Implantation and Placentation in the dasyurid marsupial, *Sminthopsis crassicaudata*

Claire Roberts

Department of Anatomy and Histology

Faculty of Medicine

University of Adelaide

South Australia

A thesis submitted for the degree of Doctor of Philosophy at the University of Adelaide

January 1995

The Fat-tailed Dunnart, *Sminthopsis crassicaudata*. Photo: Mark Newton

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Claire Roberts 28 January 1995

Acknowledgments

I would like to thank my supervisor Associate Professor Bill Breed for all his help and encouragement throughout this project and for the many hours spent reading this thesis. His comments have been invaluable. Also thanks for encouraging me to write the three already published papers during the project and for his many useful comments on the manuscripts.

I would also like to thank Dr. Graham Mayrhofer of the Department of Microbiology and Immunology of the University of Adelaide for help in raising the polyclonal antibodies to the mucoid coat and shell membrane which was so important in determining the origin of the shell membrane.

Many thanks to Chris Leigh, Gail Hermanis and Dom Panuccio for technical assistance throughout the project and to Rob Murphy for help with photography. Thanks to our laboratory manager John Cecchin for numerous acts of kindness. Thanks also to Ken Crocker and John Terlet of the Centre for Microscopical and Microstructural Analysis (CEMMSA) at the University of Adelaide for training and help on the electron microscopes.

Thanks to all the other members of the Department of Anatomy and Histology at the University of Adelaide for their friendship and help.

Finally I would especially like to thank my partner Malcolm, and our two children, Annie and Kelly, for their love, support, patience and sacrifices over the last few years. Life just wouldn't be the same without you.

Table of Contents

Abstract

This research has been performed on several aspects of the reproductive biology of female, fat-tailed dunnarts (*Sminthopsis crassicaudata*) which are small insectivorous Australian dasyurid marsupials. The study aimed to investigate the origin of the shell membrane, the stage of embryonic development at which it is lost and implantation takes place, the morphological features of fetal-maternal cellular interactions at implantation and placentation.

During early development a mucoid layer is laid down around the embryo just after fertilization in the ampulla of the fallopian tube, which may contribute to the block to polyspermy and provide an early nutrient source for the embryo. A second acellular layer, the shell membrane, is deposited around the embryo about the time it reaches the uterus less than 24h after fertilization which may give support to the cleaving blastomeres when they adhere to the thin zona pellucida. The shell membrane (SM) also allows the passage of gases and nutrients from the uterine fluid to the pre-implantation embryo and is not shed until just prior to implantation which generally occurs about two-thirds through pregnancy.

To solve the controversy of the origin of the shell membrane polyclonal antibodies were raised against the shell membrane and mucoid. Indirect immuno-fluorescence, streptavidin/biotin immunoperoxidase cytochemistry, and immuno-gold labelling revealed that precursors of the tertiary egg membranes are secreted by the luminal epithelium of the ampulla, isthmus (including the crypts), utero-tubal junction and adjacent endometrial glands. A variety of histochemical stains differentiated between the sites of mucoid and shell membrane precursor secretion. The mucoid coat consists of neutral and acidic glycoproteins and is secreted by luminal epithelial cells of the oviduct. The utero-tubal junction did not stain with any of the glycoprotein methods nor did the shell membrane although it is eosinophilic and stained positively with the red cytoplasmic stain of Masson's trichrome. Therefore, it is concluded that shell membrane precursors are secreted by non-ciliated cells of the luminal epithelium of the utero-tubal junction, adjacent glands, and by scattered glands in the anterior region of the uterus but not by any cell population of the oviduct.

Ultrastructural investigations of embryos at different times during pre-implantation development have shown that the SM is not present until zygotes have entered the utero-tubal junction and that it first has a compact granular consistency. As it thins

during blastocyst expansion it becomes fibrous in texture with fibres oriented mainly in the plane of the membrane.

The shell membrane is lost when the embryo is at the early somite stage allowing the trophoblast to become intimately associated with the maternal uterine epithelium at this time. At implantation, in the trilaminar, or vascular, region of the yolk sac (TYS), trophoblast cells adjacent to the embryo form desmosomes with uterine epithelial cells and appear to fuse with them to form hybrid cells. Later, as the placenta develops, in the bilaminar, or avascular, yolk sac (BYS) multinucleate trophoblast giant cells (TGCs) from an annular region adjacent to the sinus terminalis, penetrate the maternal epithelium by intrusion, possibly also by fusing with it. TGCs send processes down between maternal epithelial cells, break their intercellular junctions, and then form hybrid junctions. Maternal epithelial cells appear to be pushed apart by invading TGCs, which then appear to pause at the basal lamina before migrating into the stroma. Desmosomes subsequently develop between TGCs and maternal stromal fibroblasts. In the TYS placenta trophoblast is not invasive but its microvilli and larger cell processes invaginate, and interdigitate with, the highly folded maternal epithelium.

As the placenta develops TGCs in the BYS, which erode and phagocytose the maternal epithelium, migrate towards, but do not invade, the maternal capillaries; thus an endotheliochorial placenta results. In the TYS the convoluted chorion follows the contours of the highly folded endometrial epithelium but does not erode it, thus an epitheliochorial placenta is formed. The ultrastructure of trophoblast and endoderm in both placental regions suggests steroid and peptide biosynthesis as mitochondria with tubular cristae and smooth and rough endoplasmic reticulae are common.

In conclusion, the shell membrane is present for about 9 days of the 13.5 day pegnancy after which implantation proceeds. The ultrastructure of implantation and placentation of the dunnart suggests that, despite the fact that the placenta is derived from the yolk sac, its development, in particular that of the trophoblast, has many similarities with that of the eutherian chorioallantoic placenta. Although this study has largely been of a structural nature the results form the basis on which a number of functional hypotheses can be made which can be tested in the future.

Papers arising from this Thesis

Roberts C.T. and Breed W.G. (1994) Placentation in the dasyurid marsupial, *Sminthopsis crassicaudata*, the fat-tailed dunnart, and notes on placentation of *Monodelphis domestica*. Journal of Reproduction and Fertility **100**, 105-113.

Roberts C.T. and Breed W.G. (1994) Embryonic-maternal cell interactions at implantation in the fat-tailed dunnart, a dasyurid marsupial. Anatomical Record **204**, 59-76.

Roberts C.T., Breed W.G. and Mayrhofer G. (1994) The origin of the oocyte shell membrane of a dasyurid marsupial, an immunohistochemical study. Journal of Experimental Zoology **270**, 321-331.

Roberts C.T. and Breed W.G. (in preparation) The ultrastructure of the shell membrane and its origin in a dasyurid marsupial.

Roberts C.T. and Breed W.G. (in preparation) The ultrastructure of the yolk sac placenta of the fat-tailed dunnart, a dasyurid marsupial.

Roberts C.T. and Breed W.G. (in preparation) Electron-dense material at the feto-maternal interface of the yolk sac placenta of a marsupial.

Conference Communications:

1992 **Roberts C.** and Breed W.G. Placental Microscopic Structure of the Fat-tailed Dunnart (*Sminthopsis crassicaudata*): a Dasyurid Marsupial. Proceedings Australian Society for Reproductive Biology 24,125 Poster presentation.

1994 **Roberts C.T.** and Breed W.G. Marsupial viviparity - shell membrane, implantation and placentation. Proceedings Australian Society for Reproductive Biology 26, 98. Oral presentation.

1995 Boden Conference Marsupial Gametes and Embryos Thredbo, Australia Two invited papers:

Roberts C & Breed W.G. Shell membrane secretion in a dasyurid marsupial.

Roberts C. & Breed W.G. Yolk sac placentation in a dasyurid marsupial - a case of invasive trophoblast.

Figure Abbreviations

A	- apoptotic maternal epithelial cell
Al	- allantois
AN	- pyknotic nucleus of apoptotic cell
Ant	- anti-mesometrial
BC	- blastocoelic cavity
BYS	- bilaminar yolk sac
B	- blastomere
C	- ciliated cell
CF	- collagen fibrils
Cr	- crypt
DE	- degenerating epithelium
E	- uterine luminal epithelium
Em	- embryo
En	- extra-embryonic endoderm
End	- endometrium
F	- fibroblast
FCa	- fetal capillary
Fi	- fibrous material
G	- golgi complex
Gl	- gland
GE	- gland epithelium
H & E	- haematoxylin & eosin
I	- inclusion
IE	- embryonic endoderm
IS	- intracellular space
L	- lumen
Li	- lipid
Ly	- lysosome
M	- mitochondrion
Ma	- manus
MBV	- maternal blood vessel
MC	- mucoid coat
MCa	- maternal capillary
ME	- maternal epithelium
Mes	- mesometrial
My	- myometrium
N	- nucleus
P	- pes
PA	- proamnion
PAS	- periodic acid Schiff
RB	- residual body
RER	- rough endoplasmic reticulum
SM	- shell membrane
ST	- sinus terminalis
St	- stroma
T	- trophoblast
Tol. Blue	- toluidine blue
TGC	- trophoblast giant cell
TYS	- trilaminar yolk sac
S	- secretion
SC	- secretory cell
SG	- secretory granule
SM	- shell membrane
ST	- sperm tail
V	- vesicle
Va	- vacuole
Y	- yolk mass
YSC	- yolk sac cavity
ZP	- zona pellucida

Chapter 1 Literature Review

1.1 Introduction

In this thesis I first present a broad overview of what is known about marsupial pregnancy and how it compares with that of eutherians. As both these mammalian groups are viviparous this requires an introduction on the occurrence of viviparity in vertebrates. This is followed by a brief account of the differences in the mode of reproduction between the three groups of mammals, monotremes, marsupials and eutherians. Then an account of cleavage and of the extra-cellular coats laid down around the embryo during the first few days after fertilization is given. The major parts of the review are morphological descriptions of implantation and placentation in eutherians and what is known of these processes in marsupials.

The second chapter is a summary of the research proposal which includes many of the materials and methods used for the project to minimise repetition between chapters. The third to seventh chapters comprise the substance of the research and the eighth chapter is a concluding discussion of the work.

1.2 The Evolution of Viviparity in Vertebrates

Vertebrate reproduction takes four basic forms (1) ovulparity, the laying of unfertilized eggs (Guillette, 1991), (2) oviparity, the laying of fertilized eggs soon after ovulation, (3) ovoviviparity, the laying of eggs that have been retained *in utero* for a significant length of time after fertilization during which time the embryos have undergone some, but variable amounts of, development and which hatch at or soon after oviposition, and (4) viviparity, the bearing of young which have derived nutrient and gaseous exchange from the mother *in utero* and which are born exposed to the environment (Sharman, 1976; Mossman, 1987). Almost all authors who have written on this subject have slightly different interpretations of the definitions of these terms with ovoviviparity being the least agreed upon definition but for the purposes of this review the above definitions will be used. It should be noted, however, that in reality the distinctions between these forms are not as clear cut as these definitions suggest.

During vertebrate evolution the tendency for embryos to be retained within the mother's body has evolved many times and in many different ways. There are probably two major benefits of viviparity that have resulted in its evolution in a variety of vertebrate groups: (1) protection of the offspring such that they may escape predation and develop a variety of structures before having to defend themselves and

maintain homeostasis, and (2) the formation of a constant and predictable environment in which differentiation of structures and functions may occur which may not otherwise be possible (Cohen, 1977).

The earliest vertebrates laid large numbers of unfertilized eggs in which there was little or no yolk to supply nutrients to the embryo which, after fertilization, quickly developed to a free-living larval stage which derived its nutrients from the external environment (Sadleir, 1973). The development of a yolk supply within the egg was an innovation that increased the chances of survival, allowing a reduction in the number of ovulated ova which was a necessary preadaptation for the retention of embryos within the mother (Hogarth, 1976).

The first structural requirement of vertebrate viviparity is the oviduct which is present in all vertebrate classes except the cyclostomes (Porter *et al.*, 1982). The first functional requirement of vertebrate viviparity is internal fertilization (Wake, 1985) which also allows a reduction in the number of ova produced (Hogarth, 1976). Once there was internal fertilization structural modifications of the female reproductive tract evolved which allowed the retention of embryos *in utero* for longer periods which then permitted alterations to embryonic development (Guillette, 1991).

The presence of a store of yolk in oocytes as a nutritional source derived from the mother prior to ovulation (megalecithal eggs) required the development of an extra-embryonic membrane to absorb it and make it available to the embryo. This, the yolk sac, was the first of the vertebrate fetal membranes to evolve and is present in all anamniotes whose eggs are megalecithal as well as in all amniotes, reptiles, birds and mammals, irrespective of whether they are viviparous or oviparous (Luckett, 1977; Mossman, 1937, 1987).

The eggs of most teleost (bony) fishes are fertilized externally but about 500 species from 14 Families are viviparous (Schindler and Hamlett, 1993). As teleosts have no uteri viviparous species have evolved either intraluminal or intrafollicular gestations which occur within the ovary itself (Hogarth, 1976; Schindler and Hamlett, 1993). In some of these species extra-embryonic structures develop which are involved in the uptake of nutrients from the mother, including a vascular yolk sac and/or pericardial sac for respiratory exchange, and 'trophotaeniae' which are anal appendages and therefore continuous with the gut epithelium and capable of absorbing exogenous proteins (Hogarth, 1976; Schindler and Hamlett, 1993; Hollenberg and Wourms, 1994).

The majority of cartilaginous fishes (elasmobranchs) are viviparous. During pregnancy the endometrium hypertrophies and becomes extensively folded and highly vascular

under the influence of oestrogens secreted by the ovary (Hogarth, 1976). In some species the yolk is the sole nutrient supply for embryonic devlopment, while in others, particularly stingrays, there is, in addition, a contribution from the uterine secretions, histotrophe (Hamlett *et al.*, 1993). The lamnoid sharks rely on embryonic cannibalism when the yolk is exhausted and the hammerhead and requiem sharks, known as the placental sharks, develop a yolk sac placenta and therefore gain haemotrophic nutrition (Hamlett *et al.*, 1993).

Viviparity in amphibians has evolved several times (five anurans, one urodele and three caecilians) although none of these species develops a placenta (Wake, 1993). Yolk is the primary nutrient source in these species and later, embryos orally ingest endometrial secretions which are rich in carbohydrates and lipids as well as uterine epithelial cells (Wake, 1993). There are also other forms of viviparity in amphibians which involve embryonic development in what would seem to a reproductive biologist the most unlikely places such as the mouth, the skin on the back, and in the stomach, although placentation does not strictly occur (Hogarth, 1976).

Prior to the evolution of the reptiles, vertebrates relied on water to prevent dessication of their eggs and embryos. The evolution of a terrestrial lifestyle required the co-evolution of a structure to prevent such dessication, the cleidoic egg, which is always megalecithal and is covered by an acellular shell membrane and, sometimes calcareous, shell (Sadleir, 1973; Mossman, 1987). As the embryo is incubated within this egg for a variable length of time it needs all the requirements for development, a nutrient and oxygen supply and the ability to absorb them, as well as a means for storing urinary wastes (Mossman, 1987). The characteristic feature of all amniotes (reptiles, birds and mammals) is the possession of homologous fetal membranes: the amnion, chorion, allantois and yolk sac (Luckett, 1977). The yolk sac as described earlier is also shared by all other vertebrates which have a megalecithal (yolk-rich) egg and is therefore a phylogenetically old structure. The amnion secretes a fluid in which the embryo 'floats' protected from the external environment and the distorting force of gravity (Mossman, 1987). The chorion surrounds the embryo and all other fetal membranes such that physiological exchange - nutrient, gaseous and waste - takes place between mother and fetus. In most oviparous species (those that lay eggs including reptiles and birds) this is confined to gaseous exchange as the yolk is the nutrient store for the developing embryo, and the allantois is a waste receptacle. Respiratory exchange first takes place through the choriovitelline membrane and then the chorioallantoic membrane takes over this function later in ontogeny (Reeves, 1984; Metcalfe and Stock, 1993).

Viviparity in reptiles has evolved in at least 95 species of squamates (lizards and snakes) which is about 20% of this group (Shine, 1984). In all these species both choriovitelline and chorioallantoic placentation occur and a shell membrane surrounds the conceptus for at least a part of pregnancy (Blackburn, 1993; Stewart and Thompson, 1994). The "cold-climate hypothesis" has been developed to explain the evolution of viviparity amongst squamates and suggests that in cold climates the incubation temperature of eggs laid in a nest is inadequate to sustain embryonic development and that if retained *in utero* behavioural thermoregulation by the mother can achieve ideal 'incubation' temperatures and increase the chances of survival of her offspring (Shine, 1984; Guillette, 1991). However, viviparity has not evolved in the other major reptilian groups, the turtles and crocodilians, irrespective of climatic conditions. The reason for this appears to be associated with the source of calcium for the developing embryo which in these groups is the calcareous shell (Packard and Packard, 1984; Shine, 1984). In squamates the yolk provides calcium to the embryo in oviparous species and as viviparity is correlated with a reduction in shell membrane and shell deposition (Guillette, 1991) there is no deleterious effect on calcium availability in viviparous species (Packard and Packard, 1984; Shine, 1984). In those species which derive their calcium from the calcareous shell viviparity has therefore been precluded (Shine, 1984).

Embryonic birds also derive their calcium from the calcareous shell via the chorioallantoic membrane (Metcalfe and Stock, 1993) and this is also thought to be a possible reason why all birds are oviparous (Packard and Packard, 1984). However, there are other reasons which may contribute to the lack of viviparity in birds. Firstly, the fact that the retention of an egg in the avian oviduct inhibits subsequent ovulation would restrict the number of offspring to only one (Anderson *et al.* 1987). Secondly, if this was overcome the increased volume and weight of retained embryos may effect the ability to fly, and therefore to avoid predation, in birds with large clutch sizes (Blackburn and Evans, 1986). Thirdly, the cost of reproduction for egg retaining females may increase with the concurrent loss of paternal care during incubation and possibly postnatal life (Blackburn and Evans, 1986). Anderson *et al.* (1987) also believed that the resting body temperature of birds (40-41°C) is too high for normal embryonic development and that experimental incubation of avian eggs at higher than normal incubation temperatures results in a high incidence of embryonic mortality.

Viviparity in reptiles and mammals is also correlated with an increase in the secretory activity of the uterus, stimulated by the corpus luteum (Porter *et al.*, 1982). In both these groups the corpus luteum controls the luteal phase of the cycle but is absent in birds for example in which the ovarian follicle collapses a day or so after ovulation

(Porter *et al.*, 1982). The concurrent reduction in secretion of shell membrane and shell precursors (and therefore thinner shells) has made possible the modification of endometrial glandular secretion to a carbohydrate and/or lipid rich product which provides an energy source for the retained embryos, particularly early in pregnancy (Guillette, 1991).

1.3 Mammals

The primary mammalian dichotomy is between the prototherians [monotremes (platypus and echidna) and a few extinct groups] and therians (metatherians = marsupials and eutherians, Marshall, 1979). The first mammalian fossils date from the late Triassic period and are about 230 million years old (Clemens, 1977; Vaughan, 1986; Lillegraven *et al.*, 1987). It is thought that the ancestors of the prototherians diverged from the lineage that gave rise to the therians about 180 million years ago and during the Jurassic the early therians radiated on all continents (Clemens, 1977). The monotremes are distinct by the fact that they are oviparous, i.e. they lay eggs, although after the yolk is exhausted there is significant transfer of nutrients and gases between mother and embryo *in utero* prior to oviposition through the leathery shell and shell membrane which surround the developing embryo (Luckett, 1977; Hughes, 1993). Significantly, intrauterine development in the echidna proceeds for about 18 days and during this time the "egg" increases in size from about 4mm to 17mm in diameter and the embryo achieves 19-20 somites and the neural tube forms (Hughes, 1984, 1993). After oviposition in a nesting burrow (platypus) or pouch (echidna), eggs are incubated for about a further 10 days before hatching (Hughes, 1993). All therians, on the other hand, are viviparous.

The second mammalian dichotomy is that between the two groups of therian mammals (Tyndale-Biscoe and Renfree, 1987). The most recent common ancestor of marsupial and eutherian mammals is thought to have lived about 120 million years ago during the Cretaceous period (Tyndale-Biscoe, 1973; Clemens, 1977; Martin, 1977; Kemp, 1983; Vaughan, 1986; Lillegraven *et al.*, 1987). Marsupials and eutherians both began adaptive radiations about 65 million years ago during the Tertiary (Vaughan, 1986).

Metatherian and eutherian mammals are distinguished primarily by their mode of reproduction, although there are a few other less significant differences of the central nervous system and skeleton (Barbour, 1977; Marshall, 1979). The presence of the pouch is perhaps the most well-known feature of marsupials although it is not universally present in this sub-class (Sharman, 1970; Kirsch, 1977). Marsupial development does not by necessity require a pouch and those species that have a

pouch are generally those that hop, climb or dig (Kirsch, 1977), other families of marsupials merely have a shallow depression in the region of the mammary glands or no pouch at all (Tyndale-Biscoe and Renfree, 1987). The mammary glands, located within the pouch if present, are essential to the marsupial mode of reproduction with an extended period of lactation being extremely important for development of offspring that can maintain homeostasis.

For the first two/thirds of pregnancy the marsupial embryo is surrounded by the acellular shell membrane which allows the passage of nutrients from the mother to the embryo (Hughes, 1974a&b). After a short gestation in which a yolk sac placenta develops, as well as a chorioallantoic placenta in bandicoots, marsupials give birth to comparatively altricial young which are suckled for an extended period until they are capable of independent life. Eutherians, on the other hand, have a relatively longer intrauterine gestation, which is usually extended beyond the length of the oestrous cycle, and generally give birth to relatively precocial young. This is followed by a comparatively short period of lactation. The dog and ferret are exceptions to this in that pregnancy does not exceed the length of the oestrous cycle (Porter *et al.*, 1982; Renton *et al.*, 1991).

Marsupials generally have a much shorter gestation than eutherians of comparable size and life habits and the litter weight expressed as a percentage of maternal weight is much lower. It has been argued that this is due to the limitations of the marsupial female reproductive tract (Abbie, 1941; Sharman, 1965, 1970, 1976) and the presumed, but hitherto unproven, absence of mechanisms to prevent rejection of the marsupial fetus by the maternal immune system (Lillegraven *et al.*, 1987). Others have argued that marsupials, having evolved a longer period of lactation than intrauterine gestation, do not need mechanisms necessary for sustaining a long pregnancy (Kirsch, 1977; Gould, 1977). However, it has not been proven that marsupials have not evolved such mechanisms. The birth of altricial young and their subsequent development in the pouch with an extended period of lactation is no less efficient as Lillegraven *et al.* (1987) have argued, than the extended intrauterine gestation with the birth of comparatively precocial young seen in eutherians (Sharman, 1976). A popular interpretation of these differences is that marsupials have evolved an alternative reproductive strategy (Kirsch, 1977; Renfree, 1983, 1993; Tyndale-Biscoe and Janssens, 1988) which some have argued is in fact energetically less costly in more uncertain environmental conditions (Parker, 1977; Low, 1978; Hayssen *et al.*, 1985).

1.4 Early Embryonic Development in Eutherians and Marsupials

1.4.1 Cleavage

Cleavage is the process during which the relatively large zygote (one-celled fertilized egg) undergoes a series of mitotic divisions to form cells (blastomeres) of normal somatic cell size. The blastocyst of most eutherians differentiates after cleavage from the morula, a compact ball of cells which, in the mouse, are polarised and flattened against each other at about the 8-cell stage (Johnson, 1971; Johnson *et al.*, 1981). At the next division two distinct cell populations are formed as a result of the inheritance of two distinct regions of cytoplasm and plasma membrane (Johnson and Ziomek, 1981). The outer layer of cells, the trophectoderm, forms in a polarised environment with its apical cell surface exposed to the tubal/uterine lumen, although still surrounded by the zona pellucida, and its basal surface apposed to the inner aggregation of cells, the inner cell mass (ICM) cells which are loosely apposed to each other. The embryo then becomes a blastocyst with a fluid-filled cavity below the ICM. The trophectoderm becomes part of the extra-embryonic ectoderm while the ICM gives rise to the three germ layers of the embryo proper as well as the extra-embryonic ectoderm of the amnion, the extra-embryonic mesoderm of the chorion, yolk sac and allantois and the extra-embryonic endoderm of the yolk sac and allantois (Johnson and Rossant, 1981; Rossant and Frels, 1981; Denker, 1983; Kaufman, 1983). One somewhat 'primitive' group of eutherians has been found to have a very different process of early development, the Insectivora. In the few species of this order which have been studied, cleavage appears to be similar to that in marsupials (see below) such that no ICM forms and the embryo proper differentiates on the inside of the unilaminar blastocyst (Wimsatt, 1975).

During cleavage in marsupials the blastomeres flatten against the zona pellucida to form a hollow unilaminar sphere of cells also called the blastocyst (Hartman, 1916; McCrady, 1938; Selwood, 1987, 1992). The cells of this unilaminar blastocyst have been referred to as protoderm because they appear to be able to differentiate into both extra-embryonic (trophectoderm) and embryonic tissues (Hartman, 1916; McCrady, 1938). It was thought that these cells differentiate into the outer trophectoderm and an inner layer, the endoderm, which migrates along the inside of the trophectoderm. In *Dasyurus viverrinus* (the eastern quoll), the progenitors of the embryonic endoderm "the endodermal mother cells" differentiate on the inside of the unilaminar blastocyst in the "formative region" early in development (Hill, 1910). Subsequently, their progeny migrate away from the wall of the blastocyst in one hemisphere and become "internal cells" which later become the embryonic endoderm (Hill, 1910).

