed oil; Lot #119H0790, Sigma Chemical Co., St. Louis, MO, USA) and cottonseed oil (1 ml) (Sigma Aldrich, Castle Hill, NSW, Australia) treatments were administered as above.

The oil solution into which Tamoxifen was incorporated was based on that of Depo® - Testosterone. It was necessary to use 1.7 ml of excipient to dissolve the 85.7 mg of Tamoxifen therefore the ewes in this treatment group received a twice-weekly dose of 1.7 ml per injection.

The final composition of the material injected was 0.771 g Tamoxifen, 13.8 g cottonseed oil, 0.19 g benzyl alcohol and 2.02 g benzyl benzoate. This equated to a total volume of 17 ml.

At day 56 of gestation, four days prior to the date of first slaughter, pregnancy and rank status were confirmed in all ewes. Thirty-two ewes carried twins and four were diagnosed with triplets. Three of those triplet-bearing ewes had been allocated to the Androcur group and one to the testosterone group. One triplet-bearing Androcur ewe was allocated to each of the three slaughter groups and the triplet-bearing ewe treated with testosterone was allocated to the day 61 slaughter group.

Ewes were kept at the Terrace Block for the duration of the trial and were managed under similar conditions to experiment one. They were weighed at the commencement of treatment (day 36), end of the treatment period (day 61), immediately prior to euthanasia (Group A (day 61), Group B (day 117) or at parturition (Group C).

Dimensions of the mammary glands of each ewe were measured on days 35 (pre-treatment) and 58 (post treatment) of gestation, and immediately prior to slaughter. Three measurements were taken in duplicate, as described by Mellor and Murray (1985). One measurement was made from the posterior margin to the anterior margin of the udder (i.e., along the midline) and one on each side of the midline, parallel to the first measurement and immediately medial to each teat.

### 5.3.9 Blood Sampling and Assays

To monitor the concentrations of testosterone and progesterone in the maternal circulation, blood samples were collected from each ewe by jugular venipuncture as follows:

- All treatments – sampled on days 35 (baseline), 46 and 56 at 1400 h (24 hours prior to first injection) and on days 37, 38, 39, 48, 49, 58, 59 and 60 of gestation. (days 37, 48 and 58 are 24 h post-injection (pi); days 38, 49 and 59 are 48 h pi; days 39 and 60 are 72 h pi).
- Group A (day 61) – sampled at day 60 of gestation.
- Group B (day 117) – sampled on days 80, 100, (at 1400 h) and 117 (prior to euthanasia) of gestation.
- Group C (milkers) – sampled on days 80, 100, 117 and 140 (at 1400 h) and prior to morning milking on days 1, 2, 3, 4, 5, 6 and 7 of lactation.

Circulating maternal plasma concentrations of testosterone were measured in duplicate using a commercial solid-phase Testosterone RIA kit (TESTO-CTK (P3093), DiaSorin s.r.l., 13040 Saluggia (VC) Italy). Assay sensitivity was below 0.05 ng/ml at 95% confidence limit. Within and between assay coefficients of variation for testosterone were 9% and 12% respectively (based on the manufacturer's sample pools).

Maternal plasma progesterone concentrations were measured in duplicate using a commercial solid-phase RIA kit (Coat-A-Count Progesterone, Lot no. TKPG1 1567-1, Diagnostic Products Corporation, Los Angeles, California, USA). The detection limit of the assay was approximately 0.02 ng/ml at 95% confidence limit. Within and between assay coefficients of variation for progesterone were 4.7% and 6.0% respectively (based on the manufacturer's sample pools).

### 5.3.10 Tissue Collection

Ewes were euthanased by captive bolt pistol and exsanguination at 61 or 117 days of gestation, or at day 9 of lactation. Euthanasia was conducted between 0900 h and 1600 h. In ewes euthanased at days 61 and 117 of gestation the maternal mammary gland was removed, followed by the intact uterus. The fetuses were exposed and the umbilical cord tied and severed. A fetal blood sample (1.5 to 7 ml) was taken (via cardiac puncture) prior to euthanasia with sodium pentobarbitone. At day 61 of gestation, the genetic sex of fetuses was determined by chromosome harvesting to identify males whose reproductive tracts were feminised or females that were masculinised. Successful karyotyping required a minimum of 1.0 ml blood from each fetus. Body weight, crown-rump length and girth circumference were measured. The length of the penis and scrotum and the distance between the umbilicus/navel and penis, penis and scrotum, scrotum and anus, and were measured to record the extent of masculinisation. The collection of mammary tissue was similar to the procedures of experiment one. At day 117 of gestation, the major fetal organs (heart, liver, lungs, kidney, spleen) were dissected out, cleared of excess fat and connective tissue, towel dried and weighed.

Lambs born to Group C ewes were processed within 24 hours of birth. A blood sample (7 ml) was taken by venipuncture from the jugular vein prior to euthanasia by intravenous injection of sodium pentobarbitone. All internal and external body measurements, external reproductive tract measurements and mammary tissue collection are as described for the days 61 and 117 of gestation fetuses. Gestation length of the Group C ewes was the number of days between mating, as determined by crayon marks, and parturition. Group C ewes were machine-milked twice daily for 7 days, then euthanased on day 9 of lactation. The mammary gland was removed and the weight recorded for the whole and trimmed gland (including milk present in tissues). The consistency and colour of the milk secretions was noted and a 1.0 x 0.5 $cm^2$ section of parenchymal tissue was taken from the mid-section and fixed in Bouin's fixative.

### 5.3.11 Histology samples

The method used for mammary gland sampling, storage and processing is the same as described for experiment one, with one exception. The tissue sections were mounted using commercial-coated slides (Esco, Superfrost Plus, Lot 13200, Biolab Scientific, New Zealand). This promoted better adherence of the tissue from older fetuses and newborn lambs to the slide when heated under pressure. Four slides were stored for future immunohistochemical identification of androgen receptor.

### 5.3.12 Milking of ewes

Ewes whose lambs were euthanased at birth were machine milked. Milk production and composition were measured during the first 7 days of lactation. Morning milking commenced at 1000 h and afternoon milking at 1600 h. Ewes that lambed between 0900 h and 1400 h were first milked at 1600 h, that same day. Ewes that lambed between 1600 h, and 0900 h the following day, were first milked at 1000 h.

Ewes were milked in groups of 6 on a raised, modified artificial insemination (AI) platform, in a shed. Half to 1 minute prior to milking each ewe was injected via the jugular vein with 1.0 iu oxytocin (Ethical Agents Ltd., Wiri, South Auckland, New Zealand. Batch no. 980757) in 0.9 ml of physiological saline (Peterson, 1992).

The milking machine (manufactured by OTENZ, Otorohanga, New Zealand) was a small, portable, electrically powered unit, designed to milk one cow or two sheep or goats. The vacuum was set at –40 kPa and the pulsator frequency at 60 pulses per minute. The machine and milk receptacles were mounted on a trolley for mobility. After each milking the machine was rinsed with cold water. Following the afternoon milking, hot water and detergent followed by a hot water rinse were used.

After machine milking, the ewes were hand milked to remove residual milk that was not readily removed by machine because of the anatomical structure of the udder of the ewe. This was carried out by two experienced milkers.

### 5.3.13 Milk composition analyses

Following afternoon milking, the milk from each ewe that day was mixed thoroughly by gentle inversion and sub-sampled for compositional analyses. Milk samples (20 ml) were preserved by the addition of potassium dichromate ($K_2Cr_2O_7$): 0.6 mg/ml sample, then refrigerated at 4ºC until analyses were carried out at day 9 of lactation.

Milk samples were analysed for fat, protein and lactose content using a Milkoscan 104 A/B (A/S N. Foss Electric, Denmark). The instrument was calibrated by a certified testing laboratory for normal bovine milk. Since the response of the machine is linear over a restricted range of protein and fat concentrations, it was necessary to dilute the ewes' milk with a 5% lactose solution so that the concentration of fat and protein fell within the range of calibration. Readings were obtained in duplicate.

### 5.3.14 Chromosome harvesting

The methodology used to identify genetic sex of the lambs was adapted from Ansari et al. (1993). Prometaphase mitotic fetal sheep chromosomes were obtained from leukocytes grown in tissue culture. The growing medium consisted of minimum essential media alpha (7.5 ml), Pokeweed mitogen (100 µl), fetal calf serum (1.0 ml) and whole blood (0.7 ml).

After an incubation period of 48 hours, each culture was treated with colchicine (50 µl), which arrests mitosis at the metaphase. The cultures were incubated in a water bath at 39ºC for 20 minutes, centrifuged and the supernatant removed. They were then exposed to a hypertonic solution (10 ml of 0.075M potassium chloride) that makes the chromosomes swell and disperse. Half a millilitre of deionised water was added to each culture to help lyse the cells. They were incubated a further 15 minutes, centrifuged and decanted. A total of 9 ml of freshly prepared fixative (3:1 methanol:acetic acid by volume) was added before centrifuging the cells once more. The first fixation of the cells lasted half an hour. After two subsequent changes of the fixative, a few drops of the cell suspension were placed by pipette onto cold slides, air dried and stained for 10 minutes with Giemsa diluted in Sorensen's phosphate buffer at pH 6.8.

Individual chromosomes were observed using a DIC microscope (refer to experiment one for microscope details). Representative prometaphases ($2n$=54) were selected for showing complete karyotypes.

### 5.3.15 Immunohistochemistry

The methodology used to locate androgen receptors is outlined in Section 5.3.6 for experiment one.

### 5.3.16 Statistical analyses

Where parameters were measured on more than one occasion (plasma hormone concentration, milk yield and milk composition) the effects of treatment, time (day of sampling, the repeated factor) and their interactions were analysed by multivariate (repeated measures) analysis of variance.

Analysis of variance was used to determine the effects of cottonseed oil, testosterone, Androcur or Tamoxifen treatments on weights of uterine components and mammary gland, and dimensions of the mammary gland (covariate-adjusted where appropriate).

The effects of the treatments on fetal parameters were analysed by analysis of covariance (adjusted to a common fetal bodyweight). Fetal data showed treatment by stage of gestation interactions were non-significant, therefore means have been presented for "treatment by sex" cells for each slaughter date, or for "treatment" only cells when there was no effect of sex. Data are expressed as least square means and standard errors for the eight (4 treatment x 2 sex) groups of fetuses (where sex was significant) and four (4 treatment) groups of ewes.

Statistical analyses were conducted using the 'SAS' system for windows, version 6.12 (1996).

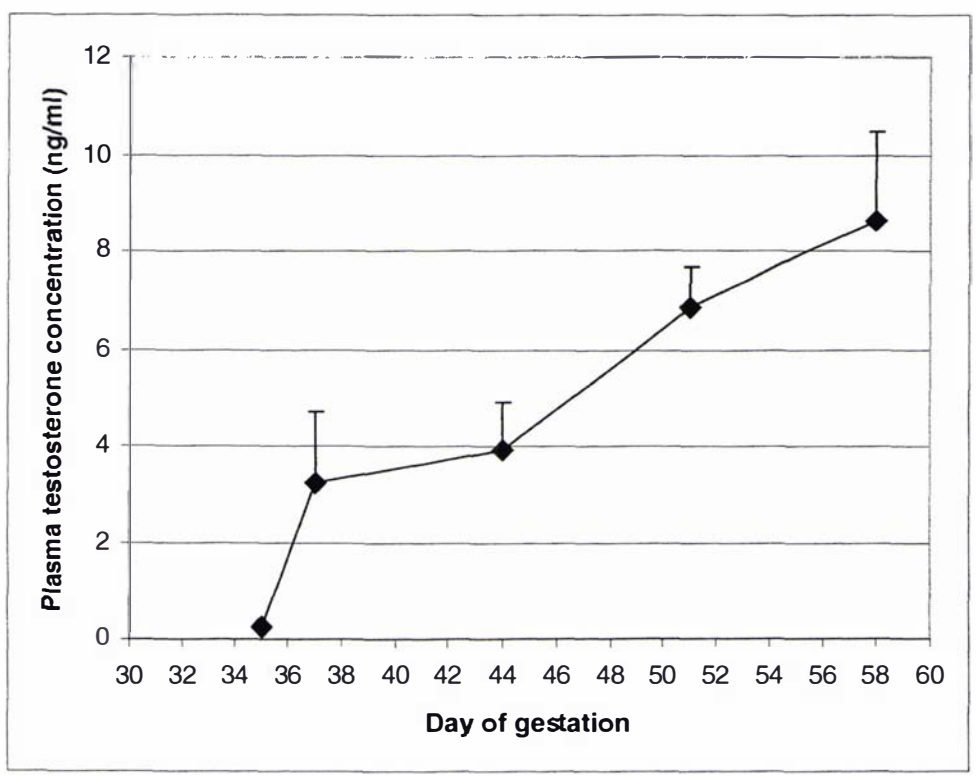
## 5.4 Results

## Experiment one

### 5.4.1 Effects of testosterone treatment upon pregnant ewes

Maternal testosterone concentrations at days 35, 37, 44, 51 and 58 are shown in Figure 5.1. Prior to the first injection, at day 36 of gestation, and at slaughter (day 140 of gestation) maternal testosterone concentrations were below 0.25 ng/ml (lowest standard). Injecting testosterone had a cumulative effect on circulating concentrations of the hormone. At day 37 (24h after the first injection) the mean plasma concentration of testosterone was 3.24±1.45 ng/ml. By day 58 (24h after the last injection) it was 8.64±1.86 ng/ml.

**Figure 5.1** Maternal plasma testosterone concentrations (ng/ml) in five ewes 24hrs after injection of testosterone cypionate (mean±SD).



### 5.4.2 Effects of maternal treatment with testosterone upon male and female fetuses

All five ewes were diagnosed as twin-pregnant at day 47 of gestation. At day 140 of gestation (slaughter) four ewes carried three sets of twins and one set of triplets and one ewe had aborted.

In female fetuses, exposure to testosterone from days 36 to 57 of gestation induced marked masculinisation of external genitalia, with the formation of a penis and scrotum. Genetically female fetuses were identified, regardless of the extent of masculinisation, by their possession of normally developed ovaries and all the derivatives of the Müllerian ducts such as fallopian tubes, uterus, cervix and an anterior vagina (blind). There was no posterior vagina. Since the ovaries remained in their normal intra-abdominal position, the scrotal sacs of the masculinised females were always empty, whereas the normal males had palpable testes present in the scrotum. Müllerian ducts terminated at the urogenital sinus and a patent urethra was present along the entire length of the penis in three of the four females.

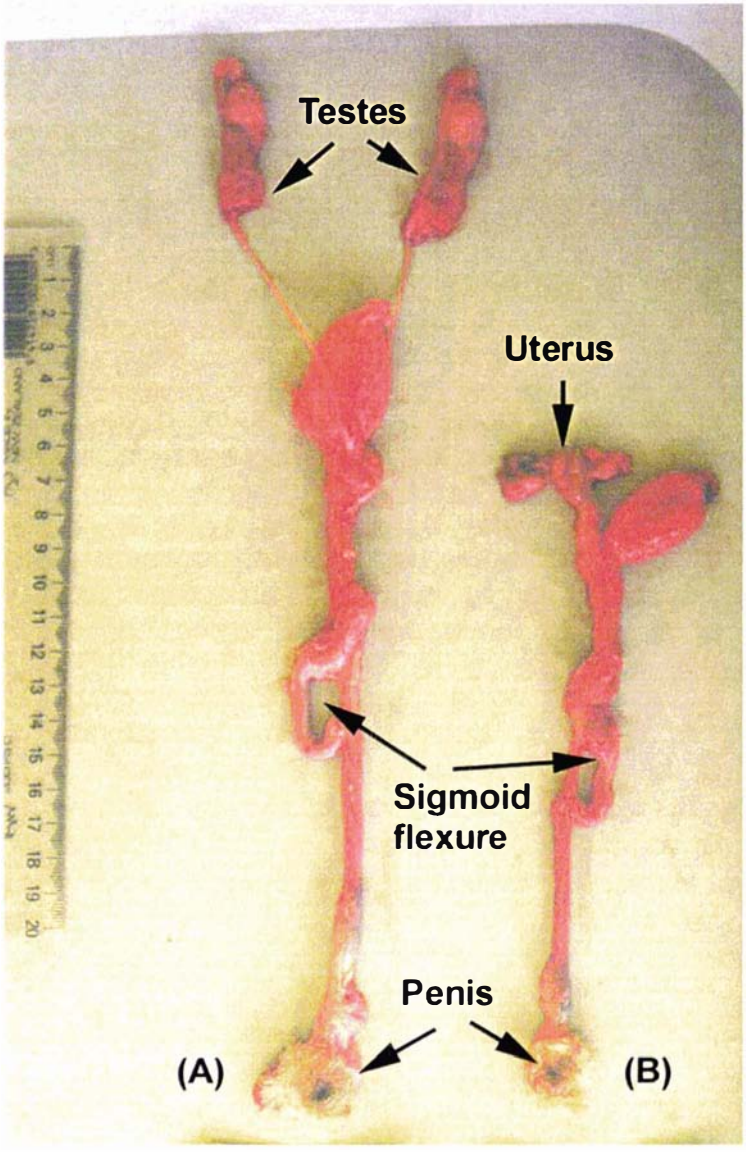
The body weight, crown-rump length (CRL), girth and the external genitalia of male and female fetuses are presented in Table 5.1. The penes and scrotal sacs of masculinised females were significantly shorter ($P<0.001$) than those of male fetuses. However, they did include a sigmoid flexure and a retractor penis muscle (Plate 5.1). Although there were no control males for comparison, the genitalia of male fetuses whose dams were treated with testosterone did not appear grossly different from males in the ontogeny trial (Chapter two). There were no significant sexual differences in body weight, crown-rump length or girth circumference.

**Table 5.1** The body weight, crown-rump length, girth and dimensions of the external genitalia (lsmean±sem) of fetal sheep at day 140 of gestation following treatment of their dams with testosterone from days 36 to 57 of gestation.

| | Sex | | Sig |
|---|---|---|---|
| Parameter | Male | Female | Sex |
| Number of fetuses | 5 | 4 | |
| Body weight (g) | 4971.6±388.3 | 4625.5±434.1 | NS |
| Crown-rump length (cm) | 54.6±1.4 | 53.5±1.5 | NS |
| Girth (cm) | 35.6±0.4 | 36.2±0.5 | NS |
| Length of penis (mm) | 14.8±0.9 | 6.0±1.0 | *** |
| Length of scrotum (mm) | 57.9±2.2 | 34.2±2.4 | *** |
| Distance from penis to anus (mm) | 155.9±7.9 | 134.6±8.9 | NS |

NS, $P>0.10$; ***, $P<0.001$; Sig = significance.

**Plate 5.1** Photomicrograph comparing the reproductive tracts of a normal male (A) and a masculinised female (B). Note the inclusion of a sigmoid flexure and a retractor penis muscle in the masculinised female. (Normal male reproductive tracts are from ontogeny trial fetuses (Chapter two)).



Mammary development, in terms of total duct area, at day 140 of gestation was greater ($P<0.05$) in female fetuses relative to male fetuses (Table 5.2). Masculinised female fetuses (Plate 5.2a) did not develop the extensive fat pad that is present in normal ewe fetuses (refer to Chapter two) (Plate 5.2b). The amount and appearance of adipose tissue was very similar to that observed in the male (Plate 5.2c). It was diffuse in nature and in close proximity to the blind scrotal sac. The udder development of the dams appeared normal but they did not show the secretory activity that would have been expected at day 140 when they were slaughtered. There was no effect on mammary development in fetal lambs.

**Table 5.2** Development of the mammary gland (lsmean±sem, $\mu m^2 x10^3$ for areas) of fetal sheep at day 140 of gestation following treatment of their dams with testosterone from days 36 to 57 of gestation.

| Parameter | Sex | | Sig |
|---|---|---|---|
| | Male | Female | Sex |
| Number of fetuses | 5 | 4 | |
| Total duct area ($\mu m^2$) | 888.6±433.8 | 2575.2±433.8 | * |
| Total lumen area ($\mu m^2$) | 362.7±270.3 | 1290.9±270.3 | † |
| Number of ducts | 59.3±26.1 | 130.9±26.1 | NS |
| Number of lumens | 50.5±24.6 | 108.7±24.6 | NS |
| Total secretory cell area ($\mu m^2$) | 526.0±218.2 | 1284.2±218.2 | † |

NS, $P>0.10$; *, $P<0.05$; † $P<0.10$; Sig=significance.

**Plate 5.2a** Photomicrograph of the mammary duct and adipose tissue morphology of a masculinised female at day 140 of gestation. (Mag x400).

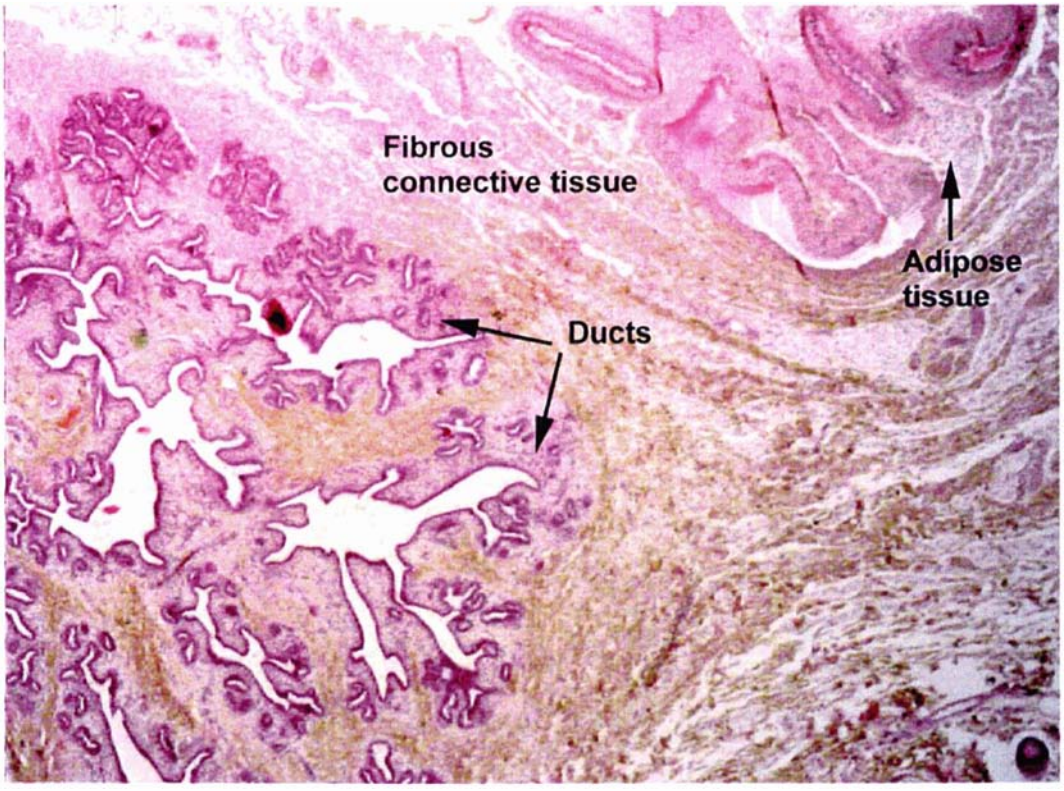



**Plate 5.2b** Photomicrograph of the mammary duct and adipose tissue morphology of a normal female fetus at day 140 of gestation (from ontogeny trial (Chapter two)). (Mag x400).

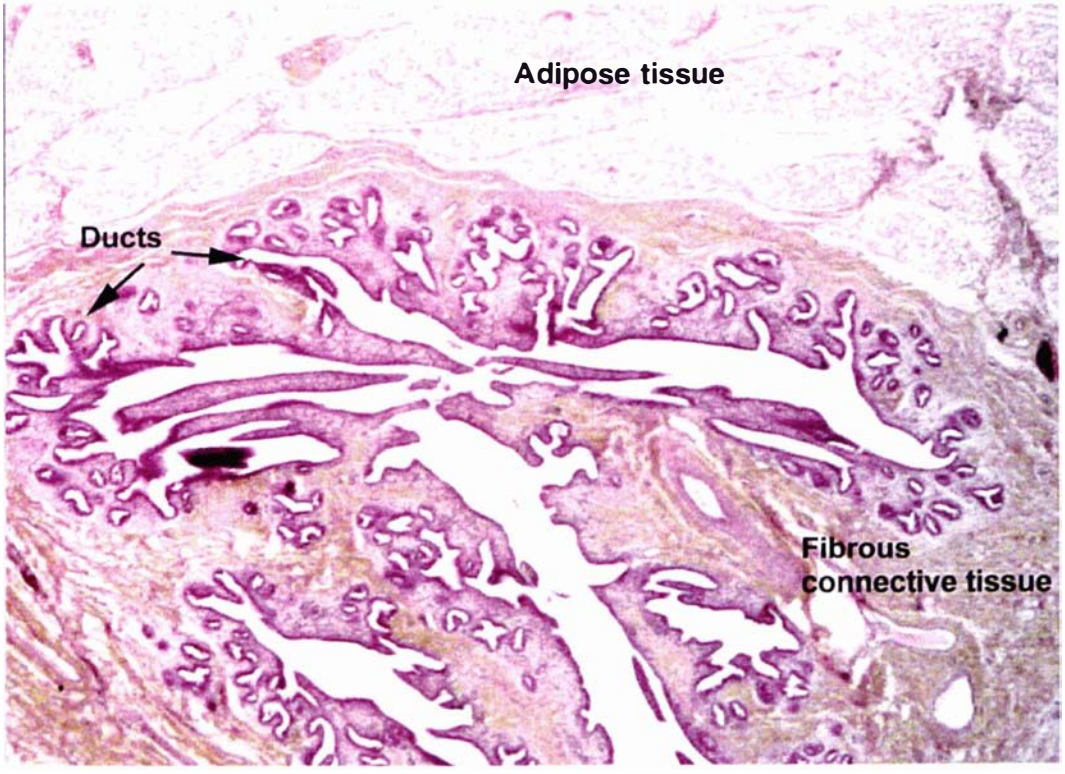
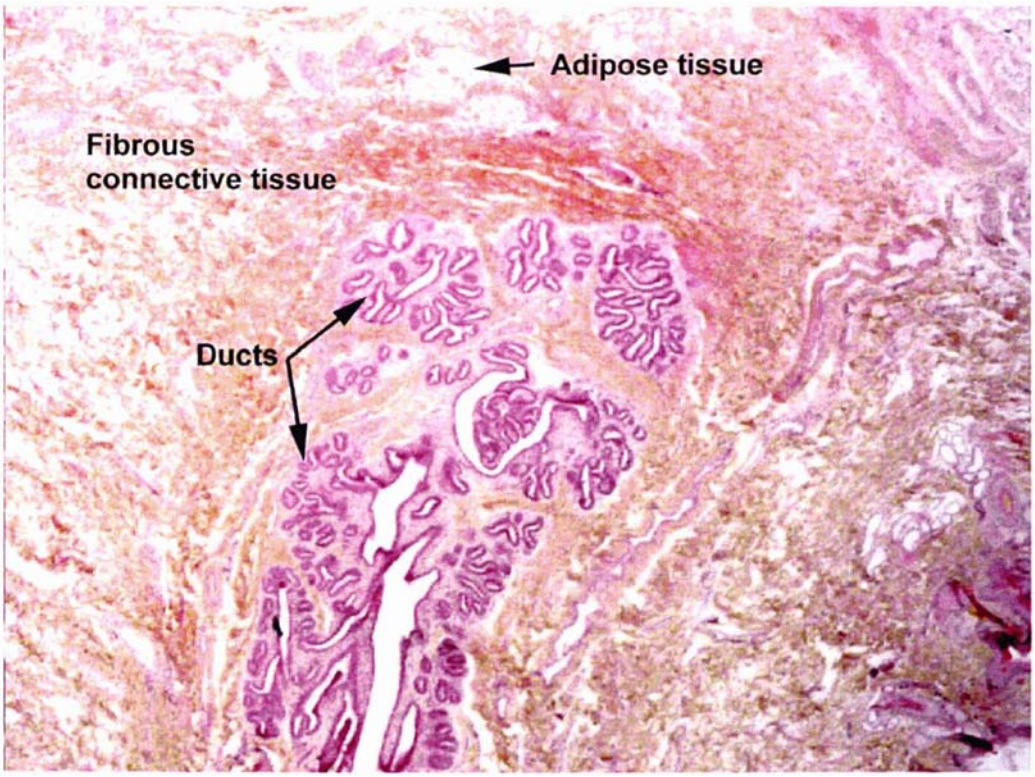


**Plate 5.2c** Photomicrograph of the mammary duct and adipose tissue morphology of a normal male fetus at day 140 of gestation (from control group). (Mag x400).



### 5.4.3 Effects of maternal treatment with testosterone upon androgen receptor immunoreactivity in male and female fetuses.

Immunoreactivity to androgen receptor antibody was observed in the epithelial cells lining the mammary ducts in both male and female fetuses. The reaction patterns described hereafter are exactly the same as those described in Chapter three: linear cell surface, cytoplasmic and nuclear.

The reaction patterns varied not only between the sexes but also between individual males and females (see appendices, Table A.4, Experiment 1; Tables A.5, A.6, A.7, Experiment 2).

The epidermis, which acted as a tissue control, showed negative immunostaining in all the glands tested (no photomicrograph shown).