In the eastern quoll Hill (1910) first noticed that cleavage results in the differentiation of two distinct cell populations which divide asynchronously. One cell type, the embryoblast, is aggregated at the 'yolky hemisphere' and is smaller than the other (trophoblast) which is not only larger and paler staining in paraffin sections but has a shorter cell cycle time (Hill, 1910; Selwood, 1992). The stage of embryonic development at which this differentiation takes place is species specific and occurs as early as the 2-cell stage in the North American opossum (Hartman, 1916) and common opossum (Hill, 1918) and at the 16-cell stage in the grey short-tailed opossum (Baggott and Moore, 1990), native cat (Hill, 1910), brown antechinus (Selwood and Young, 1983), fat-tailed dunnart (Selwood, 1987) and stripe-faced dunnart (Selwood and Woolley, 1991). However, these two distinct cell populations are not distinguishable a little later in development using phase contrast microscopy. Subsequently they reappear and the trophoblast and embryo proper differentiate (Selwood, 1992).

Studies of marsupial early embryos have generally been of a descriptive nature. Embryonic differentiation appears to be associated with the timing of mitotic division in individual blastomeres and positional effects within the early embryo, as is the case in eutherian embryos, although the details are different (for an extensive review of early marsupial embryonic development, see Selwood, 1992). Although marsupial early preimplantation embryos also have no ICM their mode of differentiation may or may not be similar to that of the Insectivora.

1.4.1 Yolk

The marsupial oocyte is microlecithal but does have a small amount of yolk-like material within the cytoplasm. Several eutherian species have oocytes with large numbers of cytoplasmic fatty droplets that are considered to be yolk, for example, the horse, pig, guinea-pig, bat (*Vesperugo noctula*), dog, cat and ferret (Hamilton and Day, 1945). In all these species the process of deutoplasmolysis (yolk extrusion) occurs during cleavage either at the time of the first division e.g. the horse (Hamilton and Day, 1945) and the Australian marsupials *Dasyurus viverrinus* (Hill, 1910), *Antechinus stuartii*, *Sminthopsis macroura* (Selwood, 1980, 1987; Selwood and Young, 1983; Selwood and Smith, 1990) and *Sminthopsis crassicaudata* (Breed and Leigh, 1990) and/or during the first few cleavage divisions as in the New World marsupials *Didelphis virginiana* and *Monodelphis domestica* (Hartman, 1916, 1919; Baggott and Moore, 1990).

The function of the yolk in mammalian embryos is unknown. Hill (1910) thought that it is merely a primitive, vestigial remnant with no function. McCrady (1938) concluded

that after deutoplasmolysis the yolk was liquefied and then taken up again and Selwood (1987) speculated that the yolk is a source of nutrition for the embryo once deutoplasmolysis has occurred. It is not clear why the yolk is extruded. Perhaps the yolk cannot be utilized by the embryo until it has been taken up by endocytosis and digested by lysosomal enzymes. There have not been any specific studies to date as to the fate of the yolk after extrusion. If the yolk is a nutritional source for early embryos it is clear that it does not last long and that the mother must provide nutrients for most of the period of intrauterine development. It is also possible that yolk extrusion may simply be required to facilitate cleavage. In all marsupial species studied to date, the yolk has disappeared by the time of the early bilaminar blastocyst stage (Selwood, 1992).

1.5 Tubal Oocyte Vestments

1.5.1 Mucoid Coat

Oogenesis is similar in eutherians and marsupials with the exception that ovulated marsupial oocytes do not have any cumulus oophorus cells around them. In marsupials the oocyte at ovulation is surrounded by the zona pellucida, the so-called secondary egg membrane, or coat, which is formed during follicular development (Hughes, 1974a). After fertilization, which takes place in the ampulla, an acellular layer, the mucoid coat, is laid down around the zygote, as well as around unfertilized eggs, as they travel down the fallopian tube. This is the first of the so-called tertiary egg coats (Hughes, 1974a) and is probably homologous to the albumen that surrounds the oocyte of the domestic hen's egg. A similar coat occurs in a few eutherian species: the horse (Hamilton and Day, 1945), the dog (Blandau, 1961) and it is particularly thick (59-129μm) around the rabbit oocyte (Greenwald, 1962; Kane, 1975).

Sperm are often found embedded within the mucoid coat of early marsupial embryos (Hughes, 1974a; Selwood, 1982; Ward and Renfree, 1988; Breed and Leigh, 1990) so it may contribute to the block to polyspermy as it is laid down very soon after fertilization such that it is extremely difficult to obtain tubal eggs without a mucoid coat (Rodger and Bedford, 1982; Selwood, 1982). This mucoid material is a strongly sulphated acidic glycoprotein which is secreted by non-ciliated goblet-like cells distributed throughout the luminal epithelium of the entire oviduct (Hughes, 1974b). The coat varies in thickness in different species (from less than 10μm in bandicoots to about 230μm in opossums, Tyndale-Biscoe and Renfree, 1987).

Hartman (1925a) suggested that the rabbit mucoid coat has no function and is a primitive feature which is shared by monotremes and marsupials. Greenwald (1962)

found that if the mucin layer of rabbit blastocysts was removed experimentally it decreased the implantation rate from 70% to less than 30% after embryo transfer to suitable recipients. Kane (1975) suggested that the mucin layer in rabbits prevents premature zona loss *in vitro* and speculated that *in vivo* it allows the 50-fold expansion of the blastocyst to occur prior to implantation and protects the blastomeres from damage. It seems then that the mucoid coat may facilitate the development of these large spherical blastocysts.

Hartman (1916) believed that the marsupial mucoid provides a source of nourishment for the early embryo. Consistent with this view, McCrady (1938) found that the mucoid was reabsorbed by the late bilaminar blastocyst stage in the North American opossum, *Didelphis virginiana*. Hughes (1974b) concluded that the mucoid is rarely present after the bilaminar blastocyst stage in all marsupial species studied.

1.5.2 Shell Membrane

A second acellular layer comes to surround the marsupial zygote (and unfertilized oocyte) by the time it reaches the uterus less than 24 hours after fertilization. This, the shell membrane (SM), is a tough permeable layer which Hill (1910) claimed was a keratin and was later described as an ovokeratin rich in disulphide bonds and to a lesser extent sulfhydryl groups (Hughes, 1969, 1974a, 1977). Hughes (1974a) demonstrated this in the brush tail possum (*Trichosurus vulpecula*) by showing that it stained positively with Alcian Blue pH 0.2 after periodic acid oxidation and also with periodic acid Schiff (PAS) after amylase digestion.

There is some controversy in the literature as to the region of the female reproductive tract responsible for secretion of the shell membrane in marsupials. Some workers (Hill, 1910; Hartman, 1916; Tyndale-Biscoe and Renfree, 1987) concluded that the shell membrane is secreted at least in part by the isthmus of the oviduct, as well as partly by the uterine glands as it gets thicker around eggs within the uterus. Hughes (1974a, 1979), from studies on the brush tail possum, believed that the shell membrane precursors were secreted only by the epithelium of the uterine glands and then some of the material somehow made its way back to the isthmus of the oviduct so that it is first deposited around the 'eggs' in this region; a somewhat implausible view. He confirmed that deposition continues to occur after embryos have entered the uterus. Lyne and Hollis (1976, 1977) found in bandicoots that the maximum thickness of the SM occurs at about the 16-cell stage which is well after the entry of embryos into the uterus. However, they did not identify the cell population(s) that secrete the SM precursors nor did they suggest where the SM first appears. Baggott and Moore (1990)

in their studies on the grey short tailed opossum (*Monodelphis domestica*) first observed the SM surrounding uterine fertilized ova which led them to speculate that its precursors are secreted by the uterine glands. By contrast, Hartman (1916) and McCrady (1938) concluded that in the North American opossum (*Didelphis virginiana*) the SM precursors are secreted by 'shell glands' of the isthmus of the oviduct but that it increases in thickness in the uterus. Breed and Leigh (1988) and Breed *et al.* (1989), from studies of the fat-tailed dunnart (*Sminthopsis crassicaudata*), speculated that the electron-dense granules that they found in epithelial cells of the crypts in the isthmus may contain SM precursors. However, as they later found that the SM that surrounds zygotes that have just entered the uterus is extremely thin while older zygotes have a much thicker SM they suggested that the SM may be secreted by uterine endometrial glands as well as by cells in the crypts of the isthmus (Breed and Leigh, 1990).

In monotremes the inner layer of the shell, which is laid down in the isthmus of the oviduct was thought to be homologous with the marsupial shell membrane (Hill and Hill, 1933; Flynn and Hill, 1939; Hill, 1941). However, this homology was questioned by Hughes (1974a, 1977) and Hughes *et al.* (1975) because they found that the uterine glands are the site of shell membrane synthesis in the possum.

Shell membranes are by no means exclusive to marsupial eggs as indicated above. In oviparous reptiles and birds their shelled eggs have a membrane between the calcareous shell and albumen layer of the egg. This has mostly been studied in birds, particularly the domestic hen (Moran and Hale, 1936; Romanoff and Romanoff, 1949; Candlish, 1972; Draper *et al.*, 1972; Wong *et al.*, 1984). Hughes (1984) suggested that because the shell membrane of birds is permeable it was important in the evolution of viviparity by allowing the ancestral embryos to absorb nutrients and gases from the maternal oviduct prior to oviposition. This, in fact, occurs in monotremes.

The avian shell membrane consists of a thick outer and a thin inner membrane between which the air sac intervenes at one pole. The outer layer consists primarily of coarse fibres of about 2-5μm thick and the inner layer has mainly finer fibres of 0.6-0.8μm diameter (Moran and Hale, 1936; Wong *et al.*, 1984). Transmission electron microscopy has shown that each fibre has a distinct electron-dense cortex and less electron-dense medulla, presumably indicating a different chemical composition between the two regions (Draper *et al.*, 1972).

The shell membrane protein was originally considered to be an "ovokeratin", that is, a protein with many cystine residues that is associated with the egg (Romanoff and Romanoff, 1949). This is now thought to be a misnomer. Hoffer (1971), in her study

of the SM of the Japanese Quail, showed that it is a disulphide and sulfhydryl rich glycoprotein. However, she concluded that it should not be called a keratin because the presence of these groups on its own is not a sufficient criterion for this characterisation. Candlish (1972) also considered that the term ovokeratin is inappropriate and suggested another term ovocapsin, as the SM is composed of an insoluble, acidic protein rich in cystine which forms many cross-linkages with other proteins, especially collagen. Wong *et al.* (1984) found in the domestic hen that collagen Type I is associated with the coarse and Type V with the fine fibres of the outer and inner SM respectively. After staining with ruthenium red they found that proteoglycan granules occur between the coarse fibres and on the outer surface of the inner SM.

In oviparous reptiles the 'uterus' has a dual function in regard to production of the egg coats. Shell membrane precursors are secreted by the uterine endometrial glands for up to 24h after ovulation and, subsequently, the luminal epithelium secretes precursors of the calcareous shell (Palmer and Guillette, 1988; Palmer *et al.*, 1993). In viviparous lizards the shell membrane is thin and composed of matted fibres which are deposited soon after fertilization (Weekes, 1927). In the viviparous garter snake the shell membrane is deposited in the uterus and persists between the extra-embryonic membranes throughout pregnancy, possibly functioning as a dialyzing membrane (Hoffman, 1970). However, in the viviparous lizards the shell membrane is shed and collects as a pad of debris at the abembryonic region of the yolk sac after the development of the placenta (Weekes, 1927, 1930, 1935; Blackburn, 1993).

The question remains: where are marsupial shell membrane precursors synthesized? It certainly seems that the endometrial glands may be responsible but is there a dual origin? If the cells of the oviduct secrete SM precursors then one can expect the marsupial SM to be homologous with the inner layer of the monotreme egg. Are the SMs of birds, reptiles, monotremes and marsupials homologous and has this character been conserved and modified to play an as yet unknown role in marsupial viviparity?

1.5.3 When is the Shell Membrane lost?

In bandicoots the shell membrane starts to get thinner by the late unilaminar blastocyst stage (Lyne and Hollis, 1976). By this time then it seems that there is no longer deposition of the shell membrane and that as the blastocyst expands it stretches and therefore becomes thinner. In all marsupial species investigated the SM is finally lost about two-thirds the way through pregnancy (Hughes, 1974a, 1984). In the tammar wallaby Denker and Tyndale-Biscoe (1986) found that the activity of alkaline

proteases of the endoderm and trophoblast of the yolk sac results in the breakdown of the shell membrane at this stage. Mechanical hatching of the embryo from the shell membrane may also play a role in the region of the trilaminar yolk sac (Denker and Tyndale-Biscoe, 1986).

Hughes (1974b, 1984) claimed, somewhat ambiguously, that embryos of the tammar wallaby have developed to the fetal stage when the shell membrane is lost. Selwood (1980) confirmed that at this stage Antechinus embryos have 8-12 somites, a rudimentary heart, cephalic differentiation of the neural tube and amniogenesis has begun. Walker and Rose (1981) described prenatal development in the red-necked wallaby (*Macropus rufogriseus rufogriseus*) and found that by the time that the shell membrane breaks down just prior to implantation the embryo has 25 paired somites, forelimb ridges, mesonephric kidneys and optic stalks.

McCrady (1938) found that in the North American opossum the shell membrane is lost as late as the stage when the manus is paddle-like which occurs on Day 10 of the 12.5 day gestation (Krause and Cutts, 1985a). The loss of the shell membrane allows the trophoblast access to the uterine epithelium so that it is not until after this time that implantation and placentation can commence.

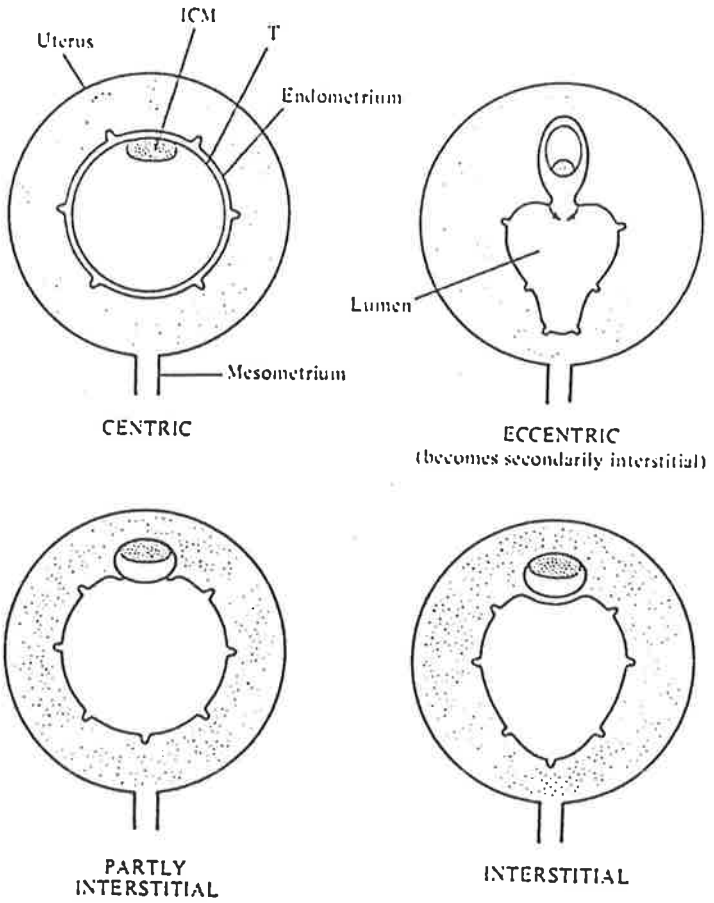
1.6 Implantation

1.6.1 Classification

In mammals placental structure is influenced by the type of implantation that occurs. Studies on eutherians have shown that implantation is either non-invasive or invasive to varying degrees. Non-invasive conceptuses e.g. those of sheep, cows, pigs and horses (Wimsatt, 1975) implant centrally (=superficially) that is, once they reach the site of implantation they expand and fill the uterine lumen. However, there are some species which exhibit central implantation and later develop an invasive placenta e.g. rabbits (Enders and Schlafke, 1971a) and marmosets (Smith *et al.*, 1987). Invasive conceptuses are classified according to the depth of invasion that they exhibit and where the attachment takes place. They show either eccentric, interstitial or partially interstitial implantation. A blastocyst that is positioned in a recess in the uterine epithelium is said to implant eccentrically (Wimsatt, 1975). Early blastocysts of humans, chimpanzees and megachiropteran bats erode the uterine epithelium and become completely embedded in the endometrium with the luminal epithelium healing over them, a process known as interstitial implantation (Wimsatt, 1975). The trophoblast may subsequently erode the endothelium of the maternal blood vessels.

Some species, however, implant eccentrically and then become secondarily interstitial e.g., rats, mice and hamsters (Wimsatt, 1975).

Figure 1.1 Implantation Patterns Observed in Eutherian Mammals (from Renfree, 1982, p.29).



Common types of implantation patterns observed in mammals: centric, eccentric, partly, or completely interstitial. Although implantation may also be mesometrial or lateral, it is most commonly antimesometrial, as shown in these diagrams. Abbreviations as in Fig. 2.1. (Redrawn from Wimsatt (1975) – see Suggested Further Reading).

Non-invasive conceptuses generally become attached to the uterus after a longer period of time in the uterine lumen than do invasive conceptuses. Prior to attachment they rely on histotrophic nutrition from the secretions of uterine glands. At this time the conceptus may grow to a considerable size e.g. the pig conceptus can grow between Days 6 and 12 from a spherical 2mm blastocyst to a membranous thread of a metre in length (Anderson, 1978). Most of this growth occurs in the extraembryonic membranes which facilitates maximum uptake of nutrients from the so-called "uterine milk". Attachment to the uterine epithelium does not occur until Day 15 in the sheep (Boshier, 1969), Day 16 in the pig (King *et al.*, 1982), Day 20 in the cow (Wathes and Wooding, 1980) and Day 21 in the horse (Enders and Liu, 1991a). By the time of implantation the ungulate embryo has developed to the somite stage or later (Hamilton and

Mossman, 1972). For example the embryonic pig at implantation has 32 somites, a beating heart that pumps blood to the yolk sac placenta and an expanding allantois (Perry, 1981).

Invasive conceptuses generally adhere to the uterine epithelium much earlier in their development e.g. human unilaminar blastocysts invade the endometrium on Day 6 of pregnancy (Boyd and Hamilton, 1970), mouse embryos consist of about 100 cells with a diameter of less than 200μm at implantation on Day 4 (Potts, 1968; Kane, 1987). However, early development of rabbit embryos, which are also invasive, is very rapid with implantation occurring at Day 6.5 by which time the blastocyst is up to 7mm in diameter, with about 80,000 cells and a visible embryonic disc (Kane, 1987).

The endometrium in many species with invasive conceptuses responds to implantation by undergoing the decidual cell reaction (DCR), a process known as decidualization. The decidual cells are derived from endometrial stromal cells which have similar morphology to undifferentiated fibroblasts (Mossman, 1987) i.e. they have little cytoplasm and few organelles (Wooding and Flint, 1994) and which "become rounded or polyhedral due to the storage of glycogen or lipids in their cytoplasm" (Mossman, 1937, p.167). These cells may be binucleate (antimesometrial decidua) or uninucleate (mesometrial decidua), polyploid in some species of rodents, and occur initially as sheaths along endometrial blood vessels (Mossman, 1987). The cytoplasm develops many intermediate filaments with numerous free ribosomes, rough endoplasmic reticulum and golgi complexes (Wooding and Flint, 1994). Decidual cells become a nutrient supply for the trophoblast and may control its invasiveness (Morriss, 1975) possibly by secreting acute-phase alpha$_2$-macroglobulin which is a protein that binds to, and inactivates, extracellular proteinases (Bell, 1979). Decidual cells may also present a permeability barrier to molecules of high molecular weight like IgG and also to maternal immunocompetent cells (Parr and Parr, 1989a).

Ramsey *et al.* (1976) found a direct relationship between the extent of trophoblast invasion and decidualization in each of three primate species, the rhesus monkey, baboon and human. In humans, in which implantation is interstitial, the trophoblast is the most invasive, with some cells penetrating the myometrium, and decidualization becomes extensive. Fibroblasts of the sub-placental endometrial stroma become large, polyhedral and packed closely together, particularly adjacent to blood vessels. However, in the rhesus monkey and baboon, which exhibit superficial implantation, only barely detectable swelling of the endometrial stroma occurs at implantation (Ramsey *et al.*, 1976). Billington (1971) found a poorly developed DCR in women with

placenta accreta, a pathological condition in which trophoblast invades deeply into the myometrium resulting in the inability to achieve placental separation *post-partum*.

Decidual tissue in the endometrium also appears to induce migration of certain populations of immunologically active cells, including lymphocytes and macrophages, to the implantation site (Bell, 1983a). Some of these small lymphocyte-like cells appear to inhibit the activity of cytotoxic T-lymphocytes and have therefore been implicated in the prevention of rejection of the antigenically foreign embryo by the maternal immune system (Bell, 1983a). Also, in laboratory rodents, granulated metrial gland cells which are derived from precursor lymphocyte-like cells of bone marrow origin are included with the decidua (Bell, 1983a&b).

In rodents decidual tissue also appears to secrete a luteotrophic substance which helps to maintain the corpus luteum of early pregnancy, although its action appears to be dependent on the presence of luteinizing hormone from the pituitary (Porter *et al.*, 1982). Decidual cells in rodents are also the source of relaxin which is synthesized under the influence of oestrogen and plays a role in remodelling the collagen architecture of the rat uterus and possibly, in the rhesus monkey at least, reduces myometrial contractions and stimulates endothelial proliferation (Porter *et al.*, 1982).

1.6.2 Embryo-Maternal Cellular Interactions at Implantation

Enders and Schlafke (1969) examined cell interactions at early implantation in six distantly related eutherian species (rat, rabbit, guinea pig, armadillo, bat and ferret). They found in all these species that the first stage of implantation is **apposition** which occurs when the trophoblast and uterine epithelium become apposed with interdigitation of microvilli from these two cell types such that the embryo "will normally not be displaced during subsequent development" (Enders and Schlafke, 1969, p.2). However, embryos at this stage of development can be flushed from the uterus with physiological saline. Species that exhibit a delay of implantation do so at this appositional stage e.g. in rodents with low oestrogen levels during lactation (Finn, 1989).

The second stage of implantation is that of **adhesion** in which the embryo resists displacement by flushing. At this time junctional complexes form between the trophoblast and maternal epithelium (Schlafke and Enders, 1975). The microvilli of the maternal epithelial cells adjacent to the embryo flatten and there are changes in the number and distribution of some of the surface molecules, especially glycoproteins, on the cell coats of both the maternal epithelium and fetal trophoblast. In the mouse, acidic glycoproteins have been shown to be present on both the blastocyst and uterine

epithelium between Days 4 and 7 *p.c.* (peri-implantation period) as a result of binding with concavalin A, ruthenium red and colloidal thorium (Enders and Schlafke, 1974). There is also evidence that at this time glycosyl transferases may catalyse the binding of sugars between oligosaccharides on the cell coats of trophoblast and uterine epithelial cells, thus forming molecular bridges between these cells (Enders and Schlafke, 1974). This adhesive stage is thought to occur in all mammalian species that exhibit implantation whether or not the conceptus is subsequently invasive.

The third stage of implantation which only occurs in species with invasive embryos is that of **epithelial penetration**. This is species specific and can occur in one of three ways: (1)intrusion into, (2)fusion with, or (3)displacement of, the maternal epithelium.

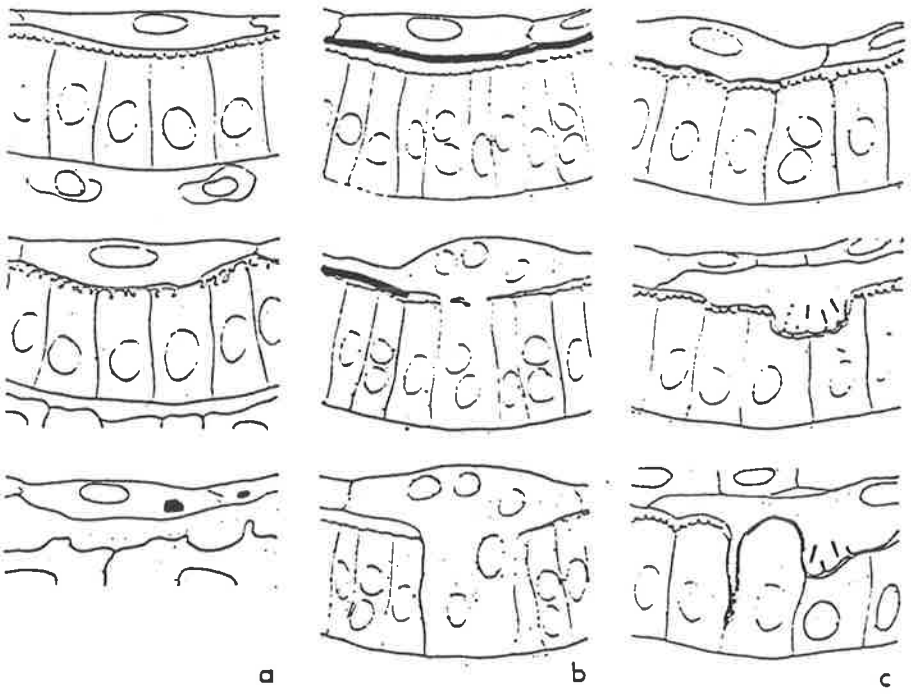
(1) Intrusive implantation is found in a variety of species including the ferret, guinea pig and primates (Schlafke and Enders, 1975; Weitlauf, 1988; Finn, 1989). It occurs when the syncytiotrophoblast penetrates between uterine luminal epithelial cells after forming tight junctions with their lateral cell membranes. It then invades the underlying endometrial stroma with little destruction of the uterine epithelium (Schlafke and Enders, 1975).

(2) Displacement implantation is found in rats, mice and several species of bats (Schlafke and Enders, 1975). It occurs when the trophoblast invades and replaces the uterine luminal epithelium. Trophoblast cells phagocytose maternal epithelial cells after the epithelium has been loosened from its basal lamina (Schlafke and Enders, 1975). The maternal epithelial cells may participate in their own destruction by programmed cell death or apoptosis (Parr *et al.*, 1987; Parr and Parr, 1989b). Recent studies in rats (Schlafke *et al.*, 1985) and mice (Blankenship and Given, 1992) have found that the decidual cells of the maternal stroma also play a part in epithelial penetration, in that decidual cell processes penetrate the uterine luminal epithelial basement membrane before the trophoblast penetrates it. However, trophoblast cell processes of lactationally delayed mouse embryos penetrate at about the same time as those of the decidual cells, presumably after having undergone additional differentiation and perhaps synthesis of proteolytic enzymes (Blankenship and Given, 1992).

(3) Fusion implantation is found in the rabbit and ruminants and is the least common method of epithelial penetration at implantation. In rabbits it involves the fusion of syncytiotrophoblastic knobs with the apical cell membranes of uterine luminal epithelial cells which have themselves become

syncytial. Consequently, the maternal epithelium loses its cytoplasmic characteristics and displays those of the more prolific syncytiotrophoblast. Eventually the maternal nuclei also disappear (Finn, 1989). After fusion in the rabbit the trophoblast penetrates the basal lamina, endometrial stroma and its blood vessels (Enders and Schlafke, 1971a). In ruminants binucleate trophoblast cells migrate and become apposed to single uterine epithelial cells and then fuse at the apical plasma membranes to form trinucleate cells. More trophoblast binucleate cells fuse with these feto-maternal hybrid cells always forming hybrid cells with odd numbers of nuclei (Wooding, 1992). Thus the uterine epithelium is replaced by a population of hybrid cells, but this is the extent of trophoblast invasion of the endometrium in ruminants.

Figure 1.2 Three Types of Epithelial Penetration at Implantation (from Schlafke and Enders, 1975).



Three types of interaction of trophoblast with uterine epithelium during penetration of this epithelium. In all three types, apposition and adhesion precede penetration. However, in *a, displacement penetration* (rat, mouse), the uterine luminal epithelium is readily dislodged from the basal lamina, and the trophoblast comes to lie along areas previously occupied by the displaced uterine cells. In *b, fusion penetration* (rabbit), syncytial trophoblast fuses with a uterine luminal epithelial cell. In *c, intrusion penetration* (ferret, others?), projections of syncytial trophoblast penetrate between uterine epithelial cells.

1.7 Placentation

1.7.1 What is a placenta?

The placenta is a temporary organ composed of both fetal and maternal tissues. The mammalian placenta has five functions, each of which is developed to a greater or lesser degree depending on the stage of pregnancy, placental type, and species. It serves the principal requirements of successful pregnancy which are: "(1) to anchor the conceptus to maternal tissues, (2) to create a physiological lifeline assuring transfer of nutrients and wastes, (3) to provide passive immunity, (4) to prevent immunologic rejection, and (5) to produce hormones" (Blackburn *et al.*, 1988 p.128). These hormones are necessary to maintain pregnancy and prepare the mother for lactation.

Anatomically, there are two types of placenta formed after the fusion of the fetal yolk sac or allantois to the fetal chorion which then make contact with the maternal uterine epithelium. These are the choriovitelline and chorioallantoic placentae, respectively. Traditionally, eutherian mammals have been called 'placentals' as distinct from marsupials. This has ignored the fact that marsupials do indeed have a placenta, although most species have a choriovitelline, or yolk sac, placenta as opposed to the well known chorioallantoic placenta of eutherians. However, not all marsupials fail to develop a chorioallantoic placenta and all eutherians, except some primates, develop a yolk sac placenta (Luckett, 1977).

Mossman (1937, p.177) defined the mammalian placenta as an "apposition or fusion of the fetal membranes to the uterine mucosa for physiological exchange." This definition is broad enough to encompass placentae of both marsupials and reptiles. Narrow definitions only include the eutherian chorioallantoic placenta as a true placenta. Flynn (1923) described the choriovitelline (yolk sac) and chorioallantoic placentae in the Peramelidae (bandicoots). He argued that the concept of a placenta must include structures such as the non-vascular yolk sac placenta of marsupials. Eutherian yolk sac placentae have important physiological functions which involve exchange between mother and fetus. Hartman (1925b) described the intimate association of the yolk sac with the uterine epithelium in the North American opossum, *Didelphis virginiana*, but failed to call this association a placenta despite the fact that he regarded it as having the function of exchange.

More recently, investigations into the functions of the yolk sac placenta in eutherians have shown that it is widespread in this subclass. It plays a significant role in fetal nutrition in the rat (Deren *et al.*, 1966; Padykula *et al.*, 1966; Freeman, Beck and Lloyd, 1981; Freeman and Lloyd, 1983) and horse between Days 10 and 40 (Steven, 1982)

and it facilitates the transfer of antibodies in rats and rabbits conferring passive immunity to the fetus *in utero* (Brambell, 1958). The yolk sac is also the site of differentiation of the primordial germ cells in mammals (Fujimoto *et al.*, 1977). Perry (1981) reported that the guinea pig retains a functional yolk sac placenta until term. The yolk sac in rats and mice also persists and serves such a significant role in fetal nutrition and gaseous exchange that these embryos can survive *in vitro* for up to 40 hours when explanted with an intact yolk sac at any time throughout the period of organogenesis (New, 1973). Williams *et al.* (1975a&b) found that the rat yolk sac takes up exogenous proteins by pinocytosis when cultured *in vitro*.

Krause and Cutts (1985b) examined the ultrastructure of the yolk sac placenta of the North American opossum (*Didelphis virginiana*) and found that both yolk sac trophoblast cells and uterine epithelial cells exhibit features characteristic of cells active in the transport of materials. These findings and others (see below) fit the broad definition of the placenta. That concept includes three types of placenta as suggested by Flynn (1923): (1) the chorioallantoic placenta, (2) the vascular (trilaminar) region of the yolk sac placenta, and (3) the bilaminar region of the yolk sac that lacks fetal blood vessels. Using this definition most reproductive biologists agree that marsupials are indeed 'placental' mammals. So, to refer to eutherians as placental mammals as distinct from metatherian mammals implies a distinction which has no foundation in reality.

1.7.2 Trophoblast: the Concept

The ectoderm of the chorion in all amniotes is the trophoblast (Blackburn *et al.*, 1988). It is derived from the ectodermal layer of the blastocyst called the trophectoderm (extra-embryonic ectoderm). Trophoblast plasticity has allowed diverse changes to occur over the 120 million years since it first evolved (Blackburn *et al.*, 1988) such that trophoblast exhibits a variety of structures and functions in different amniotes. However, the essential characteristic of trophoblast is its capacity to absorb exogenous materials. In those species that exhibit placentation (viviparous reptiles, marsupials and eutherians) the trophoblast is the major embryonic cell type involved in exchange between the mother and fetus. Blood vessels that develop in the mesoderm of the yolk sac and/or allantois lie in close proximity to trophoblast cells of the chorionic epithelium to form the choriovitelline and chorioallantoic placentae, respectively.

Luckett (1977) examined the ontogeny of amniote fetal membranes which have been conserved, although modified, during their evolution (see above). They develop from all three embryonic germ layers and Luckett regarded them, and structures derived

from them, as homologous in all amniotes and thus concluded that their morphogenesis and structure are extremely useful in determining phylogenetic relationships among amniotes. Hamilton and Mossman (1972) and Mossman (1952, 1987) had come to a similar conclusion after finding that the fetal membranes have changed much less than has adult morphology during the course of the evolution of the mammalian families and orders. Luckett (1993) developed his thesis further and suggested that relationships within and between orders of Eutheria may be determined using fetal membrane characteristics but that these need to be assessed in conjunction with a suite of other anatomical and biochemical characteristics.

Viviparity and placentation have evolved many times independently in the various vertebrate lineages as indicated previously. Viviparous reptiles commonly develop a yolk sac placenta as well as a chorioallantoic placenta (Weekes, 1935; Luckett, 1977; Thompson, 1977; Yaron, 1977; Blackburn *et al.*, 1984; Blackburn, 1993; Stewart, 1993). Stewart and Blackburn (1988) considered these to be homologous with mammalian placentae because they are derived from the same embryonic structures, despite the fact that they have evolved independently. Similarly, Blackburn *et al.* (1988) considered trophoblastic tissue (the fetal tissue that is active and often invasive in the different placental types) which is derived from extraembryonic ectoderm to be homologous in all amniotes.

Despite the common view of reproductive biologists that, at the very least, all mammalian trophoblastic tissues are homologous (Luckett, 1977; Tyndale-Biscoe and Renfree, 1987; Blackburn *et al.*, 1988) Lillegraven (1985) and Lillegraven *et al.* (1987) questioned the authenticity of the marsupial trophoblast and reopened the discussion of early mammalian evolution and hence the relationships of marsupials to eutherians. It was an anathema to these palaeontologists to consider non-eutherian placentae as true placentae.

Lillegraven (1985) and Lillegraven *et al.* (1987) argued that the marsupial trophoblast is different to that of eutherians because the marsupial unilaminar blastocyst does not develop an ICM like that of eutherians and thus differentiation of trophectoderm does not occur at this early stage of embryonic development. These workers speculated, in the "absence of pertinent experimental studies" (Lillegraven *et al.*, 1987, p.294) that, because the cells of the unilaminar blastocyst destined to become the embryo proper in marsupials are not recognisable, the marsupial trophoblast cannot be homologous with eutherian trophoblast. This is not a logical conclusion when all the evidence is considered (see below).

Firstly, Hill (1910) was convinced that he could distinguish a formative and a non-formative hemisphere in the unilaminar blastocyst of *Dasyurus viverrinus*. Similarly, Hartman (1916) found the same for *Didelphis virginiana* and Hill again (1918) identified presumptive embryonic cells in the unilaminar blastocysts of *Didelphis aurita*. Selwood (1986) studied the blastocysts of the Hill Collection which included eleven marsupial species from four families and concluded that, although marsupial embryos are polarised during cleavage, (see above) no differences between cells of unilaminar blastocysts could be found. Also the timing of differentiation between embryonic and extra-embryonic cells in the unilaminar blastocyst discernible in histological sections varies in marsupials.

In a more recent extensive review of embryonic development in marsupials Selwood (1992) has suggested that although the two cell populations are not visible histologically, they are nevertheless present. In paraffin sections of early embryos two cell types can be distinguished but a little later these differences are not apparent. The cells of unilaminar blastocysts appear morphologically similar in embryos of 32-50 cells in the Virginian opossum, 130-1000 cells in the native cat, 64-1000 cells in the brown Antechinus, 200-2200 cells in the stripe-faced dunnart, 50-643 cells in the greater glider, 65-101 cells in the red kangaroo and 75-1000 cells in bandicoots (Selwood, 1992). Selwood concluded that the expansion of the blastocyst results in compression of its cells to as little as 1-3μm thick and such flattening of epithelial cells from a variety of tissues would result in there having a similar appearance. However, Selwood has suggested that in early embryos the future embryoblast cells occur in the yolky hemisphere and at the time of endoderm differentiation trophoblast and embryoblast are easily distinguishable in whole mounts by what she described as a "suture line" at the boundary between them.

Secondly, although Lillegraven *et al.* (1987) referred to the discussion by Tyndale-Biscoe and Renfree (1987) on early embryonic development in the Insectivora they appear not to have been convinced that this confirms marsupial trophoblastic homology. Other discussions on the subject (Wimsatt, 1975; Blackburn *et al.*, 1988) argued that the eutherian insectivores are similar to marsupials in this regard. The tenrec (*Hemicentetes*) embryo develops in a very similar way to that of marsupials, while that of the elephant shrew (*Elephantulus*) shows an even later stage of development (approximately 100 cells) before presumptive embryonic and extraembryonic differentiation occurs (Wimsatt, 1975). Yet these species are eutherians and subsequently develop eutherian trophoblast tissue. The most parsimonious explanation would presume that the timing of early embryonic differentiation of trophoblast does not alter its subsequent function and that if this

applies for insectivores then, in the absence of any evidence to the contrary, it also applies to marsupial trophoblast.

Lillegraven's *et al.* (1987) second argument against marsupial-eutherian trophoblast homology is on the grounds that the eutherian trophoblast is distinguished by its role as an immunological barrier to fetal allograft rejection. They first assumed that there is no evidence for an immunological barrier in marsupial pregnancy. Walker and Tyndale-Biscoe (1978) and Rodger *et al.* (1985) immunized female tammar wallabies with skin grafts from their prospective male mates and then analysed the effect on subsequent pregnancies sired by the same male. The normal time to rejection of skin grafts from an allogeneic donor would be about 7-10 days. After preimmunization with the same histocompatability antigens graft rejection would be accelerated to perhaps 3-4 days. In tammar wallabies the embryo has been reported to implant at day 17 of the 27 day pregnancy just after the time that the shell membrane is shed (Denker and Tyndale-Biscoe, 1986). If the marsupial embryo has no immunological protection from its mother then preimmunization with paternal antigens would cause its early rejection during this 10 day period of development when it is not surrounded by the shell membrane. However, these studies found no effect on the success of pregnancy after such immunization even with repeated pregnancies to the same male (Walker and Tyndale-Biscoe, 1978; Rodger *et al.*, 1985).

More research is necessary to test the ability of the marsupial placenta to protect the fetus from the maternal immune system. It may be that the mechanisms are no different to those which occur in eutherians. Kaye (1979) concluded that viviparity and placentation in vertebrates evolved after the development of the vertebrate immune system and that all vertebrates that develop a placenta during pregnancy, by necessity, evolved mechanism(s) to prevent immunological rejection.

Lillegraven *et al.* (1987) also argued that the marsupial trophoblast is not homologous with eutherian trophoblast because it does not stimulate a decidual cell reaction (DCR). Decidualization of the endometrium is a common occurrence in eutherians with invasive placentae which serves to protect the uterus from uncontrolled trophoblast invasion (Morriss, 1975; and see above). However, in different eutherian species the DCR is extremely variable in its timing, extent and does not occur at all in some eutherians such as New World monkeys, aardvarks, pangolins, cetaceans, sirenians, perissodactyls and artiodactyls (Mossman, 1987). The assumed absence of a DCR in marsupials cannot therefore be used as an argument against trophoblast homology in marsupials and eutherians.

Thus, Lillegraven's *et al.* (1987) arguments that no trophoblast occurs in marsupials are refuted on the grounds that they do not take into account the incredible diversity in morphology of the eutherian placenta. Placental types follow taxonomic groupings, particularly at the levels of orders, suborders and superfamilies (Luckett, 1977, 1980, 1985, 1993). However, it is never argued that the variation in form of trophoblast in the different eutherian groups makes it non-homologous. Given all the arguments, it seems logical to assume that the marsupial trophoblast is homologous to the eutherian trophoblast and that the marsupial placenta is simply a variation of therian placentae of which there are two general types, chorioallantoic and choriovitelline.

1.7.3 Trophoblast Cytology

There are several morphological types of trophoblast cells which are distinguishable by their size and distribution of their nuclear material. Trophoblast may be syncytial, binucleate, multinucleate giant cells, or contain a single huge polyploid nucleus and is then called a trophoblast giant cell (TGC). Nuclear material in TGCs is aggregated into a central clump forming a single nucleus. TGCs are found in the chorionic ectoderm of both the bilaminar omphalopleure (non-vascular yolk sac placenta) and the chorioallantoic placenta (Mossman, 1987). TGCs occur in the fetal chorion of most eutherian groups, with the exception of artiodactyls. The cytoplasm of these giant cells appears vesicular in paraffin sections because of the presence of glycogen and lipids (Mossman, 1987). Another type of trophoblast cell which is diploid, and not remarkable in size, is found in the placentae of all mammals studied. All trophoblast cells are capable of taking up exogenous materials and some synthesize and secrete steroid and/or peptide hormones.

1.7.4 Eutherian Placental Classification

Eutherian chorioallantoic placentae have been classified in two ways based on their histological structure and gross anatomy. No such formal classification exists for yolk sac placentae although marsupial placentation is sometimes described using this terminology (Amoroso, 1952; Krause and Cutts, 1985b; Denker and Tyndale-Biscoe, 1986; Tyndale-Biscoe and Renfree, 1987).

1.7.4.1 Histological Classification

The extent of trophoblastic invasion determines, at least in part, the number of cell layers between fetal and maternal bloodstreams in vascularized placentae. The histological structure of the placental barrier is the basis for Grosser's system of classifying chorioallantoic placentae (Amoroso, 1952). Names have been assigned

based on the maternal tissue in contact with the fetal chorion. Using this criterion four placental types were designated:

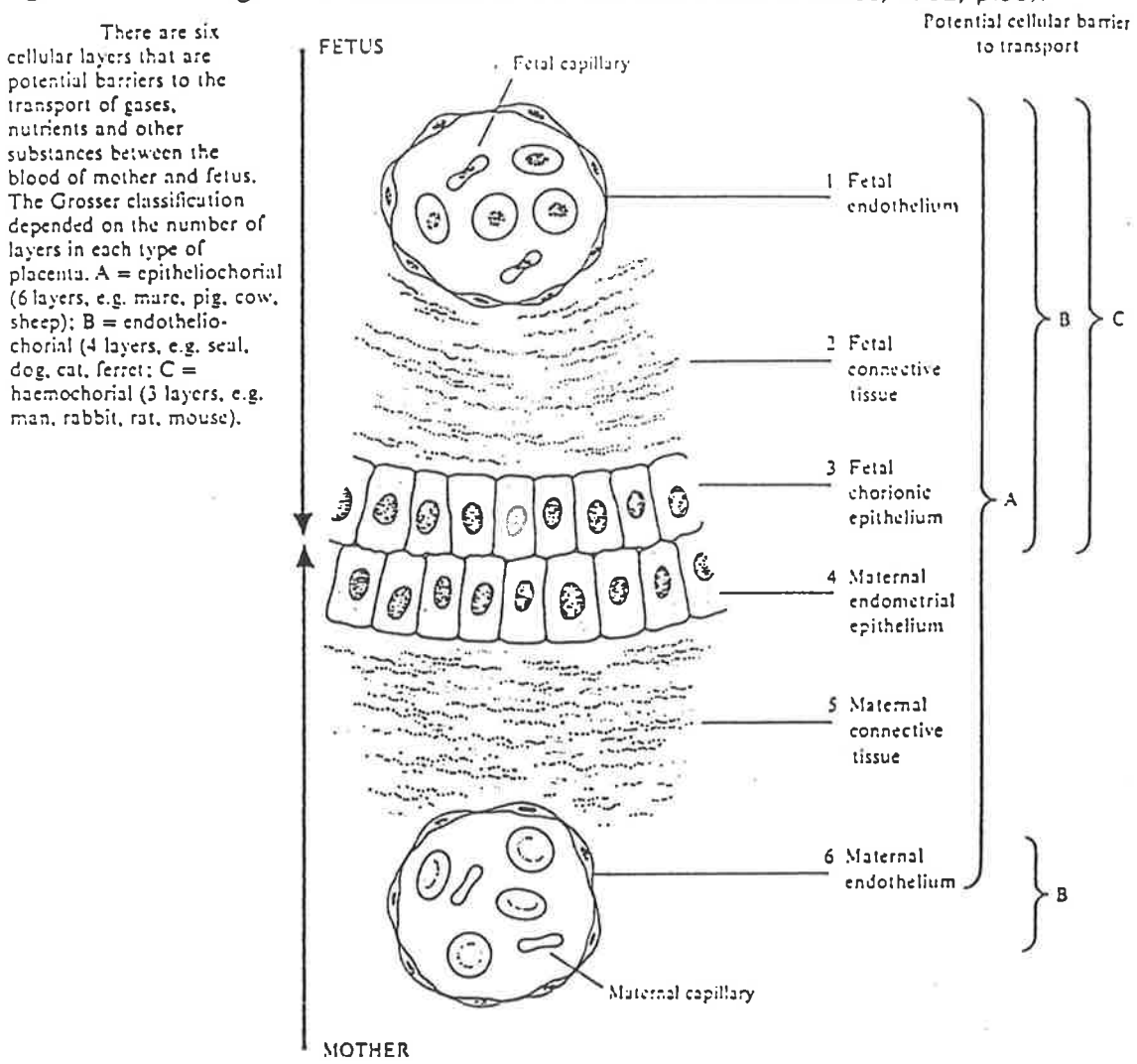
(1) Epitheliochorial (in horses and pigs) in which there is no invasion of the endometrium; the chorion (trophoblast plus somatic extraembryonic mesoderm) becomes apposed to the uterine epithelium such that there are six cell layers between fetal and maternal blood, three of fetal origin and three of maternal origin.

(2) Syndesmochorial (in ruminants) in which there is slight invasion resulting in the disappearance of the uterine epithelium and five cell layers remain between fetal and maternal blood.

(3) Endotheliochorial (in carnivores) in which there is more trophoblast invasion and fetal blood is separated from maternal blood by only four cell layers, three fetal and the fourth the endothelium of maternal blood vessels.

(4) Haemochorial (in rodents, lagomorphs and primates) in which there is also invasion and erosion of the maternal endothelium such that the fetal blood is only separated from maternal blood by three cell layers all of fetal origin i.e., the outermost fetal tissue (trophoblast) is bathed in maternal blood. The haemochorial placenta is further categorized into three types: (a) haemomonochorial, (b) haemodichorial and (c) haemotrichorial placentae which relate to the number of trophoblast cell layers present in the full-term placenta. The haemotrichorial placenta (found in rats and mice) has three layers of trophoblast. The haemodichorial placenta (found in rabbits) has two cell layers of trophoblast. The haemomonochorial placenta (found in humans) has only one trophoblast cell layer between the fetal and maternal bloodstreams at term, the syncytiotrophoblast, as well as the fetal endothelium and fetal stroma (Steven, 1975).

Figure 1.3 Histological Classification of the Placenta (from Renfree, 1982, p.50).



Although this classification system has appeared adequate for many years recent studies using the transmission electron microscope (TEM) revealed the finer structural detail of the relationships between fetal and maternal tissues. For example, a recent TEM investigation of the placenta of the insectivore *Suncus murinus* by Kiso *et al.* (1990) has shown that in the last few days of gestation (Days 24 - 30) the chorioallantoic placenta has lost the continuity of the syncytiotrophoblast between fetal and maternal capillaries. Consequently, these workers have confirmed that an endothelio-endothelial placenta occurs as suggested by Mossman and Owers (1963) and that the categorisation of the chorioallantoic placenta for this species as haemochorial (Brambell and Perry, 1945) or endothelio-chorial (Wimsatt *et al.*, 1973) are both erroneous. A second example of the refinement of our knowledge of the placenta is that of the syndesmochorial classification. Thus the cell arrangement found in the ruminant placenta, has recently been renamed a synepitheliochorial placenta because the maternal epithelium does not completely disappear but trophoblast

binucleate cells fuse with it forming a hybrid syncytium (Wooding, 1992). Obviously, the resolution of the light microscope is insufficient to accurately determine the details of the cellular layers present between maternal and fetal bloodstreams in all cases.

The number of cell layers between the maternal and fetal bloodstreams does not necessarily determine the efficiency of transfer of nutrients and gases across the placenta (Amoroso, 1952; Mossman, 1987) and therefore the potential fetal growth rate. Some species of eutherians, the horse for example, have very well developed neonates that can walk soon after birth yet the epitheliochorial placenta of these animals has the maximum possible number of cell layers between the two bloodstreams. Rodents, on the other hand, have highly invasive placentae with minimal cell layers between the two bloodstreams but the neonates in most species are comparatively altricial at birth (Eisenberg, 1981).

The criterion of adequacy of placental function in this regard is that nutrients, gases and wastes get transferred at the necessary rate for embryonic growth. Just as the histological structure of each placental type satisfies the need for adequate exchange between mother and fetus so too does the gross structural type of placenta, that is, the choriovitelline and chorioallantoic placentae. The yolk sac placentae of the rat and rabbit have been found to be the site for protein and other macromolecular uptake, including protein-bound vitamin B_{12}, by the embryo (Deren *et al.*, 1966; Padykula *et al.*, 1966). It is likely that the yolk sac placenta of marsupials is also involved in macromolecular uptake and fetal metabolism as the composition of yolk sac fluid is very different from that of uterine lumen (Renfree, 1973). There is a high concentration of amino acids, glucose and proteins of fetal origin in the yolk sac fluid of post-implantation tammar wallaby embryos whereas that of the uterine lumen contains macromolecules at much lower concentrations and of maternal origin (Renfree, 1973). It may be that wastes are not transferred to the mother in some species of marsupials and that the allantois acts as a receptacle for wastes excreted by the embryonic kidneys since the concentration of urea is high in the allantois of the tammar (Renfree, 1973). This also occurs in extant oviparous sauropsids, monotremes, ungulates and carnivores (Krause and Cutts, 1985a).