Strong immunoreactivity to androgen receptor antibody was displayed in only one male, where immunostaining was seen in all compartments of the epithelial cells lining the mammary ducts. Furthermore, the reaction pattern was continuous (Plate 5.3a). The remaining males displayed moderate linear cell surface staining (Plate 5.3b). In three of those males, the sites of immunoreactivity were randomised in a discontinuous pattern and did not appear to be associated with any particular part of the duct system. However, two males exhibited regionalised staining of the more columnar-shaped epithelial cell nuclei, but it was not a constant feature. Immunoreactivity in the masculinised females also followed a linear pattern with weak to moderate staining (Plate 5.3c). And, as with their male counterparts, the line of reactivity followed no particular pattern. Two females displayed randomised immunoreactivity in the nuclei of the larger epithelial cells (no photomicrograph shown).

Immunoreactivity to androgen receptor antibody was exhibited in the nuclei of the surrounding mesenchymal cells in most of the male glands and in one female gland. Immunostaining ranged from questionable through to moderately positive. No immunostaining was seen in the adipocytes of the developing fat pad.

**Plate 5.3a** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of a male sheep at day 140 of fetal age following treatment of the dam with testosterone between days 36 and 57 of gestation. Androgen receptor is localised in both the cytoplasmic and nuclear compartments of the epithelial cells. (Mag x400).

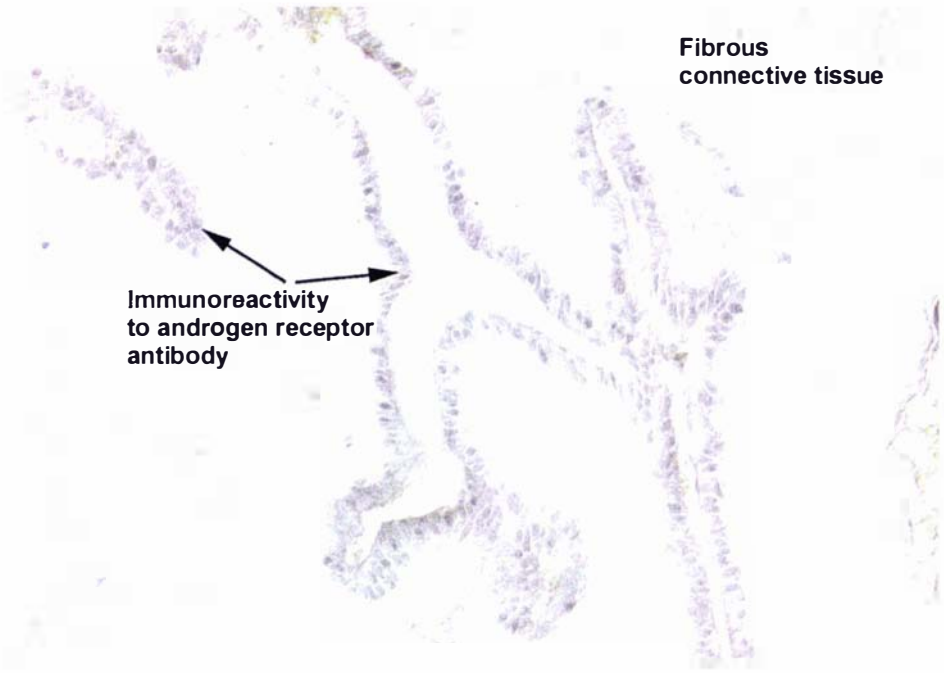



**Plate 5.3b** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of a male sheep at day 140 of fetal age following treatment of the dam with testosterone between days 36 and 57 of gestation. Androgen receptor is localised in the apical cytoplasm and immunostaining is moderately positive. (Mag x400).

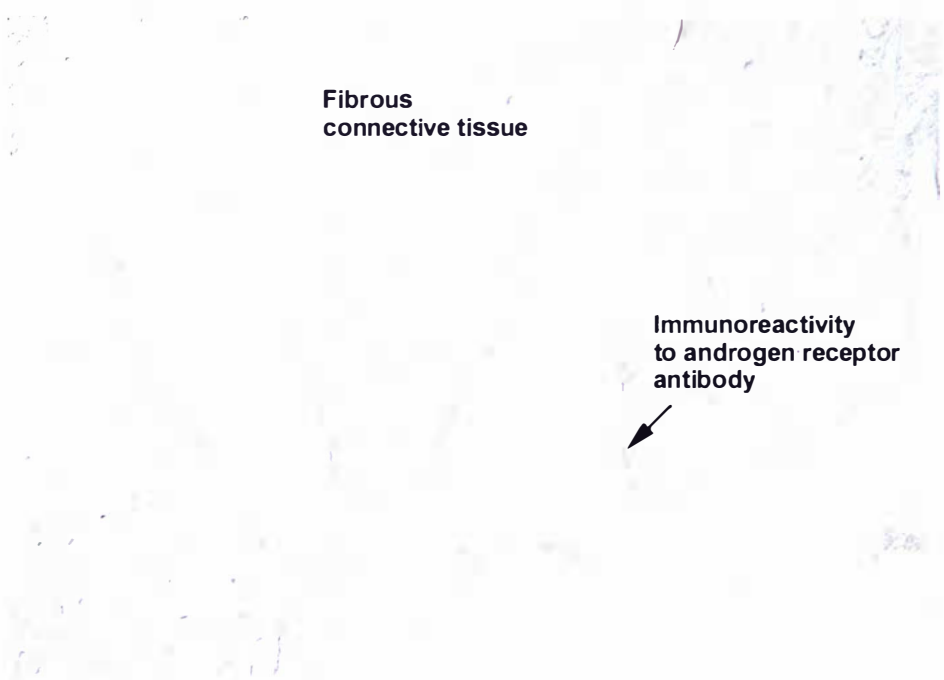
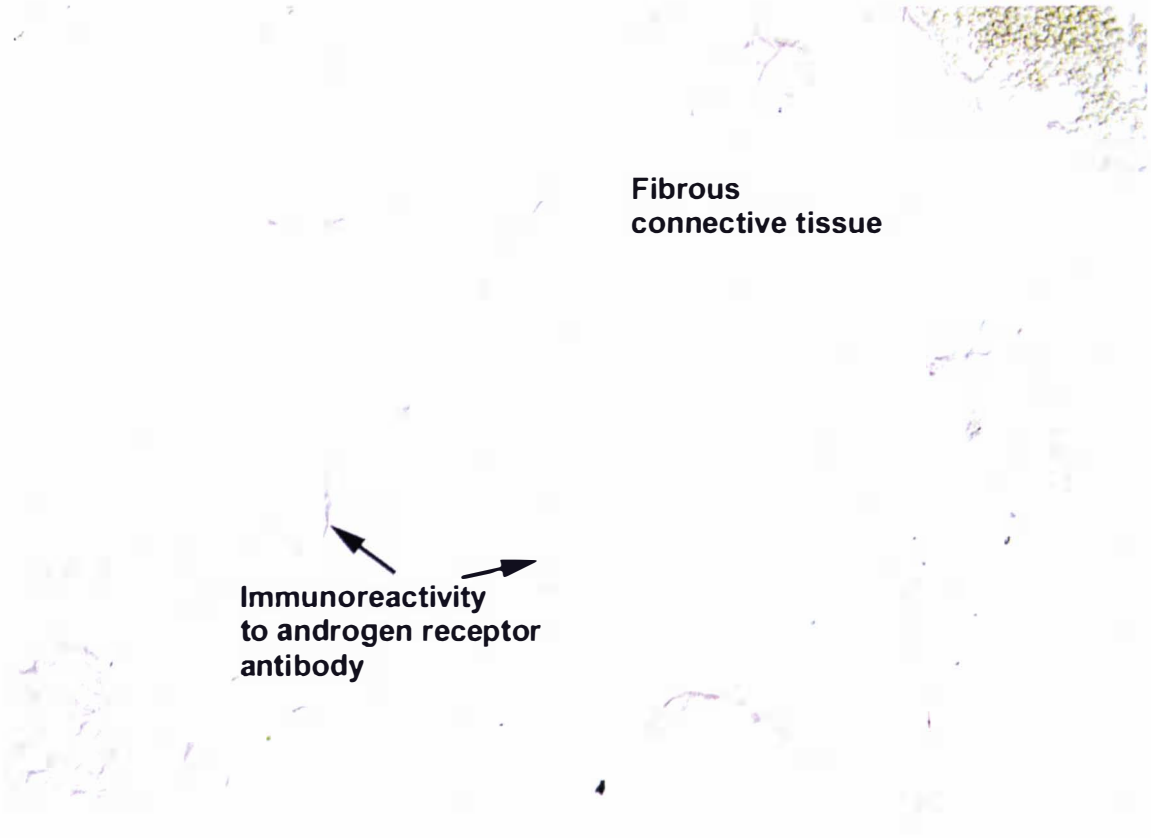


**Plate 5.3c** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of female sheep at day 140 of fetal age following treatment of the dams with testosterone between days 36 and 57 of gestation. Weak to moderate linear staining of the luminal epithelial cell surface. (Mag x400).



# Experiment two

### 5.4.4 Comparison of the effects of cottonseed oil (control), testosterone, Androcur or Tamoxifen treatment upon pregnant ewes – live weight, mammary gland and uterine characteristics.

All ewes were diagnosed as twin-pregnant at day 35 of gestation. Pregnancy status was reconfirmed at day 56 when one testosterone- and three Androcur-treated ewes were also diagnosed as carrying triplets. In the testosterone treatment group, the one triplet-bearing ewe was assigned to the day 61-slaughter group. In the Androcur treatment group, one triplet-bearing ewe was assigned to each of the three slaughter groups (days 61, 117 of gestation and birth) (Table 5.3).

During the third week of treatment, the testosterone-treated ewes showed increased aggressive behaviour. Two ewes in particular would resort to head butting when confined to a small paddock or yards on treatment days. Ewes in the other treatment groups did not show this behaviour. No objective methods were used to quantitate this behaviour.

Intrauterine death occurred only in the Tamoxifen-treated ewes (Table 5.3). Those ewes, which were allowed to continue their pregnancy through to term, all lambed after a normal gestation of approximately 145 days. However, the last lamb born to a triplet-bearing Androcur-treated ewe was stillborn (Table 5.3).

The total gravid uterus was significantly heavier ($P<0.05$) in Tamoxifen-treated ewes at day 61 of gestation (Table 5.4). This was partly due to the heavier body weight of male fetuses in particular relative to fetuses exposed to other treatments *in utero*. There were no differences between groups in maternal live weight, mammary gland dimension or mammary gland weight.

Maternal live weight, mammary gland dimension and components of the gravid uterus did not differ significantly at day 117 of gestation. Total mammary gland was lighter ($P<0.05$) in the Tamoxifen-treated ewes relative to the control ewes, but overall there was no significant difference (Table 5.5).

For the maternal parameters measured at term (parturition) there were no significant differences in gestation length, live weight, mammary gland dimension or mammary gland weight between treatment groups (Table 5.6).

**Table 5.3** The number of ewes and the number, sex and fate of their fetuses/lambs and the gestation length (lsmean±sem) following treatment from days 36 to 57 of gestation with cottonseed oil (control), testosterone, Androcur or Tamoxifen.

| Treatment group | Time of euthanasia (day of gestation) | Ewes (no.) | Rank | Cases of intrauterine death (number) | Cases of abortion or still born (number) | Fetuses/lambs viable or born | | Gestation length ‡ (days) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Male (no.) | Female (no.) | |
| Control | 61 | 3 | 3T | 0 | | 3 | 3 | |
| Testosterone | 61 | 3 | 2T, 1Tp | 0 | | 3 | 4 | |
| Androcur | 61 | 3 | 2T, 1Tp | 0 | | 0 | 7 | |
| Tamoxifen | 61 | 3 | 3T | 1 | | 3 | 2 | |
| Control | 117 | 3 | 3T | 0 | | 3 | 3 | |
| Testosterone | 117 | 3 | 3T | 0 | | 1 | 5 | |
| Androcur | 117 | 3 | 2T, 1Tp | 0 | | 4 | 3 | |
| Tamoxifen | 117 | 3 | 3T | 1 | 2 | 1 | 2 | |
| Control | Birth | 3 | 3T | 0 | 0 | 3 | 3 | 147.0±1.6 |
| Testosterone | Birth | 3 | 3T | 0 | 0 | 2 | 4 | 145.0±1.6 |
| Androcur | Birth | 3 | 2T, 1Tp | 0 | 1 | 3 | 3 | 145.0±1.6 |
| Tamoxifen | Birth | 3 | 3T | 2 | 0 | 2 | 2 | 143.7±1.6 |

T = Sets of twins; Tp = Sets of triplets.

‡ Non-significant treatment group difference.

**Table 5.4** Live weight, total gravid uterus weight and mammary gland measurements of ewes at day 61 of gestation (lsmean±sem) following treatment from days 36 to 57 of gestation with cottonseed oil (control), testosterone, Androcur or Tamoxifen.

| Parameter | Treatments | | | |
|---|---|---|---|---|
| | Control | Testosterone | Androcur | Tamoxifen |
| Number of ewes | 3 | 3 | 3 | 3 |
| Pre-treatment live weight (kg) | 58.3±2.3 | 60.8±2.3 | 60.7±2.3 | 56.2±2.3 |
| Post-treatment live weight (kg) | 65.4±2.2 | 68.1±2.3 | 66.5±2.3 | 63.5±2.4 |
| Total gravid uterus weight (g) | 2063.2±558.8[a] | 2605.8±612.4[a] | 2709.2±569.2[a] | 5007.4±639.8[b] |
| Whole trimmed mammary gland weight (g) | 180.8±34.7 | 176.4±38.1 | 142.0±35.4 | 254.5±39.8 |
| Pre-treatment mammary gland dimension (cm) | 29.6±2.4 | 26.6±2.5 | 24.4±2.5 | 27.0±2.6 |
| Post-treatment mammary gland dimension (cm) | 27.1±2.7 | 33.0±2.5 | 25.1±2.7 | 29.8±2.5 |

[ab] Means within rows with different superscripts are significantly different (P<0.05).

**Table 5.5** Live weight, total gravid uterus weight, weights of placental components and mammary gland measurements of ewes at day 117 of gestation (lsmean±sem) following treatment from days 36 to 57 of gestation with cottonseed oil (control), testosterone, Androcur or Tamoxifen.

| Parameter | Treatment | | | | Sig |
|---|---|---|---|---|---|
| | Control | Testosterone | Androcur | Tamoxifen | T |
| Number of ewes | 3 | 3 | 3 | 3 | |
| Pre-treatment live weight (kg) | 64.3±3.3 | 63.7±3.3 | 65.5±3.3 | 61.2±3.3 | NA |
| Post-treatment live weight (kg) | 66.0±3.3 | 68.3±3.3 | 71.2±3.3 | 64.3±3.4 | NS |
| Final live weight (kg) | 75.9±2.6 | 75.1±2.6 | 74.9±2.9 | 74.6±2.9 | NS |
| Pre-treatment mammary gland dimension (cm) | 24.8±2.5 | 28.9±2.5 | 28.0±2.5 | 25.7±2.5 | NA |
| Post-treatment mammary gland dimension (cm) | 24.3±1.9 | 32.1±1.9 | 28.3±1.9 | 30.0±1.9 | NS |
| Final mammary gland dimension (cm) | 41.9±2.7 | 39.8±2.6 | 41.6±2.0 | 42.0±2.4 | NS |
| Total gravid uterus weight (g) | 8734.8±694.2 | 8090.7±676.4 | 10172.4±721.9 | 8485.0±830.4 | NS |
| Weight of uterine lining + caruncles (g) | 886.5±39.3 | 831.4±41.5 | 806.0±39.9 | 856.3±68.7 | NS |
| Weight of fetal membranes + cotyledons (g) | 1305.1±103.0 | 1180.7±109.0 | 1022.2±104.8 | 1438.0±180.3 | NS |
| Trimmed whole mammary gland (g) | 515.0±63.4 | 347.4±63.5 | 461.8±68.3 | 240.5±69.0 | † |

NS, P>0.10; † P<0.10; NA = not applicable; Sig = significance; T = treatment.

**Table 5.6** Live weight, total gravid uterus weight, weights of placental components and mammary gland measurements of ewes at term, and mammary gland dimension and weight at day 7 of lactation (lsmean±sem) following treatment from days 36 to 57 of gestation with cottonseed oil (control), testosterone, Androcur or Tamoxifen.

| Parameter | Treatment | | | | Sig |
|---|---|---|---|---|---|
| | Control | Testosterone | Androcur | Tamoxifen | T |
| Number of ewes | 3 | 3 | 3 | 3 | |
| Pre-treatment live weight (kg) | 57.0±4.0 | 61.8±4.0 | 60.2±4.0 | 62.2±4.0 | NA |
| Post-treatment live weight (kg) | 70.6±2.1 | 65.9±2.1 | 68.0±2.1 | 67.1±2.1 | NS |
| Final live weight (kg) | 85.2±2.5 | 86.4±2.5 | 85.9±2.5 | 79.9±2.5 | NS |
| Pre-treatment mammary gland dimension (cm) | 28.7±3.7 | 27.7±3.7 | 30.9±3.7 | 30.0±3.7 | NA |
| Post-treatment mammary gland dimension (cm) | 27.2±2.4 | 32.9±2.4 | 28.8±2.4 | 29.7±2.4 | NS |
| Day 120 mammary gland dimension (cm) | 38.6±3.2 | 31.2±3.2 | 35.3±3.1 | 26.5±3.1 | NS |
| Mammary gland dimension at parturition (cm) | 67.3±10.4 | 73.7±9.6 | 72.7±9.9 | 57.9±11.3 | NS |
| Day 7 of lactation mammary gland dimension (cm) | 71.8±2.7 | 68.6±2.7 | 71.2±2.7 | 68.9±3.5 | NS |
| Trimmed whole mammary gland (g) (includes milk) | 1054.9±138.0 | 1121.9±138.3 | 931.4±140.2 | 825.5±179.7 | NS |

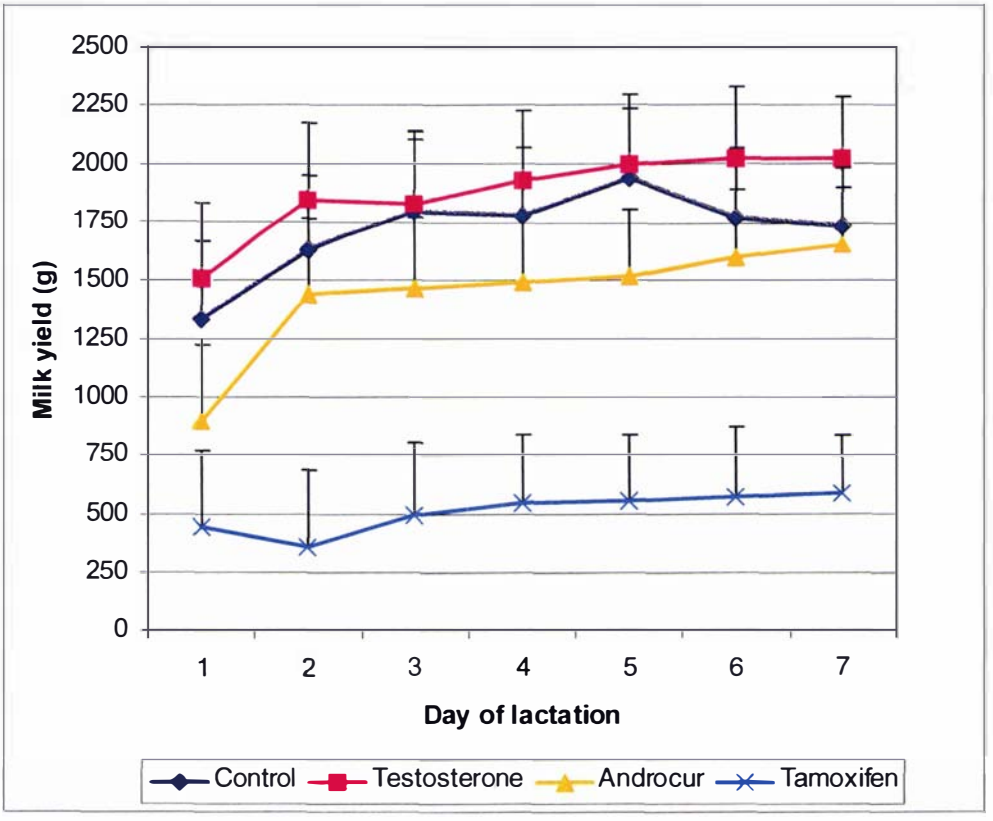
NS, P>0.10; NA = not applicable; Sig = significance; T = treatment.

### 5.4.5 Effects of cottonseed oil (control), testosterone, Androcur or Tamoxifen treatment upon pregnant ewes – milk production and composition.

All ewes produced milk, although one Tamoxifen-treated ewe dried off on day two of lactation, producing only 39 g of milk in the morning and nothing in the afternoon. She remained in the trial, producing only a few drops of milk at each milking. One Tamoxifen ewe produced similar daily milk yields to ewes in the other treatment groups, while one Tamoxifen ewe produced approximately half that of the other groups at each milking.

Milk yield of the Tamoxifen group was significantly ($P<0.05$) lower than that of all other groups at each milking (Figure 5.2). Within treatment groups, day of lactation was significant ($P<0.001$), but there was no significant treatment by time interaction.

**Figure 5.2** Daily milk yields of lactating ewes treated with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation (lsmean±sem).



Overall, the yield of milk per gram of mammary tissue at day 7 of lactation did not differ significantly between the treatment groups (Table 5.7).

**Table 5.7** Yield of milk per gram of mammary tissue at day 7 of lactation in ewes treated with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation (lsmean±sem).

| | Treatment group | | | |
|---|---|---|---|---|
| | Control | Testosterone | Androcur | Tamoxifen |
| Yield/g tissue | 1.67±0.12 | 1.92±0.13 | 1.65±0.12 | 1.17±0.17 |

Data adjusted to a common mammary gland weight.

Milk composition (fat, protein and lactose concentrations) was measured on a daily basis for each ewe. Mean milk fat, milk protein and milk lactose percentages did not differ significantly between treatment groups over the first seven days of lactation. Within treatment groups there was a significant ($P<0.001$) effect of day, but no treatment by time interaction (Figures 5.3, 5.4 and 5.5).

**Figure 5.3** Concentration of fat (percentage) in milk for the first 7 days of lactation (lsmean±sem) of ewes following treatment with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation.

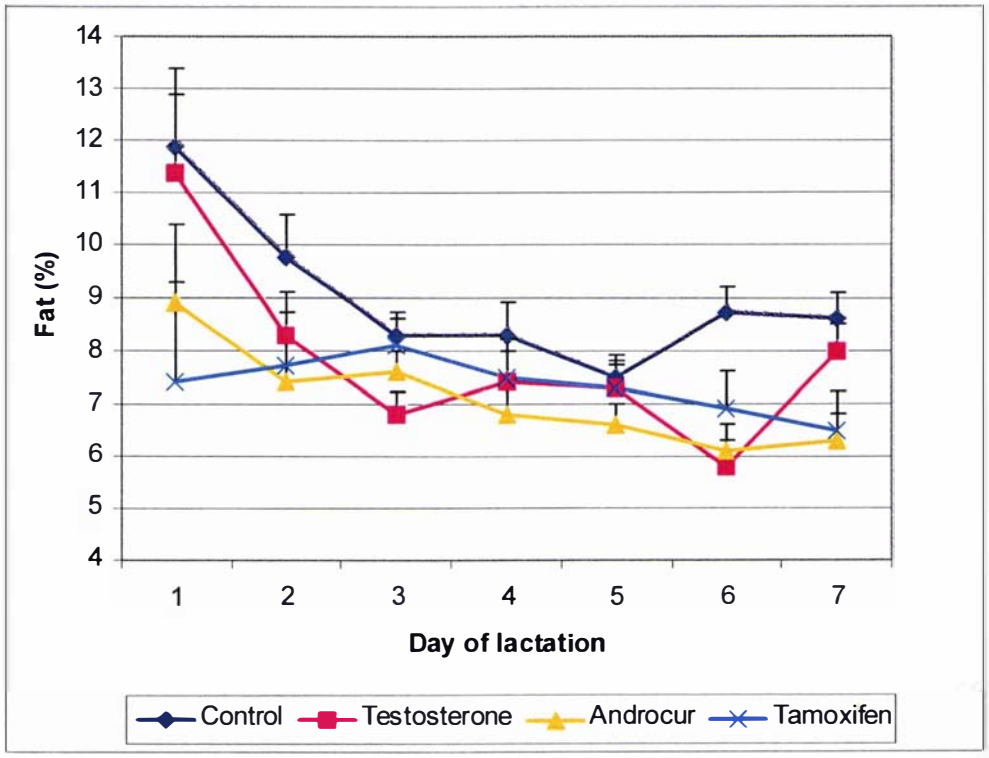


**Figure 5.4** Concentration of protein (percentage) in milk for the first 7 days of lactation (lsmean±sem) of ewes following treatment with cottonseed oil, testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation.

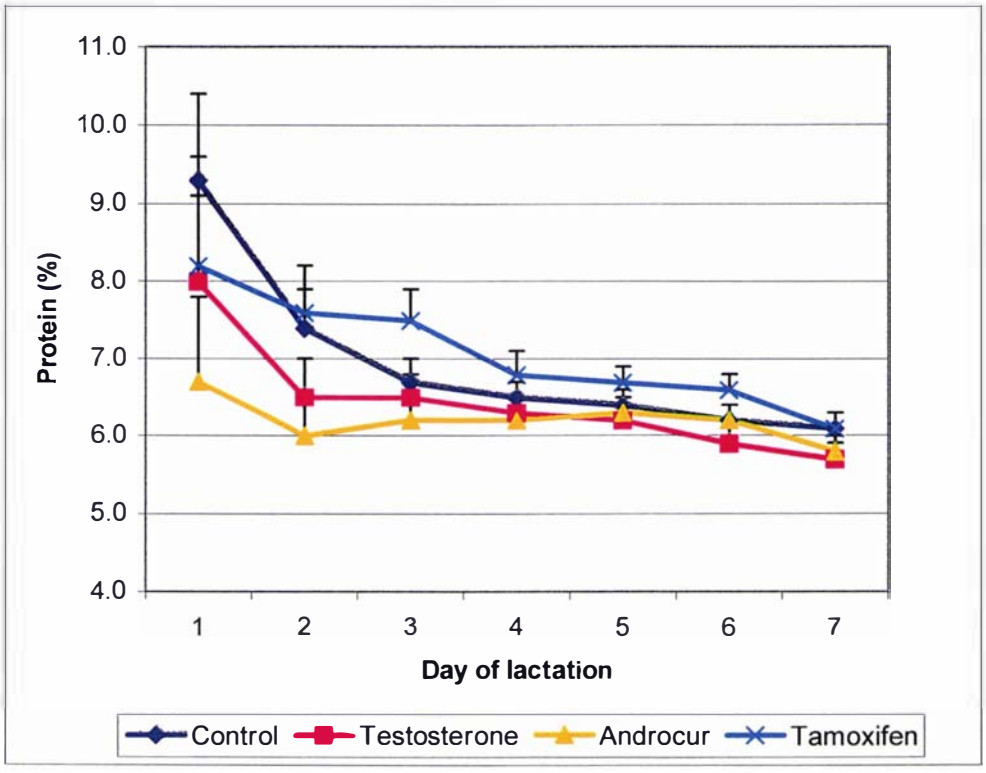
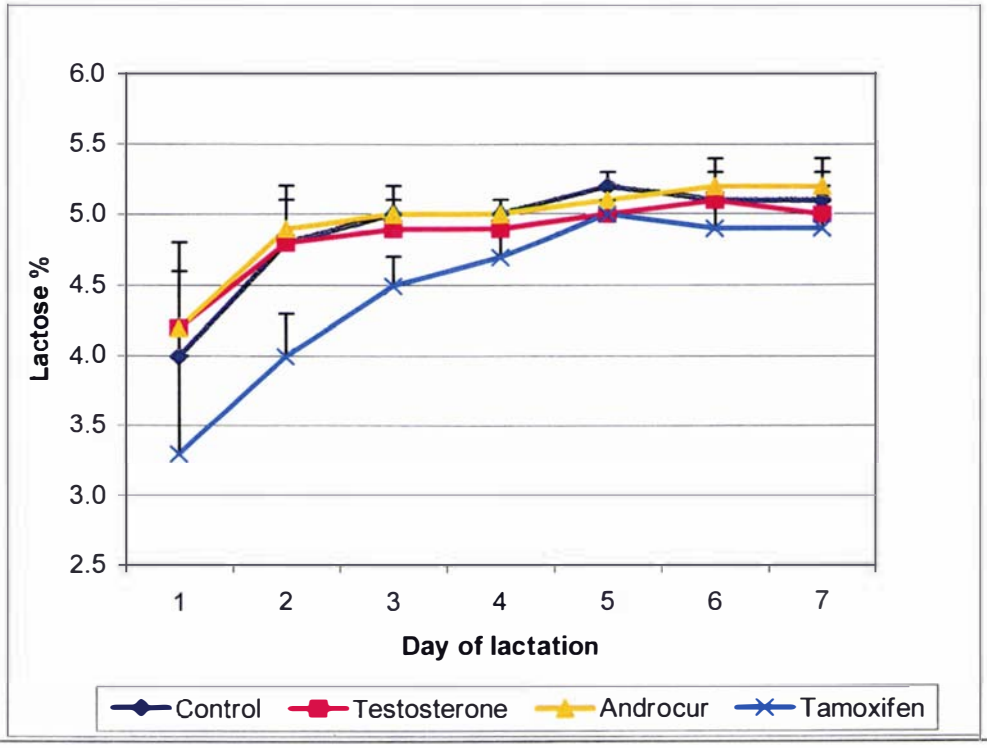



**Figure 5.5** Concentration of lactose (percentage) in milk for the first 7 days of lactation (lsmean±sem) of ewes following treatment with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 and 57 of gestation.