In humans the allantois appears as an outpocket, or diverticulum, of the caudal region (future hindgut) of the yolk sac and has no function of its own. However, the mesodermal blood vessels of the allantois become the umbilical vessels thus forming the blood supply to the placenta (Luckett, 1977), hence the name chorioallantoic placenta. Later in development the allantois becomes continuous with the urinary

bladder and eventually its lumen is obliterated, at which time it becomes the urachus and, after birth, the median umbilical ligament (Boyd and Hamilton, 1970).

1.7.4.2 Gross Classification

Gross anatomical placental classification does not necessarily coincide with the histological classification (Amoroso, 1952). The categories are:

(1) Diffuse placenta (in pigs, horses, donkeys and whales) is attached to the uterus over the entire surface of the chorion. This type is generally epitheliochorial.

(2) Cotyledonary placenta (in ruminants) has many localised sites of attachment to the uterus, for example, there are 80-90 in the sheep (Mossman, 1987). The fetal part at each site is called a cotyledon and gives the placenta its name, while the maternal part is the caruncle, and the two parts together comprise the placentome. Cotyledonary placentae are normally syndesmochorial (synepitheliochorial).

(3) Zonary placenta (in carnivores) appears as a band of attachment around the 'equator' of the fetus. This placenta is endotheliochorial. In insectivores the placenta is zonary but also haemochorial.

(4) Discoid placentae take on different histological forms but have a single disc shaped site of attachment. In megachiropteran bats (fruit bats) the placenta is endotheliochorial. In humans and rodents the placenta is haemochorial. In rhesus monkeys the placenta is also haemochorial but the gross shape is bi-discoid.

Figure 1.4 Placental Classification by Gross Anatomy (from Renfree, 1982, p.51).

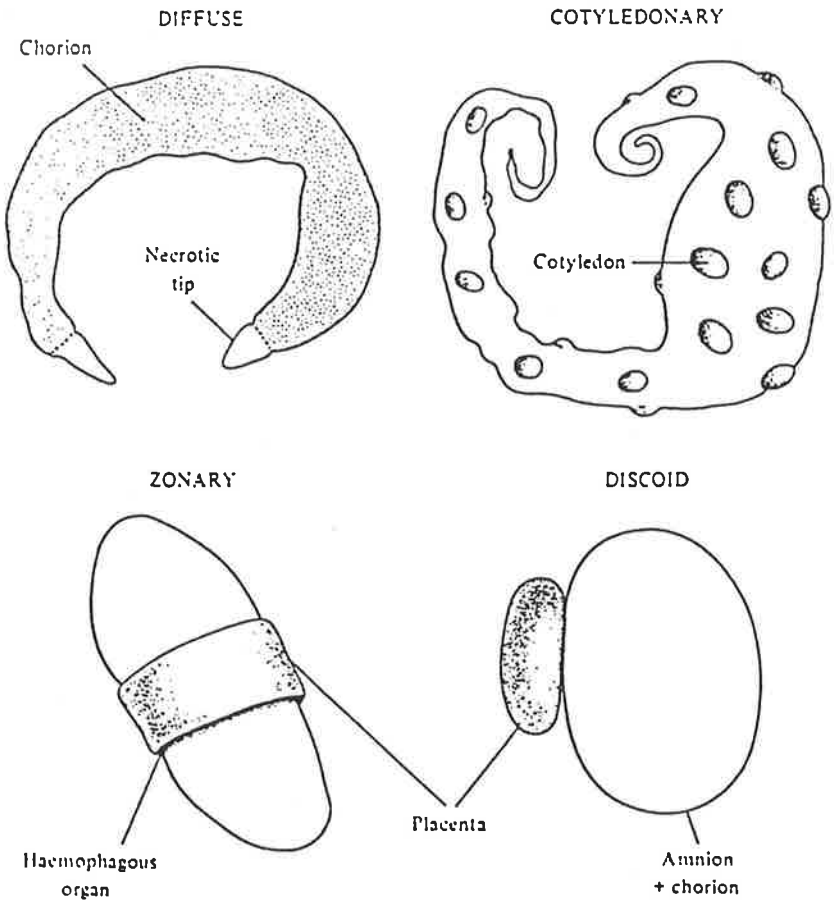


Illustration of the four main types of mammalian placenta: diffuse, cotyledonary, zonary and discoid. Diffuse placentae, where there is an attachment of the chorionic sac over its entire surface, are found in the horse, pig, camel, many dolphins and whales, and in the lemurs. Cotyledonary placentae, in which the chorion has specialized villi restricted to circular or oval areas over the chorionic sac, are characteristic of the ungulates. Zonary placentae may be complete as shown, in which the chorionic villi are restricted to an equatorial girdle, bounded by the (green) haemophagous organ, as found in the dog, cat and genet, or incomplete (circular or equatorial) regions as in ferrets, racoons, mink and bears. The haemophagous organ in these types may be central or marginal, distinguishing them from the discoid type which has no marginal effusion of blood. Finally, the discoid placenta characteristic of man, rat, mouse or guinea pig is single, and the chorionic villi are arranged in a circular plate, or may be double as in macaque monkeys.

Amoroso (1952) took great pains to avoid giving a static description of placentation and emphasized that the development of the placenta is a dynamic process that occurs over a period of time which is species specific. All embryos rely on histotrophic nutrition for at least several days and after implantation it takes some time for the definitive placenta to develop. For example, in the human on Day 6 post-fertilization trophoblast cells above the embryoblast begin to invade through the maternal epithelium. On Day 13 the cytotrophoblast has begun to push up into the syncytiotrophoblast marking the beginning of chorionic villous formation, and on Day 14 there are villi all over the surface of the chorion (Luckett, 1971). By Day 21 the stem villi have developed small blood vessels and blood cells in their mesodermal core. Once the heart begins to beat during week 4 fetal blood flows through the villous

capillary system such that physiological exchange may take place between the maternal and fetal bloodstreams (Boyd and Hamilton, 1970; Benirschke and Kaufman, 1990). During the three weeks between the embryo sinking beneath the surface of the uterine epithelium and the time that the embryonic circulation is established the embryo gains its nutrition from the glands that it has eroded as well as from the decidual cells. This is best described as histotrophic nutrition after which haemotrophe is the nutritional source (from week 4). At this time there are two layers of trophoblast (syncytiotrophoblast and cytotrophoblast), as well as the fetal stroma and fetal endothelium, intervening between the maternal and fetal blood. It is not until the third month that the characteristic discoid shape of the human placenta is evident and then in the fourth month the villous cytotrophoblast layer begins to disappear and the fetal endothelium comes to lie closely apposed to the syncytiotrophoblast (Boyd and Hamilton, 1970) thus markedly reducing the distance between maternal and fetal blood. Subsequently the placenta continues to grow with the expansion of the uterus and the decidua projects into the intervillous spaces forming septa which partially divide the placenta into lobes (Boyd and Hamilton, 1970).

1.7.5 Marsupial Placental Structure

Marsupials have very short gestation periods which result in the birth of altricial young. Pregnancy is generally accommodated within the period of one oestrous cycle and in some species pregnancy occupies the luteal phase of the cycle. In all marsupial species investigated the placenta develops relatively late in gestation. The placentae of only twenty species from eight Families of marsupials have been investigated which is less than 10% of the 250 extant marsupial species (Sharman, 1976). Most of this work has only been performed at the light microscope (LM) level of resolution and thus details of cellular structure and function are not known.

In most marsupial species the yolk sac forms the definitive placenta with only the members of the Peramelidae forming an invasive chorioallantoic, as well as a choriovitelline (yolk sac), placenta (Padykula and Taylor, 1976, 1977, 1982). Hence, in most marsupial species the yolk sac transports both gases and nutrients from the mother to the embryo and the allantois becomes a receptacle for nitrogenous wastes. Trophoblast appears to function for exchange whether it is associated with the yolk sac or allantois.

Hughes (1974b) classified marsupial placentae into three types based on the invasiveness of the trophoblast and the extent of development of the allantois:

Type 1 a non-invasive yolk sac placenta with "no implantation" and the lack of fusion of the allantois to the chorion

Type 2 a slightly invasive yolk sac placenta with "rudimentary implantation" and in these too the allantois does not fuse with the chorion

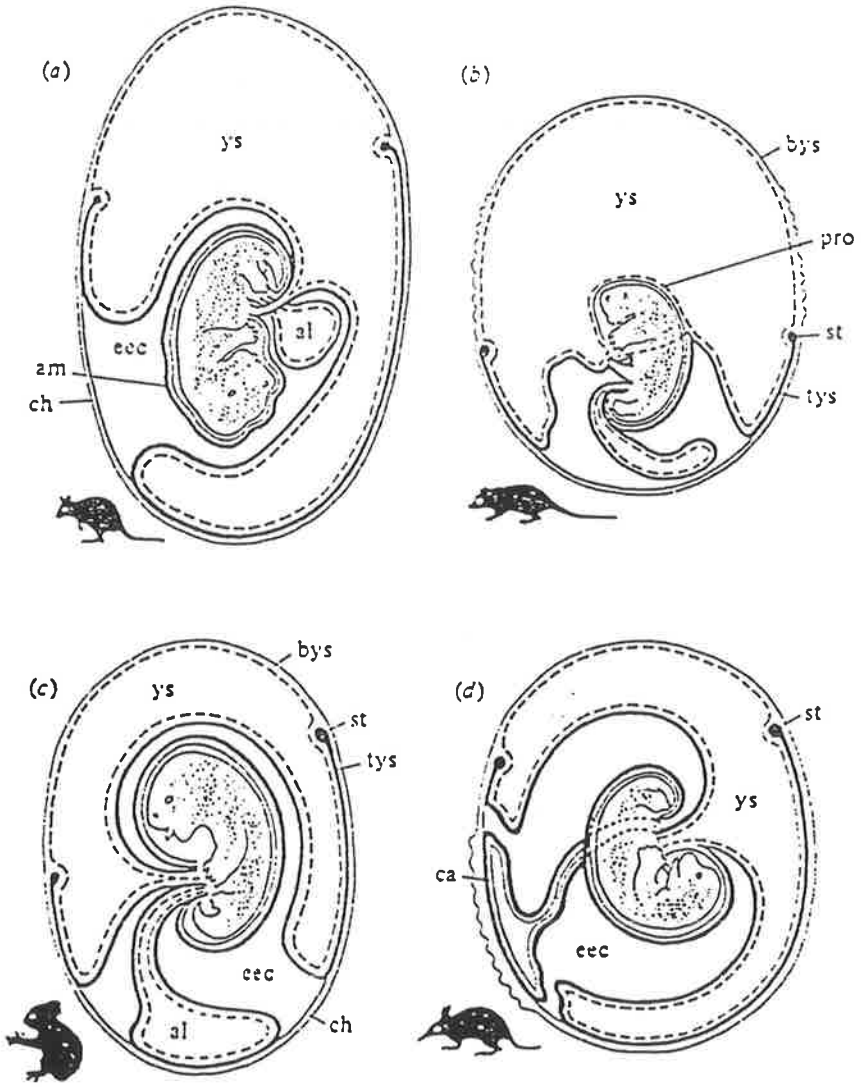
Type 3 a non-invasive yolk sac placenta followed by an invasive villous chorioallantoic placenta.

Of those species investigated the phalangerids, petaurids, macropods, didelphids and dasyurids have either Type 1 or Type 2 placentae. The allantois of species with Types 1 and 2 placentae has not been recorded as fusing with the chorion except in the koala and wombat but in these species it is not clear whether the chorioallantois is involved in feto-maternal exchange (Hughes and Green, 1994). Type 3 placentae only occur in the peramelids. Tyndale-Biscoe and Renfree (1987) have reclassified these types and allocated koalas and wombats to a separate category because their allantois fuses with the chorion but does not vascularize a placenta (Type 3) and placed the bandicoots in a fourth group (Type 4 which is the same as Hughes' Type 3).

Hughes implied that implantation only occurs if the trophoblast invades the uterine epithelium but it must be assumed that in Hughes' classification Type 1 placentation probably involves at least apposition, if not adhesion, such that implantation can be said to have occurred using the criteria of Schlafke and Enders (1975). The distinction between Types 1 and 2 then would be only the invasiveness of the placenta.

In all mammals, during early embryogenesis extra-embryonic mesoderm proliferates and migrates away from the embryonic disc intervening between the trophectoderm and the extra-embryonic endoderm of the yolk sac in an annular region. There is a limit to the extent of this mesodermal migration such that the yolk sac has an annular trilaminar region surrounding the embryo which is vascularized and the rest remains avascular with only two cell layers, the trophectoderm and the endoderm occurring. A large blood vessel, the sinus terminalis, follows an equatorial path around the yolk sac at the junction between the trilaminar region (TYS) and the bilaminar region (BYS) (Luckett, 1977). Later in development in marsupials these two regions may form placental associations with the endometrium.

Figure 1.5 Marsupial Placental Types (from Tyndale-Biscoe and Renfree, 1987, p.312).



The arrangement of the fetal membranes in species representing the four types. (*a*) Type 1: *Setonix brachyurus* at 21 days after RPY. At later stages the allantois is larger and both it and the fetus are more completely enclosed in the folds of the yolk sac. Redrawn from Sharman (1961*b*). (*b*) Type 2: *Dasyurus viverrinus*. The allantois approaches the chorion but does not fuse with it. As it retreats from the chorion at later stages its blood vessels degenerate. Over part of the bilaminar yolk sac close attachment to the endometrium occurs. Redrawn from Hill (1900*b*). (*c*) Type 3: *Phascolarctos cinereus*. The allantois makes contact with the chorion to form a chorioallantoic placenta that attaches to the uterine epithelium. Redrawn from Semon (1894). (*d*) Type 4: *Perameles nasuta*. Extensive choriovitelline and chorioallantoic placental attachments. Redrawn from Hill (1898). al, allantois; am, amnion; bys, bilaminar yolk sac; ca, chorioallantois; ch, chorion; eec, extra embryonic coelom; pro, proamnion; st, sinus terminalis; tys, vascular yolk sac; ys, yolk sac. The ectoderm is represented by a thin line, the endoderm by a dotted line, somatic mesodern medium line and the splanchnic mesoderm by a thick line.

1.7.5.1 Type 1 - Didelphidae

McCrady (1938) found that in *Didelphis virginiana* at the time of implantation the yolk sac becomes very folded and these folds sink into adjacent crypts in the endometrium although there is no invasion into the epithelium. This then is best described as a Type 1 choriovitelline placenta. McCrady (1938) thought that the marsupial allantois is degenerate and lacks the respiratory function seen in the sauropsids but, rather, it serves as a receptacle for urine. The choriovitelline placenta facilitates nutritional uptake and gaseous exchange.

Krause and Cutts (1985b) investigated placentation in the same species at the ultrastructural level during all the late stages of gestation (Day 9 to full-term). They confirmed Hughes' (1974b) Type 1 classification based on early work by McCrady (1938) and found that both uterine epithelial and trophoblast cells had features suggestive of active transport. Trophoblast cells had numerous microvilli, an "apical endocytic complex" and folded lateral and basal cell membranes, and uterine epithelial cells had many inclusions and basal mitochondria. However, there were no junctional complexes found between trophoblast and maternal epithelial cells.

1.7.5.2 Type 1 - Macropodidae, Phalangeridae and Petauridae

Sharman (1961) described placentation at the light microscopical level of resolution in five genera of diprotodont marsupials: *Trichosurus vulpecula* (Phalangeridae), *Pseudocheirus peregrinus* (Petauridae), *Protemnodon rufogrisea, Setonix brachyurus* and *Potorous tridactylus* (Macropodidae). The trophoblast in all five species was reported to be non-invasive and the uterine luminal epithelium remained intact throughout gestation. Thus they fit into Hughes' (1974b) Type 1. It should be noted that Hughes (1984) modified his classification of *Potorous tridactylus* and *Protemnodon rufogrisea* placentae, to Type 2 placentae as these species were found subsequently to have slightly invasive placentae (Shaw and Rose, 1979; Walker and Rose, 1981) although the extent of this invasion was not detailed in these studies. The early studies were sometimes of only one pregnant female at unknown stages in gestation which emphasizes the need to study placental development at a range of gestational stages, and to determine in particular its structure shortly before term, before definite conclusions can be made about the final development of maternal-fetal cellular relationships.

As there was no erosion of the uterine luminal epithelium in pregnancy in the species studied by Sharman the fetal membranes could be classified as forming an epitheliochorial yolk sac placenta. The uteri of *Trichosurus* and *Pseudocheirus* were

well-vascularized and fetal and maternal bloodstreams appeared to be closely apposed because nuclei in the intervening cell layers were apparently displaced which suggests a reduction in diffusion distance for feto-maternal exchange as occurs in the chorioallantoic epitheliochorial placenta of the pig (Enders, 1982). The uteri of the macropods were comparatively avascular (Sharman, 1961). However, TEM investigations are required to reveal the fine structural detail of the cell layers present. In all five species glycogen and other PAS-positive material emanating from the uterine glands were found between the endometrium and fetal membranes where they were separated in both vascular and non-vascular regions of the yolk sac. Embryonic nutrition was thought to be primarily histotrophic rather than haemotrophic in these species (Sharman, 1961).

1.7.5.3 Type 2 - Didelphidae

Enders and Enders (1969) investigated placentation in the four-eyed opossum (*Philander opossum*) at both the LM and TEM levels of resolution. They concluded that this species has a more "advanced" placenta than *Didelphis*. Embryos shared a common yolk sac after fusion and subsequent rupture of the bilaminar omphalopleure of neighbouring embryos. Near the sinus terminalis, in an annular zone, a modified part of the trilaminar yolk sac was found to penetrate the uterine epithelium discontinuously but the maternal endothelium remained intact throughout gestation (an endotheliochorial placenta). Trophoblast cells at this annular margin of the vascular yolk sac were giant cells with several nuclei and were closely applied to the maternal epithelial cells. Several stromal cells that looked like slightly enlarged fibroblasts were found at the sites of trophoblast invasion into the endometrium which Enders and Enders thought might "constitute a primitive decidual reaction". Late in pregnancy the trophoblast formed areas of adhesion with the endometrium and desmosomes could be seen between trophoblast and maternal epithelial cells. The trophoblast cells of the bilaminar yolk sac had features suggestive of absorptive activity while endodermal cells appeared to be involved in synthetic processes. Uterine gland secretions were found between the epithelium and trophoblast in the region of the non-vascular yolk sac.

1.7.5.4 Type 2 - Dasyuridae

Hill (1900) examined the fetal membranes of the dasyurid native cat *Dasyurus viverrinus* and found, firstly, that the allantois was not well-vascularized and that by the end of gestation it had degenerated from its previously well developed state. Secondly, the vascular yolk sac placenta followed the contours of the uterine

epithelium although the capillary systems of both the fetus and the endometrium were not well developed in this region. Hill concluded that the vascular yolk sac placenta in *Dasyurus* may only be important for gaseous exchange because of its comparatively poorly developed capillary network while the non-vascular yolk sac placenta probably was important for nutrient uptake. There was extensive invasion of the maternal epithelium in an annular area of the BYS adjacent to the sinus terminalis. Trophoblast cells became syncytial and by the last day of pregnancy they surrounded large sinuses of maternal blood. However, this author did not make it clear whether or not the maternal endothelium had been eroded by trophoblast.

Hughes (1974b) examined a single female *Sminthopsis crassicaudata* individual which "had completed at least 12 days out of the 16 days gestation" (p. 182). This assumed gestation length is not strictly correct as Selwood (1987) and Bennett *et al.* (1990) have found that the gestation period (from fertilization to birth) in this species is 13.5 days although, it is not infrequently found that birth occurs up to 16 days after copulation as sperm storage in the isthmus of the oviduct can take place for up to 3 days before ovulation in this species (Selwood, 1987; Breed *et al.* 1989) making timing of pregnancy difficult to determine accurately. Fetuses from the animal in Hughes' study (1974b) measured 2.5 mm crown-rump length (CRL). Preliminary investigations in this laboratory have shown that near term fetuses are at least 3.5 mm with an exceptional 4.5 mm fetus being found. The fetuses in Hughes' study were probably not full-term. Consequently, placental development may not have been complete.

Nevertheless, Hughes (1974b) found a similar mode of implantation in *Sminthopsis* to that of *Dasyurus*. Attachment to the uterine epithelium occurred in both the bilaminar and trilaminar yolk sac regions. Large trophoblast cells had eroded the uterine epithelium in discontinuous areas in an annular zone of the bilaminar yolk sac next to the sinus terminalis, particularly at the tops of uterine folds. Uterine epithelial cells were often found completely surrounded by cytoplasm of trophoblast cells but no fetal syncytium was found. The endothelium of the maternal blood vessels appeared to have remained intact. Attachment in the region of the trilaminar yolk sac was confined in the main to interdigitation between trophoblast and epithelial cells. The allantois was small and not fused with the chorion. As this study was only conducted at the LM level of resolution the fine detail of the cellular interactions between mother and fetus were not determined.

1.7.5.5 Type 3 - Phascolarctidae and Vombatidae

Koalas (*Phascolarctos cinereus*) and wombats (*Vombatus ursinus*) have similar placentae (Caldwell, 1884; Amoroso, 1952; Hughes, 1974b). The vascular allantois, unlike those of other marsupials discussed so far, fuses with the chorion in the full-term fetus but no chorionic villi develop and the chorion does not appear to fuse with the uterine epithelium. Similarly, there is no attachment in the region of the vascular yolk sac. In an annular zone of the non-vascular yolk sac adjacent to the sinus terminalis enlarged trophoblast cells occasionally penetrate the uterine epithelium. Implantation is constituted, for the most part, by shallow interdigitation between fetal and maternal cell processes in the non-vascular yolk sac in these species.

1.7.5.6 Type 4 - Peramelidae

The discoid chorioallantoic placenta of the peramelids (bandicoots) is unique to this marsupial family (Flynn, 1923; Padykula and Taylor, 1977). Bandicoots also develop both vascular and non-vascular yolk sac placentae but their functions in fetal and maternal exchange were considered by Hughes (1984) to be less important than those of the chorioallantoic placenta in late gestation. Flynn (1923) suggested that because the yolk sac forms earlier than the chorioallantoic placenta it has importance in the early post-implantation stage of pregnancy. Presumably, the chorioallantoic placenta of the bandicoots allows the transfer of wastes from the fetus to the mother as it does in eutherian mammals.

In bandicoots uterine epithelial cells adjacent to the yolk sac and chorioallantoic placentae are large and multinucleate. The nuclei are designated as homokaryons because they are all alike: euchromatic with conspicuous peripheral nucleoli (Padykula and Taylor, 1977). Endometrial stromal 'prongs' are found very close to the trophoblast despite the fact that there is no invasion of the uterine epithelium in the region of the yolk sac. Consequently, maternal capillaries come to lie in close proximity to fetal tissues and physiological exchange most likely occurs in these areas where the placental barrier is thinnest. The bandicoot yolk sac placenta can thus be classified as epitheliochorial. Trophoblast cells in the vascular and non-vascular regions of the yolk sac differ. In the non-vascular region trophoblast cells have long microvilli which are bathed in the secretions of the uterine glands. In the vascular yolk sac trophoblast cells have ultrastructural features that indicate that endocytotic activity occurs and that steroid and protein hormone biosynthesis takes place. These features include apical pinocytotic vesicles, smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (Padykula and Taylor, 1977, 1982).

The bandicoot chorioallantoic placenta is characterized by mutinucleate giant cells in both the trophoblast and maternal epithelium. The uterine epithelial cells are designated as heterokaryons because they have two kinds of nuclei: small euchromatic nuclei with peripheral nucleoli and large heterochromatic nuclei with central nucleoli (Padykula and Taylor, 1977, 1982). Trophoblast giant cells, on the other hand, are homokaryons (cells with one type of nucleus) in which large heterochromatic nuclei with central nucleoli are present.

In the bandicoot chorioallantoic placenta near term the trophoblast has apparently disappeared (Hill, 1898) which results in the fetal and maternal capillaries lying very close to each other. This may facilitate the rapid fetal growth at this time. Flynn (1923) and Padykula and Taylor (1977, 1982) suggested that there is evidence for fusion of trophoblast with maternal heterokaryons between the 7mm and 12mm embryo stages which would account for the presence of two distinct types of nuclei in the maternal heterokaryons at this time.

1.8 Endocrine Function During Pregnancy

1.8.1 Eutherians

Endocrine function in eutherian pregnancy is complex and varies considerably between species. Essentially, the pregnancy hormones serve to maintain pregnancy, prepare the mammary glands for lactation and play a role in parturition. These hormones are generally produced by either the corpus luteum (CL), especially in early pregnancy, and/or the placenta depending on species. In primates the feto-placental unit, i.e., trophoblast, fetal liver and fetal adrenals, synthesizes oestriol, the dominant oestrogen of primate pregnancy (Heap and Flint, 1984). In some species the CL is needed throughout pregnancy while in others it need only secrete steroid hormones for a specific period of time after which the placenta takes over its endocrine function. Pregnancy can continue following ovariectomy after a certain point in gestation in these species (Amoroso and Perry, 1977; Heap and Flint, 1984). Differences in endocrine function of the corpus luteum and placenta can occur even between very closely related species. For example, the CL is required throughout pregnancy in the goat, while pregnancy in the sheep can proceed safely without the CL after Day 50 of its 148 day gestation (Heap and Flint, 1984).