### 5.4.6 Effects of cottonseed oil (control), testosterone, Androcur or Tamoxifen treatment upon pregnant ewes – plasma testosterone and progesterone concentrations.

Maternal plasma testosterone concentrations for control and testosterone-treated ewes at days 35, 37, 38, 39, 46, 56, 60 and 80 are shown in Figure 5.6. Prior to the first injection, at day 36 of gestation, maternal testosterone concentrations were below the level of detection of the assay for both treatment groups. In the control ewes, concentrations were below 0.25 ng/ml at all days sampled. In the testosterone-treated ewes, injecting testosterone had a cumulative effect on circulating concentrations of the hormone. At day 37 (24 hrs after the first injection) the mean plasma concentration of testosterone was 1.50±0.26 ng/ml. Concentrations peaked at day 56 (24hrs prior to the last injection) (8.57±0.25 ng/ml) but by day 80, had declined to 0.73±0.06 ng/ml.

Maternal plasma progesterone concentrations at days 35, 60, 80, 100, 120, 140 of gestation and at birth, for all four treatment groups are shown in Figure 5.7. Progesterone concentrations were similar between treatment groups at days 35 (24 hours prior to the first injection) 60, 80 and 100 of gestation and at birth. At days 120 and 140 of gestation, there was a significant effect of treatment on progesterone concentrations.

**Figure 5.6** Plasma testosterone concentrations (ng/ml) in pregnant ewes following treatment with cottonseed oil (control) or testosterone from days 36 to 57 of gestation (lsmean±sem).

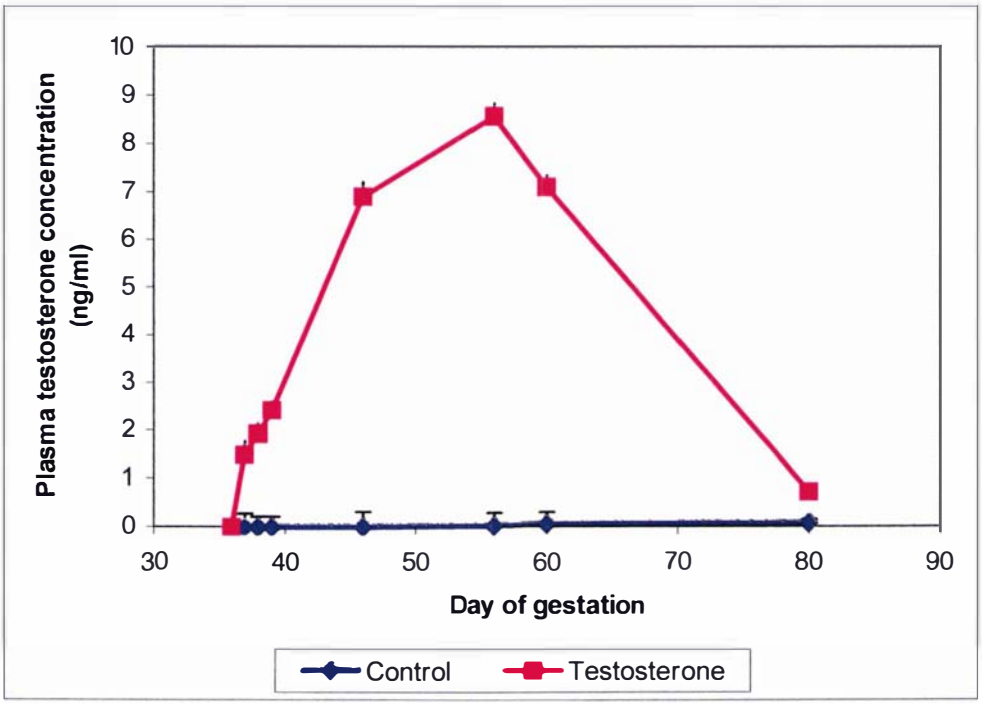
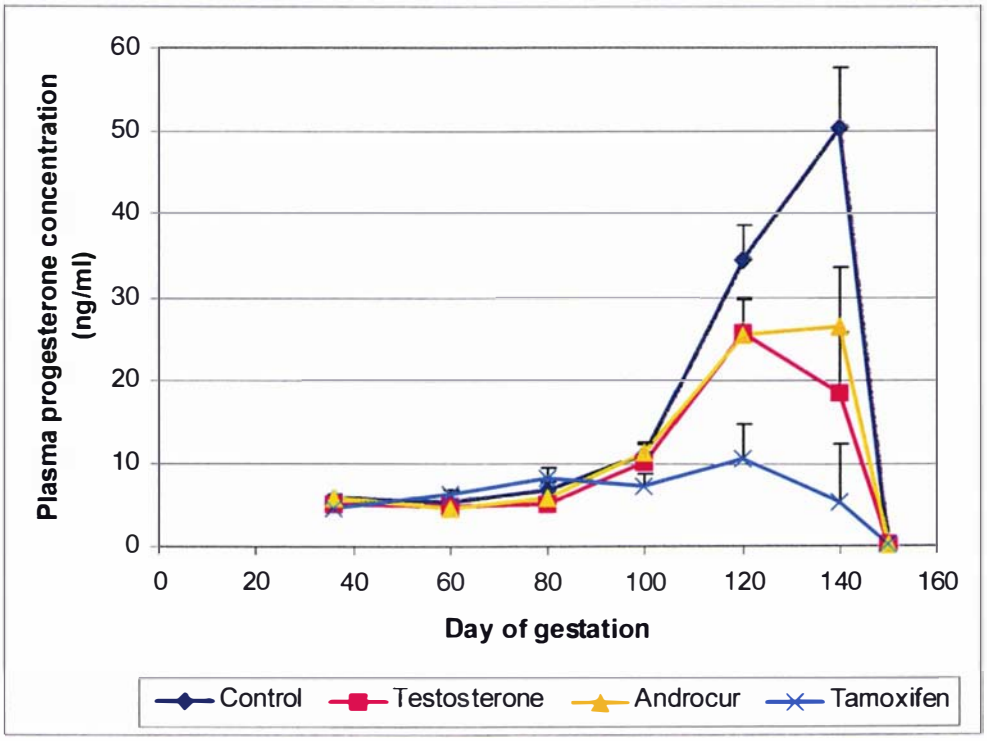



**Figure 5.7** Plasma progesterone concentrations (ng/ml) in pregnant ewes following treatment with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation (lsmean±sem).



### 5.4.7 Effects of maternal treatment with cottonseed oil (control), testosterone, Androcur or Tamoxifen upon male and female fetuses at day 61 of gestation.

In female fetuses, maternal exposure to testosterone from days 36 to 57 of gestation induced marked masculinisation of external genitalia. Karyotyping confirmed the genetic identity of female fetuses (XX chromosomes), regardless of the fact that they appeared to possess normally developed ovaries. Karyotyping also showed that all seven fetuses of the ewes treated with Androcur comprised only genetic females. Slides were stained with Giemsa and showed the 26 pairs of sheep autosomes plus the 2 sex chromosomes from a male (XY) or a female (XX) fetus (no photomicrographs shown).

Fetuses from Tamoxifen-treated ewes had abdominal swelling, and hence a significantly larger ($P<0.01$) girth relative to fetuses from other treatment groups (Table 5.8). On examination of the internal organs, the livers were enlarged with yellow colouration on the lobe edges. Furthermore, the cotyledons (fetal component of the placenta) were abnormally dark in colour and mushy in texture, falling apart on touch, although maternal caruncles appeared normal.

Male fetuses from ewes subjected to Tamoxifen treatment were significantly heavier ($P<0.01$) than their female counterparts and, males or females from the other treatment groups. Girth dimension was significantly greater ($P<0.01$) in Tamoxifen male and female fetuses, while crown-rump length did not differ significantly ($P<0.10$) between treatment groups or between males and females within treatment groups (Table 5.8). However, male fetuses from Tamoxifen-treated ewes were noticeably shorter in length and stockier in appearance than male or female fetuses from dams exposed to other treatments.

Female fetuses from ewes exposed to testosterone from days 36 to 57 of gestation developed a penis and an empty scrotal sac. The penis was not uniformly placed along the abdominal wall, with variation amongst females in its position relative to the navel (umbilicus) and scrotum. Table 5.9 compares measures of the external genitalia of masculinised female fetuses with males from the control, testosterone and Tamoxifen treatment groups. At day 61 of gestation, male fetuses from testosterone-treated ewes had significantly longer ($P<0.05$) penes than control males or females from testosterone-treated ewes. Also, the distance between the navel and the penis was longer ($P<0.05$) in females whose dams were exposed to testosterone relative to males from the control, testosterone or Tamoxifen groups. There was no significant effect of treatment on body weight, crown-rump length or girth circumference.

Table 5.10 shows mammary gland measures of fetuses from the four treatment groups at day 61 of gestation. There was a significant effect of treatment ($P<0.01$) and sex ($P<0.001$) on total lumen area with fetal males from Tamoxifen-treated ewes having a smaller lumen area than all other fetuses. The number of ducts and number of ducts with lumens did not differ between the treatment groups. There was no treatment by sex interaction for any of the parameters measured.

**Table 5.8** The body weight, crown-rump length and girth circumference of fetal sheep at day 61 of gestation following treatment of their dams with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation (lsmean±sem).

| Parameter | Control | | Testosterone | | Androcur | | Tamoxifen | |
|---|---|---|---|---|---|---|---|---|
| | Male | Female | Male | Female | Male | Female | Male | Female |
| Number of fetuses | 3 | 3 | 3 | 4 | 0 | 7 | 3 | 2 |
| Body weight (g) | 79.0±4.0$^{a}$ | 73.8±4.0$^{ab}$ | 74.4±4.2$^{ab}$ | 69.4±3.7$^{ab}$ | - | 67.9±2.6$^{b}$ | 97.5±5.1$^{c}$ | 78.6±4.9$^{ab}$ |
| CRL (mm) | 155.4±4.6 | 151.9±4.6 | 151.9±4.6 | 151.2±4.1 | - | 154.8±3.5 | 136.3±6.3 | 140.4±5.7 |
| Girth (mm) | 90.8±3.2$^{a}$ | 92.2±3.2$^{a}$ | 91.9±3.2$^{a}$ | 91.7±2.8$^{a}$ | - | 92.8±2.4$^{a}$ | 108.0±4.4$^{a}$ | 103.6±3.9$^{a}$ |

$^{abc}$ Means within rows with different superscripts are significantly different (P<0.05).

**Table 5.9** Body weight and, body and external genitalia dimensions of male and masculinised* female fetal sheep at day 61 of gestation following treatment of their dams with cottonseed oil (control), testosterone or Tamoxifen from days 36 to 57 of gestation (lsmean±sem).

| Parameter | Control | Testosterone | | Tamoxifen |
|---|---|---|---|---|
| | Male | Male | Female | Male |
| Number of fetuses | 3 | 3 | 4 | 3 |
| Body weight (g) | 79.0±5.5 | 73.5±7.6 | 68.5±7.5 | 98.8±12.8 |
| Crown-rump length (mm) | 157.6±4.7 | 154.1±4.7 | 153.3±4.6 | 138.8±6.2 |
| Girth circumference (mm) | 92.1±3.7 | 93.4±3.7 | 93.7±3.6 | 107.8±4.9 |
| Distance from navel to penis (mm) | 2.21±0.49[a] | 2.12±0.50[a] | 4.80±0.5[b] | 2.43±0.65[a] |
| Distance from penis to scrotum (mm) | 13.97±1.40 | 13.22±1.42 | 11.33±1.39 | 14.91±1.85 |
| Distance from scrotum to anus (mm) | 12.23±0.70 | 11.91±0.71 | 11.46±0.69 | 10.63±0.92 |
| Length of penis (mm) | 1.26±0.16[a] | 1.91±0.16[b] | 1.25±0.16[a] | 1.28±0.21[ab] |
| Length of scrotum (mm) | 7.11±0.58 | 6.82±0.58 | 6.05±0.57 | 5.36±0.76 |

* Females that show masculine morphology of the external genitalia.

[ab] Means within rows with different superscripts are significantly different ($P<0.05$).

**Table 5.10** Mammary gland development (log transformed – $\log_e$ of areas) of male and female fetal sheep at day 61 of gestation following treatment of their dams with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation (lsmean±sem).

| Parameter | Control | | Testosterone | | Androcur | Tamoxifen | |
|---|---|---|---|---|---|---|---|
| | Male | Female | Male | Female | Female | Male | Female |
| Number of fetuses | 3 | 3 | 3 | 4 | 7 | 2 | 2 |
| Total duct area ($\log_e \mu m^2$) | 11.25±0.11 | 11.74±0.11 | 11.45±0.11 | 11.71±0.10 | 11.89±0.09 | 11.38±0.19 | 11.77±0.14 |
| Total lumen area ($\log_e \mu m^2$) | 7.56±0.37$^{a}$ | 9.09±0.36$^{bc}$ | 8.04±0.36$^{ab}$ | 9.89±0.38$^{bc}$ | 10.08±0.28$^{c}$ | 5.25±0.64$^{e}$ | 8.10±0.46$^{ab}$ |
| Total secretory area ($\log_e \mu m^2$) | 11.22±0.10 | 11.67±0.10 | 11.40±0.10 | 11.62±0.09 | 11.75±0.08 | 11.44±0.18 | 11.74±0.13 |
| Number of ducts ($\log_e$) | 0.78±0.34 | 1.34±0.38 | 0.51±0.37 | 0.66±0.33 | 1.33±0.29 | 1.07±0.64 | 1.51±0.47 |
| Number of ducts with lumens ($\log_e$) | 0.49±0.28 | 0.58±0.27 | 0.25±0.28 | 0.40±0.29 | 0.28±0.21 | 0.78±0.49 | 0.05±0.35 |

$^{abcde}$ Means within rows with different superscripts are significantly different ($P<0.05$).

### 5.4.8 Effects of maternal treatment with cottonseed oil (control), testosterone, Androcur or Tamoxifen upon male and female fetuses at day 117 of gestation.

At day 117 of gestation, the reproductive tracts of the masculinised females were exactly as those described for experiment one. Hence there was no need for karyotyping. The genitalia of male fetuses whose dams were exposed to testosterone did not appear grossly different from those males in the control, Androcur or Tamoxifen treatment groups. There was no apparent feminisation of male fetuses whose dams were treated with Androcur.

Preliminary analysis of the organ data showed that sex effects were non-significant. Thus, means have been presented for treatment cells only. There was no overall effect of treatment on fetal body weight, crown-rump length, girth measurements or organ weights (Table 5.11). However, fetuses whose dams were treated with Tamoxifen had significantly ($P<0.05$) heavier kidneys than fetuses from all other treatment groups.

Table 5.12 shows the effects of the treatments on body characteristics, mammary gland weight and external genitalia of male and masculinised female fetal sheep at day 117 of gestation. Tamoxifen and testosterone males were not included in this analysis as there was only one male representative from each of the two treatment groups. There was no effect of treatment on body weight, crown-rump length or girth circumference. Distance, along the abdominal wall, of the penis relative to the navel and scrotum was significantly affected by treatment. The penes of masculinised females were significantly ($P<0.001$) more posterior relative to the navel than those of the control and Androcur males.

The distance from the penis to the scrotum was shorter ($P<0.01$) in masculinised females than in males from the other groups. Length of the penis and scrotum were significantly affected by treatment, being shorter in females whose dams were treated with testosterone than in males from the Control or Androcur groups ($P<0.001$ and $P<0.01$, respectively). Mammary gland weight did not differ between the treatment groups indicating that females whose dams were exposed to testosterone showed masculinisation not only of the external genitalia but also of the mammary gland.

Treatment of pregnant ewes with cottonseed oil, testosterone, Androcur or Tamoxifen had no effect on fetal mammary development at day 117 of gestation (Table 5.13). However, there were significant treatment ($P<0.001$) and sex ($P<0.001$) effects on mammary gland weight. Female glands were heavier ($P<0.001$) than male glands in the control and Androcur groups. However, the mammary gland weights of testosterone females did not differ significantly from those of Androcur males, but were lighter ($P<0.001$) than those of control males.

**Table 5.11** The body weight, crown-rump length and girth circumference of fetal sheep at day 117 of gestation following treatment of their dams with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation (lsmean±sem).

| Parameter | Treatment | | | | Sig |
|---|---|---|---|---|---|
| | Control | Testosterone | Androcur | Tamoxifen | T |
| Number of fetuses | 6 | 5 | 7 | 2 | |
| Body weight (g) | 2495.3±103.9 | 2336.9±109.0 | 2371.3±95.7 | 2124.2±172.9 | NS |
| Crown-rump length (mm) | 446.1±9.7 | 449.7±9.9 | 448.4±8.4 | 430.8±16.3 | NS |
| Girth (mm) | 282.3±3.5 | 281.3±3.5 | 283.5±3.0 | 283.95±5.8 | NS |
| Heart (g) | 17.6±0.7 | 16.6±0.7 | 16.9±0.6 | 19.4±1.1 | NS |
| Lungs (g) | 77.8±3.1 | 81.3±3.2 | 83.5±2.7 | 67.8±5.3 | NS |
| Liver (g) | 90.4±4.0 | 89.9±4.1 | 81.2±3.5 | 102.1±6.8 | NS |
| Kidney (g) | 15.7±1.1 | 15.3±1.1 | 15.9±0.9 | 21.2±1.8 | NS |
| Spleen (g) | 4.0±0.4 | 4.1±0.4 | 3.8±0.4 | 5.1±0.7 | NS |

NS, P>1.0; Sig = significance; T = treatment. All parameters adjusted to a common sex.

**Table 5.12** Body weight, mammary gland weight and, body and external genitalia dimensions of male and masculinised* female fetal sheep at day 117 of gestation following treatment of their dams with cottonseed oil (control), testosterone or Androcur from days 36 to 57 of gestation (lsmean±sem).

| Parameter | Control | Testosterone | Testosterone | Androcur | Tamoxifen |
|---|---|---|---|---|---|
| | Male | Male | Female | Male | Male |
| Number of fetuses | 3 | 1 | 5 | 4 | 1 |
| Body weight (g) | 2493.1±131.3 | - | 2311.5±102.6 | 2116.4±134.6 | - |
| Crown-rump length (mm) | 419.5±14.1 | - | 445.3±9.7 | 456.4±14.6 | - |
| Girth circumference (mm) | 286.8±4.3 | - | 278.5±2.8 | 282.7±4.2 | - |
| Distance from navel to penis (mm) | 11.9±2.2[a] | - | 43.5±1.5[b] | 9.6±2.3[a] | - |
| Distance from penis to scrotum (mm) | 48.6±5.4[b] | - | 5.6±3.7[a] | 51.4±5.6[b] | - |
| Distance from scrotum to anus (mm) | 55.8±4.8 | - | 58.2±3.3 | 51.9±5.0 | - |
| Length of penis (mm) | 10.9±0.8[b] | - | 5.5±0.6[a] | 12.21±0.9[b] | - |
| Length of scrotum (mm) | 42.1±3.5[b] | - | 26.8±2.4[a] | 42.2±3.6[b] | - |
| Mammary gland weight (g) | 2.8±0.2 | - | 2.0±0.2 | 2.3±0.2 | - |

*Females that show masculine morphology of the external genitalia.

[ab] Means within rows with different superscripts are significantly different (P<0.05).

**Table 5.13** Mammary gland development (log transformed – $\log_e$ of areas) of male and female fetal sheep at day 117 of gestation following treatment of their dams with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation (lsmeans±sem).

| Parameter | Control | | Testosterone | | Androcur | | Tamoxifen | |
|---|---|---|---|---|---|---|---|---|
| | Male | Female | Male | Female | Male | Female | Male | Female |
| Number of fetuses | 3 | 3 | 1 | 5 | 4 | 3 | 1 | 2 |
| Total duct area ($\log_e$ $\mu m^2$) | 14.34±0.22 | 14.35±0.23 | - | 14.41±0.17 | 14.36±0.20 | 14.53±0.23 | - | 14.72±0.28 |
| Total lumen area ($\log_e$ $\mu m^2$) | 13.60±0.28 | 13.63±0.29 | - | 13.79±0.21 | 13.77±0.25 | 13.92±0.28 | - | 13.32±0.35 |
| Total secretory area ($\log_e$ $\mu m^2$) | 13.67±0.26 | 13.69±0.27 | - | 13.63±0.20 | 13.55±0.24 | 13.75±0.26 | - | 14.23±0.33 |
| Number of ducts ($\log_e$) | 4.22±0.26 | 4.14±0.28 | - | 3.99±0.20 | 3.84±0.24 | 4.11±0.27 | - | 4.75±0.33 |
| Number of ducts with lumens ($\log_e$) | 4.02±0.26 | 4.01±0.28 | - | 3.78±0.20 | 3.68±0.24 | 3.94±0.27 | - | 4.62±0.33 |
| Mammary gland weight ($\log_e$ g) | 1.07±0.08$^b$ | 2.09±0.08$^c$ | - | 0.73±0.06$^a$ | 0.76±0.07$^a$ | 1.97±0.08$^c$ | - | 2.00±0.10$^c$ |

$^{abc}$ Means within rows with different superscripts are significantly different ($P<0.05$).

### 5.4.9 Effects of maternal treatment with Cottonseed oil (control) or Testosterone or Androcur or Tamoxifen upon male and female lambs at birth.

There was no effect of sex on any of the parameters measured; hence the presentation of data by treatment cells only. Lambs from the control ewes had higher ($P<0.001$) mean body weights than lambs whose dams were treated with testosterone or Androcur or Tamoxifen (Table 5.14). Although of similar mean body weight to the Androcur lambs, subjective assessment of body condition found the Tamoxifen lambs to be of a smaller and thinner stature. This was especially obvious when compared to the larger lambs from the control and testosterone treatment groups. Crown-rump length, girth circumference and organ weights were not significantly different between lambs from the four treatment groups.

**Table 5.14** The body weight, crown-rump length, girth circumference and organ weights of lambs at birth following treatment of their dams with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation (lsmean±sem).

| Parameter | Treatment | | | |
|---|---|---|---|---|
| | Control | Testosterone | Androcur | Tamoxifen |
| Number of lambs | 6 | 6 | 7 | 4 |
| Birth weight (g) | 5403.2±169.3[a] | 4472.5±170.8[b] | 3979.5±159.6[c] | 3640.2±235.1[c] |
| CRL (mm) | 508.0±22.7 | 523.0±15.3 | 489.3±15.1 | 485.6±26.6 |
| Girth (mm) | 387.3±11.1 | 399.6±7.5 | 396.1±7.3 | 382.2±13.0 |
| Heart (g) | 42.6±3.9 | 35.9±2.7 | 31.8±2.6 | 28.1±4.6 |
| Lungs (g) | 80.2±7.5 | 68.8±5.1 | 76.1±5.0 | 67.5±8.8 |
| Liver (g) | 102.9±12.3 | 92.6±8.3 | 84.3±8.1 | 86.2±14.4 |
| Kidney (g) | 27.4±3.0 | 21.8±2.0 | 20.9±2.0 | 23.4±3.5 |
| Spleen (g) | 7.0±1.2 | 6.2±0.8 | 7.2±0.8 | 4.6±1.4 |

[abc] Means within rows with different superscripts are significantly different ($P<0.05$).

Measurements of the external genitalia of male lambs from all four treatment groups with testosterone-exposed female lambs are presented in Table 5.15 There were treatment effects on distance from the navel to the penis and on penis and scrotum lengths. The position of the penis relative to the navel differed in the masculinised females, being significantly ($P<0.001$) further posterior than penes of males from all treatment groups. The penes were shorter ($P<0.05$) in females whose dams were treated with testosterone relative to testosterone and Androcur males, but did not differ from control or Tamoxifen males. Scrotum length was significantly ($P<0.01$) shorter in testosterone-exposed female lambs relative to male lambs from all treatment groups.

Table 5.16 shows measures of mammary development of lambs at birth. Sex had a significant effect on all the parameters measured. Within treatment groups, females had significantly ($P<0.01$) greater total duct and secretory cell areas than males, except in the testosterone group, where the gland size of males and masculinised females did not differ.

Overall, both treatment ($P<0.01$) and sex ($P<0.001$) had significant effects on mammary gland weight. Furthermore, a significant ($P<0.001$) treatment by sex interaction reflected a divergence between the treatment groups where the mammary gland of masculinised females was significantly lighter than those of females in all other treatment groups.

**Table 5.15** Body weight, mammary gland weight and, body and external genitalia dimensions of male and masculinised female lambs at birth following treatment of their dams with cottonseed oil (control), testosterone, Androcur, or Tamoxifen from days 36 to 57 of gestation (lsmean±sem).

| Parameter | Control | Testosterone | | Androcur | Tamoxifen |
|---|---|---|---|---|---|
| | Male | Male | Female | Male | Male |
| Number of fetuses | 3 | 2 | 4 | 4 | 2 |
| Body weight (g) | 5432.7±232.5$^{c}$ | 4738.6±280.6$^{bc}$ | 4370.9±205.2$^{ab}$ | 3851.9±198.0$^{a}$ | 3608.6±307.3$^{a}$ |
| Crown-rump length (mm) | 533.8±44.2 | 525.9±33.7 | 526.4±23.2 | 465.5±28.9 | 442.6±46.4 |
| Girth circumference (mm) | 385.6±22.8 | 402.7±17.4 | 397.5±12.0 | 391.2±14.9 | 370.6±24.0 |
| Distance from navel to penis (mm) | 17.8±6.0$^{a}$ | 14.1±4.6$^{a}$ | 39.9±3.2$^{b}$ | 12.3±4.0$^{a}$ | 5.2±6.3$^{a}$ |
| Distance from penis to scrotum (mm) | 48.2±9.5 | 42.9±7.2 | 40.0±5.0 | 62.4±6.2 | 64.6±10.0 |
| Distance from scrotum to anus (mm) | 74.0±13.3 | 34.3±10.2 | 69.0±7.0 | 66.3±8.7 | 57.5±14.0 |
| Length of penis (mm) | 10.5±1.3 | 11.6±1.0 | 8.9±0.7 | 12.7±0.8 | 11.4±1.4 |
| Length of scrotum (mm) | 53.1±6.1$^{ab}$ | 58.2±4.6$^{b}$ | 32.9±3.2$^{a}$ | 56.0±4.0$^{b}$ | 54.5±6.4$^{b}$ |
| Mammary gland weight (g) | 3.0±0.6 | 2.8±0.5 | 2.4±0.3 | 2.0±0.4 | 1.2±0.7 |

$^{abc}$ Means within rows with different superscripts are significantly different ($P<0.05$).

**Table 5.16** Mammary gland development (log transformed – $\log_e$ of areas) of male and female lambs at birth following treatment of their dams with cottonseed oil (control), testosterone, Androcur or Tamoxifen from days 36 to 57 of gestation (lsmean±sem).

| Parameter | Control | | Testosterone | | Androcur | | Tamoxifen | |
|---|---|---|---|---|---|---|---|---|
| | Male | Female | Male | Female | Male | Female | Male | Female |
| Number of fetuses | 3 | 3 | 2 | 4 | 4 | 3 | 2 | 2 |
| Total duct area ($\log_e$ $\mu m^2$) | 13.19±0.40 | 14.84±0.38 | 13.50±0.41 | 14.36±0.28 | 14.31±0.31 | 15.73±0.34 | 14.53±0.48 | 15.75±0.52 |
| Total lumen area ($\log_e$ $\mu m^2$) | 12.58±0.43 | 14.50±0.42 | 12.69±0.44 | 13.90±0.31 | 13.80±0.33 | 15.23±0.35 | 13.95±0.52 | 15.22±0.56 |
| Total secretory area ($\log_e$ $\mu m^2$) | 12.38±0.45 | 13.56±0.43 | 12.90±0.46 | 13.17±0.32 | 13.40±0.35 | 14.78±0.37 | 13.67±0.54 | 14.86±0.58 |
| Number of ducts ($\log_e$) | 3.77±0.56 | 4.77±0.63 | 4.01±0.57 | 3.76±0.40 | 3.51±0.42 | 5.35±0.45 | 3.96±0.65 | 5.27±0.71 |
| Number of ducts with lumens ($\log_e$) | 3.67±0.64 | 4.73±0.72 | 3.92±0.65 | 3.48±0.45 | 3.41±0.48 | 5.22±0.52 | 3.73±0.74 | 5.21±0.81 |
| Mammary gland weight ($\log_e$ g) | $0.97\pm0.18^{ab}$ | $2.51\pm0.18^{c}$ | $1.01\pm0.19^{b}$ | $0.84\pm0.13^{ab}$ | $0.72\pm0.14^{ab}$ | $2.30\pm0.15^{c}$ | $0.36\pm0.22^{a}$ | $2.03\pm0.24^{c}$ |

$^{abc}$ Means within rows with different superscripts are significantly different ($P<0.05$).

### 5.4.10 Effects of maternal treatment with cottonseed oil (control), testosterone or Androcur upon immunoreactivity to androgen receptor antibody in male and female fetuses at days 61 and 117 of gestation and in lambs at birth.

The reaction patterns described hereafter are exactly the same as those described in Experiment 1: linear cell surface, cytoplasmic and nuclear. The predominant pattern of immunoreactivity to androgen receptor (AR) antibody, irrespective of the treatment group, was cytoplasmic and the intensity of immunostaining ranged from weakly to strongly positive. The changes in the expression of AR in the mammary gland of fetuses from the three treatment groups are summarised in the Appendices (Tables A.5, A.6, A.7).

Immunoreactivity to androgen receptor antibody was not seen in the epithelial cells of secondary ducts, regardless of treatment, at day 61 of fetal age. Immunostaining of the mesenchymal cells in the surrounding dermis and hypodermis ranged from questionable to weakly positive in all males and testosterone females (photomicrograph not shown).