1.8.2 Marsupials

The secretory activity of the corpus luteum in marsupial pregnancy appears to be necessary for early embryonic differentiation (Tyndale-Biscoe and Hinds, 1989; Hinds

and Selwood, 1990), parturition (Renfree and Young, 1979), the survival of neonates and for the establishment of lactation (Young and Renfree, 1979). It is the source of circulating progesterone (Renfree, 1980) and, therefore, it appears that the function of the CL in marsupial pregnancy is little different from that in many eutherian species. Bandicoots are unusual in that the CL of pregnancy continues to secrete progesterone until day 20 of lactation and persists morphologically until Day 45, inhibiting ovulation during early lactation (Gemmell, 1981).

Amoroso and Perry (1977) reported that, in all marsupials, anatomical and histological similarities in the female reproductive tract and mammary glands "indicate an endocrinological equivalence of the pregnant and non-pregnant states" (p.359). Numerous investigations cited by these authors concluded that, in some marsupial species, there are no detectable differences in post-oestrus vaginal smears and mammary gland development or peripheral plasma progesterone levels between pregnant and non-pregnant females at equivalent post-ovulatory stages. In addition, it is possible to transfer embryos into non-pregnant females at equivalent post-ovulatory stages without interrupting their development.

Harder and Fleming (1981) found that the luteal phase of the oestrous cycle in *Didelphis virginiana* has similar profiles of circulating oestradiol and progesterone to those of a pregnant cycle. Thus, they concluded that the female opossum shows no endocrine recognition of pregnancy although ovarian hormones are required for parturition since it is inhibited by ovariectomy. Hinds and Tyndale-Biscoe (1982) concluded that tammar wallabies also do not exhibit an endocrine recognition of pregnancy as they found that plasma progesterone levels were not significantly different between pregnant and non-pregnant animals. These conclusions are premised on the assumption that the maternal recognition of pregnancy should be measurable systemically.

However, other studies on endocrine function in marsupial pregnancy have shown that, in some species at least, the placenta is capable of producing steroid hormones (Renfree, 1977; Bradshaw *et al.*, 1975; Heap *et al.*, 1980) which have a local effect on the uterine tissue (Renfree, 1972, 1980). Renfree (1972) showed that the embryo and/or placenta had a local morphogenic effect on the pregnant uterus of the tammar as the presence of the embryo in the uterus stimulates endometrial growth as well as uterine-specific protein synthesis in the pregnant uterus but not in the contralateral non-pregnant uterus. Later, it was found that the yolk sac stimulates the endometrium in tammars in the absence of the embryo (Renfree, 1977). Incubated yolk sac membrane of tammars (Heap *et al.*, 1980) and quokkas (Bradshaw *et al.*, 1975;

Renfree, 1980) can also synthesise progesterone *in vitro*. Shaw and Rose (1979) also found in the potoroo that the pregnant uterus is histologically quite different after implantation than the contra-lateral non-pregnant uterus which has a similar appearance to the uteri of non-pregnant animals in the luteal phase of the oestrous cycle. In bettongs, too, there is a significant histological difference in the pregnant endometrium when compared to that of non-pregnant females which is thought to be due to the effect of the feto-placental unit and constitutes a maternal recognition of pregnancy (Rose, 1992). There is evidence, then, to suggest that the yolk sac placenta of marsupials does synthesise and secrete hormones, especially progesterone, but that these probably have only local effects on the endometrium without significantly raising peripheral plasma levels.

Tyndale-Biscoe *et al.* (1983) suggested that the tammar embryo plays a role in parturition as they found a rapid decrease in progesterone levels just prior to this time which is induced by a luteolytic agent (prolactin) which is of maternal origin. More recent studies have shown that prostaglandin F2alpha stimulates both prolactin release and birth behaviour in a variety of marsupial species (Renfree, 1993a). At parturition in the tammar circulating oestradiol levels rise as a result of increasing Graafian follicle secretory activity (Harder *et al.*, 1984) associated with the impending post-partum oestrus. Thus the typical change in the oestrogen : progesterone ratio at parturition seen in many eutherians also occurs in tammars. It is also thought, but not yet proven, that the fetal adrenal secretes cortisol which induces the cascade of hormonal events which lead to parturition in marsupials (Renfree, 1993a) as it does in a number of eutherian mammals (Challis and Olson, 1988). As the marsupial fetus is involved in the endocrine control of parturition it is unlikely that it is undergoing immunological rejection at birth as has been argued by Gould (1977) or that it is born before immunological rejection can occur as suggested by Lillegraven *et al.* (1987).

1.9 Conclusion

It is clear that the marsupials have a very different reproductive strategy to that of eutherians. They exhibit a few apparently "primitive" features i.e. retention of the shell membrane of unknown origin and function and yet also exhibit a clearly defined period, albeit brief, of implantation and placentation. The fragmentary evidence available at present clearly indicates that as in eutherians, marsupial species differ in the types of implantation that occur. Clearly as the foregoing review shows there have been remarkably few in depth studies of implantation and placentation of any marsupial species and those investigations that have been performed have generally raised more questions than they have answered.

My project involves a detailed study of the origin of that enigmatic structure the shell membrane, and the subsequent cellular changes that occur at implantation and placentation in a dasyurid marsupial. It is hoped that this morphological investigation will facilitate the design of critical experiments that will shed light on the functions of feto-maternal interactions in this other infraclass of mammals.

Chapter 2 Research Project

2.1 Introduction

In all amniotes there is at least one extracellular coat surrounding ovulated oocytes and early embryos. In marsupials oocytes are enclosed only by the zona pellucida, an extracellular layer of ovarian origin, having lost the cumulus cells which surrounded them during pre-ovulatory maturation (Hughes, 1974a, 1984). After fertilisation a further two acellular layers are deposited around the early embryo, as well as around unfertilised oocytes. First the mucoid coat, which is analogous to the albumen layer of the hen's egg, and then the shell membrane, which is analogous, and possibly homologous, to the tough shell membrane lining the shell of the domestic hen's egg, are laid down by the time that embryos start to cleave in the uterus (Hughes, 1974a, 1984).

Oviparous and ovoviviparous amniotes retain a shell membrane whereas the viviparous eutherians have lost it (Luckett, 1977; Mossman, 1987). The presence of the shell membrane around the marsupial embryo does not preclude intrauterine gestation but presumably may have influenced the type of viviparity that has evolved in marsupials.

As there is disagreement in the literature as to the origin of the shell membrane in marsupials, the first part of this project involves the determination of the cell populations responsible for its synthesis and secretion, as well as a histochemical and ultrastructural study of its composition. Polyclonal antibodies were raised to the extra-cellular coats of the early embryo (mucoid and shell membrane) and immunocytochemistry, at both the light and electron microscopical levels of resolution were employed.

In marsupials implantation cannot occur until after the loss of the shell membrane (Hughes, 1974b, 1984). In eutherians implantation occurs at a variety of stages of embryonic development, from the early blastocyst in rodents and humans to somite stages in ungulates (Boyd and Hamilton, 1952). Therefore I aimed to determine in the dunnart at what stage of embryonic development the shell membrane is lost and hence the stage at which implantation occurs.

Most of the investigations to date on implantation and placentation in marsupials have been conducted at the light microscopical level of resolution. Research on marsupial implantation has been confined to determining whether trophoblast is

invasive or not and there have been no detailed studies on the cellular interactions between maternal and embryonic cells at this stage of marsupial development.

Most of the marsupial species investigated appear to develop a non-invasive yolk sac placenta. The dasyurids are a relatively old marsupial lineage (Clemens *et al.*, 1989) and it may be that their placental structure is closer to the ancestral condition than that of other families. The yolk sac placentae of the two dasyurids that have been superficially investigated have both been found to be invasive. In the eastern quoll, *Dasyurus viverrinus*, trophoblast, from an annular region of the bilaminar yolk sac adjacent to the sinus terminalis, erodes the maternal uterine luminal epithelium and subsequently surrounds the maternal capillaries (Hill, 1900). It is not clear from this early light microscopical study whether the maternal endothelium is replaced by trophoblast resulting in a haemochorial placenta or if no erosion of the endothelium occurs, resulting in an endotheliochorial placental association.

As there is a similarity of placental structure between species within each particular mammalian family (Mossman, 1952, 1987; Luckett, 1977, 1993) *Sminthopsis crassicaudata*, the fat-tailed dunnart, is likely to have a similar placenta to that of the quoll. An ultrastructural study of implantation and the development of the placenta in the dunnart will reveal the structural details of the cell layers between maternal and fetal blood in the development of the invasive placenta in this species. Investigations on the placenta of the dunnart have so far been confined to a light microscopical study of a single pregnant female (Hughes, 1974b), the gestational stage of which was not accurately known.

This project, then, aimed to achieve a description of the origin and structure of the shell membrane, the cellular processes involved in implantation and placentation in the fat-tailed dunnart, *Sminthopsis crassicaudata*, a dasyurid marsupial. Animals were sacrificed at various stages of gestation to elucidate the timing and site of shell membrane deposition, the developmental stage at which the shell membrane is lost, the embryonic-maternal cell interactions at implantation, and the structural changes during the development of the placenta. A light microscopical (LM) study was first carried out followed by a transmission electron microscopical (TEM) examination of selected regions of placentae at various gestational ages. Investigations into the morphology, using both light and electron microscopy were performed. TEM studies were necessary to determine the invasiveness of the trophoblast and the mode by which it penetrates the uterine luminal epithelium. Histochemical techniques were used to investigate maternal and fetal secretions during placentation.

A preliminary study, using mainly paraffin sections, was conducted during my Honours year and a few of the micrographs obtained at that time are included in this thesis for orientation purposes. I have also re-interpreted some of the data from my Honours thesis in the light of new information. The Animal Ethics Committee of the University of Adelaide approved all aspects of this project and work was carried out under approval numbers: M/16/90, M/30/92 and M/30/92A.

2.2 Animals

All fat-tailed dunnarts, (*Sminthopsis crassicaudata*, Dasyuridae), were obtained from the colony housed in the Department of Genetics at the University of Adelaide. This species is a small insectivorous marsupial which has a large distribution over much of the Australian mainland, particularly south of the tropics. Adults weigh about 16g with a head and body length of 90mm and a tail of about 60mm in which fat is stored (Bennett *et al.*, 1990). The oestrous cycle of females is 31.1±0.7 days and litters are born 13.5 days after copulation (Smith *et al.*, 1978) although sperm storage in the isthmus of the oviducts can occur for up to 3 days prior to ovulation (Selwood, 1987; Breed *et al.*, 1989). At birth neonates weigh about 16mg and there can be up to 15 embryos born although there are only 10 teats in the female's pouch (Bennett *et al.*, 1990).

All animals were maintained in a 'long days' lighting regime of 16h of light and 8h of darkness with lights out at 2.30p.m. Food and water, as described by Bennett *et al.* (1990), were supplied *ad lib.*

For the studies on the shell membrane, most females were housed in groups of five in standard rat cages and primed with gonadotrophins (see below) to induce ovulation and their eggs recovered from the reproductive tract. Some females were primed and then paired with a male three days after priming. For the implantation and placentation studies females were placed in pairs with a male four days after having had their previous litter removed or weaned. The pair was then housed in a small standard laboratory mouse cage (28cm by 15cm by 9cm). Early embryos from a few of these unprimed, but mated, females were included in the shell membrane study.

2.3 Induced Ovulation by Priming with Gonadotrophins

For the studies on the shell membrane female dunnarts were given 1 i.u. of pregnant mare serum gonadotrophin (PMSG) (Folligon, 50i.u./ml, Intervet) by intra-peritoneal injection to induce ovulation (Rodger *et al.*, 1992). They were placed with a male 3 days later and daily urine samples were checked for the presence of sperm to

determine whether mating had taken place. In the Honours study urine samples were checked for the presence of nucleated and cornified vaginal epithelial cells and polymorphonuclear leucocytes to determine whether ovulation had taken place (Selwood, 1987, 1989). However, it was found that in this species these cellular changes are not a good indicator of the stage of the oestrous cycle in many females, as no clear patterns of changing cellular composition were observed that were constant between females nor was there any correlation of these factors with oestrous behaviour determined by the occurrence of mating. In later groups of females this method was abandoned and only the presence of sperm was recorded. Females were sacrificed by an intra-peritoneal overdose of sodium pentobarbitone (Nembutal, Bomac Laboratories, Sydney) and their uteri were dissected and everted into a watch glass of 0.1M phosphate buffered saline (PBS, pH 7.4). Early embryos were viewed with a dissecting microscope. Some unmated females were sacrificed 4-6 days after priming which was the period during which ovulation was most likely to occur (Rodger *et al.*, 1992).

2.4 Selection of Pregnant Animals

Females required for the morphological studies of implantation and placentation were not primed, but allowed to mate with a male at natural oestrus. This was necessary because priming dunnarts with gonadotrophins reduces the likelihood that embryos will develop to term (Rodger *et al*, 1992). Similar studies in mice have revealed that embryonic development is retarded and abnormal, and there are a significantly greater number of resorptions in pregnant females which were primed with gonadotrophins (Ertzeid and Storeng, 1992).

Young adult females which had their pouch young removed or weaned were paired with males 3 days later. For determination of mating each morning at about 8.30 a.m. urine samples were obtained from each female and these were examined using a phase contrast microscope. The number of sperm present, if any, was recorded. The day that sperm were present in the smear was regarded as Day 1 of pregnancy. The pair of animals was maintained together for three more days and then the male was removed, the female remaining in the same cage in isolation until sacrifice or its return to the Department of Genetics if pregnancy had not taken place. In some cases smears were performed on these three days whereas in others no further smears were taken because of a low pregnancy rate in the early study and it was thought that such handling might interfere with the success of pregnancy.

2.5 Dissections and Processing

Immediately after death each animal was dissected and blood was removed through a puncture in the right atrium and spun in a Hettich Mikroliter centrifuge at 15,000r.p.m. for five minutes after which the serum was pipetted off into an Eppendorf tube and placed in a freezer at -18°C. The ovaries, oviducts and uteri (after having been slit with fine scissors) and, in some cases, the lower reproductive tract, were removed and immediately fixed by immersion at 4°C for 2-4h in 3% glutaraldehyde / 3% paraformaldehyde in 0.2M phosphate buffer (pH 7.4) to which 2.5% polyvinyl pyrrolidine had been added.

One uterus was then dissected longitudinally with the aid of a dissecting microscope taking great care not to damage the embryos. After dissection, photographs were taken of the uterus of some animals. At this point the stage of embryonic development and the arrangement of the fetal membranes were ascertained.

In most animals pieces of endometrium at the sites of embryonic attachment and adjacent to these sites in the first uterus were selected for TEM, cut into 1mm cubes and fixed for a further 2h at 4°C. The whole uteri of other animals were fixed for 8-24h at 4°C and then dissected. The small pieces of endometrium were then washed in 0.2M phosphate buffer, post-fixed in 1% osmium tetroxide (OsO_4) for 2h, and washed again. Tissue from most animals was then *en bloc* stained with 1% uranyl acetate in maleate buffer for 1.5h. The pieces of uterus were then dehydrated with a graded series of alcohols, cleared in propylene oxide, embedded in TAAB TK3 epoxy resin (TAAB Laboratories, Berkshire) and polymerized for at least 48 hours at 60°C.

In most cases the second undissected uterus, or selected parts thereof, with their blastocysts or attached embryos of more than 2mm crown-rump length (CRL), were fixed for 24h at room temperature in routine TEM fix (see above) and processed for light microscopy (LM) the following day. LM processing was done in a Shandon Duplex Processor in which tissue was dehydrated in a series of alcohols, then cleared in Safsolv (Ajax Chemicals, Adelaide) and finally infiltrated with molten paraffin wax. The tissue was then embedded in paraffin wax (Paraplast Plus) using a Tissue Tek II wax dispenser. Some of the larger uteri also required extra infiltration using a vacuum oven at 25mm Hg and 60°C for 20-40min.

2.6 Sectioning and Staining

Thick plastic sections (0.5 μm) were cut using a Reichert-Jung Ultracut ultramicrotome with a glass knife and stained with 0.025 % toluidine blue in 0.5 % sodium tetraborate

and examined with a light microscope. Areas of interest were then selected, the blocks trimmed, and thin sections with silver/gold interference colours (70-90μm) were cut with a diamond knife (Diatome). Sections were then picked up onto uncoated copper/palladium grids (200 mesh) and stained with saturated uranyl acetate in 70% alcohol and then lead citrate (Reynolds, 1963). Thin sections were then viewed at 80 kV on a JEOL 100S or JEOL 100CX transmission electron microscope (TEM).

Paraffin blocks were cut into 7μm serial sections using a Leitz Lab Tek rotary microtome, floated onto a warm water bath and picked up onto albumenized glass slides and dried in an oven at 37-40°C for 24h. Sections were finally stained with one of a variety of histochemical stains (see later).

Chapter 3 The Origin of the Shell Membrane

3.1 Introduction

In marsupials fertilization occurs in the ampulla of the oviduct, and shortly afterwards the acellular mucoid coat is laid down around the zygote, as well as around unfertilized eggs. This is the first of the so-called tertiary egg membranes (Hughes, 1974a) and may be homologous to the albumen layer that surrounds the oocyte of the hen's egg. A similar layer of mucinous material occurs around tubal eggs in a few eutherian species such as the horse (Hamilton and Day, 1945) and dog (Blandau, 1961), and is particularly thick (59-112μm) around the rabbit oocyte (Kane, 1975).

A second acellular layer, the shell membrane, comes to surround the marsupial zygote by the time it reaches the uterus less than 24 hours after fertilization or ovulation. This is a tough permeable layer, which has been described as consisting of "ovokeratin", or as having a "keratin base" (Hill, 1910) and has been considered to be rich in disulphide bonds and, to a lesser extent, sulfhydryl groups (Hughes, 1974a, 1977).

The presence of the shell membrane in marsupials has evolutionary significance because the shell membrane evolved concurrently with the cleidoic egg seen in oviparous reptiles and birds and is also present in viviparous reptiles and monotremes. However, eutherians are the only amniotes which do not develop a shell membrane (Luckett, 1977; Mossman, 1987). The presence of the shell membrane does not preclude intrauterine gestation but presumably has had an effect on the type of viviparity that has evolved in reptiles and marsupials.

There is disagreement as to the cell population(s) responsible for shell membrane (SM) production in marsupials. Some workers (Hill, 1910; Hartman, 1916; Tyndale-Biscoe and Renfree, 1987) have been of the opinion that it is secreted by the isthmus of the fallopian tube and uterine glands. Hartman (1916) and McCrady (1938) reported that in the North American opossum (*Didelphis virginiana*) the SM precursors are secreted by "shell glands" of the isthmus. However, the SM increases in thickness after the embryos enter the uterus which suggests uterine secretion as well.

Hughes (1974a), after histochemical studies on the tertiary egg membranes of the brush tail possum, believed that the shell membrane precursors were secreted by uterine glands and then somehow made their way to the isthmus of the oviduct to be first deposited around embryos and oocytes in this region. He nevertheless found that deposition continues to occur after embryos enter the uterus. Lyne and Hollis (1976,

1977) found that in bandicoots the maximum thickness of the SM occurs during the development of the unilaminar blastocyst, well after zygotes have entered the uterus. However, they did not identify the cell populations that secrete it, nor did they suggest where in the reproductive tract the SM first appears. Baggott and Moore (1990) found in the grey short-tailed opossum (*Monodelphis domestica*) that the first evidence of SM surrounding fertilized ova occurred in the uterus which led them to speculate that SM precursors are secreted by the uterine glands.

By raising polyclonal antibodies to the tertiary egg coats, including shell membranes, and by employing both indirect immunofluorescence cytochemistry on fresh shell membranes, as well as histochemical and immunoperoxidase staining of paraffin sections of the reproductive tract, I have been able to identify specifically the region of the female reproductive tract responsible for secretion of SM precursors in the fat-tailed dunnart, *Sminthopsis crassicaudata*.

3.2 Materials and Methods

3.2.1 Oocyte Retrieval from Dunnarts

Female dunnarts were primed with pregnant mare serum gonadotrophin as previously described (see page 43) to induce ovulation. Some females were placed with a male 3 days later and daily urine samples were checked for the presence of sperm. Females were sacrificed by an overdose of sodium pentobarbitone (Nembutal, Bomac Laboratories, Sydney) on days 1 and 2 *p.c.* or on days 4-6 after priming (if unmated) and their uteri were removed, placed in phosphate buffered saline (PBS, pH 7.6), and everted. Embryos and oocytes were recovered with the aid of a dissecting microscope.

3.2.2 Polyclonal Antibody Production against Marsupial Egg Coats

Polyclonal antibodies were raised against whole mucoid and shell membranes in a 10-12 week old male Balb/C mouse. One hundred and fifty four egg cell coats were collected over a period of six months from primed female dunnarts. The shell membranes of embryos or oocytes were pierced with 21 gauge needles on 1ml syringes and the oocytes or blastomeres inside were scraped and flushed out with PBS. Some mucoid remained attached to the inside surface of the SMs as these two "membranes" are intermixed at their interface (Breed *et al.*, 1989; Breed and Leigh, 1990). The egg coats (ECs) were then picked up with a finely drawn mouth pipette and placed in fresh PBS in Eppendorf tubes and frozen at -18°C until required. After thawing,

pooled ECs were divided into three aliquots, frozen in 100µl of PBS in 1ml Beam capsules and then refrozen at -18°C until immunization.

It was estimated from protein extracted from 12 shell membranes prior to SDS-PAGE that one hundred and fifty four ECs would consist of about 2.4µg protein. The primary immunization was prepared by emulsifying 100µl Complete Freund's Adjuvant (Sigma, Australia) with one 100µl aliquot of ECs by using a Branson Sonifier Cell Disperser fitted with a microprobe, using short bursts totalling about 10s. Virtually all of the 200µl dose was collected into a 1ml insulin syringe with 30G needle (Terumo, Melbourne) and injected intraperitoneally.

Three weeks later a booster dose of a second aliquot of ECs prepared in Freund's Incomplete Adjuvant was injected subcutaneously at two sites along the dorsum of the mouse. A test bleed of 200µl blood was taken 10 days later from the retro-orbital sinus of the mouse after ether anaesthesia. A sample of blood was also taken from a normal negative control male Balb/C mouse of the same age. Three weeks after the first booster, the mouse received the second booster subcutaneously and retro-orbital bleeds were taken 7 and 10 days after the third injection. Finally, the mouse was exsanguinated at 14 days. Serum was stored at -18°C until required.

3.2.3 Indirect Immunofluorescence for Detection of Anti-egg Coat Antibodies

Immune mouse serum was titrated for antibodies against egg coat proteins by immunofluorescence. Uterine oocytes, obtained from female dunnarts primed with PMSG as described above, were placed in PBS, incubated in 1% BSA in PBS for 30min and then incubated at room temperature in dilutions of serum from the immunized mouse (1:10, 1:20, 1:40 or 1:80 in PBS) in a humid chamber for 1h. Negative controls were incubated in either normal mouse serum or 1% BSA in PBS. All oocytes were then washed and incubated in a 1:20 dilution of FITC-conjugated $(Fab')_2$ sheep anti-mouse IgG (Silenus, Hawthorn, Australia) diluted 1:20 in PBS for 30min and then washed again. Oocytes were subsequently mounted in antifade (Slow Fade, Molecular Probes) and viewed with an Olympus BH phase and fluorescence microscope using FITC filters and dichroic mirror. Oocytes were photographed with a x10 objective using Kodak TMAX 400 film.

3.2.4 Immunoperoxidase Cytochemistry of Uterus and Oviduct

Female dunnarts primed as described above, were sacrificed at either oestrus or one day after ovulation when oocytes/embryos had entered the uterus. Fresh uterus, utero-tubal junction, isthmus, ampulla, liver, kidney, skin and small intestine were

dissected and fixed in Bouin's fixative for 1-3 days at room temperature. Tissues were dehydrated by passing them by hand through a series of alcohols. They were then cleared in cedar wood oil and vacuum embedded in paraffin wax (Paraplast, Oxford Labware) at 56°C. Seven micron serial sections were cut and some of the sections were stained with haematoxylin and eosin. Adjacent suitable sections were selected for immunoperoxidase cytochemistry.

Paraffin sections of each tissue were immunostained using an Immuno Tag Peroxidase Anti-Peroxidase System (Immunon, Pittsburgh) with the mouse polyclonal anti-EC antiserum as the primary antibody. Sections were dewaxed, hydrated, washed in 0.1M phosphate buffered saline pH 7.4 (PBS) and endogenous peroxidase was blocked with 3% H_2O_2 for 30min. Then sections were incubated in the kit protein blocking agent for 30min. They were then incubated for 2h at room temperature in a humid chamber in anti-EC which had been diluted 1:10 in PBS and preadsorbed 1:1 with dunnart serum for 2h. Appropriate positive (uterine oocytes) and negative (normal mouse serum or PBS only) controls of reproductive tract sections were performed and negative controls from liver, kidney, small intestine and skin from a female dunnart were included. Sections were washed, incubated with biotinylated anti-mouse IgG for 1h, washed again and incubated in streptavidin PAP for 1h at room temperature in a humid chamber. Washed sections were then incubated in amino-ethylcarbazole (AEC) with 0.001% H_2O_2 for 5min, washed and counterstained with haematoxylin. Finally, slides were mounted in Gel Tol (Immunon, Pittsburgh) aqueous mounting medium.

3.2.5 Histochemistry

Female dunnarts were primed with PMSG and sacrificed as above 4-6 days after priming. Their oviducts and uteri were removed, fixed in either Bouin's fixative or 10% buffered formalin, processed and embedded in paraffin wax. Serial sections were cut at 7μm and every fifth slide was stained with haematoxylin and eosin. Intervening slides were stained with either periodic acid Schiff (PAS), PAS after amylase digestion, alcian blue at pH 1.0 or 2.5, Masson's trichrome, alcian blue pH 0.2 after performic acid oxidation, dihydroxy-dinapthyl-disulphide (DDD), or ferric ferricyanide using the methods of Drury and Wallington (1980).

3.3 Results

3.3.1 Indirect Immunofluorescence Cytochemistry

Indirect immunofluorescent staining of unfixed uterine oocytes/embryos with anti-EC at 1:20 and 1:40 dilutions produced bright fluorescence of the shell membrane (Figs. 3.1 and 3.2). In contrast, oocytes/embryos incubated with 1:20 dilutions of normal mouse serum or with 1% BSA in PBS in place of primary antibody did not exhibit fluorescence (Figs. 3.3 and 3.4).

3.3.2 Immunoperoxidase Cytochemistry

The polyclonal anti-EC antiserum stained several cell populations in the reproductive tract. These were the luminal epithelium of the ampulla, the luminal epithelium and crypts of the isthmus of the oviduct (Figs. 3.5 and 3.6), fibrous material coating the oviducal epithelium and present within the lumen, the luminal epithelium of the utero-tubal junction and some adjacent endometrial glands (Figs. 3.7 and 3.8).

The majority of immunoreactive cells were labelled in their apical cytoplasm only, although occasionally the whole cytoplasm of individual glandular epithelial cells was stained (Fig. 3.9). Secretion in the apical glandular lumina was also positively immunostained (Fig. 3.9). The basal region of the glands was negative. A few cells of the uterine luminal epithelium were also immunolabelled, together with a thin layer on the apical surface of some of these cells. Positively stained fibrous secretory material, presumably from the endometrial glands, was found in the uterine lumen.

Staining patterns of the oviduct were similar in females at oestrus and in those with uterine "eggs" (Fig. 3.10). However, although not quantified, there appeared to be more immunoreactive endometrial glands in females in which uterine oocytes/embryos were present. Despite this, immunolabelling was generally confined to the endometrium at the cranial pole of the uterus, near the utero-tubal junction.

The specificity of immunostaining for the mucoid and shell membrane in reproductive tract tissues was shown by the failure of the anti-EC antiserum to stain sections of liver, kidney, skin or small intestine from female dunnarts bearing uterine oocytes/embryos.

3.3.3 Histochemistry

Table 3.1 shows the results of immunocytochemical and histochemical staining of the tertiary egg membranes and of the epithelial lining and secretions of the female reproductive tract of the dunnart.

Table 3.1 Immunocytochemical and histochemical staining of tertiary egg membranes and of the epithelium of the female reproductive tract of the dunnart.

Stain	Mucoid	SM	Ampulla	Isthmus	UTJ	Uterus	RBCs
Anti-EC	+++	+++	+++	+++	+++	++[a]	-
PAS	+++	-	+++	+++	-	+[b]	-
PAS, amylase digestion	+++	-	+++	+++	-	-	-
Alcian Blue pH 1.0	+++	-	+++	+++	-	+[b]	-
Alcian Blue pH 2.5	+++	-	+++	+++	-	+[b]	-
PAO - Alcian Blue pH 0.2	++	-	++	++	-	+[b]	-
DDD	-	-	-	-	-	-	+
Ferric ferricyanide	-	-	-	-	-	-	+
H & E - eosin		+++					+
Trichrome - light green	+++	-					-
Trichrome -red cyto stain	-	+++					+

[a]Scattered glands and occasional luminal epithelial cells at the tubal pole of the uterus were stained.
[b]The lumina of occasional large dilated endometrial glands were stained.

The mucoid layer of fertilized and unfertilized oocytes was PAS positive (Fig. 3.11) and this reaction was amylase resistant. It was also alcian blue positive at both pH 1.0 and 2.5 (Fig. 3.12), and after performic acid oxidation it stained weakly with alcian blue at pH 0.2. The luminal epithelium of the ampulla and isthmus, as well as the isthmic crypts and occasional mucinous material within the uterine lumen, was also stained by PAS and alcian blue (Figs. 3.13 and 3.14). However, these histochemical methods did not stain the luminal epithelium of the utero-tubal junction nor the

Figures 3.1 and 3.2 Uterine embryo incubated in 1:20 dilution of mouse anti-EC antiserum.

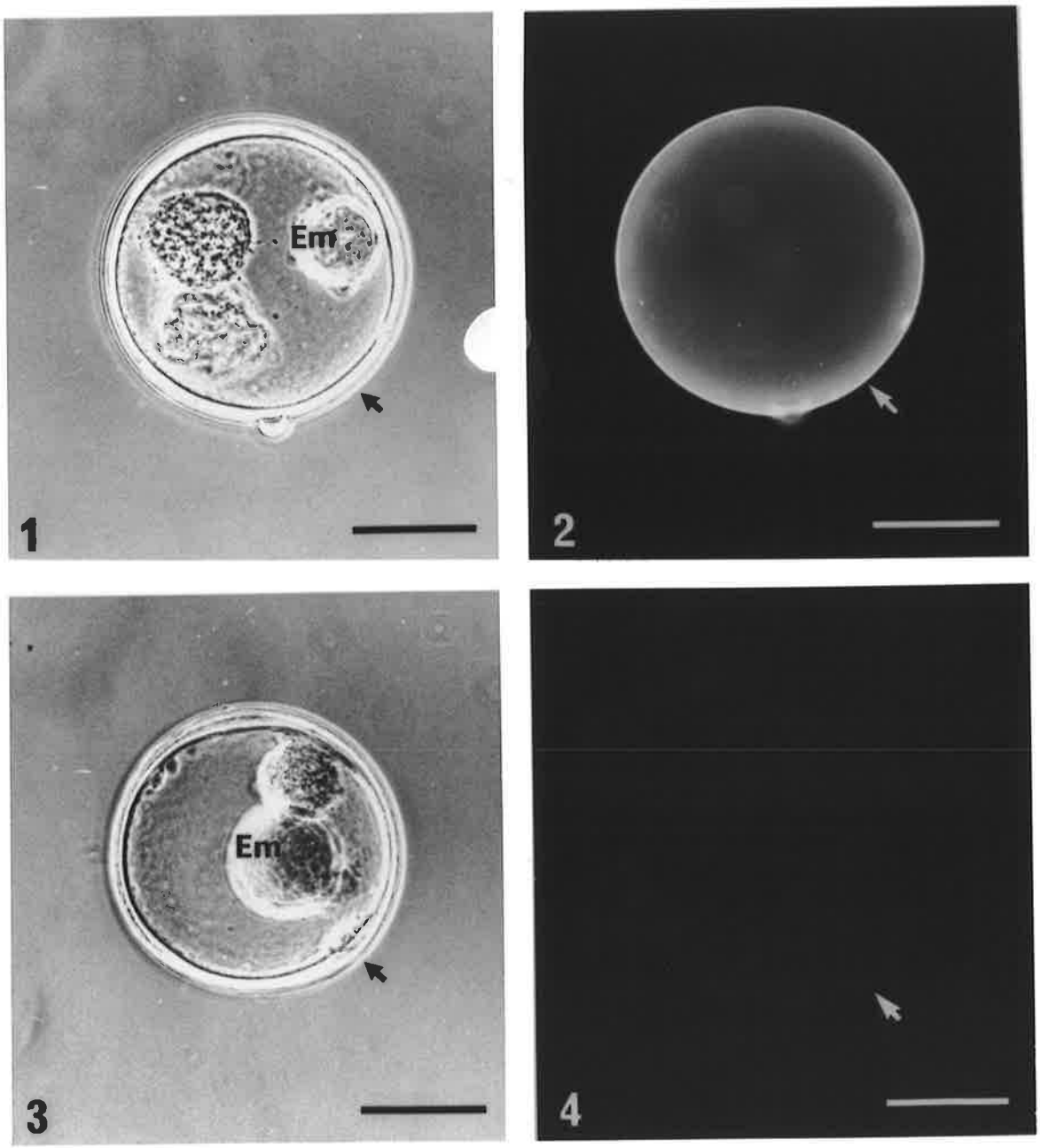
Fig. 3.1 Embryonic cell (Em), shell membrane (arrow), phase contrast. Bar = 100μm.

Fig. 3.2 Shell membrane (arrow) fluoresces brightly. Bar = 100μm.

Figures 3.3 and 3.4 Control uterine embryo incubated in 1:20 dilution of normal mouse serum.

Fig. 3.3 Embryonic cell (Em), shell membrane (arrow), phase contrast. Bar = 100μm.

Fig. 3.4 Shell membrane (arrow) does not fluoresce. Bar = 100μm.


Em
1
2
Em
3
4

Figures 3.5 and 3.6 Immuno-staining of sections of isthmus of female with uterine oocytes/embryos using anti-EC antiserum.

Figure 3.5 Negative control isthmus incubated in 1:20 dilution of normal mouse serum shows no immunoreactivity, lumen (L). Bar = 50μm.

Figure 3.6 Isthmus incubated in 1:20 dilution of anti-EC antiserum shows immunostaining in luminal epithelial cells and crypts (Cr), lumen (L) Bar = 10μm.

Figures 3.7 and 3.8 Immuno-staining of sections of utero-tubal junction of female at oestrus.

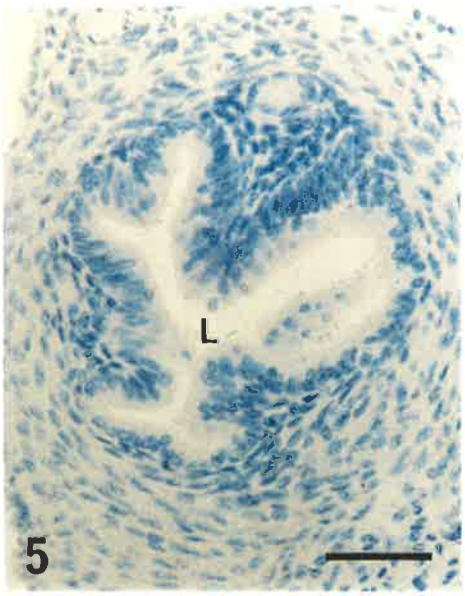
Figure 3.7 Negative control utero-tubal junction incubated in 1:20 dilution of normal mouse serum shows no immunoreactivity, gland (Gl), lumen (L). Bar = 25μm.

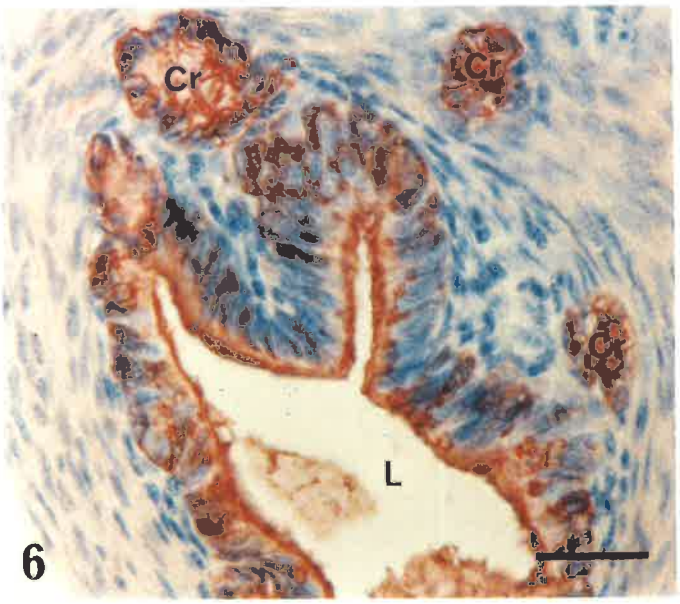
Figure 3.8 Utero-tubal junction incubated in 1:20 dilution of mouse anti-EC shows immunostaining in some luminal epithelial cells, on both the luminal surface and in an adjacent gland (Gl), lumen (L). Bar = 20μm.

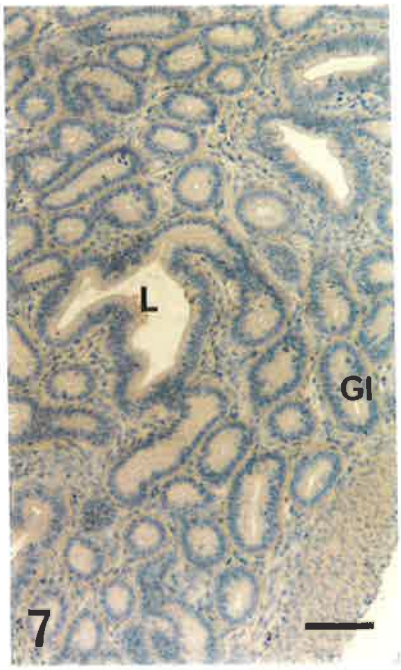
Figures 3.9 and 3.10 Uterine endometrium from females with uterine oocytes incubated in 1:20 dilution of mouse anti-EC.

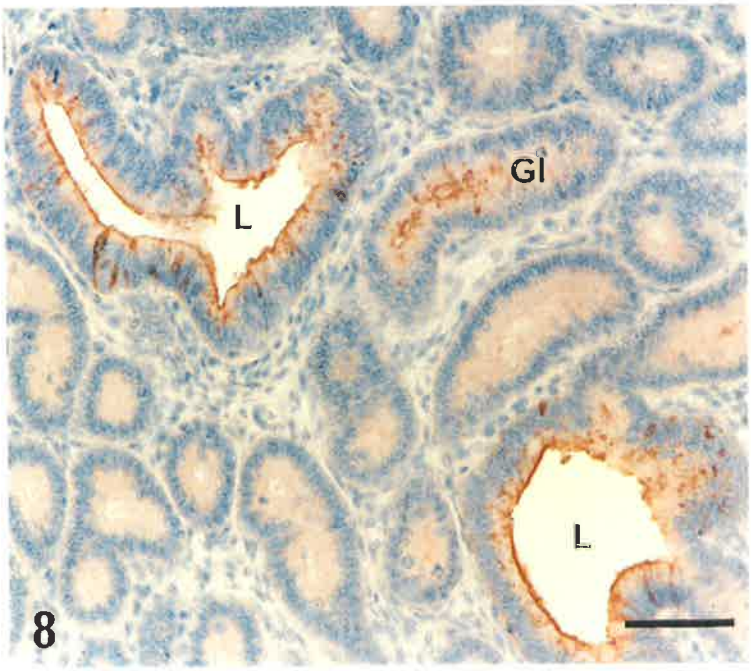
Figure 3.9 Endometrial glands (Gl) show immunostaining commonly in apical cytoplasm of epithelium but occasionally throughout the cytoplasm. Secretion in gland lumina (L) is also immunoreactive. Bar = 10μm.

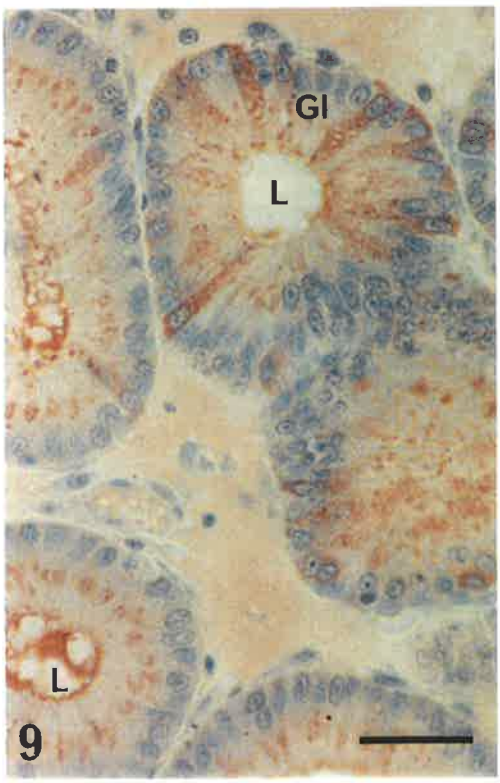
Figure 3.10 Endometrium from the cranial pole of uterus shows more immunoreactive glands (Gl) than in females at oestrus, luminal epithelium (E). Bar = 20μm.

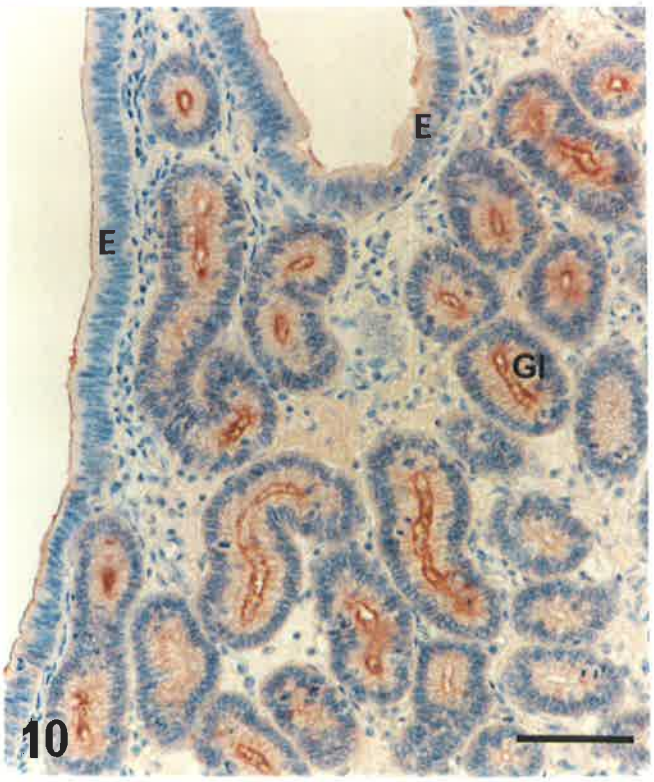

L
5


Cr
Cr
Cr
L
6


L
Gl
7


Gl
L
L
8


Gl
L
L
9


E
E
Gl
10

Figures 3.11 - 3.16 Histochemical staining of embryos, isthmus and utero-tubal junction of females with uterine oocytes.

Figure 3.11 Mucoid coat (arrows) of uterine embryo is PAS positive and shell membrane (arrowheads) PAS negative. Bar = 10μm.

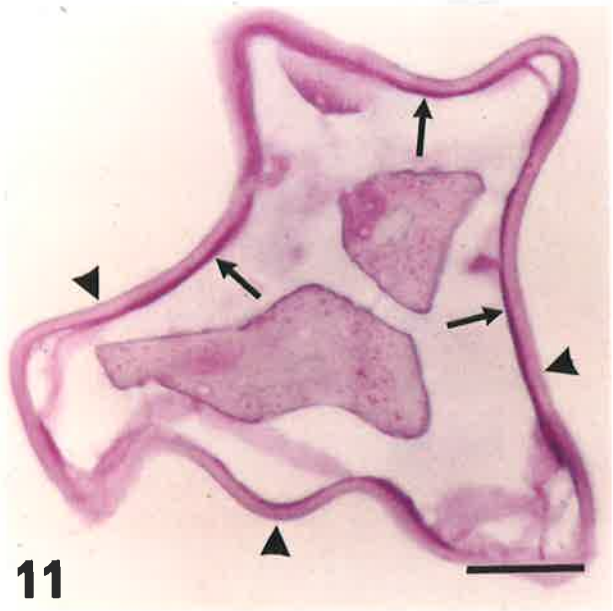
Figure 3.12 Mucoid coat (arrow) of uterine embryo is alcian blue (pH 2.5) positive and shell membrane (arrowhead) alcian blue negative. Bar = 10μm.

Figure 3.13 Luminal epithelium of isthmus and crypts (arrows) of female with uterine oocytes are both alcian blue (pH 2.5) positive, lumen (L). Bar = 10μm.

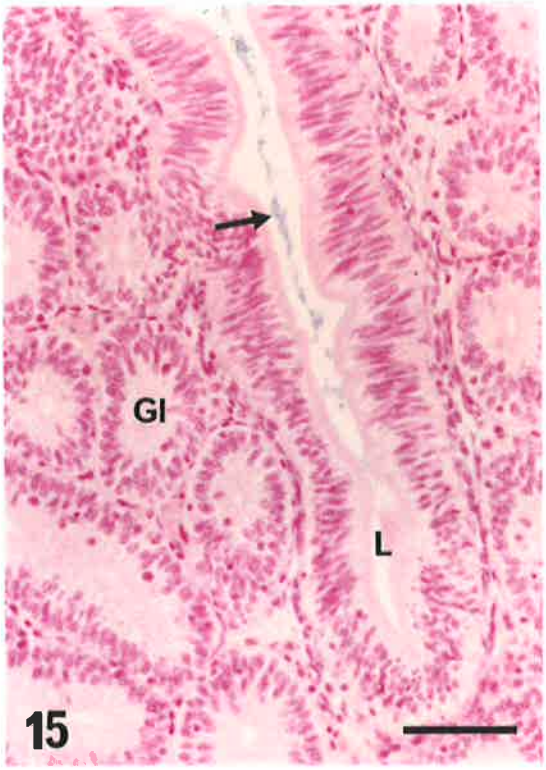
Figure 3.14 Luminal epithelium of isthmus of female with uterine oocytes is weakly PAS positive and crypts (arrows) are more strongly PAS positive, lumen (L). Bar = 20μm.

Figure 3.15 Luminal epithelium of utero-tubal junction and adjacent glands (Gl) of female with uterine oocytes are alcian blue (pH 2.5) negative but mucinous material (arrow) in the lumen (L) is positive. Bar = 20μm.

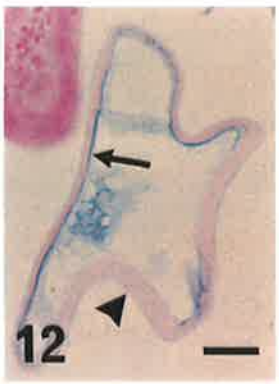
Figure 3.16 Luminal epithelium of utero-tubal junction and adjacent glands (Gl) of female with uterine oocytes are PAS negative but mucinous material (arrow) in the lumen (L) is positive. Bar = 20μm.



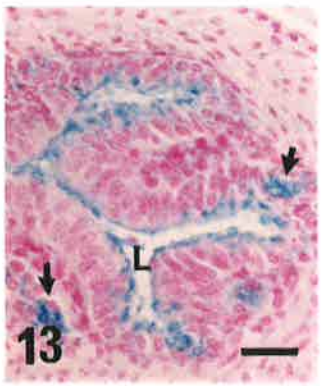
11



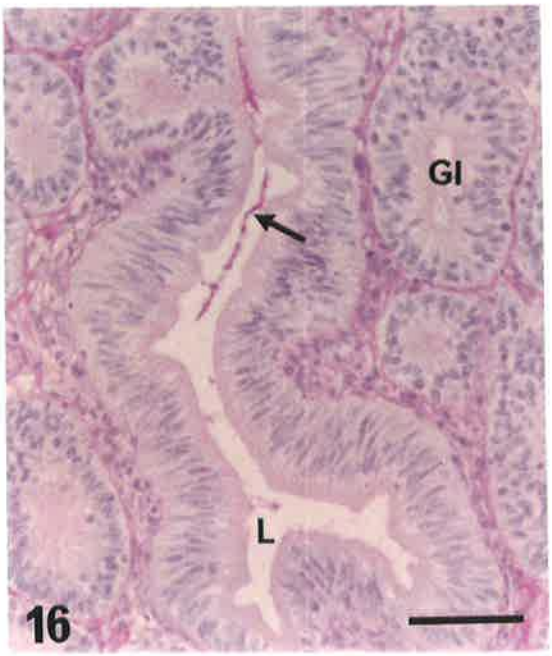
15



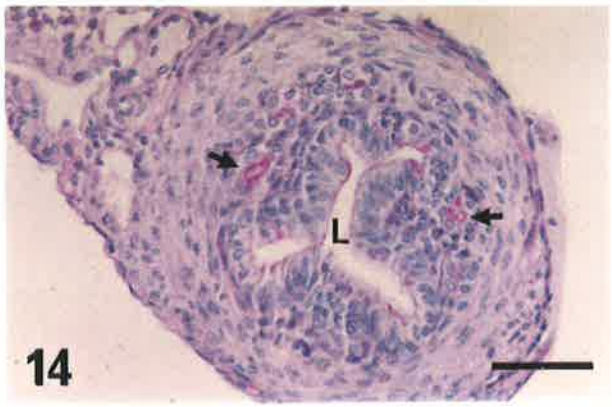
12



13



16



14

adjacent glands (Figs. 3.15 and 3.16). The mucoid also stained with light green with the Masson's trichrome method.

In contrast, the shell membrane was only stained by eosin and the red cytoplasmic stain of the trichrome method. None of the methods for either acidic glycoproteins, disulphide bonds, or sulfhydryl groups stained the shell membrane.

3.4 Discussion

These results show that, in the dunnart, strongly sulphated, acidic glycoprotein mucoid precursors are synthesized by luminal epithelial cells of the ampulla and isthmus, as well as by those of the isthmic crypts. This is consistent with Hughes' (1974a) conclusion that in marsupials mucoid precursors are secreted by non-ciliated goblet-like cells of the luminal epithelium of the entire oviduct. The secretion forms a layer of variable thickness around ovulated oocytes in different marsupial species (less than 10μm in bandicoots to about 230μm in opossums, Tyndale-Biscoe and Renfree, 1987).