By day 117 of gestation, the apical plasmalemma and adjacent cytoplasm of the ductal epithelial cells were immunopositive in fetal males from the control group and in those whose dams were treated with testosterone or Androcur. Immunostaining varied in intensity from weakly to moderately positive (Plate 5.4a). Selected epithelial cell nuclei were seen to stain in some of the testosterone and Androcur males, but immunoreactivity in the epithelial cell cytoplasm was reduced in these males relative to all other males (Plate 5.4b). Whereas females from testosterone-treated dams followed a similar pattern of reactivity and intensity of staining to the males (Plate 5.4c), females from the control and Androcur groups exhibited mainly weak cell surface immunoreactivity to androgen receptor antibody (Plate 5.4d). The mesenchymal cells in the control and Androcur females were either non-reactive or very weakly stained while those of the males and testosterone females exhibited nuclear staining.

For most of the male sections analysed at birth, immunoreactivity to androgen receptor antibody was weaker in the epithelial cells compared to that exhibited at day 117 (Plate 5.5a). However, one Androcur male showed continuous, strong immunoreactivity deep in the apical cytoplasm of the epithelial cells lining the secondary ducts and the teat canal (Plate 5.5b). In those females in which immunoreactivity was still seen at birth, the pattern was exclusively linear cell surface (Plate 5.5c). Immunostaining in the testosterone females was generally more intense than that observed in the control or Androcur females, but less intense than that seen at day 117 of fetal age (Plate 5.5d). The fibroblastic cells of the connective tissue were either non-reactive or weakly immunostained, irrespective of treatment group.

**Plate 5.4a** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of a male sheep at day 117 of fetal age following treatment of the dam with cottonseed oil (control) from days 36 to 57 of gestation. Moderately positive immunostaining of the apical cytoplasm. (Mag x400).

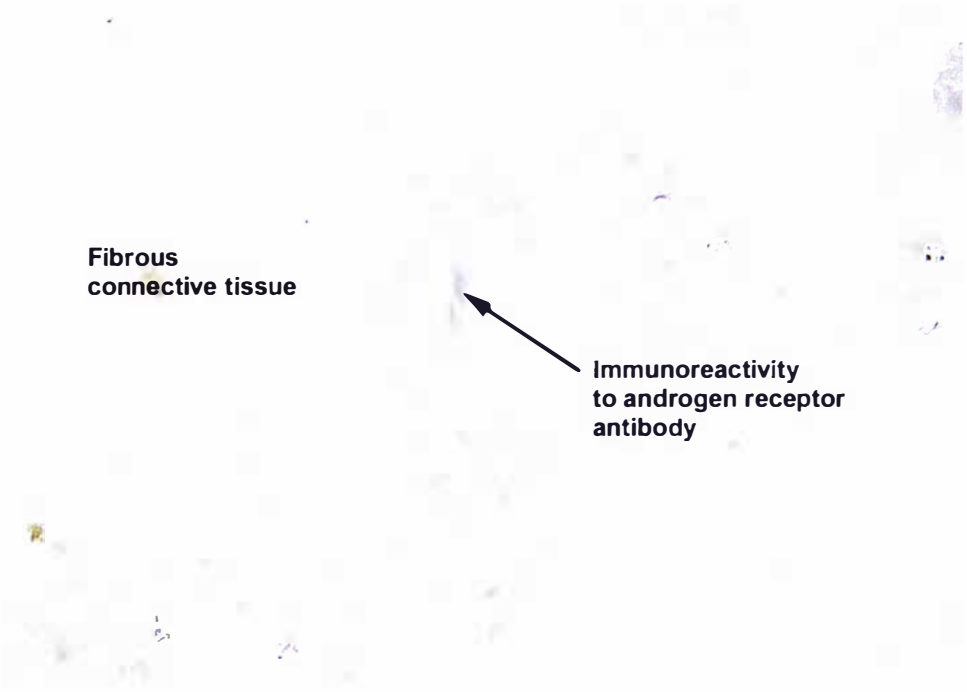



**Plate 5.4b** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of a male sheep at day 117 of fetal age following treatment of the dam with Androcur (anti-androgen) from days 36 to 57 of gestation. Selected staining of the epithelial cell nuclei. (Mag x400).

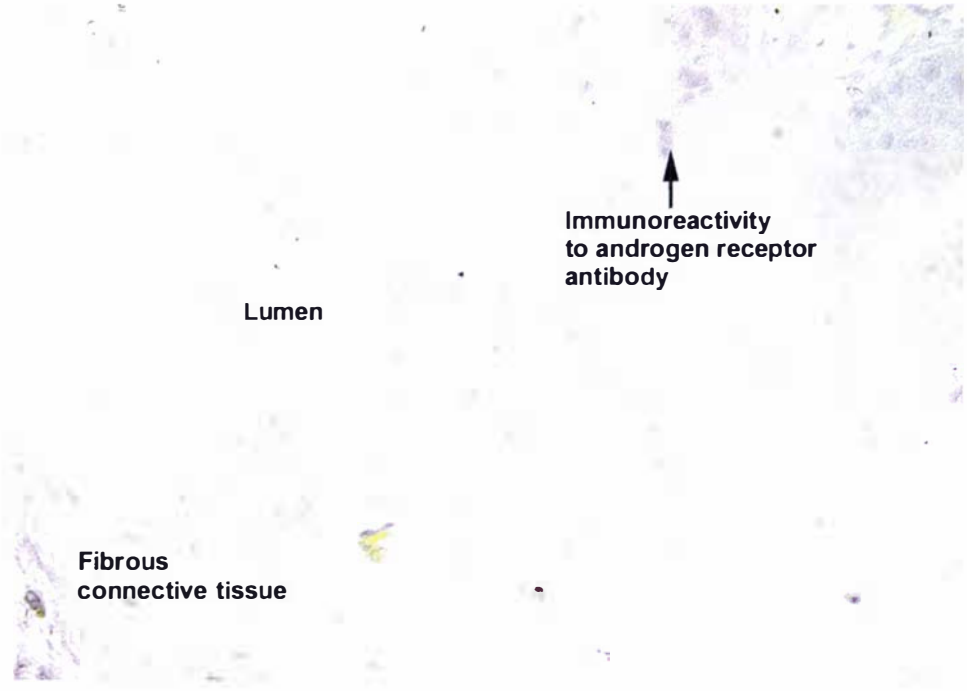


**Plate 5.4c** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of a female sheep at day 117 of fetal age following treatment of the dam with testosterone from days 36 to 57 of gestation. Moderately positive immunostaining of the apical cytoplasm. (Mag x400).

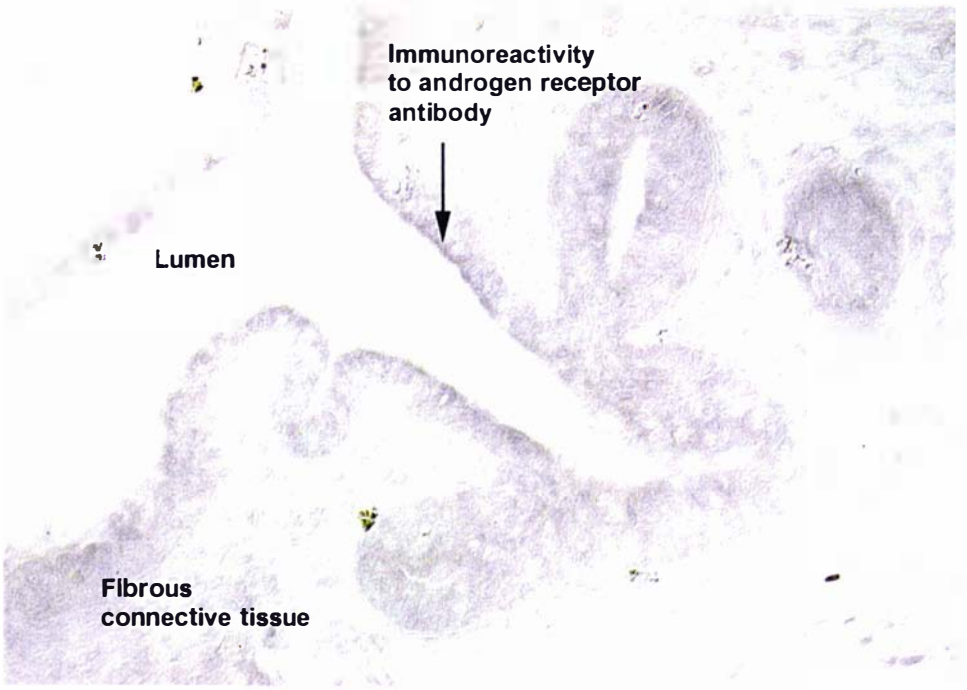



**Plate 5.4d** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of a female sheep at day 117 of fetal age following treatment of the dam with cottonseed oil (control) from days 36 to 57 of gestation. Weak to moderate linear immunostaining of the luminal epithelial cell surface. (Mag x400).

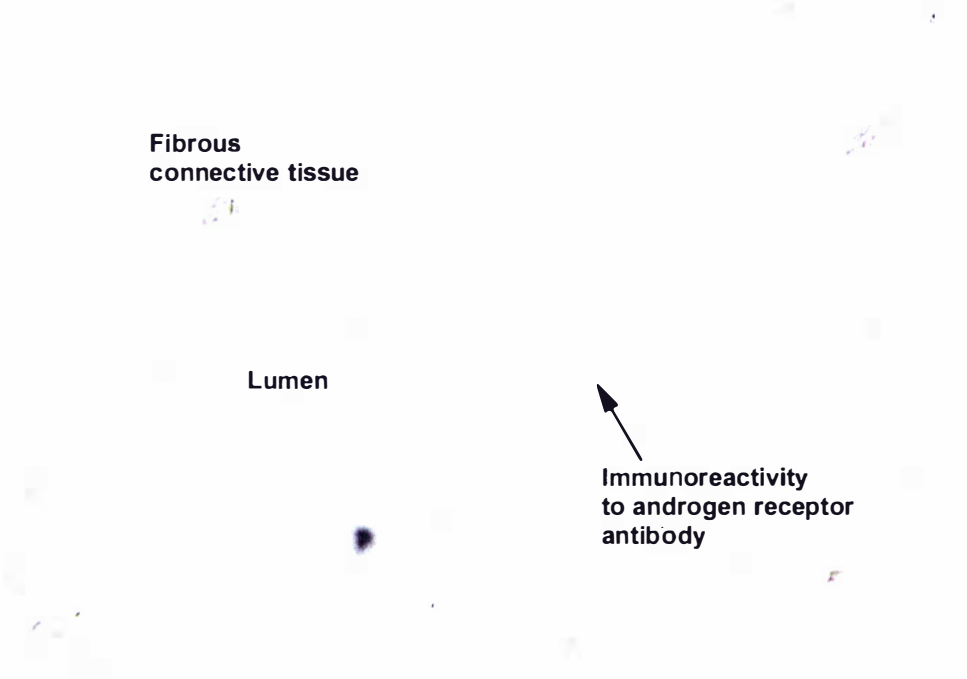


**Plate 5.5a** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of a male lamb at birth following treatment of the dam with cottonseed oil (control) from days 36 to 57 of gestation. Moderately positive immunostaining of the apical cytoplasm and nuclear membrane. (Mag x400).

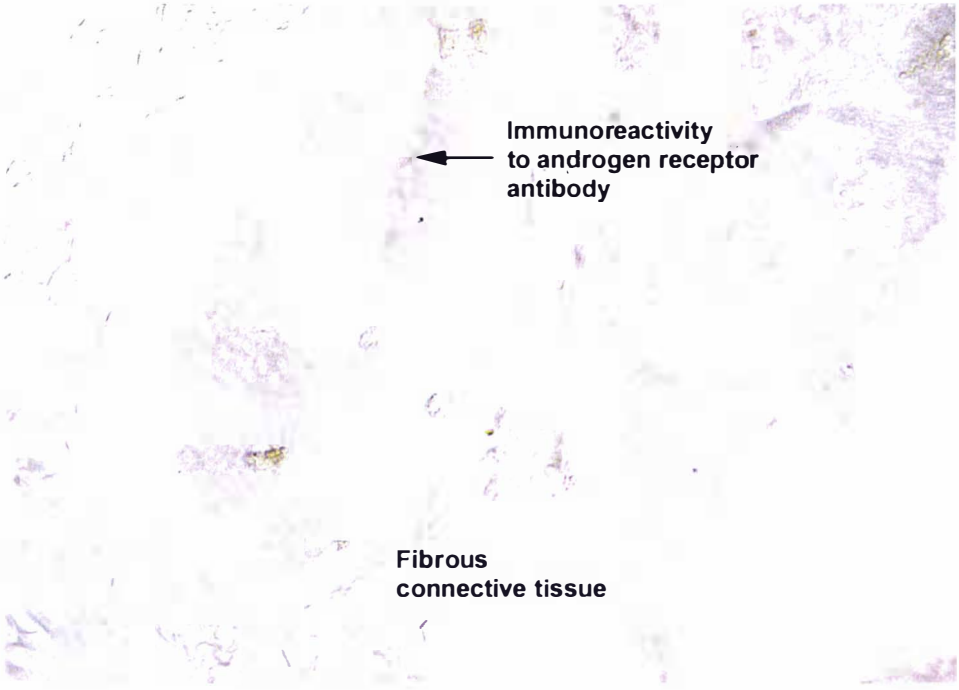



**Plate 5.5b** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of a male lamb at birth following treatment of the dam with Androcur (anti-androgen) from days 36 to 57 of gestation. Intense immunostaining of the apical cytoplasm. (Mag x400).

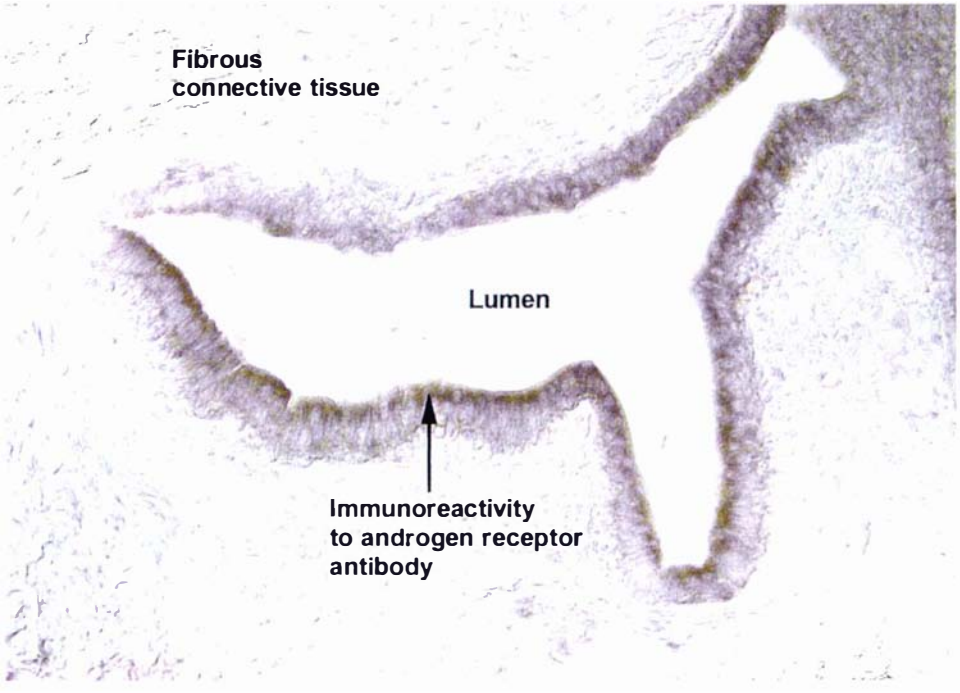


**Plate 5.5c** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of a female lamb at birth following treatment of the dam with cottonseed oil (control) from days 36 to 57 of gestation. Moderate linear immunostaining of the luminal epithelial cell surface. (Mag x400).

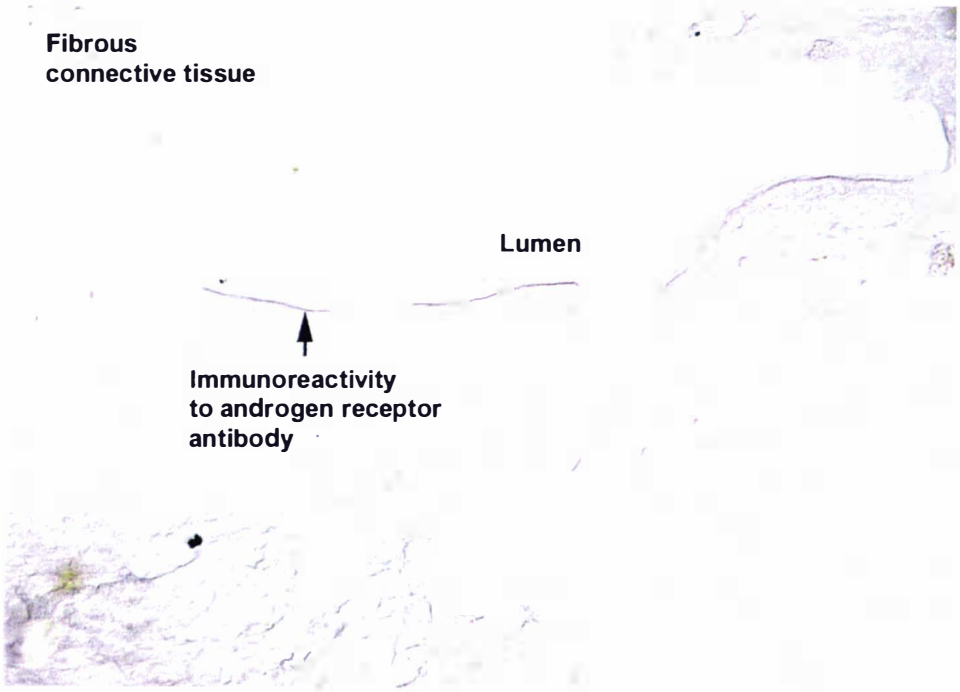
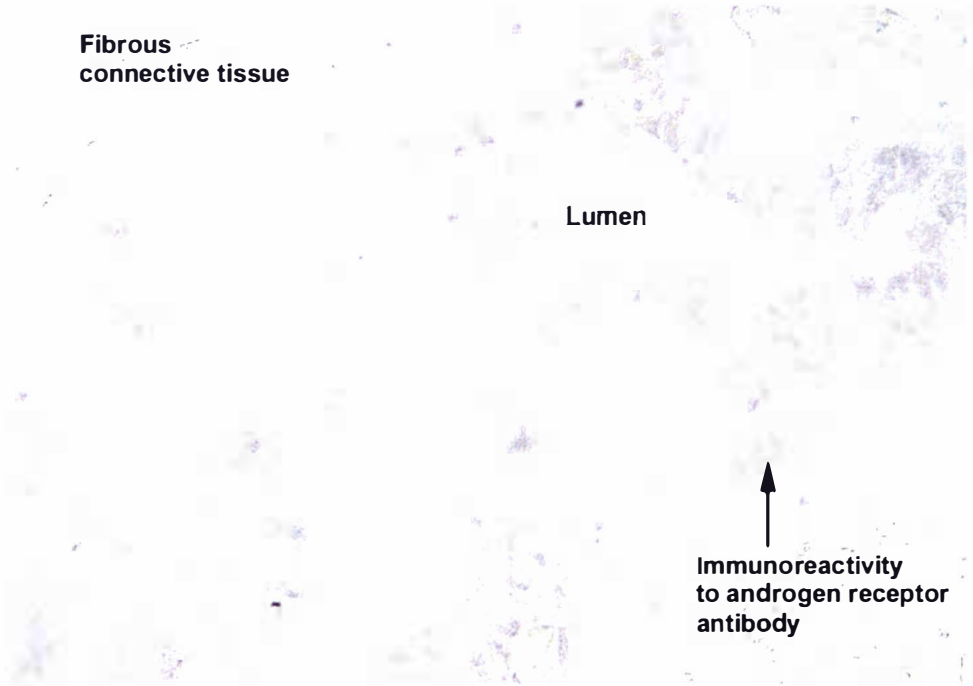



**Plate 5.5d** Photomicrograph of immunoreactivity to androgen receptor antibody in the mammary gland of a female lamb at birth following treatment of the dam with testosterone from days 36 to 57 of gestation. Weak to moderate immunostaining of the apical cytoplasm. Some epithelial cell nuclei are immunostained. (Mag x400).



## 5.5 Discussion

The developing mammary gland of the ovine male is somewhat distinct from the rodent mammary gland, in that its morphological development continues beyond the stage of the mammary bud. However, relative to the gland of the ovine female, adipose tissue is far less abundant in the male from the outset and epithelial development, in terms of total duct area, does not progress beyond that observed in both sexes at day 120 of gestation (Chapter two).

Immunohistochemical analyses (Chapter three) confirmed an association between the developmental divergence in total duct area in the mammary glands of ovine males and females, and the intensity of immunostaining for androgen receptor antibody. To determine indirectly that inhibition of mammary gland growth in the male is dependent on its exposure to testosterone, two experiments were undertaken. A preliminary study was necessary to demonstrate, as reported previously, that exposure to testosterone during the period for sexual differentiation would result in masculinisation of the external genitalia of the female offspring (Wilson and Tarttelin, 1978; Tarttelin, 1986; Wood et al., 1991; 1995). It was also hypothesised that, if androgens were responsible for sexual differentiation of the ovine mammary gland, those females exposed to testosterone *in utero* would show immunoreactivity to androgen receptor antibody and a developmental pattern of mammary growth similar to that observed in ontogeny (Chapters two and three) and control males. To further demonstrate that the morphological development of the normal male mammary gland is dependent on androgens, a treatment group in which the pregnant dams were administered cyproterone acetate, an androgen antagonist (Neumann, 1994), was included in experiment two. It also seemed of interest to investigate the effects of anti-oestrogens on mammary development in the female fetus, given their widespread abundance in the environment and their potential to alter reproductive tract and mammary gland development (Colburn et al., 1993; McLachlan, 1993; Hilaviki-Clarke et al., 1998). Thus the inclusion of a treatment group in which ewes were administered tamoxifen, an oestradiol receptor blocker (Furr and Jordan, 1984).

Testosterone treatment of ewes, in the form of cypionate (total dose of 800 mg from days 36 to 57 of gestation) was identical to that of the 'early group' of Wood et al. (1995). However, injections started 6 days later (day 36 as opposed to day 30) as accurate diagnosis of pregnancy is difficult to determine before day 35 of gestation with real-time ultrasound (Ward and Rosa, personal communication).

Treatment of the pregnant dam with exogenously administered testosterone caused partial masculinisation of the mammary gland of female fetuses at day 140 of gestation and in lambs at birth. The trend towards a male pattern of mammary gland development was associated with a greater intensity of immunostaining for androgen receptor antibody in female fetuses exposed to testosterone relative to control females, particularly at day 117 of gestation. Hence, the trend for partial masculinisation not only applies to the morphological development of the mammary

gland but also to the steroid hormone receptors which potentially regulate that development. That maternal treatment with testosterone induced a male pattern in mammary gland development and androgen receptor localisation suggests that there was *in utero* exposure to testosterone (as opposed to the treatment causing a primary effect in the dam, which then caused a secondary effect in the fetus). At birth in the present study, males and females from all treatment groups showed a similar pattern of immunoreactivity to that reported in the ontogeny trial for both sexes at day 140 of gestation (Chapter three).

In experiment two, there was no difference in total duct area between masculinised females and controls, or males or females from other treatment groups at day 61 of fetal age. While there were also no differences in total duct area across the treatment groups in 117-day-old fetuses, mammary gland weight was significantly lower in testosterone females relative to all other females. The reduction in the weight of the mammary gland was most likely due to the development of the fat pad where adipose tissue was far less abundant in the masculinised female relative to that of females from all other treatment groups at day 117 of fetal age. At birth, while the weight of the mammary gland continued its downward trend, partial inhibition of ductal development was observed in the female offspring of ewes treated with testosterone. It is tempting to speculate that the depletion of the fat pad and hence, the amount of interlobular fibrous connective tissue, suppressed paracrine regulation of epithelial growth (Chapter four) in female fetuses whose dams were subjected to testosterone treatment.

Administration of testosterone to pregnant ewes also induced marked masculinisation of the external genitalia of the female offspring while the ovaries remained in their normal intra-abdominal position and the scrotal sacs were empty. The genitalia of male fetuses and lambs did not appear grossly different from the control males. The effect on the reproductive organs confirmed earlier reports that exposure to testosterone during the period of maximum sensitivity (Clarke et al., 1974), induces masculinisation of the external genitalia (Tarttelin, 1986; Wood et al., 1991, 1995). However, the effect on mammary morphogenesis, until now, has only been observed in the rodent, where, in female fetuses exposed to testosterone propionate on day 12 of pregnancy, mammary growth was inhibited to the extent that it did not differ from that of the male (Hoshino, 1965). Androgen administered on day 15, 16 or 17 did not impair mammary growth, which suggests that the gland is no longer responsive to the masculinising effects of testosterone (Hoshino, 1965).

The degree to which prenatal androgens masculinised the external genitalia of individual females varied markedly. The penes of the masculinised females were not uniformly placed along the abdominal wall, with variation amongst females in their position relative to the navel and scrotum (Wood et al., 1991; 1995). In the most masculinised females (i.e., those resembling control and testosterone males), the penis lay immediately caudal to the navel. However, there was a tendency for the penis to be positioned further from the navel in females that had small bifid scrotums. It is not clear as to why the appearance of the external genitalia was not uniform across the masculinised females. All pregnant females received the same dose of testosterone

during the same period of gestation, and no individual differences were observed in circulating plasma concentrations of maternal testosterone. There was no relationship between the extent of masculinisation and the sex of the co-twin. For instance, females who were co-twins to males were not masculinised to a greater degree relative to those who were co-twin to a female.

Several studies have clearly demonstrated that control of reproductive anatomy, physiology, behaviour and neuroendocrine function in sheep is largely dependent on the presence or absence of testosterone at critical periods during development (Clarke et al., 1976; Tarttelin, 1986; Wood et al., 1991; 1995, Wood and Foster, 1998; Robinson et al., 1999). As a result of exposure of the female fetus to exogenous androgens during the first half of a 147-day gestation, these sexually dimorphic traits are permanently masculinised, and the females are rendered infertile. As already shown for reproductive function, one concept emerging from this study is that of a unique period of sensitivity to androgens for sexual differentiation of the developing mammary gland. Testosterone cypionate was administered to ewes during the period of sensitivity to androgens (Clarke et al., 1976), which results in complete masculinisation of the external genitalia of the female offspring. This period also coincides with onset of gonadal testosterone secreting activity in the normal male (Attal, 1969; Mauleon, 1977; Lun et al., 1998). During the treatment period maternal plasma concentrations of testosterone rose steadily to peak at day 56. Elevated concentrations persisted three to four weeks following the final injection (day 55), which ensured that the treatment encompassed the period of genital masculinisation in the sheep (Clarke et al., 1976). However, there are two factors that suggest responsiveness of the mammary gland to the effects of androgens occurs later than that of reproductive anatomy. First, the ontogeny data (Chapter two) did not begin to show differential patterns in mammary growth in males and females until day 80 of fetal age, which coincided with the emergence of androgen receptors in the epithelial cells of the mammary ducts and, hence, sensitivity to the masculinising effects of testosterone. Second, partial inhibition of mammary development was seen only at day 140 and at birth in testosterone-treated females. It may be deduced therefore that the masculinising effect on the mammary gland requires exposure to testosterone at least until day 120 of gestation, the point of no return, in terms of epithelial growth, in the male. It is also probable that the suppression of epithelial growth may not depend solely on testosterone, but may involve local mediation by growth factors, such as IGF-I (Chapter four). Reduced epithelial growth, as opposed to complete inhibition, in the testosterone-treated females relative to the control and ontogeny females, may also reflect the increase in circulating concentrations of fetal and maternal oestrogens in late pregnancy (Findlay and Cox, 1970; Challis and Patrick, 1981). Certainly, the presence of oestrogen receptors in the ductal epithelial cells of the fetal female infers sensitivity to oestrogenic stimuli, which may override the effects of testosterone.

Treatment of the pregnant ewe with the anti-oestrogen tamoxifen had no effect on mammary gland development in the female offspring at any of the stages examined. This is in agreement with the rodent model, which states that oestrogen plays no role in development of the fetal mammary gland (Korach et al., 1996). It is also consistent with the known variability in the

biological effects of tamoxifen between species and even between organs, tissues and cell types in the same species (Furr and Jordan, 1984). Tamoxifen acts as an oestrogen receptor activator in the mammary gland of the fetal mouse, where, on administration to the pregnant mother between days 15 and 20 of gestation, it increased the density of terminal end buds (Hilakivi-Clarke et al., 1998). Conversely, the administration of the anti-oestrogen MER-25 to pregnant mice from days 13 to 18 of gestation resulted in suppressed nipple differentiation and reduced volume of the mammary epithelial tissue in 19-day-old female fetuses (Jean-Faucher et al., 1977). The series of mouse experiments by Raynaud (reviewed 1961, 1971) established that the sensitivity of the mammary gland to oestrogen is determined by fetal age. Hence, the stage of gestation at which the anti-oestrogen treatment was administered may account for the opposing effects reported by the two aforementioned studies. Treatment in the latter study encompassed the period of maximum sensitivity of the mammary gland to the effects of oestrogens (days 12 to 14 of gestation) as described by Raynaud.

The failure of the present study to show an effect of tamoxifen on mammary gland development may also be attributed to the stage of gestation at which the treatment was administered. Although the ontogeny study demonstrated extensive nuclear staining in the cells of the fibroblasts at day 60 of fetal age, epithelial oestrogen receptors were only observed in all females at 100 days of fetal age (Chapter three). It is therefore likely that an epithelial response to oestrogenic stimuli may not occur until late gestation.