In the present study of the fat-tailed dunnart, fertilized and unfertilized oocytes found in both the ampulla and isthmus had a mucoid layer outside the zona pellucida but no shell membrane. Hence, deposition of the shell membrane occurs further down the reproductive tract, a conclusion that is consistent with previous work in our laboratory which found that embryos recovered from the utero-tubal junction were surrounded by a very thin shell membrane (Breed and Leigh, 1990).

Streptavidin-biotin PAP-labelled antibodies raised against egg coat proteins stained some epithelial cells of both the ampulla and isthmus of the oviduct, as well as epithelial cells of the utero-tubal junction and adjacent glands. Furthermore, binding of the labelled antiserum to the shell membrane of intact oocytes/embryos indicates that at least some of the antibodies were directed against the shell membrane component of the tertiary egg coats. However, because mucoid could not be separated from the shell membranes in the preparation of the antigens, the antibodies were probably raised against epitopes of both mucoid and shell membrane components. Immunoreactivity could therefore indicate the presence of either mucoid and/or shell membrane precursors in cells and secretions of the reproductive tract.

Histochemical staining distinguished between the ampullary/isthmic secretions and those of the utero-tubal junction and uterus. Positive staining for acidic glycoproteins was essentially confined to the ampulla and isthmus while immunostaining with the

anti-EC antiserum extended beyond these areas to the utero-tubal junction and some glands and luminal epithelial cells at the cranial pole of the uterus. Neither the latter areas, nor the acellular shell membrane, stained with any of the histochemical stains and therefore it seems likely that in the dunnart shell membrane synthesis is confined to the utero-tubal junction and nearby endometrial glands.

Hughes (1974a) suggested that, because the shell membrane of the brushtail possum stained positively with alcian blue at pH 0.2 after performic acid oxidation, it is rich in disulphide bonds and, to a lesser extent, sulfhydryl groups. This study on the dunnart shows that the shell membrane did not stain with either alcian blue pH 0.2 after performic acid oxidation, DDD, nor by the ferric ferricyanide method for sulfhydryl groups. Therefore, I suggest that the shell membrane of the dunnart does not have disulphide bonds or sulfhydryl groups in high enough concentration to be detected by these histochemical methods. Hughes found that the possum shell membrane was also PAS positive, whereas I found that the dunnart SM is PAS negative. The mucoid is PAS positive in both species and, as electron micrographs of dunnart embryos indicate an intermixing of mucoid and shell membranes at their interface (Breed and Leigh, 1990), light microscopical observations at low magnification could misinterpret the SM as being PAS positive.

The fact that the SM is eosinophilic and also stains with the red cytoplasmic stain of the trichrome method suggests that it contains some basic proteins and that the physical structure of the shell membrane allows molecules of a limited size to penetrate, and bind to, it. Hughes and Shorey (1973) showed that the brush tail possum shell membrane is permeable to both toluidine blue (molecular weight 306) and horse radish peroxidase (molecular weight 40,000) but not to ferritin (molecular weight 460,000).

The shell that surrounds the monotreme egg is composed of three layers, all of which apparently allow the passage of nutrients and consequently intrauterine growth of the embryo takes place. This is evident by the fact that the ovulated oocyte measures about 4mm in diameter, while the newly laid egg is about 17mm in diameter (Luckett, 1977; Hughes, 1993). It has been suggested that the inner basal layer is secreted by the tubal glands of the isthmus of the oviduct, the second, the rodlet layer, by the utero-tubal junction and the third, which is leathery in texture, by the uterine glands (Hill, 1941). The first two layers are considered homologous to the avian shell membrane and the third to the avian calcareous shell (Hill, 1941).

As Hughes (1974a) found that the possum shell membrane was only synthesized by endometrial glands in the uterus, he concluded that it must be homologous with the third layer of the monotreme shell (Hughes, 1984). The finding that the shell membrane in the dunnart is secreted by epithelial cells of the utero-tubal junction questions this generalization. Rather, it would appear that the dasyurid shell membrane, is homologous with the second, or rodlet, layer of the monotreme egg shell.

In oviparous reptiles the "uterus" has a dual function in regard to production of the egg coats. Shell membrane precursors are secreted by the uterine endometrial glands for up to 24h after ovulation and, subsequently, the luminal epithelium secretes precursors of the calcareous shell (Palmer and Guillette, 1988; Palmer *et al.*, 1993). Nevertheless, this shell can be highly expandable as in some species the egg increases in size after oviposition which is probably due to the particular fibrillar construction of the shell (Mossman, 1987). In viviparous lizards the shell membrane is thin and composed of matted fibres which are deposited in the oviduct soon after fertilization (Weekes, 1927). However, in birds, the bilaminar shell membrane, which is rich in disulphide bonds and sulfhydryl groups, is secreted in the isthmus region of the oviduct (Hoffer, 1971). In the past, the presence of these sulfhydryl groups has led to the conclusion that the shell membrane proteins belong to the keratin family of proteins and the shell membrane has therefore been described as being composed of an ovokeratin (Hughes, 1974a; Dumont and Brummett, 1985). However, this designation has been disputed since the mere presence of cystine or cysteine does not necessarily mean that a protein is a keratin and for this reason Hoffer (1971) suggested that the shell membrane proteins should not be classified as an ovokeratin. Candlish (1972) suggested the term, ovocapsin, because of the presence of collagen and other proteins (see also Wong *et al.*, 1984), as well as carbohydrate groups in the shell membrane of the domestic hen. It seems that the term ovokeratin is probably also a misnomer for the marsupial shell membrane.

Spermatozoa are often found embedded in the mucoid coat of early marsupial embryos (Selwood, 1982; Ward and Renfree, 1988; Breed and Leigh, 1990). Functionally, the mucoid coat may contribute to the block to polyspermy, as it is laid down very soon after fertilization (Rodger and Bedford, 1982; Selwood, 1982). It has also been suggested that it may provide a nutrient source for early embryos (Hartman, 1916).

The function of the shell membrane in marsupials remains an enigma. Its presence in all sub-mammalian amniotes is indicative of its phylogenetic age, but it appears to have been lost early in the evolution of eutherian mammals, before divergence of the

major orders. In marsupials there is no *a priori* reason to believe that it is merely a vestige of evolutionary history. Although eutherians are invariably viviparous and have completely lost the shell membrane, its absence is not a necessary condition for this type of embryonic development, as it allows the exchange of nutrients, gases and wastes for up to two-thirds of pregnancy in all marsupials (Hughes, 1977, 1984). In some species of viviparous reptiles the shell membrane is present throughout pregnancy while in others it breaks down with the development of the placenta (Blackburn, 1993). In the viviparous garter snake, the shell membrane is deposited in the "uterine region" of the oviduct and persists between the extra-embryonic membranes and uterine epithelium throughout pregnancy, possibly functioning as a dialyzing membrane (Hoffman, 1970). However, in the viviparous lizards the shell membrane is shed after the development of the placenta and collects as a pad of debris at the abembryonic region of the yolk sac where it is phagocytosed by chorionic epithelial cells (Weekes, 1927, 1930, 1935; Blackburn, 1993; Stewart and Thompson, 1994).

The marsupial shell membrane is necessary for normal blastocyst development, as embryos cultured after their investments have been enzymatically removed fail to form unilaminar blastocysts (Selwood, 1989). In marsupials, an inner cell mass does not form during early embryonic development. Instead, blastomeres flatten and adhere to the inside of the zona pellucida before forming junctional complexes with each other (Selwood, 1987, 1992). In the absence of the egg membranes there is no physical structure to which blastomeres may adhere. In birds, too, the vitelline envelope, which is analogous to the mammalian zona pellucida, serves as a substrate to which the extra-embryonic epiblast of the expanding blastocyst is apposed as it migrates around to encompass the yolk (Dumont and Brummett, 1985). Although a little thicker than that of monotremes, the marsupial zona pellucida is thin compared to that of eutherians and after removal of the oocyte the marsupial zona collapses and is also very rapidly degraded by serine proteases (Bedford, 1991). In particular, the zona pellucida of the fat-tailed dunnart is completely dispersed after only 4-10s incubation in 0.1% trypsin or 0.1% chymotrypsin (Bedford and Breed, 1994). Consequently, it appears that the shell membrane may give strength and structural integrity which the zona alone cannot provide to the developing marsupial embryo. Hence, in marsupials the shell membrane may be required to maintain embryonic shape and integrity during cleavage and blastocyst formation.

Chapter 4 Ultrastructure of the Shell Membrane

4.1 Introduction

In eutherians, several glycoproteins are synthesized by non-ciliated oviducal cells and are secreted into the tubal fluid in which the embryos reside for 2-4 days (Mastroianni, 1969). There have been many studies on glycoprotein secretion by the rabbit oviduct that forms a mucin layer surrounding tubal embryos (Greenwald, 1962, 1969; Brower and Anderson, 1969; Fredricsson, 1969; Jansen and Bajpai, 1982).

The immunocytochemical results on the dunnart shell membrane (see chapter 3; Roberts *et al.*, 1994) have shown that at the light microscopical level there appear to be differences in the cell populations responsible for secretion of the extra-cellular coats of the oocyte. Breed and Leigh (1988) and Breed *et al.* (1989) speculated that, in the fat-tailed dunnart (*Sminthopsis crassicaudata*), the electron-dense secretory granules that they found in epithelial cells of the crypts in the isthmus may contain shell membrane precursors (SM) as they appeared different to secretory granules of the ampulla and degranulated at the time that embryos passed through that region (W.G. Breed, unpublished observations). Later, Breed and Leigh (1990) found that the SM that surrounds zygotes that have recently entered the uterus is extremely thin compared to the much thicker SM of older embryos. They suggested that the SM may be secreted by uterine endometrial glands, as well as by cells lining the crypts of the isthmus. However, these cell populations were not described.

Not only are there differences of opinion about which population of cells secretes the shell membrane but, also, there are inconsistencies between reports of its ultrastructure. Krause and Cutts (1983) described the North American opossum SM as a mat of interwoven electron-dense fibres which have a homogeneous appearance. Their observations were made of embryos on day 9 of the 12.5 day pregnancy when there are 5-8 somites and the yolk sac vesicle measures about 6.5mm in diameter However, Hughes (1984) described the ultrastructure of the brush-tailed possum SM as being a "dense granular lattice" although he did not indicate the stage of development at which his observations were made. Recently, Renfree (1993) described the shell membrane of a diapausing tammar wallaby unilaminar blastocyst as having three layers, a fact which has not previously been referred to in the literature. Studies of recently fertilized eggs of the fat-tailed dunnart in this laboratory have made no mention of it as being layered (Breed *et al.*, 1989; Breed and Leigh, 1990). Therefore, the aim of this investigation was to determine whether the shell membrane is in fact layered and also whether changes occur during development of the embryo.

Polyclonal antibodies to the extra-cellular coats of the oocyte which were raised in the mouse have been employed to examine the ultrastructure of the cell populations which synthesize and secrete the precursors of the mucoid coat and shell membrane. A morphological account of the changes in the ultrastructure of the shell membrane during embryonic development is also given.

4.2 Materials and Methods

4.2.1 Ultrastructure

Female dunnarts primed as described above (see page 43), were sacrificed 4-6 days later both at oestrus and when oocytes had entered the uterus. Some unprimed females were also killed 1-4 days after sperm were found in the urine. Other females were killed at a variety of times up to 14.5 days *post coitum* (*pc*). Pre-implantation embryos were recovered by dissecting uteri and everting their contents into a watch glass containing PBS (pH 7.4).

Pieces of ampulla, isthmus, utero-tubal junction and uterus as well as embryos were fixed and processed as described above for routine transmission electron microscopy (see page 45). Subsequently, 0.5µm thick plastic sections were cut and stained. Ultrathin sections were cut from selected blocks and stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed at 80kV in a JEOL 100S or 100CX transmission electron microscope.

4.2.2 Immunogold Labelling

A group of fresh zygotes recovered from the uterus were immunogold labelled prior to osmication and embedding in resin. Components of the Immuno Tag Peroxidase Anti-Peroxidase System (Immunon, Pittsburgh) used above for paraffin sections (see chapter 3, Roberts *et al.*, 1994) with the mouse polyclonal anti-EC antiserum as the primary antibody were used. Oocytes were picked up and transferred in PBS to glass histological slides on which the incubations were performed. They were incubated in the kit protein blocking agent for 30min, then for 2h at room temperature in a humid chamber with anti-EC which had been diluted 1:10 in PBS and preadsorbed 1:1 with dunnart serum for 2h. Oocytes were then washed in PBS, incubated with biotinylated anti-mouse IgG for 1h, washed again, and then incubated with streptavidin gold (10nm) for 1h and washed in PBS. Finally, they were processed as above for routine TEM and embedded in TAAB TK3 resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and viewed as above.

For immunogold labelling studies tissue from the ampulla, isthmus, utero-tubal junction and uterus from females which had mated but not ovulated (oestrus), as well as from females with embryos which had entered the uterus, were immersed in 3% paraformaldehyde / 0.25% glutaraldehyde in 0.1M phosphate buffer pH 7.4 for 1-4h, dehydrated in a series of alcohols, and embedded in LR White hard resin (Structure Probe, West Chester). Later, 0.5µm thick plastic sections were cut and stained as above. Ultrathin sections were cut from selected blocks and placed on nickel grids and allowed to dry. Sections were blocked for 30min in 0.02M tris buffered saline [to which 0.01M glycine and 1% BSA had been added (TBS)]. They were then incubated for 2h at room temperature in a humid chamber in drops of anti-EC which had been preadsorbed 1:1 with *Sminthopsis* serum and diluted with TBS giving a final dilution of 1:20. The grids were then washed in TBS and incubated in anti-mouse IgG gold (10nm) for 1h and finally washed in TBS and distilled water. After air drying grids were stained with uranyl acetate and lead citrate and viewed at 80kV with a JEOL 100S or JEOL 100CX transmission electron microscope.

4.3 Results

4.3.1 Ultrastructure

A cleaving uterine embryo which was dehydrated in acetone and critical point dried for scanning electron microscopy shows that the shell membrane has a smooth homogeneous outer surface when observed at low magnification (Breed and Leigh, 1992; Breed, 1994) (Fig. 4.1). During antigen preparation the shell membranes remained in one torn piece while the oocytes/cleaving embryos were scraped out and appeared quite tough.

In one animal, fertilised oocytes found in the ampulla of the oviduct had a mucoid coat in which there were spermatozoa, but no shell membrane was present (Figs. 4.2 and 4.3). The simple columnar luminal epithelium of the ampulla consisted of a mixture of ciliated and non-ciliated cells. The cytoplasm of the ciliated cells was more electron-dense than that of non-ciliated cells and their apical cytoplasm had large numbers of electron-dense mitochondria with lamellar cristae, presumably to supply energy to the cilia, and occasional golgi complexes (Fig. 4.4). The apical cytoplasm of non-ciliated secretory cells had numerous secretory granules with a heterogeneous matrix and regions of varying electron density (Fig. 4.4). The cytoplasm of non-ciliated secretory cells was also comparatively free of organelles and less electron-dense, with fewer mitochondria, occasional golgi complexes, and a little rough endoplasmic reticulum.

The nuclei of both cell types were predominantly euchromatic (Fig. 4.3). Ovulated oocytes in the lumen of the ampulla were surrounded by amorphous mucoid material which had been produced by exocytosis of secretory granules of the non-ciliated epithelial cells (Fig. 4.5).

In a second female, embryos found in the isthmus lacked a shell membrane (Fig. 4.6). The luminal epithelium of the isthmus had a similar ultrastructure to that of the ampulla except that in the isthmus crypts were present and there were fewer ciliated luminal epithelial cells than in the ampulla. Secretory granules in the epithelium of the isthmic crypts were more electron-dense than those in the ampulla and they were also homogeneous (Fig. 4.7). Although not quantified, the numbers of these electron-dense granules appeared to have decreased in females which had cleaving uterine embryos as compared with those at oestrus although occasional cells had a few remaining after embryos had passed through to the uterus (Fig. 4.8). Also, at the later stage, the apical cytoplasm of epithelial cells in the crypts contained several residual bodies (Fig. 4.9). At oestrus there appeared to be fewer electron-dense secretory granules present in luminal epithelial cells than in those of the crypts and by the time embryos had reached the uterus the luminal epithelial cells of the isthmus were virtually devoid of granules (Fig. 4.10). In the luminal epithelium there were only occasional ciliated cells.

In another female, embryos which were recovered from the region of the utero-tubal junction, were surrounded by a mucoid layer and a very thin irregular shell membrane of uneven thickness (0.1-0.5μm) and consistency (Figs. 4.11 and 4.12). This animal was killed 29.5h after it had been seen mating which suggests that sperm transport, fertilization in the ampulla, transport to the utero-tubal junction, and the beginnings of shell membrane deposition, took place in less than this time. Many sperm and other cellular debris were seen adjacent to the embryos.

At the junction between the isthmus and utero-tubal junction, secretory granules of non-ciliated cells change from electron-dense and homogeneous to electron-lucent. Occasional glands in the transitional region between the isthmus and utero-tubal junction had a mixture of cells with electron-dense granules and those with electron-lucent granules (Fig. 4.13). Glandular epithelial cells had many more secretory granules than those in the lumen (Fig. 4.14). Ciliated cells had abundant polyribosomes, apical golgi complexes, elongate mitochondria with lamellar cristae and patches of unidentified granular material (Fig. 4.15).

The luminal epithelium of the utero-tubal junction had comparatively few ciliated cells and, once embryos had entered the uterus, occasional sperm tails were seen in the

Figure 4.1 Scanning electron micrograph (SEM) of the shell membrane (SM) of an embryo recovered from the uterus shows that it has a smooth homogeneous surface. Bar = 50μm.

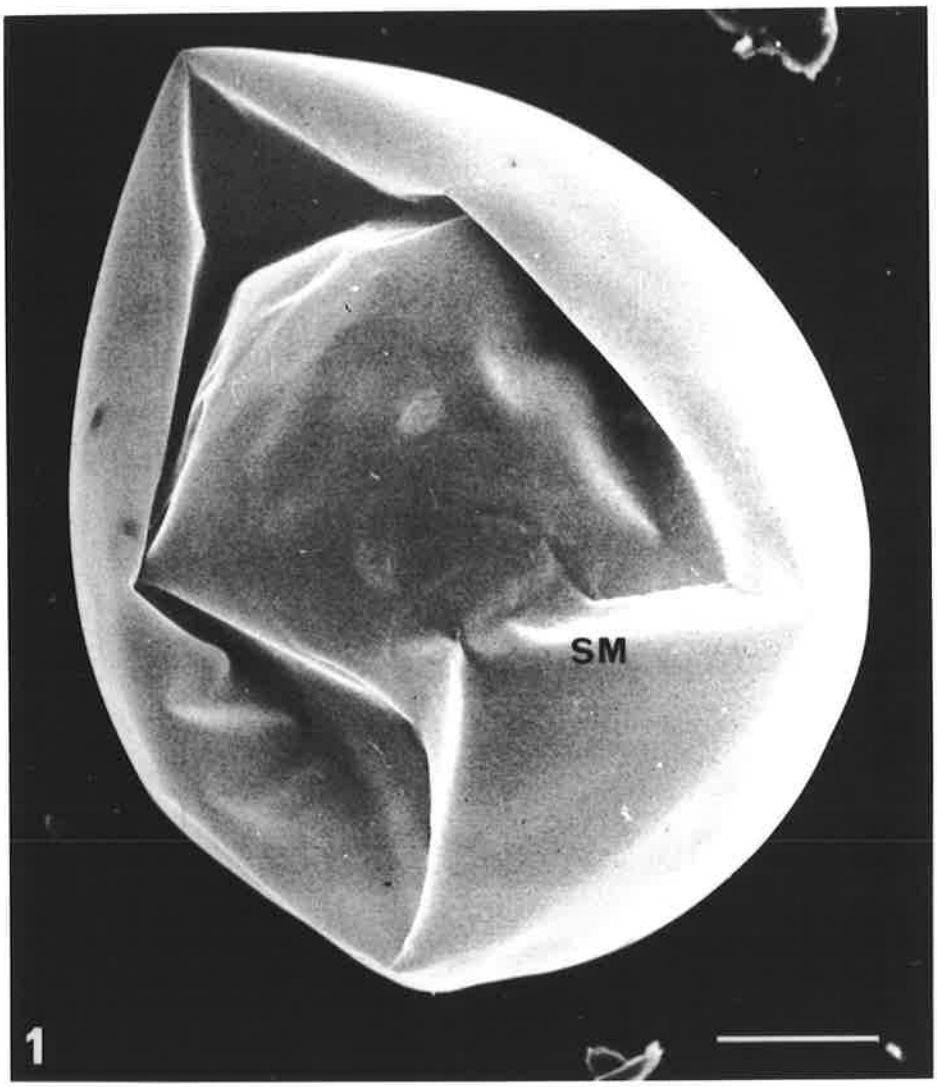
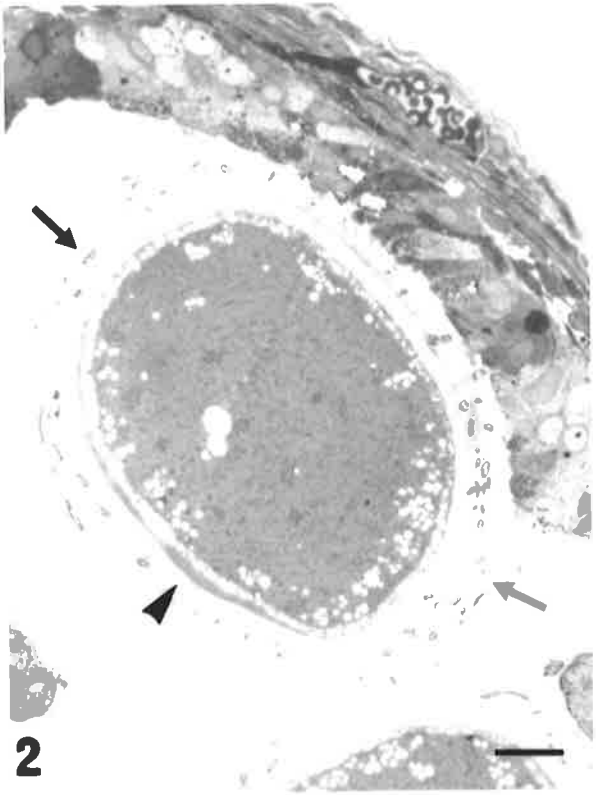

SM
1

Figure 4.2 A recently fertilized oocyte in the ampulla of the oviduct shows that a mucoid coat (arrows), with sperm embedded within it, already surrounds the zygote. The mucoid coat between two zygotes is continuous; zona pellucida (arrowhead). Bar = 50μm.

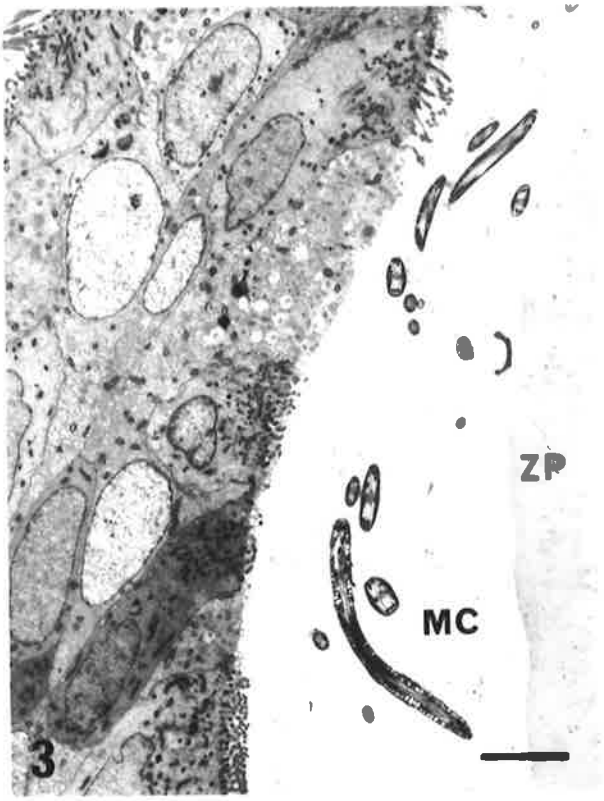
Figure 4.3 The upper zygote from Fig. 4.2 shows the homogeneous zona pellucida (ZP) surrounded by an electron-lucent diffuse mucoid coat (MC) with embedded sperm profiles. Ciliated cells are common in the epithelium. Bar = 5μm.

Figure 4.4 Luminal epithelium of ampulla at the time of fertilization shows that both ciliated and non-ciliated secretory cells are present. Ciliated cells (C) have abundant elongate mitochondria with lamellar cristae, free ribosomes and small golgi complexes in their apical cytoplasm. Cross sections of cilia (arrowheads) and basal bodies (arrows) are numerous. Non-ciliated secretory cells (SC) which are comparatively electron-lucent have many heterogeneous secretory granules which virtually fill the apical cytoplasm. There are few short irregular microvilli. Bar = 1μm.