In contrast to its lack of effect on fetal mammogenesis, tamoxifen had profound effects on the mortality of those fetuses exposed to it. The greatest losses, through intrauterine death and stillbirth, were observed at day 117 of gestation and at birth. On examination of the uterine components, the fetal cotyledons were found to be much darker in colour and had a tendency to fall apart, hence, unlike their control counterparts, they were very difficult to separate cleanly from the maternal caruncles. This effect of tamoxifen treatment on the outcome of pregnancy is consistent with reports in fetal rats and rabbits. Tamoxifen treatment of rats during early pregnancy (days 9 to 11) resulted in impairment of fetoplacental development and a higher incidence of growth retarded fetal development and fetal death compared to RU486 (an anti-progesterone) (Sadek and Bell, 1996). Administration of tamoxifen to rabbits from day 10 of pregnancy resulted in considerable embryonic loss whilst administration from day 20 caused premature parturition and abortion (Furr et al., 1976). As reported in rabbits, the effects observed in the present study were associated with significantly reduced plasma progesterone concentrations in the pregnant ewes treated with tamoxifen relative to the control ewes. Hence, tamoxifen may interfere with the maintenance of pregnancy. Progesterone is also required for secretory cell development in the maternal mammary gland during gestation. Since the secretory cells proliferate on the ducts, one would expect that a reduction in duct development would ultimately result in fewer secretory cells. Although the proportion of parenchymal versus stromal tissue comprising each gland was not measured, the trimmed weight of the udders did not differ significantly between treatment groups. Tamoxifen treatment during pregnancy did, however, affect the subsequent lactational performance of the ewes, when lactation was

assessed as daily milk yield. Milk yield of the tamoxifen group was significantly lower than that of all other groups at each milking, which would suggest a reduced number of secretory cells in the mammary glands of these ewes. One could speculate that mammary progesterone receptors in the tamoxifen-treated ewes during late pregnancy may have been lower relative to those in the control ewes.

An interesting observation at day 61 of gestation was yellow secretions, the consistency of runny custard, in the mammary glands of the tamoxifen ewes while a milky white fluid, the consistency of homogenised milk, was seen in the glands of the testosterone-treated ewes. At day 117 of gestation, the secretion from the control and Androcur ewes was assumed to be mainly immunoglobulin. When secretions were present in the mammary glands of the testosterone- and tamoxifen-treated ewes, they were white in colour and very watery, not at all viscous. In experiment one, although the udder development of the testosterone-treated ewes appeared normal, they did not show the secretory activity that would have been expected at day 140 of gestation, when they were euthanased. This is reminiscent of anecdotal accounts of both ewes and cows occasionally failing to lactate following an apparently normal pregnancy.

The treatment of pregnant ewes with cyproterone acetate was not sufficient to induce development in the female direction. It did not influence the appearance of the mammary glands in the male offspring, nor did it affect the normal sexual dimorphism of the reproductive tract. This is in contrast to rodents where the feminising effects of cyproterone acetate were seen in the male offspring of rats injected daily from the 13$^{th}$ day of pregnancy. At all stages of development examined, the male mammary gland followed the female pattern of morphogenesis (Elger and Neumann, 1966; Elger et al., 1966). Cyproterone acetate blocks the effect of endogenously produced androgens in the mammary gland by means of competitive inhibition. Hence, the stimulating effect of androgens on androgen-dependent organs such as the rodent mammary gland is weakened or destroyed by cyproterone acetate (Neumann, 1994). The doses of cyproterone acetate used in this study were chosen to approximate the clinical dose (300 mg per week) used in man (without orchidectomy) to treat inoperable prostatic carcinoma (Neumann, 1994). Ewes were administered 200 mg per week based on the assumption that a Romney ewe weighs approximately 30% less than that of an average man. It is possible that the dose was inadequate, as 10 mg per day at days 13 and 14 of pregnancy was required to induce an effect in the male offspring of adult female Sprague-Dawley rats (Neumann and Elger, 1966). Once again, the stage of fetal development at the time of maternal treatment is critical in terms of inducing an effect. Exogenous treatment of ewes around the time of androgen receptor appearance in the epithelial cells of the fetal mammary gland (days 80 to 120) may have induced a further proliferation of glandular tissue in the fetal male.

Overall, these data are consistent with the notion that alterations in the differentiation of fetal reproductive and mammary tissues are a result of *in utero* exposure to endocrine-disrupting chemicals that either mimic or inhibit the action of the gonadal steroid hormones. Partial inhibition of epithelial growth in the female offspring of ewes treated with testosterone not only

demonstrated that androgens are critical determinants of the male phenotype but reflected the crucial role of the androgen receptor in controlling the timing of the event. The poor lactational performance of the tamoxifen-treated ewes also make it unequivocally clear that anti-oestrogenic compounds interfere with mammary development and hence the potential for milk secretion. This gives cause for concern considering the widespread distribution of oestrogens and anti-oestrogens in the environment. However, they must be able to get into the female in quantities sufficient to have an effect.

# CHAPTER SIX

# THE EFFECT OF THE MATERNAL ENVIRONMENT ON DEVELOPMENT AND GROWTH OF THE FETAL MAMMARY GLAND

# 6 Preamble

A series of studies were conducted at Massey University to examine the effects of modifying the maternal environment on fetal growth, and hence birth weights and lamb survival (Cooper et al., 1998; Kenyon et al., 1999; Tarsono, 2000). During the course of those studies, the opportunity arose to collect fetal mammary tissue from three such experiments. This allowed for testing of the hypothesis that altering the maternal environment, through pre-lamb shearing or nutrition affects mammary gland size.

## 6.1 Abstract

This study was conducted to examine the effects of pre-lamb shearing and maternal nutrition at different stages of gestation on fetal growth and mammary gland development.

Mammary tissue from female ovine fetuses was collected from three different trials designed to:

1. Improve lamb birth weight through mid- to late-pregnancy shearing (1997).
2. Test the effect of maternal nutrition during early and mid-gestation on fetal growth (1996).
3. Test the effect of manipulation of feed intake throughout pregnancy on lamb birth weight (1998).

In trial one, 180 mixed-aged Border Leicester x Romney ewes were randomly allocated to a factorial experiment involving two shearing treatments (shorn on day 69 of pregnancy or unshorn) and two pregnancy ranks (single or twin). At day 140 of gestation, no significant differences were observed in fetal mammary gland development, as measured by total duct area, or in fetal bodyweight, between the shorn and unshorn groups.

In trial two, 60 mixed-age Romney ewes were allocated to one of three feeding levels, 0.5 (low), 1.0 (control) and 1.5 (high) times maintenance from days 21 to 101 of gestation. At day 101 of gestation, ewe live weights were 42.8±1.3, (low), 52.0±1.3 (control) and 69.6±1.3kg (high) ($P<0.001$). However, fetal body weights and mammary gland development did not differ between the treatment groups.

In trial three, 89 Romney ewes were allocated to either a 1.0 x maintenance (M) (n=60) or 1.5 x maintenance (H) (n=29) level of feeding at day 19 of pregnancy. At day 47 of pregnancy, 30 M ewes were moved to the high feeding level to give 3 treatment groups (MM, MH and HH) (n=30, 30 and 29 ewes/group). At day 101, 10 MM, 10 MH and 9 HH ewes were euthanased. Total duct areas (least square means±sem) of the fetal mammary glands were 1089.1±125.9, 1549.9±140.9 and 1612.6±119.2 $\mu m^2 \times 10^3$ ($P<0.05$) for MM, MH and

HH groups, respectively. There was an overall treatment effect on fetal bodyweight ($P<0.05$) but ewe live weights were not significantly different.

At day 101, the remaining ewes were reallocated to nutritional treatments (M and H) to give six groups of 10 ewes each with overall treatments of MMM, MMH, MHM, MHH, HHM and HHH. At day 140 of pregnancy, total duct areas (lsmeans±sem) were 1760.6±574.6, 4369.91±598.5, 3025.1±724.3, 3719.8±558.3, 3771.2±572.0 and 4640.5±725.5 $\mu m^2 \times 10^3$ ($P<0.05$), respectively. No significant differences in fetal body weights or fetal mammary gland weights were observed, despite the two and a half-fold variation in total duct area.

Immunohistochemistry demonstrated the presence of insulin-like growth factor-I receptor (IGF-IR) in the epithelial cells of the fetal mammary gland, irrespective of maternal nutritional status. While the depth of IGF-IR immunostaining tended to be greater in the control and high treatment groups relative to the low group at day 101 of gestation in trial two, the intensity of staining was highly variable between individuals. In trial three, the intensity of immunostaining was greater in the MH and HH treatment groups relative to the MM group at day 101. By day 140, the depth and intensity of immunostaining was greater in the HHH group relative to the MMM group.

These results have highlighted that the development and growth of the mammary gland is sensitive to the effects of maternal nutritional status from the earliest stages of fetal life. Furthermore, while the energy supply *per se* may not be important, the influence of energy intake or the ewes' energy status on hormonal parameters may be of real importance in the development of the fetal mammary gland. The reduction in total duct area accompanied by the down regulation of IGF-IR observed at day 140 in response to a restricted maternal diet, without an effect on fetal body or mammary gland weights, demonstrates that the hierarchy of nutrient partitioning can be dramatically altered in the fetal sheep. Moreover, it suggests that it is low maternal nutrition throughout pregnancy that is detrimental to development of the fetal mammary gland.

The conflicting results at day 101 of gestation may indicate that the plane of maternal nutrition prior to or around the time of conception may be important in terms of fetal development. It also lends support to the growing hypothesis that maternal nutrition during pregnancy can alter physiological function in the offspring without necessarily affecting size at birth.

Finally, this research raises the possibility of impaired capacity for milk production in adult life as a result of altered structure and function of the mammary gland during fetal life.

## 6.2 Introduction

The amount of milk produced by a lactating female is a function of the amount of secretory tissue in the mammary gland and its activity. Studies comparing rates of secretion of milk

between species (Linzell, 1972) and within species (Davis et al., 1983) indicate that many animals already secrete milk at a rate that approaches the upper limit of the secretory activity of mammary gland tissue. Therefore, increasing the mass of secretory tissue present at parturition is the major means available for increasing the milk production potential of individual animals.

The size of the udder at parturition is most likely determined by events occurring at the three stages in the life of the female when mammary gland tissue grows rapidly, namely, during fetal development, around the time of puberty and during pregnancy. Although the greatest proportion of secretory tissue in the udder is laid down during pregnancy, events that occur around the time of puberty can have a substantial effect on subsequent milk production of the dairy cow. For example the milk production of cows that were grown very rapidly during their prepubertal growth spurt, was reduced by up to 40% in comparison with cows that were grown at more moderate rates (Little and Kay, 1979). This illustrates that the gland is vulnerable to environmental influences at this time and that differences in growth or development at this critical period may have a disproportionate effect on the final size of the gland.

There is very little evidence of an *in utero* effect on fetal and subsequent mammary gland development, but a number of models have been shown to differentially influence fetal growth and development in general. Shearing of ewes during mid- to late pregnancy commonly increases lamb birth weights, but the response is inconsistent across treatments and trials (Kenyon et al. 1999). In some cases shearing at day 70 of pregnancy is twin-lamb specific (Morris and McCutcheon, 1997) and in others, it only influences the birth weight of single-born lambs (Morris et al., 2000). Furthermore, the response is associated with an increase in thyroid hormone concentrations (triiodothyronine ($T_3$)) in the maternal circulation (Morris et al., 2000) and non-insulin dependent uptake of glucose by the feto-placental unit (Revell et al., 2000). The sensitivity of lamb birth weight, particularly of twins, to the dam's plane of nutrition in late pregnancy is well known (Wallace, 1948; Mellor, 1983). However, up until 100 days of gestation, fetal growth appears to be largely insensitive to variations in maternal nutrition or other potential restraints, suggesting that it occurs at rates close to the genetic potential (Parr et al., 1986; Heasman et al., 1999). Nevertheless, Cooper et al. (1998) concluded that while fetuses do not require high levels of nutrition in early gestation, moderate levels of maternal nutrition might enhance fetal growth. There are few data to indicate a differential growth effect of nutrition on individual organs and tissues as many researchers still use temporal as opposed to allometric relationships. However, one example of a fetal body component for which there is a differential effect due to nutrition is the skeleton, which shows relative insensitivity to maternal food restriction (Robinson et al., 1999).

It is well known that fetal insulin-like growth factor-I (IGF-I) affects fetal growth (Gluckman et al., 1997) and that diet is one of the most effective factors influencing circulating concentrations of IGF in fetal and adult sheep (Bauer et al., 1995). Plasma concentrations of IGF-I are clearly dependent on nutritional status (Gluckman et al., 1987) through both GH-dependent and independent mechanisms. In the fetal lamb, undernutrition leads to a fall in plasma IGF-I

concentrations (Bassett et al., 1990). Furthermore, the much higher concentrations of IGF-II in the fetus, which strongly correlate with glucose supply, suggest that this IGF may act in both endocrine and paracrine modes to modulate growth dependent upon the availability of glucose (Owens, 1991). A local role for IGF production in early ovine mammary development has been suggested after transcripts for the IGF type I receptor were located in the mammary epithelium from days 70 to 140 of gestation (Forsyth et al., 1999).

The objective of this study was to determine whether pre-lamb shearing or restriction of food intake of pregnant ewes at various stages of pregnancy, have an effect on fetal mammary gland development and, if so, whether these effects are associated with the localisation of insulin-like growth factor-I receptor.

## 6.3 Materials and Methods

All procedures were approved by the Massey University Animal Ethics Committee. Ewes were obtained from a commercial flock on the Animal Research Unit (ARU), Massey University. The trials were conducted at Massey Universities Keeble Farm, 7 km south of Palmerston North (latitude 40.23°S and longitude 178.37°E). All ewes were pregnant to the first cycle of a progesterone-synchronised oestrus. Real-time ultrasound confirmed pregnancy status and rank.

## Trial one

### 6.3.1 Animals and Treatments

One hundred and eighty, pregnant mixed age Border Leicester x Romney ewes, were randomly allocated to a factorial experiment incorporating two shearing treatments (shorn at day 69 of pregnancy, or unshorn) and two pregnancy ranks (single or twin). Ewes were fed *ad libitum* (average pasture cover of 3200 kg DM/ha) from day 70 until euthanasia at day 140 of pregnancy (n=10 ewes per group) or lambing.

## Trial two

### 6.3.2 Animals and Treatments

One hundred and fifty mixed-age Romney ewes were allocated to three nutritional treatment groups. The treatment groups involved feeding levels of 0.5 (low), 1.0 (control) and 1.5 (high) times maintenance from days 21 to 100 of gestation. Maintenance requirements for a 50 kg ewe were assumed to be 0.9 kg DM/ewe/day (10 MJ ME/day) at an energy concentration of 11 MJ ME/kg DM (Robinson, 1983). At day 42 of gestation, ultrasound scanning confirmed pregnancy rank. Ten single-bearing and ten twin-bearing ewes (n=60 in total) were allocated to each of the

three nutritional groups after stratification by live weight. At a mean day 101 (days 99-103) of pregnancy all ewes and their fetuses were euthanased.

## Trial three

### 6.3.3 Animals and Treatments

One hundred and sixty pregnant mixed-age Romney ewes were allocated to two nutritional treatment groups (1.0 x maintenance (M) or 1.5 x maintenance (H), where n=55 or 105, respectively) from days 19 to 47 of pregnancy. Maintenance requirements of 11 MJ ME/day (Geenty and Rattray, 1987) at an energy concentration of 11 MJ ME/kg DM, were based on an average initial ewe live weight of 55 kg.

The objective was to use only twin-bearing ewes. However, at pregnancy diagnosis (day 47 of gestation) only 59 ewes were twin-pregnant to the first mating. Therefore, 30 single-bearing ewes were incorporated to make a total treatment number of 89 ewes. The remaining 71 ewes were merged into Massey University's commercial flock. At day 47 of pregnancy, 30 M ewes were retained on maintenance (MM), 30 M ewes were moved to a high feeding level (MH) and 29 H ewes remained on the high plane (HH). Each treatment group consisted of 21 twin-bearing and 10 single-bearing ewes, except HH, which consisted of 17 twin-bearing ewes. At a mean day 101 (days 100-102) of pregnancy, 10 ewes (6 single- and 4 twin-bearing) from each of the three treatment groups (MM, MH, HH), and their fetuses were euthanased.

The remaining 59 ewes from the three treatment groups (n=19 or 20 per group) were split again at day 101 of gestation. Of the 19 (HH) ewes, 9 remained at a high level of feeding (HHH) and 10 moved to maintenance (HHM) for the period days 101-140. Of the 20 (MH) ewes, 10 remained on a high level of feeding (MHH) and 10 dropped back to maintenance (MHM). Of the 20 (MM) ewes, 10 moved to a high level of feeding (MMH) and 10 remained at maintenance (MMM) (refer to Table 6.1).

At a mean day 140 (days 137 to 141, inclusive) of pregnancy, all 59 ewes and their fetuses were euthanased.

### 6.3.4 Tissue collection – all trials

Ewes were weighed before slaughter and the fetuses were weighed after they were euthanased.

Ewes and fetuses were euthanased following the procedures outlined in Chapter two. The number of female fetuses collected from each trial and selected for analyses, is presented in Table 6.1.

**Table 6.1** The number of female mammary glands collected and selected for analysis for each of the three trials*.

| Treatment groups | Number of female glands collected | | Number of female glands selected for analysis |
|---|---|---|---|
| | Singles | Twins | |
| Trial one (day 140) | | | |
| Shorn | 3 | 3 | 6 |
| Unshorn | 1 | 3 | 4 |
| Trial two (day 101) | | | |
| Low | 5 | 8 | 4 |
| Control | 5 | 9 | 4 |
| High | 5 | 7 | 4 |
| Trial three (day 101) | | | |
| MM | 2 | 4 | 6 |
| MH | 4 | 1 | 5 |
| HH | 4 | 2 | 6 |
| Trial three (day 140) | | | |
| MMM | 1 | 4 | 5 |
| MMH | 1 | 4 | 5 |
| MHM | 1 | 4 | 5 |
| MHH | 1 | 4 | 5 |
| HHM | 1 | 5 | 6 |
| HHH | 0 | 4 | 4 |

*In trials one and three, all the mammary tissue collected was analysed. In trial two, mammary tissue was randomly selected from 15 single females and analysed.

At euthanasia (mean day 101 of gestation for Trials 2 and 3, and mean day 140 for Trials 1 and 3), mammary glands were dissected off the female fetuses, weighed, fixed in Bouin's solution and then preserved in 70% ethanol. Left and right glands from each fetus were embedded in paraffin wax. Sections 7 μm thick were cut running through and parallel to the long axis of the teat in the anterior-posterior plane and laid on glass microscope slides. The sections were stained with haematoxylin and eosin (H&E). Slides with extra sections were stored in dust-free boxes for immunohistochemical identification of insulin-like growth factor-I receptor.

The number of ducts and lumens, the areas ($\mu m^2$) of individual ducts and their lumens and hence the total duct and lumen areas ($\mu m^2$) were measured on the sections of the left glands using the image analyser programme, Sigmascan (see Chapter two). The total secretory cell areas ($\mu m^2$) were calculated by subtracting the lumen areas from the respective duct areas.

### 6.3.5 Immunohistochemistry – all trials

The methodology used to locate IGF-IR is outlined in Chapter four, section 4.3.3.

The method used for examining the intensity of immunohistochemical staining is the same as described in Chapter three.

### 6.3.6 Statistical analyses – all trials

Analysis of variance was used to determine the effects of pre-lamb shearing at day 140 of gestation and maternal nutrition at days 101 or 140 of gestation on fetal mammary gland development. All measures of mammary development were adjusted to a common fetal body weight and rank, where applicable. Data are expressed as least squares means and standard errors for the treatment groups. Statistical analyses were conducted using the 'SAS' system for windows, version 6.12 (1996).

## 6.4 Results (morphological)

### Trial one

Shearing during mid-pregnancy (day 69) had no effect on the measures of fetal mammary gland development (Table 6.2). Furthermore, ewe live weight and fetal body weight did not differ significantly between the shorn and unshorn treatment groups.

**Table 6.2** Effect of shearing ewes at day 69 of gestation on development of the female fetal mammary gland at day 140 of gestation (lsmean±sem.).

| Parameter | Shearing regimen | | Significance |
|---|---|---|---|
| | Shorn | Unshorn | Treatment |
| Number of fetuses | 6 | 4 | |
| Ewe live weight (kg) | 69.6±2.3 | 66.7±3.2 | NS |
| Fetal bodyweight (g) | 5184.8±123.8 | 5171.5±181.2 | NS |
| Fetal mammary gland (g) | 10.6±0.7 | 12.0±1.0 | NS |
| Total duct area ($\mu m^2 \times 10^3$) | 4422.0±494.5 | 3790.3±743.6 | NS |
| Total lumen area ($\mu m^2 \times 10^3$) | 1538.0±216.5 | 1337.9±325.6 | NS |
| Total secretory cell area ($\mu m^2 \times 10^3$) | 2884.1±335.5 | 2452.4±504.5 | NS |
| Number of ducts | 284.1±59.9 | 288.7±90.1 | NS |
| Number of ducts with lumens | 207.2±43.8 | 199.6±65.9 | NS |

NS = not significant (P>0.10).

### Trial two

Mammary glands from 27 female fetuses (singles and twins) were collected from trial two. Twelve mammary glands (4 per treatment group) were randomly selected from the single females for analysis. The data for weight of ewes, fetuses and mammary gland for the non-selected and selected groups are shown in Tables 6.3 and 6.4, respectively. Analysis of the data from the two groups indicated that there were no statistically significant differences between the means.

**Table 6.3** The effect of maternal nutrition on ewe live weight, fetal body weight and fetal mammary gland weight at day 101 of gestation of female fetuses (singles and twins) not selected for analysis.

| Parameter | Maternal nutritional regimen | | |
|---|---|---|---|
| | Low (0.5M) | Control (1.0M) | High (1.5M) |
| Number of fetuses | 9 | 10 | 8 |
| Ewe live weight (kg) | 45.4±2.8[a] | 54.8±2.8[b] | 76.6±2.9[c] |
| Fetal bodyweight (g) | 1566.4±102.1 | 1280.4±74.7 | 1002.5±125.2 |
| Fetal mammary gland weight (g) | 5.3±0.5 | 5.1±0.4 | 5.1±0.4 |

[abc] Means within rows with different superscripts are significantly different (P<0.05).

There was a highly significant (P<0.001) effect of plane of nutrition from day 21 of pregnancy on the body weight of the ewes at day 101. The weight of the ewes on the low plane was 17.7% lower and those on the high plane 33.3% higher than the controls (Table 6.4). However, measures of fetal body weight and mammary gland development did not differ between the treatment groups.

**Table 6.4** Effect of maternal nutrition in early pregnancy on development of the mammary gland of female fetal sheep (singles only) at day 101 of gestation (lsmean±sem., $x10^3$).

| Parameter | Nutritional regimen | | |
|---|---|---|---|
| | Low (0.5M) | Control (1.0M) | High (1.5M) |
| Number of fetuses | 4 | 4 | 4 |
| Ewe live weight (kg) | 42.8±1.3[a] | 52.0±1.3[b] | 69.6±1.3[c] |
| Fetal bodyweight (g) | 1377.4±229.3 | 1228.2±86.9 | 976.4±277.8 |
| Fetal mammary gland weight (g) | 4.5±0.5 | 4.9±0.5 | 4.4±0.5 |
| Total duct area ($\mu m^2 \times 10^3$) | 1155.3±155.5 | 1066.9±157.7 | 1051.7±159.0 |
| Total lumen area ($\mu m^2 \times 10^3$) | 566.0±81.6 | 364.1±82.7 | 471.7±83.4 |
| Total secretory cell area ($\mu m^2 \times 10^3$) | 589.3±106.1 | 702.8±107.5 | 580.1±108.4 |
| Number of ducts | 28.5±9.1 | 43.7±9.2 | 32.8±9.3 |
| Number of ducts with lumens | 20.0±7.8 | 34.1±7.9 | 26.1±8.0 |

[abc] Means within rows with different superscripts are significantly different (P<0.05).

## Trial three

A high plane of maternal nutrition from days 19 to 101 of gestation (HH) or movement from maintenance feeding to a high level at day 47 of gestation (MH), significantly increased total duct area ($P<0.05$) and total secretory cell area ($P<0.01$) of the fetal mammary gland in comparison with the MM group (Table 6.5.). Although no difference in maternal live weight was observed, fetal body weight was significantly increased ($P<0.05$) in the MH group relative to the MM group. Other indicators of mammary development did not differ between treatment groups.

There was a significant treatment by rank interaction for ewe live weight ($P<0.05$), reflecting a continued decrease in the live weight of twin-bearing MH ewes even after they were moved to a high plane from days 47 to 101.

Ewe live weight, fetal body weight and indicators of fetal mammary gland development at day 140 of gestation are presented in Table 6.6. Raising the maternal feeding level from maintenance to high (MMH), remaining at high (HHH) or decreasing to maintenance from a high level of feeding (HHM), from day 101 of gestation significantly increased ($P<0.05$) total duct area compared to fetuses whose dams remained at maintenance (MMM). Furthermore, the number of ducts were significantly ($P<0.01$) increased in the (HHH), (HHM), (MHM) and (MMH) treatment groups relative to the maintenance (MMM) group. Although fetal body weight did not differ between the treatment groups, it had a significant ($P<0.01$) effect on all the measures of mammary gland development, except total lumen area, recorded.

**Table 6.5** Effect of maternal nutrition treatment on development of the mammary gland of fetal sheep at day 101 of gestation (lsmean±sem., $x10^3$ for areas).

| Parameter | Maternal nutritional regimen | | |
|---|---|---|---|
| | Maintenance (MM) | Maintenance High (MH) | High (HH) |
| Number of fetuses | 6 | 5 | 6 |
| Ewe live weight (kg) | 52.7±2.4 | 54.5±3.1 | 60.9±2.4 |
| Fetal bodyweight (g) | 1127.4±55.9[a] | 1372.1±61.3[b] | 1222.8±58.9[a] |
| Fetal mammary gland weight (g) | 6.5±0.4 | 6.1±0.5 | 7.2±0.4 |
| Total duct area ($um^2$) | 1089.1±125.9[a] | 1549.9±140.9[b] | 1612.6±119.2[b] |
| Total lumen area ($um^2$) | 462.5±78.8 | 702.2±88.3 | 586.3±74.6 |
| Total secretory cell area ($um^2$) | 626.6±69.5[a] | 847.7±77.9[ab] | 1026.2±65.9[b] |
| Number of ducts | 43.1±8.1 | 51.6±9.0 | 59.7±7.6 |
| Number of ducts with lumens | 34.5±6.1 | 44.7±6.8 | 45.2±5.8 |

[ab] Means within rows with different superscripts are significantly different ($P<0.05$).

**Table 6.6** Effect of maternal nutrition treatment on development of the mammary gland in fetal sheep at day 140 of gestation (lsmean±sem., $x10^3$ for areas).

| Parameter | Maternal nutritional regimen | | | | | |
|---|---|---|---|---|---|---|
| | MMM | MM*H* | M*H*M | M*HH* | *HH*M | *HHH* |
| Number of fetuses | 5 | 5 | 5 | 5 | 6 | 4 |
| Ewe live weight (kg) | 63.5 ± 4.0 | 60.9 ± 4.0 | 62.1 ± 4.0 | 67.6 ± 4.0 | 61.3 ± 4.0 | 69.5 ± 4.4 |
| Fetal bodyweight (g) | 4725.6 ± 275.2 | 4541.9 ± 292.4 | 4772.6 ± 289.6 | 4440.2 ± 290.2 | 4462.0 ± 291.3 | 4130.6 ± 344.5 |
| Fetal MG weight (g) | 11.5 ± 0.8 | 13.0 ± 0.8 | 11.2 ± 1.0 | 11.5 ± 0.8 | 11.2 ± 0.8 | 11.0 ± 1.0 |
| Total duct area ($um^2$) | 1760.6 ± 574.6a | 4369.9 ± 598.5b | 3025.1 ± 724.3ab | 3719.8 ± 558.3b | 3771.2 ± 572.0b | 4640.5 ± 725.5b |
| Total lumen area ($um^2$) | 892.3 ± 319.2 | 1767.4 ± 332.4 | 1486.7 ± 402.3 | 1883.5 ± 310.1 | 1507.5 ± 317.7 | 2149.6 ± 403.0 |
| Total secretory cell area ($um^2$) | 868.3 ± 314.5a | 2602.5 ± 327.5c | 1538.4 ± 396.4bd | 1836.2 ± 305.5ab | 2263.7 ± 313.0cd | 2490.8 ± 397.1bd |
| Number of ducts | 62.7 ± 28.3a | 327.1 ± 31.7c | 170.5 ± 32.2bd | 129.9 ± 33.0ab | 235.6 ± 30.9cd | 220.0 ± 32.2bc |
| Number of ducts with lumens | 48.7 ± 19.1a | 228.9 ± 21.4b | 123.1 ± 21.8ab | 96.9 ± 22.3ab | 177.9 ± 20.8b | 159.9 ± 21.8b |

[abcd] Means within rows with different superscripts are significantly different ($P<0.05$).

M = maintenance (1.0M), H = high (1.5M), planes of nutrition.

## 6.5 Results (immunohistochemical)

To localise insulin-like growth factor-I receptors (IGF-IR) to specific cell types, immunohistochemistry was performed on paraffin-embedded sections of mammary gland tissue from fetal sheep in nutrition trials two and three. Although there was no effect of maternal nutritional regimen on fetal body weight or measures of fetal mammary gland development in trial two, the fetal mammary tissues were histochemically analysed because there was a large effect on maternal body weight, clearly indicating the expected response to treatment. Trial three showed an effect on the weight of the fetus and the size of the mammary gland. Immunohistochemical analysis was not performed on the tissue samples from trial one because no differences were found in maternal live weight or in measures of fetal parameters. Positive immunoreactivity was confirmed when staining was present beyond the actual surface of the cells, and the cells stained darker than the surrounding tissue. The skin (epidermis) and sweat glands of the mammary gland acted as a control for edge artefact. The reaction patterns described hereafter, are exactly the same as those described in Chapter three: linear cell surface, cytoplasmic and nuclear. The distribution of IGF-IR in the mammary gland of fetal sheep from nutrition trials two and three are presented in the appendices (Table A.8, Trial 2; Tables A.9, A.10, Trial 3).

### Trial two

Immunoreactivity to IGF-IR antibody was seen in the luminal epithelial cells of the secondary ducts of the mammary glands of the 101-day-old fetuses (range = 99 to 103 days, inclusive) irrespective of the nutritional regimens to which their dams had been subjected from days 21 to 101 of gestation. In all three-treatment groups, immunostaining extended beyond the cell surface and into the apical cytoplasm but was not continuous around the lumen perimeter. In general, the depth of staining into the apical cytoplasm and the intensity of immunostaining were greater in the control and high groups relative to the low group (Plates 6.1, 6.2, and 6.3). However, variability in staining intensity was evident between animals within each treatment group.

In all treatment groups, immunoreactivity was observed in the nuclei of the fibroblasts that resided in close contact with the ducts and ranged from weakly to moderately positive. The fibroblasts of the deep hypodermis were either non-reactive or faintly immunostained. The adipoblasts within the lobules of developing adipose tissue were weakly to moderately immunoreactive, with immunostaining restricted to the nucleus. It was not possible to establish definite differences in the intensity of staining of the cells of the fibrous connective tissue and adipose tissue between the treatment groups (photomicrographs not presented).

Across the treatment groups, the epidermis exhibited moderately positive immunoreactivity along the outer edge or stratum corneum. However, the intensity of staining ranged from superficial to strong (photomicrographs not presented).

**Plate 6.1** Photomicrograph of a secondary duct from the mammary gland of a fetal sheep at day 101 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a low (0.5M) plane of nutrition from days 21 to 101 of gestation. Weak to moderate immunostaining of the apical cytoplasm. (Mag x400).

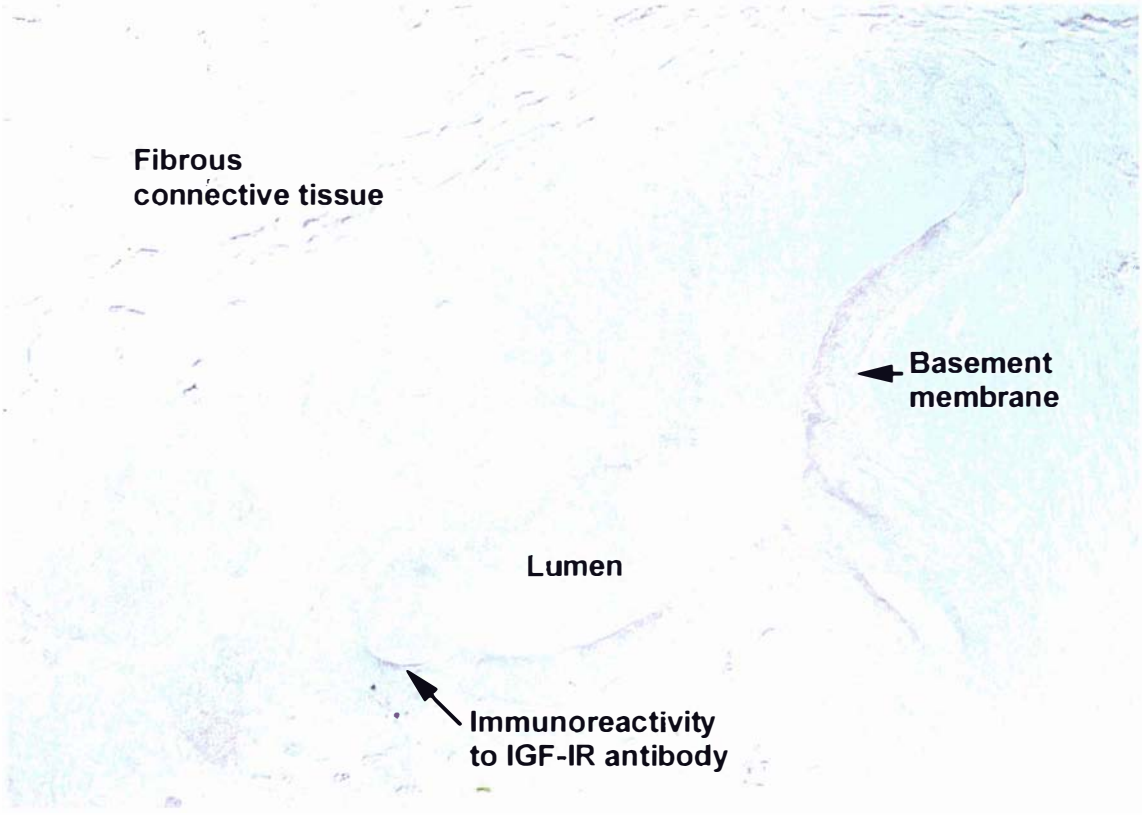


**Plate 6.2** Photomicrograph of a secondary duct from the mammary gland of a fetal sheep at day 101 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a maintenance (1.0M) plane of nutrition from days 21 to 101 of gestation. Moderate immunostaining of the apical cytoplasm. (Mag x400).

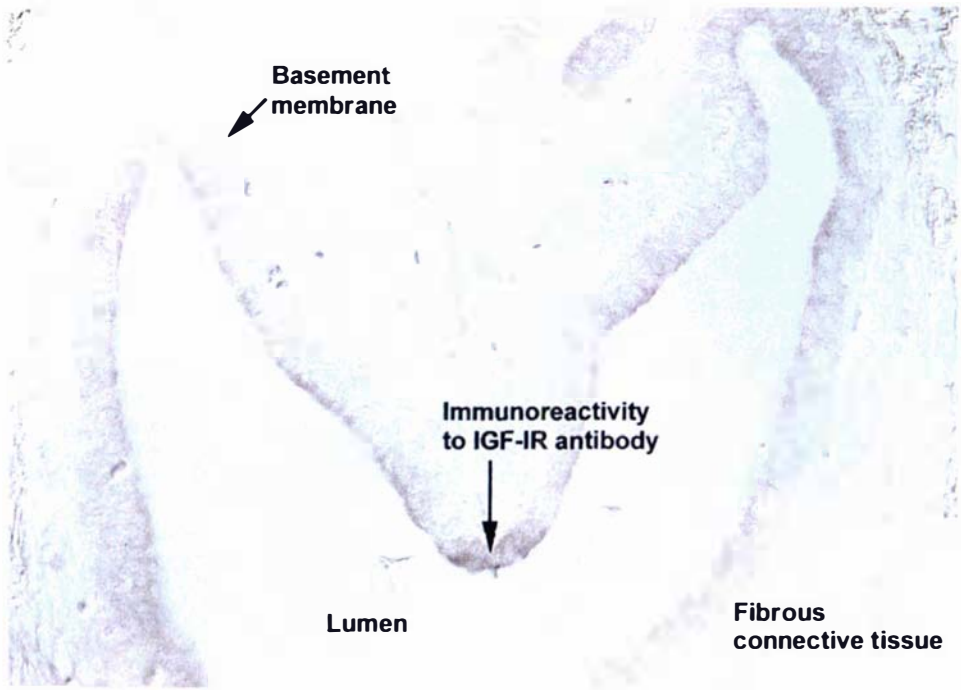
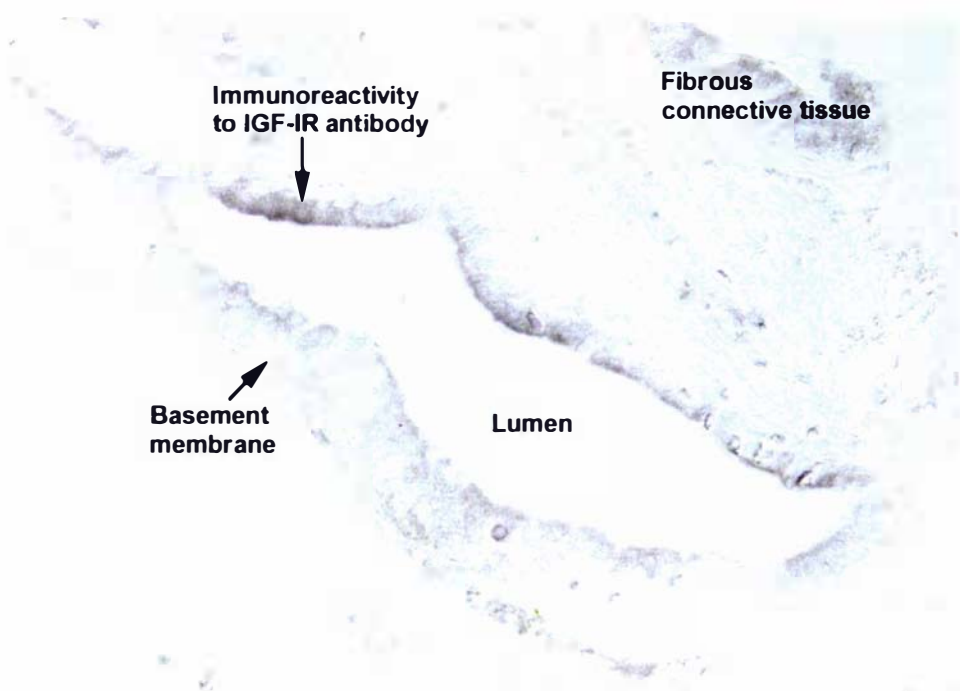



**Plate 6.3** Photomicrograph of a secondary duct from the mammary gland of a fetal sheep at day 101 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a high (1.5M) plane of nutrition from days 21 to 101 of gestation. Intense immunostaining of the apical cytoplasm and, in some cells, of the nuclear membrane. (Mag x400).



## Trial three

Immunoreactivity to IGF-IR antibody was exhibited in the epithelial cells of the fetal mammary glands at a mean day 101 (days 100 to 102, inclusive) of gestation irrespective of the maternal nutritional regimen. However, the pattern and intensity of immunostaining differed between the three treatment groups. In those fetuses whose dams were subjected to a maintenance diet (MM) from days 19 to 101 of gestation, immunoreactivity was superficial along the luminal cell surface and extended beyond the cell membrane into the apical cytoplasm. However, the intensity of staining varied between individual fetuses within this treatment group. While four fetuses showed discontinuous, fine granular cytoplasmic reactivity (Plate 6.4), two fetuses exhibited a coarser, more intense granular cytoplasmic immunoreactivity, which was almost continuous around the lumen perimeter (Plate 6.5).

Consistent and intense, mainly linear cell surface immunoreactivity with the antibody to IGF-IR was found in the mammary epithelial cells of those fetuses that were subjected to a high plane of nutrition (HH) *in utero* from days 19 to 101 of gestation (Plate 6.6). The pattern of immunoreactivity and the intensity of staining in the mammary epithelial cells of fetuses from the MH treatment was variable (Plate 6.7). Immunoreactivity ranged from the apical margins through to incorporating the entire apical cytoplasm and the nuclear membrane. In the glands of two fetuses from the MH group, staining of the epithelial cells encompassed the perimeter of the nuclear membrane. Overall, while a greater intensity of staining was observed in the MH and HH groups relative to the MM group, variability existed between individual animals within each treatment group.

Whereas the fibroblasts of the dermis showed weak to moderate immunostaining of their nuclei in the MM and MH treatment groups, the intensity of staining of fibroblasts in the HH treatment group ranged from moderate to strong. While no immunoreactivity was seen in the fibrous connective tissue of the developing fat pad in the MM treatment group, nuclear staining of the hypodermal fibroblasts ranged from weak to moderate in the MH group and moderate to strong in the HH group. Cytoplasmic and nuclear staining was observed in the adipoblasts of the developing adipose tissue irrespective of the nutritional regimen. The MH and HH groups exhibited weak to moderate, mainly nuclear, staining of the adipose tissue whereas weak cytoplasmic and nuclear staining was seen in the MM group.

The epidermis showed immunoreactivity in all treatment groups. Immunostaining was intense around the nuclei and along the basement membrane of the stratum spinosum in the MM group. However, it was the stratum corneum that reacted in the MH and HH groups. Whereas the intensity of staining ranged from superficial through to strong in the MH group, the HH group displayed moderately positive immunoreactivity of the stratum corneum.

**Plate 6.4** Photomicrograph of a secondary duct from the mammary gland of a fetal sheep at day 101 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a maintenance (1.0M) plane of nutrition from days 19 to 101 of gestation. Fine granular cytoplasmic immunoreactivity in the epithelial cells. (Mag x400).

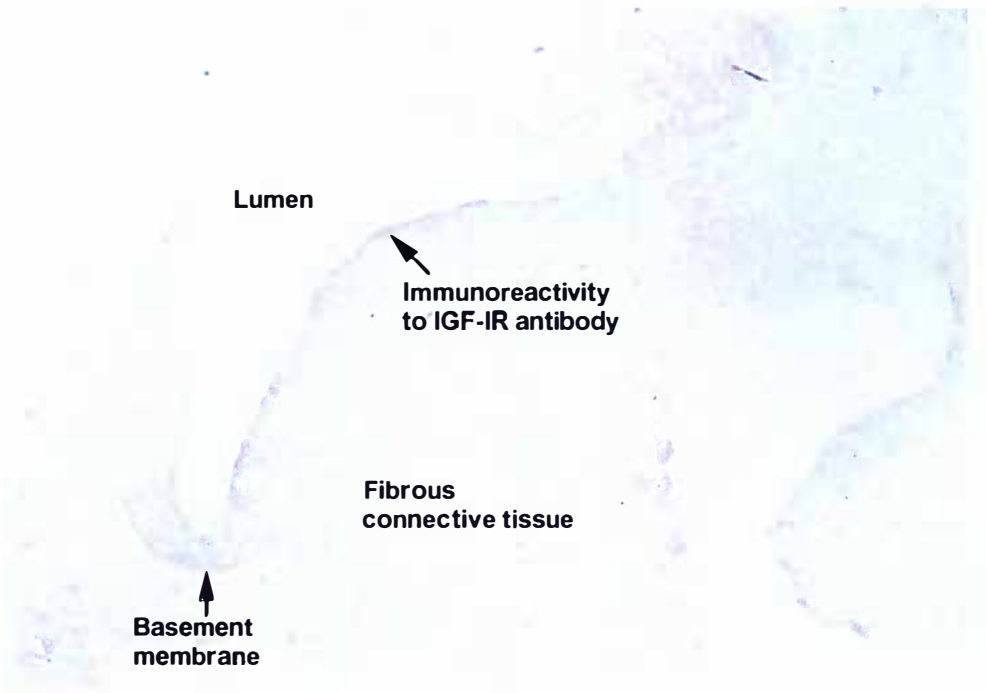



**Plate 6.5** Photomicrograph of a secondary duct from the mammary gland of a fetal sheep at day 101 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a maintenance (1.0M) plane of nutrition from days 19 to 101 of gestation. Coarse granular cytoplasmic immunoreactivity in the epithelial cells. (Mag x400).

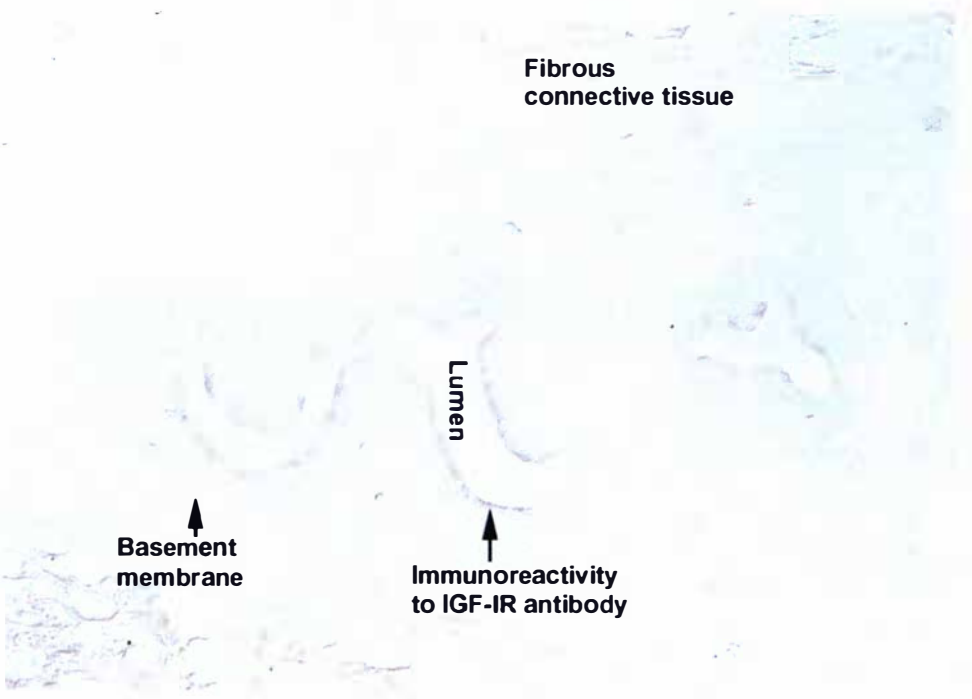


**Plate 6.6** Photomicrograph of a secondary duct from the mammary gland of a fetal sheep at day 101 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a high (1.5M) plane of nutrition from days 19 to 101 of gestation. Intense, mainly linear cell surface immunoreactivity. (Mag x400).

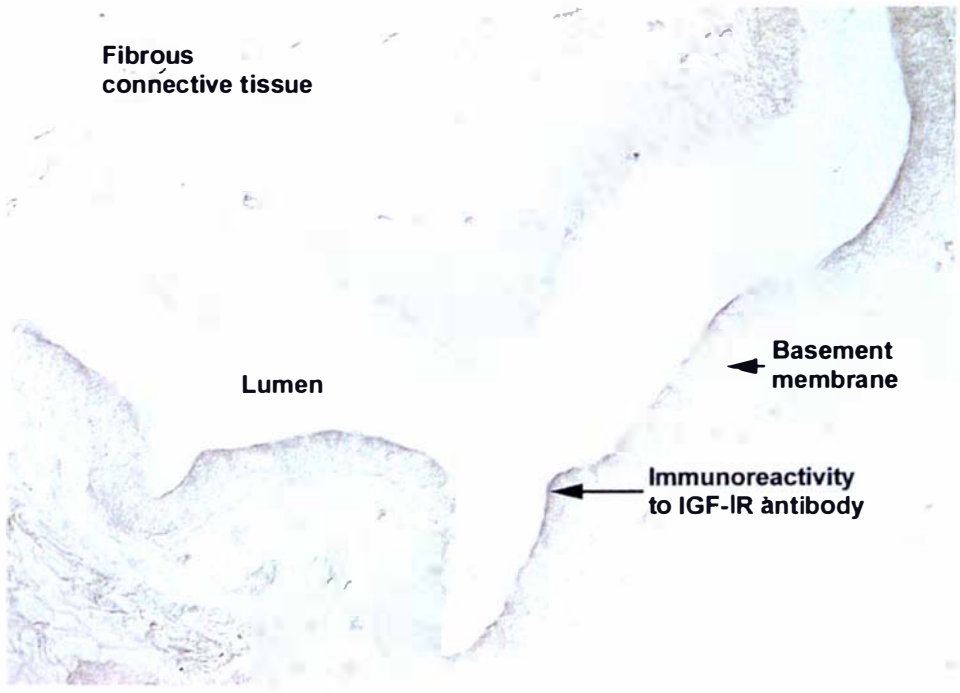
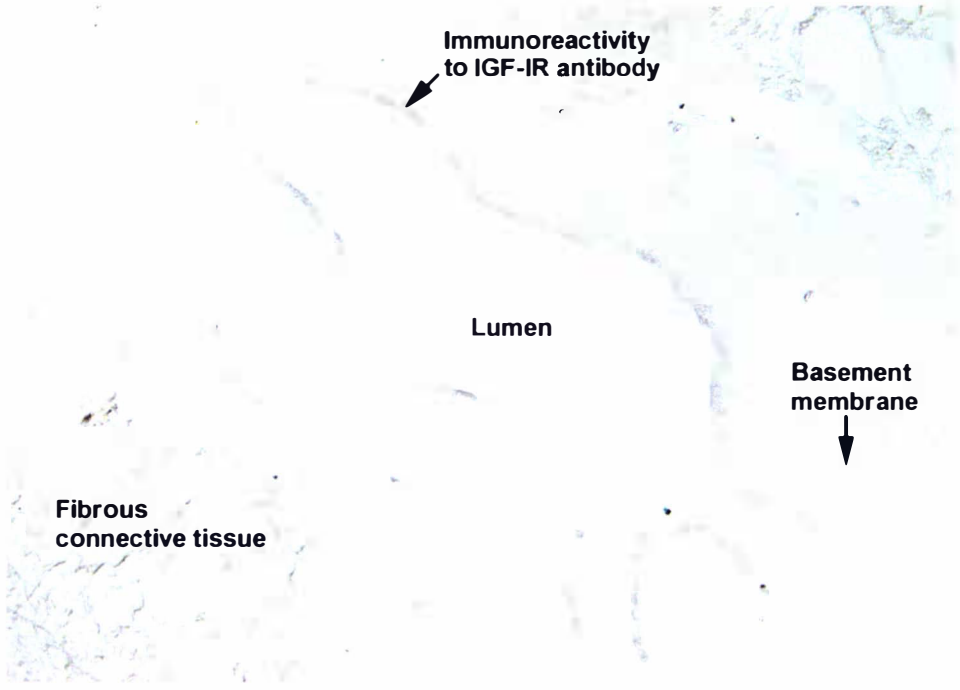



**Plate 6.7** Photomicrograph of a secondary duct from the mammary gland of a female sheep fetus at day 101 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a maintenance (M) diet from days 19 to 47 of gestation, followed by a high (1.5M) plane of nutrition from days 47 to 101 of gestation. Moderately positive immunoreactivity extending beyond the epithelial cell surface. (Mag x400).



Immunoreactivity to IGF-IR antibody was also seen in the multi-layered ductal epithelial cells of the fetal mammary glands at a mean day 140 (days 137 to 141, inclusive) of gestation irrespective of the maternal nutritional regimen. Whereas fetuses from dams subjected to a high (HHH) maternal plane of nutrition from days 19 to 140 of gestation showed intense immunostaining of the epithelial apical cytoplasm (Plate 6.8), the fetuses whose dams were subjected to a maintenance (MMM) diet throughout pregnancy exhibited very faint immunostaining, which was confined to the cell surface (Plate 6.9).

The pattern of immunoreactivity and the intensity of immunostaining in treatment groups MMH, MHM, MHH and HHM fell somewhere in-between those of the two extremes, namely MMM and HHH. Due to the variability in the pattern of immunoreactivy and the intensity of immunostaining between individual animals within each treatment group, it was not possible to rank these groups in terms of increasing immunoreactivity/immunostaining. The descriptions that follow give the commonly found trends exhibited by animals within each of these four treatment groups (photomicrographs not shown).

The luminal surface of the epithelial cells was faintly immunostained in the MHH treatment group and immunoreactivity was discontinuous around the lumen. The pattern of immunoreactivity was similar between treatment groups MHM and HHM, namely linear cell surface with some animals showing reactivity around the apical margins of the cytoplasm. The intensity of immunostaining differed in that fine granulated staining of the cell membrane and adjacent cytoplasmic margins were seen in the MHM group, whereas the HHM group displayed a coarser granulated staining of the apical cytoplasm of the epithelial cells. In both groups, immunoreactivity was usually discontinuous around the luminal surface of the duct.

In the MMH treatment group, the epithelial cell surface showed immunoreactivity to IGF-1R antibody. In some MMH animals, immunostaining extended into the apical cytoplasm, but reactivity was not consistent across ducts and ranged from weakly to moderately positive. One animal exhibited perinuclear and nuclear staining in some ducts.

**Plate 6.8** Photomicrograph of a secondary duct from the mammary gland of a fetal sheep at day 140 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a high plane of nutrition from days 19 to 140 of gestation. Intense immunostaining of the epithelial apical cytoplasm. (Mag x400).

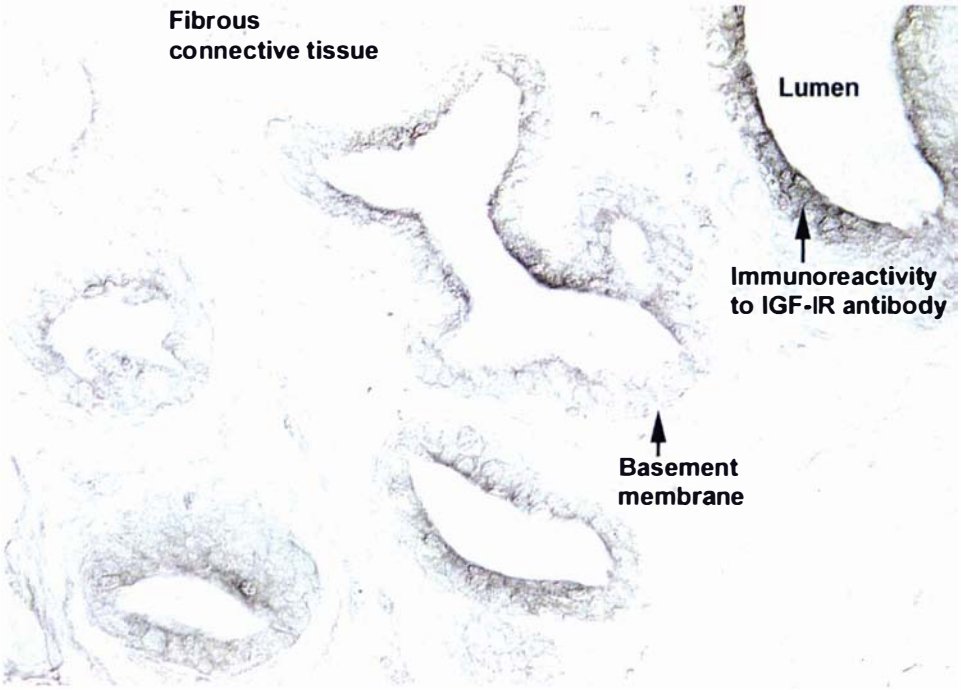
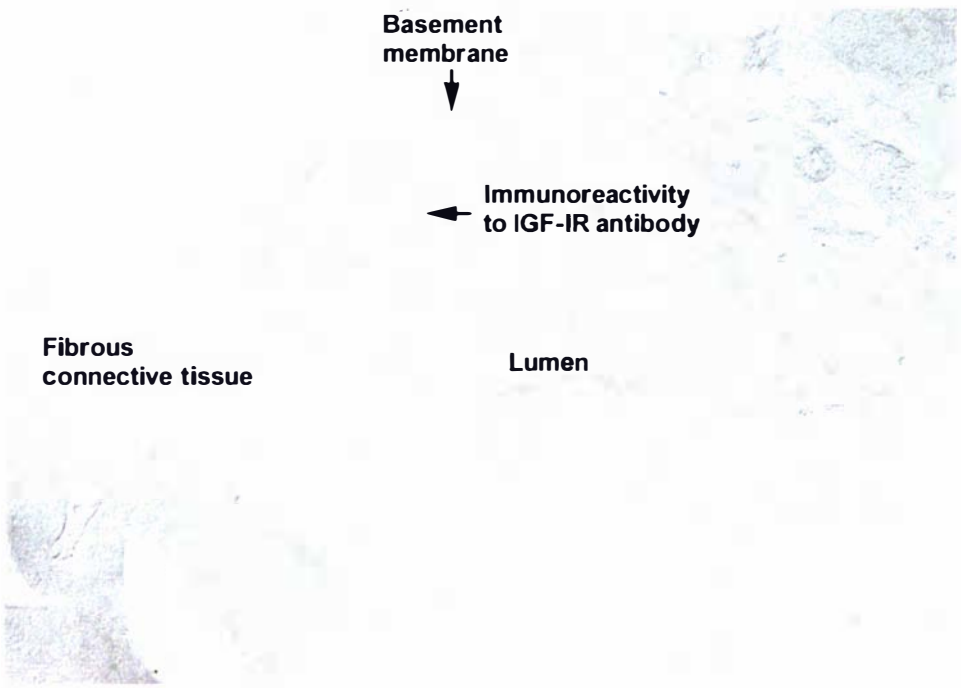



**Plate 6.9** Photomicrograph of a secondary duct from the mammary gland of a fetal sheep at day 140 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a maintenance plane of nutrition from days 19 to 140 of gestation. Very faint immunostaining of the epithelial cell surface. (Mag x400).



The cells of the fibrous connective tissue and adipose tissue were essentially non-reactive in the MMM treatment group. The fibroblasts in the MHH and HHM groups were either non-reactive or showed weakly positive immunoreactivity of the cell nucleus. The cells of the adipose tissue were non-reactive. Light to moderate nuclear immunostaining was observed in the fibroblasts of the MHM and MMH groups. However, while the cells of the adipose tissue were non-reactive in the MHM group, nuclear and cytoplasmic immunostaining of the adipoblasts was evident in some MMH animals. Moderate to strong cytoplasmic and nuclear immunostaining was seen in the adipoblasts of the HHH group (Plate 6.10) while immunoreactivity in the fibroblasts ranged from weakly to strongly positive. More intense nuclear immunostaining was observed in the fibroblasts that resided in close contact with the epithelial ducts, rather than in those of the deep hypodermis (Plate 6.11).

Although the intensity of immunostaining was variable, the epidermis showed immunoreactivity in all six treatment groups. Immunostaining was observed through all layers of the epidermis and ranged from weakly to strongly positive. Staining was weakly positive in the HHH group, moderately positive in the MMM, MMH and MHM groups, and varied significantly between animals in each of the MHH and HHM groups, ranging from weak to strong. A similar diversity in the intensity of immunostaining was found in the cells of the sebaceous glands, and in the inner and outer root sheaths of the primary wool follicles (Plate 6.12).

**Plate 6.10** Photomicrograph of adipose tissue from the mammary gland of a sheep fetus at day 140 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a high (days 19 to 140 of gestation) plane of nutrition. Moderate to strong cytoplasmic and nuclear immunostaining in the adipoblasts. (Mag x400).

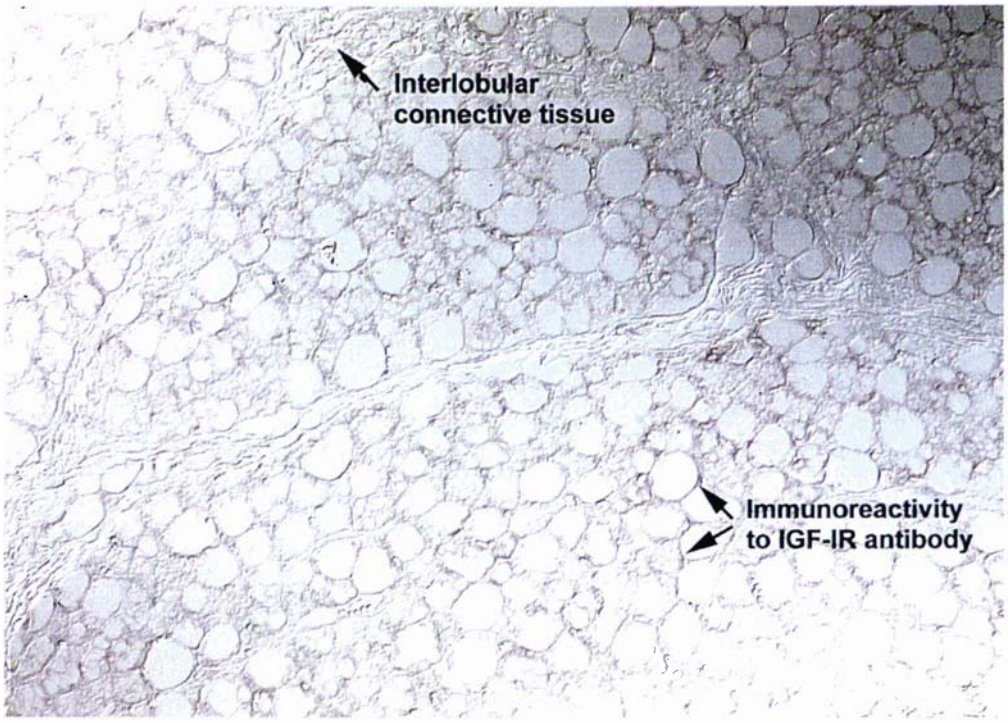



**Plate 6.11** Photomicrograph of fibrous connective tissue from the mammary gland of a sheep fetus at day 140 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a high (days 19 to 140 of gestation) plane of nutrition. Intense nuclear immunostaining in the fibroblasts surrounding the epithelial ducts. (Mag x400).

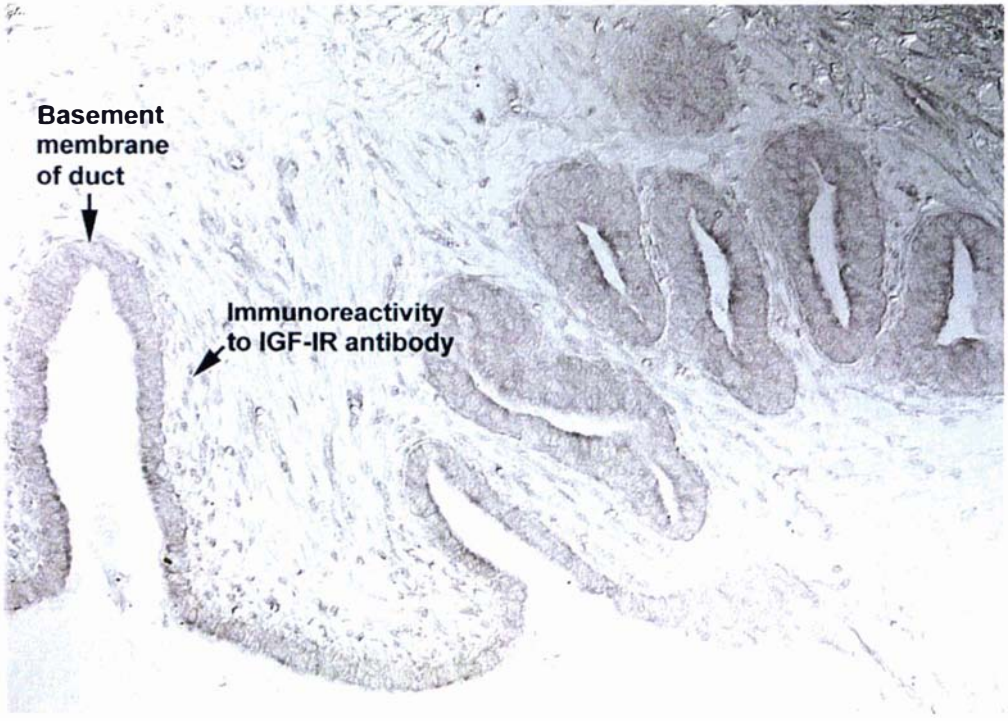
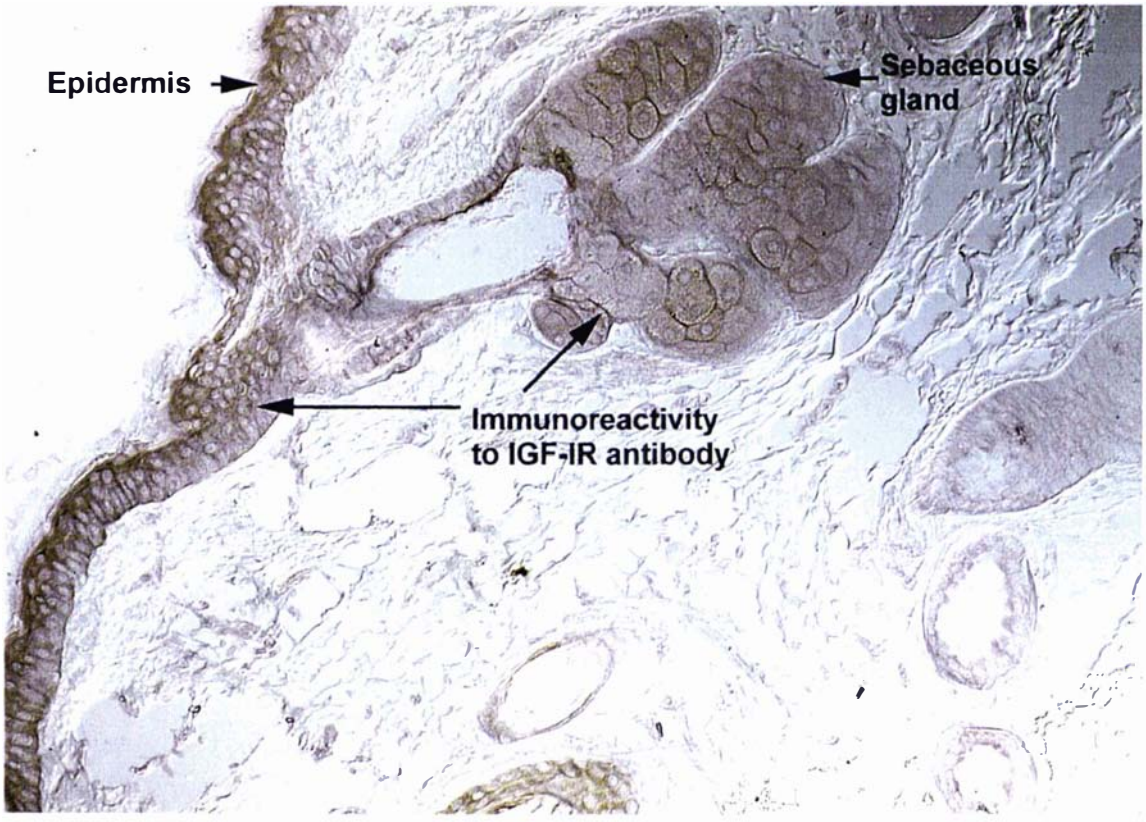


**Plate 6.12** Photomicrograph of epidermis and wool from the mammary gland of a sheep fetus at day 140 of gestation. Immunoreactivity to IGF-IR antibody after exposure of the dam to a high (days 19 to 140 of gestation) plane of nutrition. The cells of the sebaceous glands and the epidermis are staining intensely. (Mag x400).



## 6.6 Discussion

This experiment was designed to examine the effects of pre-lamb shearing, or changing maternal feeding levels at different stages of gestation, on fetal growth. There is evidence from other studies that altering the maternal environment through changes in nutrition (Mellor, 1983), season (Jenkinson et al., 1995), temperature (Alexander and Williams, 1971), shearing (Kenyon et al., 1999) and hormones (Jenkinson et al., 1999), to name a few, differentially influences fetal growth and size at birth. However, there are no data on the effects on fetal mammary gland development. Hence the opportunity existed to test the hypothesis that mammary gland size and/or development would be influenced by those treatments that were expected to affect fetal growth.

The nutritional treatments in trial two generated a difference in ewe live weight between the three groups. However, fetal body weights were not significantly different at day 101 of gestation. Furthermore, no differences were observed in mammary gland size. These results are indicative of the small nutritional requirement the fetus has relative to the dam in early gestation. The similarity in fetal weight between the treatment groups is consistent with the notion that fetal growth in early to mid-pregnancy is largely insensitive to variations in maternal nutrition (Wallace, 1948; Heasman et al., 1999). However, some studies have reported a significant reduction in the weight of fetal lambs from ewes which were severely underfed until 90 days of gestation (Everitt, 1967). Despite the apparent normality of the fetal mammary gland, a high plane of maternal nutrition tended to enhance immunoreactivity to IGF-IR antibody in the mammary epithelial cells relative to the low group. This suggests that the fetal somatotrophic axis may be sensitive to the direct or indirect effects of maternal dietary intake from the earliest stages of life.

Recent studies have shown that the plane of nutrition around the time of conception may have major implications in terms of fetal development. For example, a modest reduction in maternal food intake, from 60 days before until 30 days after conception in single-bearing ewes, resulted in a significant reduction in gestation length, which was associated with accelerated fetal hypothalamic-pituitary-adrenal axis maturation. Interestingly fetuses from the restricted group were of a similar size to those from the well-fed control group (Bloomfield et al., 2003). All ewes in trial two were well fed (*ad-lib*) on good quality autumn pasture prior to the allocation of nutritional treatments, which commenced at day 20 of gestation. Moreover, ewes were not randomly assigned to each treatment group, but allocated to a group after stratification of live weight, which may have biased the results. It is tempting to speculate, based on the Bloomfield data, that maternal food intake around the time of conception resulted in activation of the fetal somatotrophic axis without an effect on fetal body weight or mammary gland size and composition.

In trial three, at day 101 of fetal age the size of the mammary gland, as measured by total duct area, was one and a half times greater in fetuses whose dams had been exposed to a high level of feeding from days 19 to 101 relative to those whose dams remained at maintenance (Table 6.5). This trend continued into late gestation (Table 6.6), where, by day 140 of gestation, fetuses from ewes exposed to MMH, MHH, HHM or HHH had at least twice the duct area of those on a maintenance diet (MMM) throughout pregnancy ($P<0.05$). This substantial difference in the amount of parenchymal tissue present occurred without any significant effect on fetal weight or gland weight. The glands were able, however, to compensate and increase the deposition of parenchymal tissue by a high plane of nutrition at any time during pregnancy with the possible exception of the MHM treatment. It appears that mammary glands from fetuses whose dams remained at maintenance until days 47 or 101, were able to compensate for earlier treatments during late gestation with the possible exception of the MHM group.

Thus the growth of the parenchymal tissue in the mammary gland is severely retarded in fetuses whose dams are restricted to a maintenance diet throughout pregnancy. In view of the similarity in ewe, fetal or mammary gland weight between the treatment groups, it is unlikely that a lack of nutrients is directly responsible for the differences in amount of parenchymal tissue in the mammary glands. Rather the effect is probably mediated by endocrinological changes in the fetus and possibly the dam.

As this study is the first to demonstrate that maternal nutritional status has a major impact on the growth of the fetal mammary gland, it is appropriate to examine the putative mediators of this outcome. Bassett et al. (1990) demonstrated the importance of nutrient availability on both growth rate and IGF-I concentrations in the ovine fetus, which led to the establishment of IGF-I as a determinant of fetal growth (Gluckman et al., 1997). Within the fetal tissues, IGF-II, which acts via the IGF-I receptor, tends to play a more prominent role than IGF-I in mediating the effects of nutrition on fetal growth in early pregnancy. Thereafter, the late gestation rise in fetal cortisol levels leads to an increase in circulating IGF-I, which, in turn, then mediates the nutritional response (Li et al., 1993). Interestingly, this correlates well with the expression of mRNA for IGF-I and IGF-II in the mesenchymal cells of the fetal mammary gland, whereby IGF-II was more highly expressed than IGF-I up until day 140 of gestation (Forsyth et al., 1999). In this connection, we demonstrated the differential expression of the IGF-I receptor (IGF-IR) on the luminal epithelial cells comprising the ducts of the mammary gland of the female sheep at days 101 (Plates 6.5 and 6.6) and 140 (Plates 6.8 and 6.9) of fetal age. This is in agreement with the immunohistochemical detection of IGF-IR reported in Chapter three and the *in situ* data for the expression of IGF-IR in the epithelial tissue of the fetal ovine mammary gland (Forsyth et al., 1999). Furthermore, in trial three, the increased intensity of IGF-IR immunostaining closely mirrored the increased total duct area associated with a higher maternal dietary intake (i.e., MMM vs HHH treatment groups).

The combined results suggest that throughout pregnancy the maternal diet controls fetal mammary gland growth indirectly by modifying the expression of maternal and fetal endocrine

mechanisms that influence nutrient uptake and utilisation by the fetus, which, in turn, alters the expression of fetal IGF-IR in the mammary epithelium. These receptors could be a target for locally produced or circulating IGFs from the fetal circulation, but not maternally derived IGF-I, which does not cross the placenta in physiologically important quantities (Brown and Thorburn, 1989). Fetal IGF-I concentrations are reduced following maternal starvation but can be quickly reversed by elevations in fetal glucose or fetal insulin (Oliver et al., 1996). Fetal plasma IGF-I concentrations showed a high correlation with the total body weight of the fetuses and with the weights of organs (liver, thymus and spleen) when these are changed by maternal under-nutrition in late pregnancy (Bauer et al., 1995). A similar effect was mirrored in the expression of the IGF-IR in this study. For example, the MM group at day 101 of gestation exhibited weaker immunoreactivity to IGF-IR antibody relative to the MH or HH groups. However, when the MM group was moved to a high plane of nutrition from day 101 (MMH), immunohistochemical analyses at day 140 showed a tendency for up-regulation of IGF-IR that reflected an intensity of immunostaining closer to that seen in the HHH group.

One could hypothesize that IGF-I receptors, present in the epithelial cells lining the mammary ducts (present study; Chapter three; Forsyth et al., 1999), are down-regulated in response to a nutritionally mediated decline in fetal glucose concentrations. However, given that no reduction in fetal body or mammary gland weight was observed at day 140 of gestation in those animals under a restricted maternal nutritional regimen suggests that nutrient partitioning is such that it favours optimal fetal growth at the expense of a non-vital component – the epithelial cells of the mammary gland. This theory is also substantiated by the lack of a nutritional effect on the weight of vital organs such as the liver, heart and kidneys (Tarsono, 2000). The body weights of fetuses whose mothers were subjected to either a restricted or *ad-lib* diet during late pregnancy (days 100 to 124) were not significantly different at day 124 of gestation (Bauer et al., 1995). Furthermore, when fetal organ weights were corrected for total fetal bodyweight no organ weight differences were seen (Bauer et al., 1995). As a result of under-nutrition of the ewe from days 50 to 140 of gestation, the progeny to 1.4 years of age had a 2-10% decrease in secondary to primary wool follicle ratios, and a 3% decrease in wool production at 1.4 years (Kelly et al., 1996). These effects were detected despite mean lamb birth weights of 5.0 and 5.5 kg from the two feeding treatments (sub-maintenance vs maintenance) being substantially higher than those of most other studies. In Everitt's (1967) study, lamb birth weights were in the range of 2.5 to 4.2 kg. However, the data from trial three suggests that it is the continued low nutrition that is detrimental to development of the secretory epithelial cells of the fetal mammary gland.

Other points to consider are the determinants of the response in the IGF-IR and the mammary gland to maternal food restriction. It may be that the IGF-IR and the mammary gland are responding to two different signals. In fetuses from ewes subjected to a maintenance diet throughout pregnancy, GH may act via systemic IGF-I to distribute and optimise substrate use for fetal growth, while local synthesis of IGFs may be important components in the response of the mammary epithelium.

Pre-lamb shearing had no effect on fetal mammary gland size (Trial 1). However this is consistent with the results obtained in Trial 3 where mammary gland development was only restricted in fetuses whose dams were on a maintenance plane of nutrition from day 47 of pregnancy whereas in Trial 1 all ewes were allowed *ad libitum* access to pasture from day 69 of pregnancy. Thus it is probable that the intake of unshorn ewes was adequate to allow full development of the mammary gland and that the increased intake of the shorn ewes conferred no further advantage. Furthermore in this connection the total area of secretory cells in the mammary glands of the fetuses in Trial 1 were similar to those in the MMH, MHM and HHH groups of Trial 3 and considerably greater than those of the MMM group.

Of key importance is the concept that lifetime milk production potential may be programmed during prenatal life, given the significant reduction in the amount of parenchymal tissue present in the mammary gland of fetuses whose dams were subjected to a restricted plane of nutrition throughout pregnancy. It remains to be established whether these prenatal effects on the development of the fetal mammary gland are translated into impaired lactational performance in adult life.

Given that maternal nutrition in the periconceptual period can alter physiological function and growth responses of the fetus to limited nutrients in late gestation, without affecting fetal size (Harding, 1997; Hawkins et al., 2000; Oliver et al., 2001; Bloomfield et al., 2003) it might be necessary for future trials to also focus on events around the time of conception.

# CHAPTER SEVEN

# GENERAL DISCUSSION AND CONCLUSIONS

Mammogenesis is a complex process involving extensive epithelial proliferation and morphogenesis within a matrix of connective and adipose tissue, which, following pregnancy, generally results, after parturition, in the synthesis and secretion of milk. These developmental processes are initiated during fetal life and continue, in repeated cycles of differentiation, proliferation and involution, throughout adult life in the female. The amount of milk produced by the adult gland during lactation is highly correlated with secretory tissue mass (Linzell, 1966). Since the secretory cells proliferate on the ducts that have developed earlier in life one could expect that a reduction in duct development during fetal life would ultimately result in fewer secretory cells and hence, reduced milk yield from the mammary gland at the adult stage.

The male versus female model was used to establish patterns of fetal mammary gland development in the sheep as one could say with confidence that at some stage along the developmental path a difference would occur. The histomorphological studies conducted by Turner (1930, 1931), Wallace (1953), Martinet (1962) and Sonstegard (1972) reported differences between patterns of mammary development in males and females as early as the mammary bud stage in cattle and the formation of secondary ducts and adipose tissue in sheep. Hence, there are critical periods along the developmental line where morphological changes are observed, and, at which, altered development may have a long-term effect.

Two critical stages of mammary development were identified in the fetal sheep in the present studies. First, the differentiation of preadipocytes in the male gland lagged behind that of the female from day 80 of fetal age, and as a consequence, adipose tissue was much less abundant in the male thereafter (Chapter two). Second, while epithelial growth was similar in males and females at days 40, 60, 80, 100 and 120 of fetal age, total duct area at day 140 had doubled in the female, but failed to progress beyond that seen at day 120 in the male (Chapter two).

The sex differences observed in the histomorphogenesis of the gland were reflected in a relative growth analysis of mammary development. The growth of the mammary gland in the female followed the general development of the fetus, while in the male mammary growth slowed down from day 80 to 140 of fetal age, exhibiting negative allometry (Chapter two). In the volumetric data reported by Martinet (1962), the male gland followed the general development of the fetus and the female gland slowed down from about day 70 of gestation, but continued to exhibit positive allometry. Thus the differences observed between males and females in the patterns of development of the mammary gland were similar across the two studies even though the effect of sex on the growth patterns differed between the two studies. The reasons for this discrepancy are unclear. They were also consistent with the gross anatomical and histological observations of the ovine fetal mammary gland described briefly by Wallace (1953).

The histomorphogenesis and combined temporal and allometric relationships described in the present studies illustrate the gradual divergence in the morphological development of the mammary gland of male and female fetal sheep (Chapter two). This is in contrast to the fetal mouse, where sexual dimorphism of the mammary gland begins and ends with the mammary

bud as a result of androgens secreted by the male testes (Raynaud, 1971; Kratochwil, 1971). The localisation of androgen receptors (AR) in the ovine gland suggests the involvement of androgens (primarily testosterone) in the determination of sex differences in the ontogenesis of the prenatal mammary gland (Chapter three). Moreover, the positive association between developmental divergence and the intensity of immunostaining for AR lends further support to a role for androgens. However, unlike the mouse where androgen receptors are located exclusively in the dermal mesenchymal cells (Heuberger et al., 1982; Wasner et al., 1983), androgen receptors are also present in the epithelial cells of the ducts from day 80 of fetal age in the ovine gland (Chapter three). It is tempting to speculate that testosterone acts directly on epithelial androgen receptors from day 80 of fetal age to prevent further differentiation of the mammary epithelium. Furthermore, immunohistochemical data (Chapter four) suggests a role for insulin-like growth factor-I receptor (IGF-IR) in mediating the growth suppressing effect of testosterone in the mammary gland of the fetal male sheep.

Increased IGF-IR immunostaining was observed in both the fibroblasts and epithelial cells of the growing ducts of the male from day 60, reaching a peak between days 100 and 120 of fetal age (Chapter four). This was consistent with the pattern of immunoreactivity observed for androgen receptor (Chapter three). However, the fibroblasts of the deep hypodermis and the adipoblasts of the developing fat pad showed greater immunoreactivity to IGF-IR antibody than to AR antibody (Chapters three and four). This lends further support to the suggestion that suppression of mammary growth, particularly that of the adipose and fibrous connective tissues, in the fetal male sheep may involve mediation by the IGFs.

To provide indirect evidence that the pattern of mammary morphogenesis in the male was, in part, dependent on its exposure to testosterone, it was proposed to inject pregnant ewes with testosterone during the period of sensitivity of the fetal reproductive tract to gonadal steroids (Clarke et al., 1976). Although this approach could not prove cause and effect, it provided a non-invasive model which was easy to produce and reliable, with respect to obtaining masculinisation of the external genitalia of the female offspring. Testosterone treatment of pregnant ewes from days 36 to 57 of gestation resulted in partial masculinisation of the mammary gland, in terms of total duct area, of the female offspring at day 140 of fetal age and at birth. This is consistent with the data from Chapter two where sex differences in total duct area were not evident until day 140. While no differences were observed in total duct area at day 117 of fetal age, mammary gland weight was lower in female fetuses from testosterone-treated dams relative to all other females and continued to be lower as gestation progressed (Chapter five). A necessary part of the successful progression of mammary gland growth is an adequately developed mammary fat pad. Differential patterns in adipose tissue development are evident at day 80 of fetal age in sheep and by day 140 the female has developed a more extensive fat pad than the male (Wallace, 1953; Chapter two). The same is true for the masculinised female, where the size and complexity of the fat pad is similar to that of a normal male at day 140 of fetal age (Chapter five). The fat pad has no room to proliferate in male (Anderson, 1985) and masculinised female (Chapter five) fetuses due to the close proximity of

the mammary gland to the scrotal sacs. This is consistent with the lower weight of the mammary gland observed from day 117 of fetal age (Chapter five). The fat pad appears crucial as a space for future epithelial growth and, as a site of synthesis of growth factors, which are now thought to mediate epithelial-mesenchymal interactions in prenatal mammary gland organogenesis (Finch et al., 1995; Forsyth et al., 1999). The findings of the present studies indicate that the fat pad may also be important in stimulating prenatal allometric mammary growth, a proposal consistent with the findings in the prepubertal ewe lamb (Hovey, 1996).

To lend further support for a role for androgens in the incomplete development of the mammary gland was the association between "masculinisation" of the mammary gland and the intensity of androgen receptor immunostaining (Chapter five). Immunoreactivity to AR antibody in the mammary gland of masculinised females followed a similar pattern to that seen in male fetuses from all other treatment groups. It would be of interest to examine the distribution of IGF-IR in the mammary gland of the masculinised female given the differential patterns observed in IGF-IR immunoreactivity between normal males and females in Chapter four.

The combined results of experiments from Chapters three and five strongly suggest that androgens play a major role in the control of mammary morphogenesis in the fetal male sheep. But is it solely the absence of testosterone that allows the female mammary gland to proceed in its development? The presence of oestrogen receptors in the mesenchymal cells of the ovine fetal mammary gland during development of the mammary bud agrees with the rodent model (Narbaitz et al., 1980). However, unlike the female rodent, where epithelial oestrogen receptors are not present until day 3 of postnatal age (Haslam and Nummy, 1992), ovine females first exhibit oestrogen receptors in the epithelial cells of the growing ducts between days 80 and 100 of fetal age (Chapter three). This would indicate that the mammary epithelium could respond to oestrogen stimulation, the placenta being the most likely source of oestrogen production (Findlay and Seamark, 1973). The concentration of oestrogen in the fetal plasma begins to increase from about day 70 of gestation, and rises rapidly between days 90 and 120 of gestation reflecting the pattern of increasing placental production (Findlay and Cox, 1969; Findlay and Seamark, 1973). This corresponds to the stage of gestation when sexual dimorphism becomes evident in the mammary gland of the fetal sheep (Martinet, 1962; Chapter two) and suggests a direct role for oestrogen in stimulating ductal elongation, particularly in the late gestation fetus. Arguing against the idea that oestrogen alone is involved in prenatal ovine mammary development, total duct area and mammary gland weight did not differ between female fetuses whose dams were treated with Tamoxifen (an anti-oestrogen) and female fetuses from dams injected with cottonseed oil (control) (Chapter five). However, it is possible that Tamoxifen did not actually get into the fetus in quantities sufficient to have an effect. Furthermore, the number of fetal mammary glands available for study was small due to the adverse effects of maternal Tamoxifen treatment on pregnancy outcome.

The presence of the IGF-I receptor in the epithelial cells of the mammary ducts (Chapter four) confirms the *in situ* expression data for IGF-IR mRNA reported by Forsyth et al., (1999). It also

suggests that oestrogens may act indirectly, mediated by oestrogen-induced IGF-I or IGF-II from the underlying mesenchymal cells, to stimulate mammary ductal elongation. A role for locally synthesised IGF-I or IGF-II in the fetal ruminant mammary gland is likely given the distribution of mRNA for these mammary mitogens in the mesenchymal cells of the mammary gland of female sheep from days 70 to 140 of fetal age (Forsyth et al., 1999). However, it is not clear as to why the IGF-IR protein was observed in the mesenchymal cells in the present study when Forsyth et al., (1999) did not detect expression of IGF-IR mRNA in the mammary stroma. Consistent with the possibility of a role for IGF-I in mediating early mammary development is the demonstration of increasing immunoreactivity to IGF-I receptor antibody from days 60 to 120 of fetal age in the female sheep (Chapter four). Moreover, the fibroblasts of the connective tissue and the adipoblasts of the developing fat pad expressed IGF-IR protein (Chapter four), the increased immunoreactivity of which corresponded with the onset of rapid growth of the female fat pad (Chapter two). It is certainly possible that the differentiation of adipose tissue in the fetal female represents a response to an increased local availability of growth factors such as IGF-I or IGF-II.

The effect of maternal nutrition during fetal life on adult performance is becoming increasingly recognised. For example, poor nutrition during fetal life has the potential to reduce both the quantity and quality of wool in young Merino sheep (Kelly et al., 1996). It also has adverse effects on fetal growth, and hence birth weights and lamb survival (Mellor, 1983). The present studies are the first to report that a low plane of maternal nutrition (1.0 x maintenance) throughout pregnancy is detrimental to development of the fetal mammary gland. Fetal mammary growth, in terms of total duct area, was more than two-fold greater in fetuses whose dams were exposed to a high plane of nutrition (1.5 x maintenance) throughout pregnancy than in those fetuses whose dams remained at maintenance (Chapter six). This substantial difference in the amount of epithelial tissue present occurred without any significant effect on fetal weight, linear dimensions or gland weight. Moreover, most of the glands were able to initiate compensatory growth by a high plane of maternal nutrition at any time during pregnancy. In view of the similarity in ewe, fetal or mammary gland weights between the treatment groups, it is likely that endocrinological changes in the fetus regulate the distribution of limited nutrients during periods of maternal under-nutrition (Chapter six). The involvement of the fetal somatotrophic axis in the mediation of the nutritional response is supported by data that showed increased plasma GH and decreased plasma IGF-I concentrations in both the ewe and its fetus during maternal under-nutrition in late gestation (Bauer et al., 1995). It would have been of interest to examine the patterns of fetal plasma GH, IGF, glucose and insulin concentrations in fetuses from ewes subjected to a low plane of nutrition throughout pregnancy. Unfortunately, fetal blood samples were not collected in the present studies.

Consistent with the role of IGFs in mediating the effects of nutrition on fetal growth (Li et al., 1993; Bauer et al., 1995), was the differential expression of the IGF-IR on the epithelial cells of the mammary ducts at days 101 and 140 of fetal age (Chapter six). However, the lack of effect of a restricted maternal nutritional regimen on fetal growth or mammary gland weight at day 140

of gestation suggests two mechanisms of action. First, GH may act via systemic IGF-I to distribute and optimise substrate use for fetal growth and the development of vital organs. Second, synthesis of IGFs within the fetal mammary gland may be important regulators in the response of the mammary epithelium. Considering the importance of the mammary fat pad in stimulating parenchymal growth (Hovey, 1996), it would be of interest to know whether the fat pad of fetuses from nutritionally restricted ewes was also reduced in total area. And if so, whether this represented a decrease in the local availability of growth factors such as IGF-I.

It is tempting to speculate that the target size of the mature gland is programmed during fetal life and that inhibition of gland size by continued maternal nutrient restriction during intrauterine life may affect eventual milk yield in later life. The mammary gland of the fetal sheep provides a nutritionally sensitive model with which to study the causes, consequences and permanence of prenatal restriction of mammary growth. Furthermore, it would be expected that the earlier in life epithelial growth is restricted, the more likely it is to have a long-term effect, in terms of secretory cell mass and hence, subsequent lactational performance.

In conclusion, these studies have demonstrated that critical aspects of mammary differentiation are established during fetal life. Quantitative data and histomorphological examination have identified the development of adipose tissue as a crucial factor in determining the amount of epithelial tissue that is grown during fetal mammogenesis and hence, the capacity to produce milk in later life. Immunohistochemical analyses have confirmed an association between androgen and oestrogen receptor immunoreactivity and the differential patterns in mammary morphogenesis between males and females. Moreover, the similarity in the pattern of mammary development and in the localisation of androgen receptors in female fetuses whose dams were treated with testosterone have provided overwhelming evidence of androgen involvement in the sexual dimorphism of the ovine gland. Immunohistochemistry also lends further support to recent reports suggesting a role for the IGF-I receptor in mediating early mammary development. In late gestation, the association of epithelial oestrogen receptors with a dramatic increase in total duct area in the female gland suggests a direct role for oestrogen in stimulating epithelial growth.

Of major importance, from both an agricultural and clinical perspective, is the impact of long-term maternal under-nutrition on the growth and development of the fetal mammary gland. The marked reduction in the amount of epithelial tissue present in the fetal gland at birth, as a consequence of alterations in maternal nutrition throughout gestation, may ultimately impact on future milk yield and hence, the ability of the ewe to meet the energy requirements of her offspring.

# APPENDICES

**Table A.1** Patterns of immunoreactivity to androgen receptor antibody in the mammary gland of sheep at days 40, 60 and 80 of fetal age.

| Fetus | | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|---|
| ID | Sex | Int[a] | Pattern | Int[a] | Pattern |
| Day 40 | | | | | |
| 7380 | M | - | No reactivity | + | Nuclear – around bud |
| 7382 | M | - | No reactivity | +++ | Nuclear – around bud |
| 7390 | M | - | No reactivity | ++ | Nuclear – around bud |
| 7392 | M | - | No reactivity | ++ | Nuclear – around bud |
| 7378 | F | - | No reactivity | ++ | Nuclear – around bud |
| 7384 | F | - | No reactivity | + | Nuclear – around bud |
| 7386 | F | - | No reactivity | ++ | Nuclear – around bud |
| 7388 | F | - | No reactivity | ++ | Nuclear – around bud |
| 7395 | F | - | No reactivity | +++ | Nuclear – around bud and in dermis |
| Day 60 | | | | | |
| 7404 | M | - | No reactivity | - | No reactivity |
| 7406 | M | - | No reactivity | - | No reactivity |
| 21414 | M | - | No reactivity | - | No reactivity |
| 7397 | F | - | No reactivity | - | No reactivity |
| 7399 | F | - | No reactivity | - | No reactivity |
| 7401 | F | - | No reactivity | - | No reactivity |
| 7402 | F | - | No reactivity | - | No reactivity |
| 7408 | F | - | No reactivity | - | No reactivity |
| 7412 | F | - | No reactivity | - | No reactivity |
| Day 80 | | | | | |
| 19173 | M | +++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 19174 | M | +++ | Cytoplasmic | +++ | Nuclear – dermal fibroblasts |
| 19175 | M | +++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 19176 | M | ++ | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| 5342 | F | ++ | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 18338 | F | ++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 19169 | F | +++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 19170 | F | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 19172 | F | ++ | Cytoplasmic | + | Nuclear – dermal fibroblasts |

Int[a] = intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Sex: M = male, F = female

**Table A.1** continued. Patterns of immunoreactivity to androgen receptor antibody in the mammary gland of sheep at days 100, 120 and 40 of fetal age.

| Fetus | | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|---|
| ID | Sex | Int[a] | Pattern | Int[a] | Pattern |
| Day 100 | | | | | |
| 7477 | M | +++ | Cytoplasmic | +++ | Nuclear – dermal fibroblasts |
| 19239 | M | ++ | Cytoplasmic | +++ | Nuclear – dermal fibroblasts |
| 20082 | M | +++ | Nuclear | ++ | Nuclear – dermal fibroblasts |
| 5347 | M | +++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 7473 | F | +++ | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| 7475 | F | ++ | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 18998 | F | ++ | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| 5350 | F | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 20085 | F | ++ | Linear cell surface | + | Nuclear – dermal fibroblasts |
| Day 120 | | | | | |
| 18976 | M | ++ | Cytoplasmic | +++ | Nuclear – dermal fibroblasts |
| 19161 | M | +++ | Cytoplasmic and nuclear | ++ | Nuclear – dermal fibroblasts |
| 19163 | M | ++ | Cytoplasmic and nuclear | ++ | Nuclear – dermal fibroblasts |
| 20088 | M | +++ | Cytoplasmic | +++ | Nuclear – dermal fibroblasts |
| 5353 | F | + | Linear cell surface | - | No reactivity |
| 5354 | F | ++ | Linear cell surface | - | No reactivity |
| 18979 | F | ++ | Linear cell surface | - | No reactivity |
| 18984 | F | + | Linear cell surface | - | No reactivity |
| 20090 | F | + | Linear cell su face | - | No reactivity |
| Day 140 | | | | | |
| 19079 | M | + | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| 19166 | M | ++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 19167 | M | ++ | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 19165 | M | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 20093 | M | ++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 5360 | F | + | Linear cell surface | - | No reactivity |
| 5362 | F | - | No reactivity | - | No reactivity |
| 18989 | F | - | No reactivity | - | No reactivity |
| 18990 | F | ++ | Cytoplasmic and nuclear | - | No reactivity |

Int[a] = intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Sex: M = male, F = female

**Table A.2** Patterns of immunoreactivity to oestrogen receptor alpha antibody in the mammar gland of sheep at days 40, 60 and 80 of fetal age.

| Fetus | | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|---|
| ID | Sex | Int[a] | Pattern | Int[a] | Pattern |
| Day 40 | | | | | |
| 7380 | M | - | No reactivity | + | Nuclear – around bud |
| 7382 | M | - | No reactivity | +++ | Nuclear – around bud |
| 7390 | M | - | No reactivity | ++ | Nuclear – around bud |
| 7392 | M | - | No reactivity | +++ | Nuclear – around bud |
| 7378 | F | - | No reactivity | ++ | Nuclear – around bud |
| 7384 | F | - | No reactivity | ++ | Nuclear – around bud |
| 7386 | F | - | No reactivity | +++ | Nuclear – around bud |
| 7388 | F | - | No reactivity | +++ | Nuclear – around bud |
| 7395 | F | - | No reactivity | ++ | Nuclear – around bud |
| Day 60 | | | | | |
| 7404 | M | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 7406 | M | - | No reactivity | + | Nuclear – dermal fibroblasts |
| 21414 | M | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 7397 | F | - | No reactivity | +++ | Nuclear – dermal fibroblasts |
| 7399 | F | - | No reactivity | +++ | Nuclear – dermal fibroblasts |
| 7401 | F | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 7402 | F | - | No reactivity | + | Nuclear – dermal fibroblasts |
| 7408 | F | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 7412 | F | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| Day 80 | | | | | |
| 19173 | M | - | No reactivity | + | Nuclear – dermal fibroblasts |
| 19174 | M | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 19175 | M | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 19176 | M | - | No reactivity | + | Nuclear – dermal fibroblasts |
| 5342 | F | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 18338 | F | - | No reactivity | ++ | Nuclear – fibroblasts (D/H) |
| 19169 | F | ++ | Cytoplasmic | +++ | Nuclear – dermal fibroblasts |
| 19170 | F | - | No reactivity | + | Nuclear – dermal fibroblasts |
| 19172 | F | - | No reactivity | ++ | Nuclear – dermal fibroblasts |

Int[a] = intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Mesenchymal cells: D = dermis, H = hypodermis; Sex: M = male, F = female.

**Table A.2** continued. Patterns of immunoreactivity to oestrogen receptor alpha antibody in the mammary gland of sheep at days 100, 120 and 140 of fetal age.

| Fetus | | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|---|
| ID | Sex | Int[a] | Pattern | Int[a] | Pattern |
| Day 100 | | | | | |
| 7477 | M | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 19239 | M | ++ | Linear cell surface | ++ | Nuclear – dermal fibroblasts |
| 20082 | M | +++ | Nuclear | ++ | Nuclear – dermal fibroblasts |
| 5347 | M | ++ | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| 7473 | F | + | Linear cell surface | ++ | Nuclear – dermal fibroblasts |
| 7475 | F | + | Linear cell surface | ++ | Nuclear – dermal fibroblasts |
| 18998 | F | +++ | Nuclear | ++ | Nuclear – dermal fibroblasts |
| 5350 | F | ++ | Cytoplasmic | ++ | Nuclear – fibroblasts/fat cells |
| 20085 | F | +++ | Cytoplasmic | +++ | Nuclear – dermal fibroblasts |
| Day 120 | | | | | |
| 18976 | M | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 19161 | M | ++ | Linear cell surface | ++ | Nuclear – dermal fibroblasts |
| 19163 | M | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 20088 | M | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 5353 | F | ++ | Nuclear | ++ | Nuclear – dermal fibroblasts |
| 5354 | F | +++ | Cytoplasmic | ++ | Nuclear –fibroblasts/fat cells |
| 18979 | F | +++ | Linear cell surface | +++ | Nuclear – dermal fibroblasts |
| 18984 | F | ++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 20090 | F | + | Linear cell surface | ++ | Nuclear – dermal fibroblasts |
| Day 140 | | | | | |
| 19079 | M | - | No reactivity | - | No reactivity |
| 19166 | M | - | No reactivity | - | No reactivity |
| 19167 | M | + | Linear cell surface | - | No reactivity |
| 19165 | M | + | Linear cell surface | - | No reactivity |
| 20093 | M | - | No reactivity | - | No reactivity |
| 5360 | F | +++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 5362 | F | ++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 18989 | F | ++ | Linear cell surface | ++ | Nuclear – dermal fibroblasts |
| 18990 | F | +++ | Cytoplasmic | +++ | Nuclear – dermal fibroblasts |

Int[a] = intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Mesenchymal cells: D = dermis, H = hypodermis; Sex: M = male, F = female

**Table A.3** Patterns of immunoreactivity to IGF-I receptor antibody in the mammary gland of sheep at days 60 and 80 of fetal age.

| Fetus | | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|---|
| ID | Sex | Int[a] | Pattern | Int[a] | Pattern |
| Day 60 | | | | | |
| 7404 | M | ++ | Cytoplasmic | ++ | Nuclear – fibroblasts (D/H) |
| 7406 | M | + | Cytoplasmic | + | Nuclear – fibroblasts (D/H) |
| 21414 | M | ++ | Cytoplasmic – distal end of primary duct | + | Nuclear – fibroblasts (D/H) |
| 7397 | F | ++ | Cytoplasmic | + | Nuclear – fibroblasts (D/H) |
| 7399 | F | +++ | Cytoplasmic | +++ | Nuclear – fibroblasts (D/H) |
| 7401 | F | ++ | Cytoplasmic | + | Nuclear – fibroblasts (D/H) |
| 7402 | F | ++ | Cytoplasmic | ++ | Nuclear – fibroblasts (D/H) |
| 7408 | F | + | Cytoplasmic | + | Nuclear – fibroblasts (D/H) |
| 7412 | F | ++ | Cytoplasmic | +++ | Nuclear – fibroblasts (D/H) |
| Day 80 | | | | | |
| 19173 | M | +++ | Cytoplasmic | ++ | Nuclear (F, A); cytoplasmic (F) |
| 19174 | M | + | Cytoplasmic | +++ | Nuclear (F, A) |
| 19175 | M | ++ | Cytoplasmic | ++ | Nuclear (F, A); cytoplasmic (F) |
| 19176 | M | ++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 5342 | F | ++ | Cytoplasmic + perinuclear | +++ | Nuclear (F, A) |
| 18338 | F | +++ | Cytoplasmic | ++ | Nuclear (F, A); cytoplasmic (F) |
| 19169 | F | +++ | Cytoplasmic + perinuclear | +++ | Nuclear (F, A) |
| 19170 | F | + | Cytoplasmic | ++ | Nuclear (F, A); cytoplasmic (F) |
| 19172 | F | ++ | Cytoplasmic | ++ | Nuclear (F, A); cytoplasmic (F) |

Int[a] = intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Sex: M = male, F = female.

Mesenchymal cells: F = fibroblasts, A = adipoblasts, D = dermis, H = hypodermis.

**Table A.3** continued. Patterns of immunoreactivity to IGF-I receptor antibody in the mammary glands of sheep at days 100, 120 and 140 of fetal age.

| Fetus | | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|---|
| ID | Sex | Int[a] | Pattern | Int[a] | Pattern |
| Day 100 | | | | | |
| 7477 | M | +++ | Cytoplasmic | +++ | Nuclear (F, A) |
| 19239 | M | ++ | Cytoplasmic and nuclear | ++ | Nuclear (F, A); cytoplasmic (F, A) |
| 20082 | M | ++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 5347 | M | +++ | Cytoplasmic and nuclear | + | Nuclear (F, A); cytoplasmic (F, A) |
| 7473 | F | +++ | Cytoplasmic | ++ | Nuclear (F, A); Cytoplasmic (F, A) |
| 7475 | F | ++ | Linear cell surface | +++ | Nuclear (F, A) |
| 18998 | F | ++ | Linear cell surface | ++ | Nuclear (F, A); cytoplasmic (F) |
| 5350 | F | + | Linear cell surface | ++ | Nuclear (F, A); cytoplasmic (F, A) |
| 20085 | F | ++ | Cytoplasmic | +++ | Nuclear (F, A) |
| Day 120 | | | | | |
| 18976 | M | ++ | Linear cell surface | + | Nuclear (F, A); cytoplasmic (F, A) |
| 19161 | M | ++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 19163 | M | ++ | Linear cell surface | + | Nuclear (F, A); cytoplasmic (F) |
| 20088 | M | + | Linear cell surface | + | Nuclear (F, A); cytoplasmic (A) |
| 5353 | F | ++ | Cytoplasmic + perinuclear | ++ | Nuclear (F, A) |
| 5354 | F | +++ | Cytoplasmic + perinuclear | +++ | Nuclear (F, A) |
| 18979 | F | ++ | Cytoplasmic + perinuclear | +++ | Nuclear (F, A); cytoplasmic (A) |
| 18984 | F | +++ | Cytoplasmic | ++ | Nuclear (F, A); cytoplasmic (F) |
| 20090 | F | ++ | Linear cell surface | ++ | Nuclear (F, A); cytoplasmic (A) |
| Day 140 | | | | | |
| 19079 | M | ++ | Linear cell surface | + | Nuclear (F, A); cytoplasmic (A) |
| 19166 | M | ++ | Linear cell surface | + | Nuclear (F, A); cytoplasmic (A) |
| 19167 | M | + | Cytoplasmic | ++ | Nuclear (F, A) |
| 19165 | M | + | Linear cell surface | + | Nuclear (F, A); cytoplasmic (F, A) |
| 20093 | M | ++ | Linear cell surface | + | Nuclear (F, A); cytoplasmic (A) |
| 5360 | F | + | Cytoplasmic | ++ | Nuclear (F, A); cytoplasmic (A) |
| 5362 | F | ++ | Linear cell surface | ++ | Nuclear (F, A); cytoplasmic (A) |
| 18989 | F | +++ | Cytoplasmic | +++ | Nuclear (F, A) |
| 18990 | F | ++ | Cytoplasmic + perinuclear | ++ | Nuclear (F, A); cytoplasmic (A) |

**Table A.4** Patterns of immunoreactivity to androgen receptor antibody in the mammary gland of sheep at day 140 of fetal age following treatment of their dams with testosterone from days 36 to 57 of gestation (Experiment one).

| Fetus | | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|---|
| ID | Sex | Int[a] | Pattern | Int[a] | Pattern |
| 93/5 | | | | | |
| A | M | +++ | Cytoplasmic and nuclear | ++ | Nuclear – dermal fibroblasts |
| B | M | + | Linear cell surface | - | No reactivity |
| C | F | ++ | Linear cell surface | - | No reactivity |
| 100/4 | | | | | |
| A | F | + | Linear cell surface and random nuclear | - | No reactivity |
| B | M | ++ | Linear cell surface | ++ | Nuclear – dermal fibroblasts |
| 360/4 | | | | | |
| A | F | ++ | Linear cell surface | + | Nuclear – dermal fibroblasts |
| B | M | ++ | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 474/4 | | | | | |
| A | F | + | Linear cell surface | - | No reactivity |
| B | M | ++ | Linear cell surface and random nuclear. | + | Nuclear – dermal fibroblasts |

Int[a] = intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Sex: M = male, F = female.

**Table A.5** Patterns of immunoreactivity to androgen receptor antibody in the mammary gland of sheep at day 61 of fetal age following treatment of their dams with cottonseed oil (control), testosterone or Androcur from days 36 to 57 of gestation (Experiment 2).

| Fetus | | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|---|
| ID | Sex | Int[a] | Pattern | Int[a] | Pattern |
| Control | | | | | |
| 21404 | M | - | No reactivity | + | Nuclear – dermal fibroblasts |
| 21405 | M | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 21408 | M | - | No reactivity | - | No reactivity |
| 21403 | F | - | No reactivity | - | No reactivity |
| 21406 | F | - | No reactivity | + | Nuclear – dermal fibroblasts |
| 21407 | F | - | No reactivity | - | No reactivity |
| Testos | | | | | |
| 21419 | M | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 21413 | M | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 21414 | M | - | No reactivity | - | No reactivity |
| 21410 | F | - | No reactivity | + | Nuclear – dermal fibroblasts |
| 21411 | F | - | No reactivity | - | No reactivity |
| 21412 | F | - | No reactivity | ++ | Nuclear – dermal fibroblasts |
| 21415 | F | - | No reactivity | + | Nuclear – dermal fibroblasts |
| Androc | | | | | |
| 21416 | F | - | No reactivity | - | No reactivity |
| 21417 | F | - | No reactivity | + | Nuclear – dermal fibroblasts |
| 21418 | F | - | No reactivity | - | No reactivity |
| 21419 | F | - | No reactivity | - | No reactivity |
| 21420 | F | - | No reactivity | + | Nuclear – dermal fibroblasts |
| 21421 | F | - | No reactivity | - | No reactivity |
| 21422 | F | - | No reactivity | - | No reactivity |

Int[a] = intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Sex: M = male, F = female. Fetus ID: Testos = testosterone, Androc = Androcur.

**Table A.6** Patterns of immunoreactivity to androgen receptor antibody in the mammary gland of sheep at day 117 of fetal age following treatment of their dams with cottonseed oil (control), testosterone or Androcur from days 36 to 57 of gestation (Experiment 2).

| Fetus | | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|---|
| ID | Sex | Int[a] | Pattern | Int[a] | Pattern |
| Control | | | | | |
| 21784 | M | +++ | Cytoplasmic | - | No reactivity |
| 21785 | M | ++ | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| 21787 | M | ++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 21786 | F | + | Linear cell surface | - | No reactivity |
| 21788 | F | ++ | Cytoplasmic | - | No reactivity |
| 21789 | F | + | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| Testos | | | | | |
| 21795 | M | ++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 21790 | F | + | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 21791 | F | ++ | Linear cell surface | - | No reactivity |
| 21792 | F | ++ | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| 21793 | F | + | Cytoplasmic and nuclear | ++ | Nuclear – dermal fibroblasts |
| Androc | | | | | |
| 21796 | M | ++ | Cytoplasmic and nuclear | + | Nuclear – dermal fibroblasts |
| 21798 | M | ++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 21799 | M | ++ | Cytoplasmic and nuclear | + | Nuclear – dermal fibroblasts |
| 21802 | M | +++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 21797 | F | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 21800 | F | ++ | Linear cell surface | - | No reactivity |
| 21801 | F | + | Linear cell surface | - | No reactivity |

Int[a] = intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Sex: M = male, F = female. Fetus ID: Testos = testosterone, Androc = Androcur.

**Table A.7** Patterns of immunoreactivity to androgen receptor antibody in the mammary gland of lambs at birth following treatment of their dams with cottonseed oil (control), testosterone or Androcur from days 36 to 57 of gestation (Experiment 2).

| Fetus | | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|---|
| ID | Sex | Int[a] | Pattern | Int[a] | Pattern |
| Control | | | | | |
| 21945 | M | + | Linear cell surface | + | No reactivity |
| 21946 | M | + | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| 21947 | M | + | Linear cell surface | - | Nuclear – dermal fibroblasts |
| 21937 | F | - | No reactivity | - | No reactivity |
| 21938 | F | + | Linear cell surface | + | No reactivity |
| 21948 | F | - | No reactivity | - | Nuclear – dermal fibroblasts |
| Testos | | | | | |
| 21933 | M | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 21941 | M | ++ | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| 21934 | F | + | Cytoplasmic | - | No reactivity |
| 21935 | F | ++ | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| 21936 | F | ++ | Cytoplasmic | - | No reactivity |
| 21942 | F | + | Cytoplasmic | + | Nuclear – dermal fibroblasts |
| Androc | | | | | |
| 21932 | M | +++ | Cytoplasmic | ++ | Nuclear – dermal fibroblasts |
| 21950 | M | ++ | Linear cell surface | - | No reactivity |
| 21951 | M | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 21952 | M | + | Linear cell surface | + | Nuclear – dermal fibroblasts |
| 21931 | F | + | Linear cell surface | - | No reactivity |
| 21943 | F | - | No reactivity | - | No reactivity |
| 21944 | F | - | No reactivity | - | No reactivity |

Int[a], intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Fetus ID: Testos = testosterone, Androc = Androcur; Sex: F = female, M = male;

**Table A.8** Patterns of immunoreactivity to IGF-I receptor antibody in the mammary glands of female sheep at day 101 of fetal age whose dams were subjected to different nutritional regimens in early to mid pregnancy (Trial 2).

| Treatment | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|
| Fetus | Int[a] | Pattern | Int[a] | Pattern |
| Low | | | | |
| 19044 | ++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 19045 | + | Cytoplasmic | + | Nuclear (F, A) |
| 19046 | + | Cytoplasmic | + | Nuclear (F, A) |
| 19047 | ++ | Cytoplasmic | + | Nuclear (F, A) |
| Control | | | | |
| 19038 | ++ | Cytoplasmic | + | Nuclear (F, A) |
| 19039 | ++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 19040 | +++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 19041 | ++ | Cytoplasmic | + | Nuclear (F, A) |
| High | | | | |
| 19032 | ++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 19033 | ++ | Cytoplasmic | + | Nuclear (F, A) |
| 19034 | +++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 19035 | ++ | Cytoplasmic | + | Nuclear (F, A) |

Int[a], intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Mesenchymal cells: F = fibroblast, A = adipoblast

**Table A.9** Patterns of immunoreactivity to IGF-I receptor antibody in the mammary glands of female sheep at day 101 of fetal age whose dams were subjected to different nutritional regimens in early to mid pregnancy (Trial 3).

| Treatment | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|
| Fetus | Int[a] | Pattern | Int[a] | Pattern |
| MM | | | | |
| 19050 | ++ | Cytoplasmic | + | Nuclear (F, A), cytoplasmic (A) |
| 19051 | + | Cytoplasmic | + | Nuclear (F, A), cytoplasmic (A) |
| 19052 | + | Cytoplasmic | + | Nuclear (F, A), cytoplasmic (A) |
| 19053 | ++ | Cytoplasmic | + | Nuclear (F, A), cytoplasmic (A) |
| 21135 | +++ | Cytoplasmic | ++ | Nuclear (F, A), cytoplasmic (A) |
| 21138 | ++ | Cytoplasmic | + | Nuclear (F, A), cytoplasmic (A) |
| MH | | | | |
| 19054 | ++ | Cytoplasmic | + | Nuclear (F, A) |
| 19055 | +++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 19056 | ++ | Cytoplasmic + perinuclear | ++ | Nuclear (F, A) |
| 19057 | +++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 21134 | ++ | Cytoplasmic + perinuclear | + | Nuclear (F, A) |
| HH | | | | |
| 19058 | +++ | Linear cell surface | ++ | Nuclear (F, A) |
| 19059 | +++ | Linear cell surface | ++ | Nuclear (F, A) |
| 19060 | ++ | Cytoplasmic | + | Nuclear (F, A) |
| 19061 | ++ | Linear cell surface | + | Nuclear (F, A) |
| 21136 | +++ | Cytoplasmic | ++ | Nuclear (F, A) |
| 21137 | ++ | Linear cell surface | + | Nuclear (F, A) |

Int[a], intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Mesenchymal cells: F = fibroblast, A = adipoblast

**Table A.10** Patterns of immunoreactivity to IGF-I receptor antibody in the mammary glands of female sheep at day 140 of fetal age whose dams were subjected to different nutritional regimens throughout pregnancy (Trial 3).

| Treatment | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|
| Fetus | Int[a] | Pattern | Int[a] | Pattern |
| MMM | | | | |
| 19062 | ++ | Linear cell surface | + | Nuclear (F, A) |
| 19063 | + | Linear cell surface | - | No reactivity |
| 19064 | + | Linear cell surface | - | No reactivity |
| 21139 | + | Linear cell surface | - | No reactivity |
| 21148 | + | Linear cell surface | - | No reactivity |
| MMH | | | | |
| 19065 | + | Linear cell surface | + | Nuclear (F, A) |
| 19066 | ++ | Linear cell surface | ++ | Nuclear (F, A) |
| 19067 | ++ | Cytoplasmic + nuclear | ++ | Nuclear (F, A), cytoplasmic (A) |
| 21142 | + | Linear cell surface | + | Nuclear (F, A) |
| 21147 | + | Cytoplasmic | ++ | Nuclear (F, A), cytoplasmic (A) |
| MHM | | | | |
| 19068 | ++ | Linear cell surface | ++ | Nuclear (F) |
| 19069 | + | Cytoplasmic | + | Nuclear (F) |
| 19070 | + | Cytoplasmic | ++ | Nuclear (F) |
| 21140 | + | Linear cell surface | + | Nuclear (F) |
| 21145 | + | Linear cell surface | + | Nuclear (F) |

Int[a], intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Mesenchymal cells: F = fibroblast, A = adipoblast

**Table A.10 continued** Patterns of immunoreactivity to IGF-I receptor antibody in the mammary glands of female sheep at day 140 of fetal age whose dams were subjected to different nutritional regimens throughout pregnancy (Trial 3).

| Treatment | Epithelial cells | | Mesenchymal cells | |
|---|---|---|---|---|
| Fetus | Int[a] | Pattern | Int[a] | Pattern |
| **MHH** | | | | |
| 19071 | + | Linear cell surface | + | Nuclear (F) |
| 19072 | + | Linear cell surface | - | No reactivity |
| 19073 | + | Linear cell surface | - | No reactivity |
| 21141 | ++ | Linear cell surface | + | Nuclear (F) |
| 21146 | + | Linear cell surface | + | Nuclear (F) |
| **HHM** | | | | |
| 19074 | ++ | Cytoplasmic | + | Nuclear (F) |
| 19075 | ++ | Cytoplasmic | + | Nuclear (F) |
| 19076 | + | Linear cell surface | - | No reactivity |
| 19929 | ++ | Linear cell surface | + | Nuclear (F) |
| 21143 | ++ | Linear cell surface | + | Nuclear (F) |
| 21144 | + | Linear cell surface | - | No reactivity |
| **HHH** | | | | |
| 19077 | ++ | Cytoplasmic | +/++ | Nuclear (F,A), cytoplasmic (A) |
| 19925 | +++ | Cytoplasmic | +++ | Nuclear (F,A), cytoplasmic (A) |
| 19928 | ++ | Cytoplasmic | +/++ | Nuclear (F,A), cytoplasmic (A) |
| 21479 | +++ | Cytoplasmic | ++ | Nuclear (F,A), cytoplasmic (A) |

Int[a], intensity of staining: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Mesenchymal cells: F = fibroblast, A = adipoblast.

Treatment: M = maintenance (1.0M) level of nutrition, H = high (1.5M) level of nutrition.

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