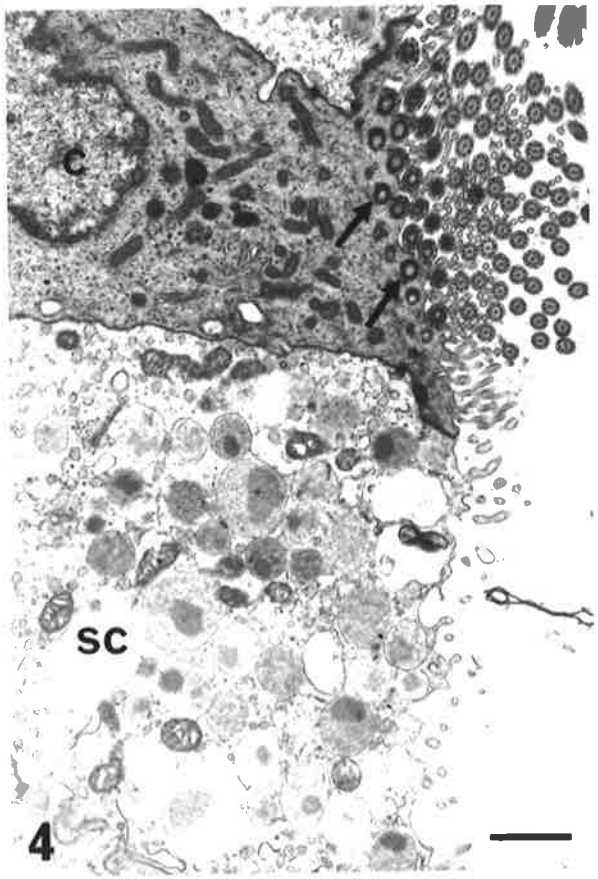
Figure 4.5 Amorphous material in the lumen of the ampulla (arrowhead) has recently been secreted from a non-ciliated cell with numerous apical secretory granules (arrows). It becomes part of the mucoid coat. Bar = 0.5μm.



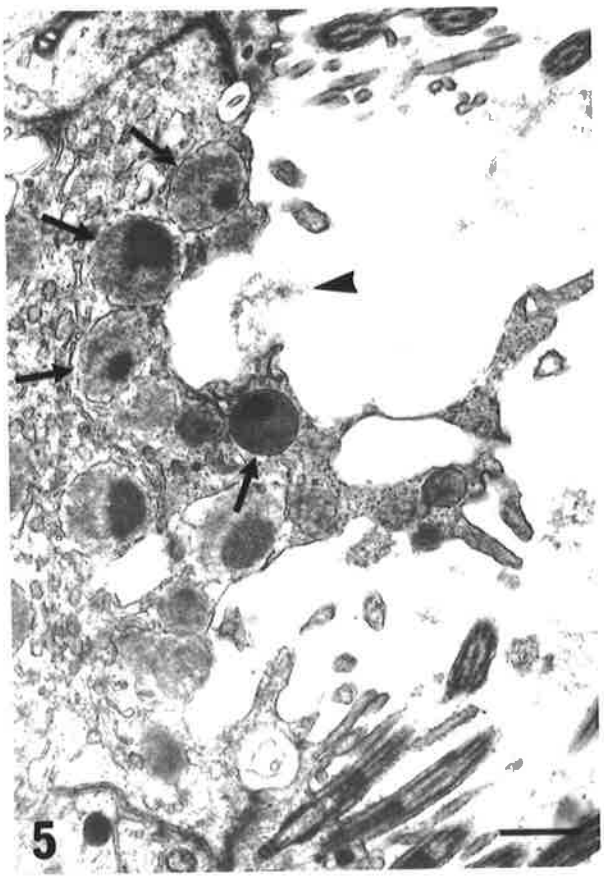
2



3



4



5

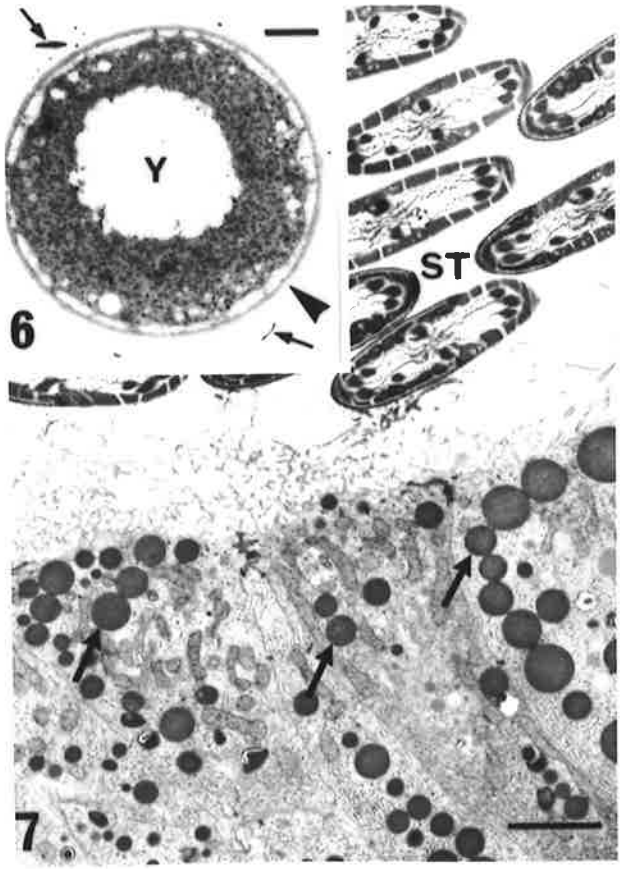
Figure 4.6 Embryo recovered from the isthmus of the oviduct is surrounded by a zona pellucida (arrowhead), a barely visible diffuse mucoid coat in which sperm (arrows) are embedded but no shell membrane is present; yolk mass (Y). Bar = 50μm.

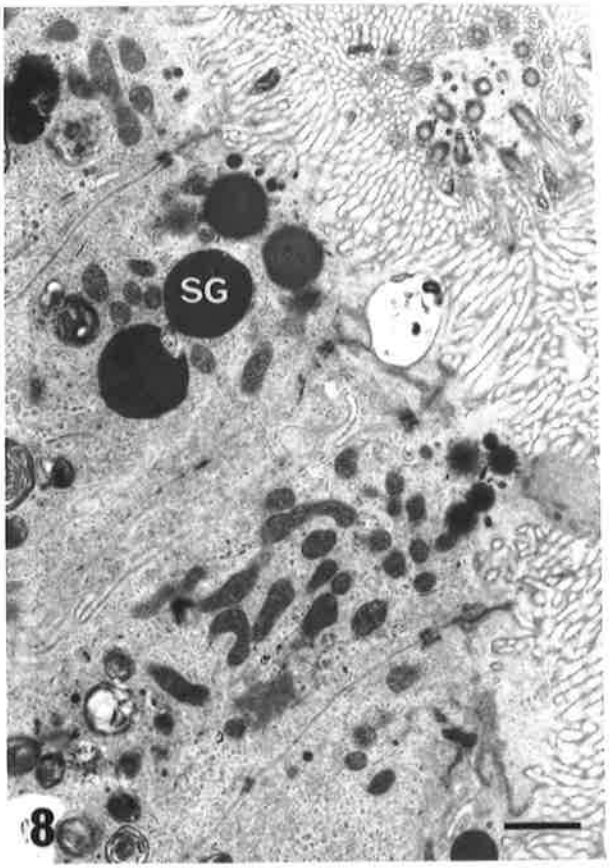
Figure 4.7 Numerous sperm are stored in the crypts of the isthmus at oestrus; sperm tails (ST). Non-ciliated cells have abundant electron-dense homogeneous secretory granules (arrows). Bar = 2μm.

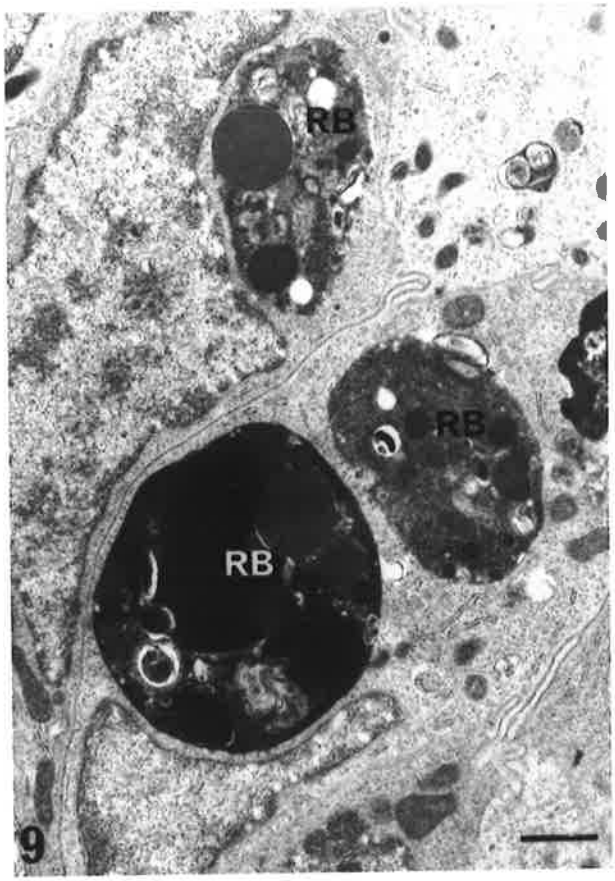
Figure 4.8 Secretory cells from a crypt of the isthmus of a female which had uterine embryos show that most secretory granules have disappeared by this time although some remain in occasional cells. Sperm have also left the crypts. Bar = 1μm.

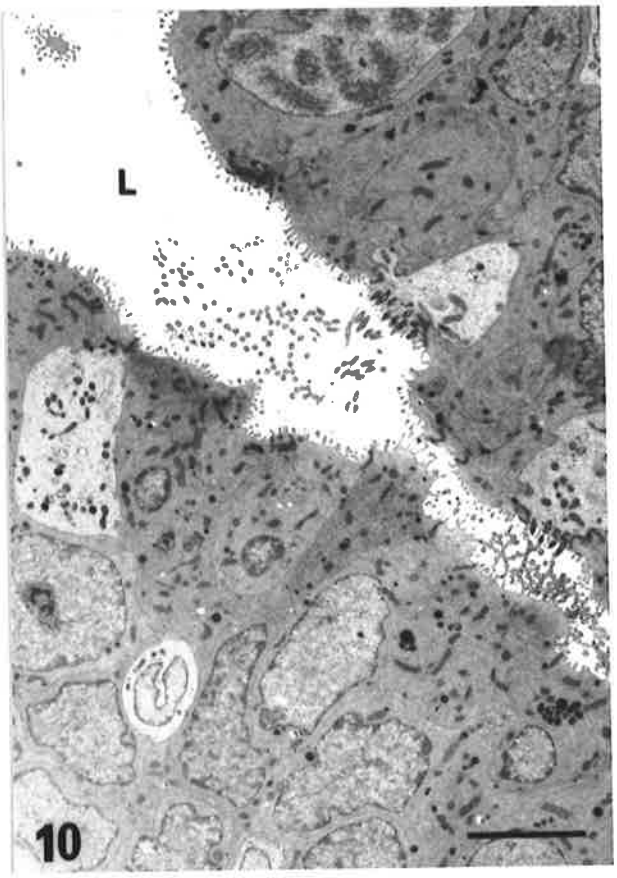
Figure 4.9 Non-ciliated cells of the isthmus of the oviduct after embryos have entered the uterus often contain residual bodies (RB) some of which contain round structures which resemble the secretory granules. Bar = 1μm.

Figure 4.10 After embryos have entered the uterus luminal epithelial cells of the isthmus are devoid of secretory granules; lumen (L). Most cells are non-ciliated. Bar = 5μm.


Y
ST
6
7


SG
8

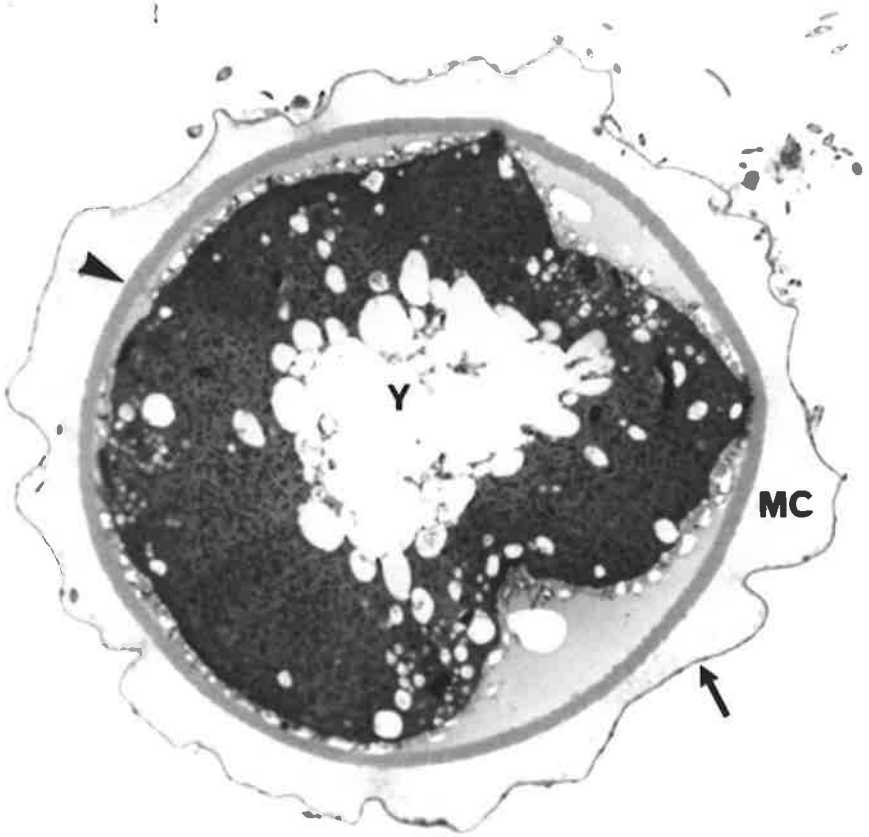

RB
RB
RB
9


L
10

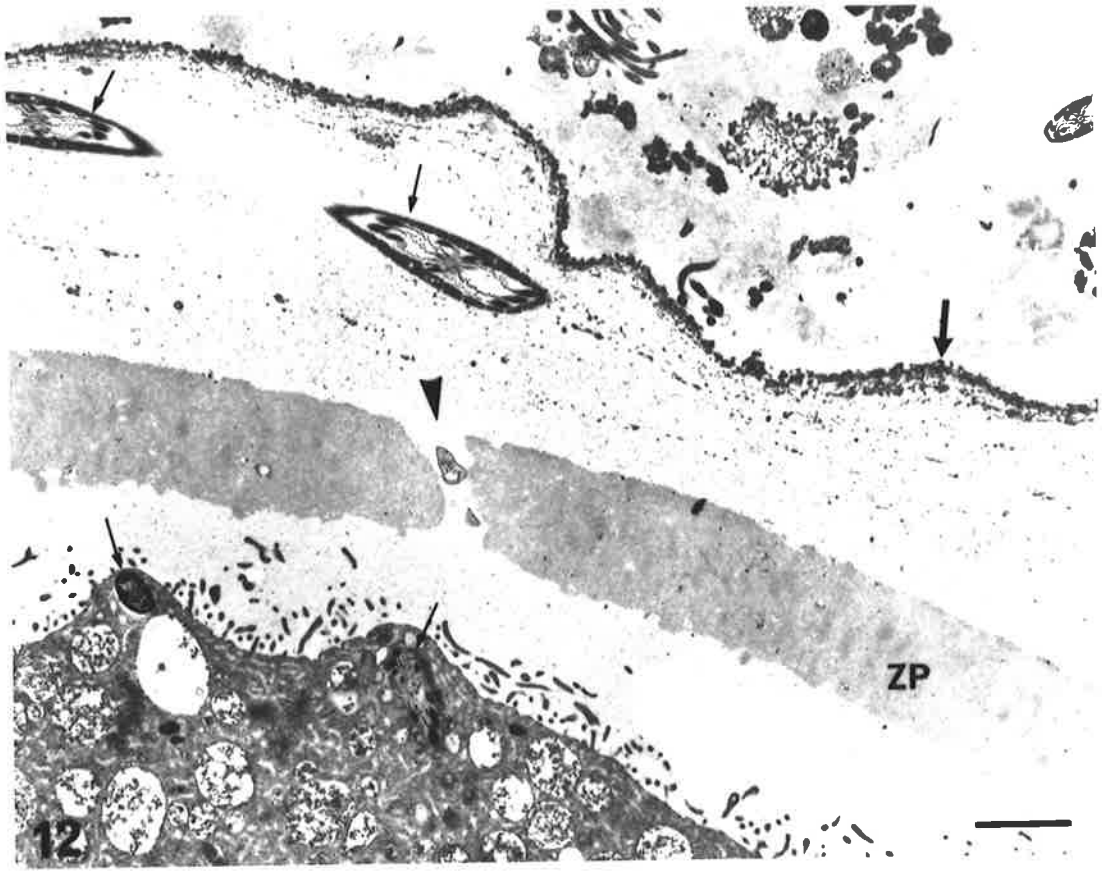
Figures 4.11 and 4.12 Embryo recovered from the utero-tubal junction 29.5h after female had been seen mating.

Figure 4.11 Light micrograph shows that the embryo is surrounded by a zona pellucida (arrowhead), a diffuse mucoid coat (MC) and a thin irregular shell membrane (arrow); yolk mass (Y). Bar = 100μm.

Figure 4.12 Electron micrograph shows that the electron-dense shell membrane (large arrow) is presumably in the initial stages of deposition and is lumpy and of irregular thickness. Shell membrane material is adjacent to cellular debris including cilia outside the embryo. The zona pellucida (ZP) is homogeneous and moderately electron-dense but has a hole which was probably made by the fertilizing sperm (arrowhead). Sperm tails (small arrows) are seen in the cytoplasm of the zygote as well as embedded in the mucoid coat (MC). There appears to be a mixing of mucoid and shell membrane components at their interface. Bar = 2.5μm.



11



12

lumen. Non-ciliated luminal epithelial cells consisted of two types. One was obviously secretory with numerous electron-lucent secretory granules with a few which were electron-dense and had a similar appearance to the more abundant granules in the adjacent glands. Secretion in the lumen of the utero-tubal junction appeared to have precipitated adjacent to the apical plasmalemma with cross sections of cilia apparently embedded within it (Figs. 4.16 and 4.17). The second type of non-ciliated cell had similar cytoplasmic features to those of the ciliated cells (Fig. 4.16).

Embryos which had been in the uterus for an unidentified time, but which were still at the one-cell stage of development, were found with an electron-dense, compact, granular shell membrane which was 1.5-2µm thick (Fig. 4.18). The mucoid layer was also granular but much more diffuse than the shell membrane. This granular appearance of the shell membrane remained during cleavage. Cleaving embryos on Days 3 and 4 *pc* were collected with a shell membrane of similar appearance and thickness but the mucoid layer had become more diffuse than previously (Fig. 4.19).

Four uterine expanded unilaminar blastocysts collected from a female 14.5 days after mating were approximately 750µm in diameter. By the time that the unilaminar blastocyst formed the shell membrane had a more fibrous appearance and had become slightly thinner measuring about 1.2µm (Fig. 4.20). At this time the mucoid layer was present only in patches, and the zona pellucida had disappeared.

By the bilaminar blastocyst stage, when the embryo was approximately 1.8mm in diameter, the SM, which was 1.2µm thick, had a fibrous appearance with many fibres oriented in the plane of the membrane (Fig. 21). In some regions the outer surface of the shell membrane was quite granular (Fig. 4.22). However, there was no evidence of layering within the shell membrane.

Primitive streak stage embryos with elongated embryonic disc regions recovered from two different females killed on days 11 and 12 *pc* measured 3mm in diameter. These embryos were still surrounded by a shell membrane which was 1.5-1.8µm thick. Even at the outer surface the shell membrane was fibrous. At this stage only a few cells of the endometrial glands had electron-lucent secretory granules with most having a different ultrastructural appearance (see chapter 5).

4.3.2 Pre-embedding immuno-gold labelling of fresh oocytes

The outer surface of the shell membrane of oocytes recovered from the uterus incubated with anti-EC antibodies was labelled with gold particles (Fig. 4.23) which confirms

that some of the polyclonal antibodies were directed against shell membrane epitopes. Negative controls were unlabelled.

4.3.3 Immuno-gold labelling of female reproductive tract

After immunogold incubations with anti-EC polyclonal antibodies, irregular secretory granules of varying electron density in the apical cytoplasm of non-ciliated epithelial cells of the luminal epithelium of the ampulla from females at oestrus were heavily labelled (Figs. 4.24 and 4.25). Gold labelling was also found over amorphous material in the lumen of the ampulla (Fig. 4.26). Ciliated cells adjacent to the labelled non-ciliated cells were negative. By the time that embryos have entered the uterus the immunoreactive secretory granules in the ampulla have been shed. A little immunogold labelling was found in occasional non-ciliated epithelial cells over granules that appeared to have just been exocytosed (Figs. 4.27 and 4.28). Scant immunogold labelling was also found on a little amorphous material in the ampullary lumen.

In females at oestrus, round homogeneous electron-dense secretory granules in the apical cytoplasm of both luminal and crypt non-ciliated epithelial cells in the isthmus of the oviduct were also labelled with gold particles (Figs. 4.29 and 4.30). These granules had a different appearance to those positively labelled in the ampulla. Again amorphous material in the lumen was similarly immunoreactive (Fig. 4.31). Ciliated epithelial cells were not labelled.

In the utero-tubal junction of females at oestrus scant immunoreactivity was found in the apical cytoplasm of occasional non-ciliated luminal epithelial cells and in adjacent glands. After embryos had entered the uterus immunogold labelling was found over electron-lucent secretory granules in the apical cytoplasm of occasional luminal epithelial cells and in adjacent glands (Figs. 4.32 and 4.33). The ultrastructural features of the immunoreactive luminal and glandular epithelia was similar. The cytoplasm of occasional glands was filled with immunoreactive granules of varying sizes (Fig. 4.34). Gold labelling was also found on amorphous material within the lumen of the utero-tubal junction, particularly adjacent to epithelial microvilli.

In females killed at oestrus the luminal epithelia of the uterus and endometrial glands, were generally not immunoreactive. However, after embryos had entered the uterus immunogold labelling of these epithelia was increased. Immunoreactive secretory granules were morphologically similar to those of the utero-tubal junction, as were the luminal and glandular epithelia in which they were found.

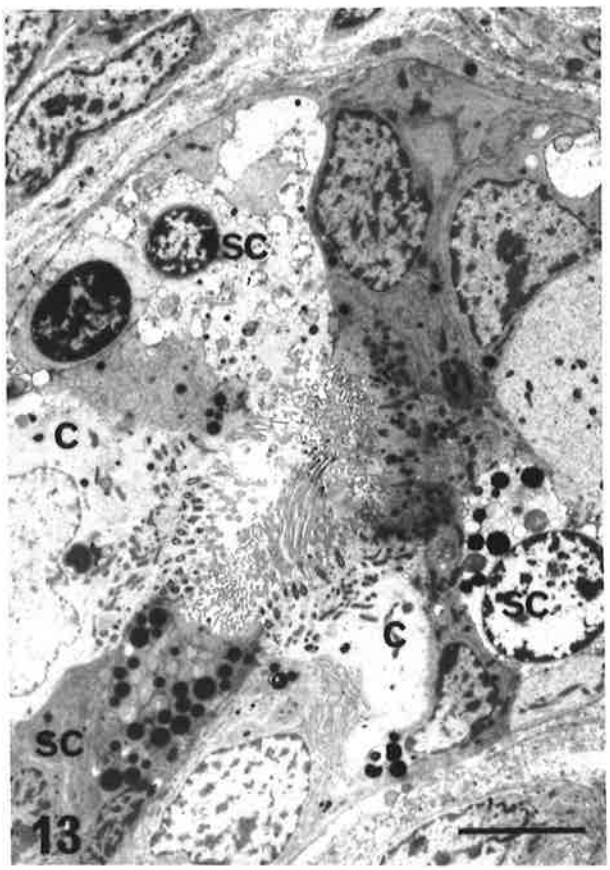
Figure 4.13 Gland from the utero-tubal junction has a mixture of non-ciliated secretory cells (SC) some of which have electron-dense secretory granules resembling those of the isthmus of the oviduct and in some the whole cytoplasm is more electron-lucent, more typical of the majority of glands from this region. Numerous ciliated cells (C) are present in the gland. Bar = 5μm.

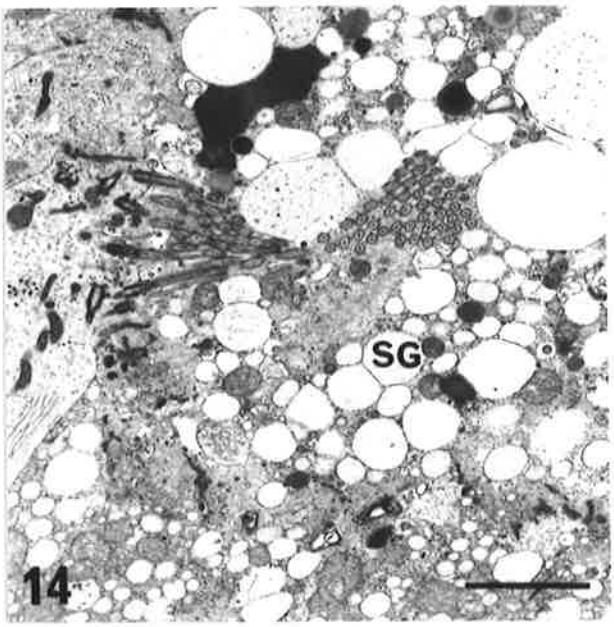
Figure 4.14 Typical non-ciliated secretory cells in glands of the utero-tubal junction have abundant electron lucent secretory granules (SG) which are filled with fine granular material. Bar = 2.5μm.

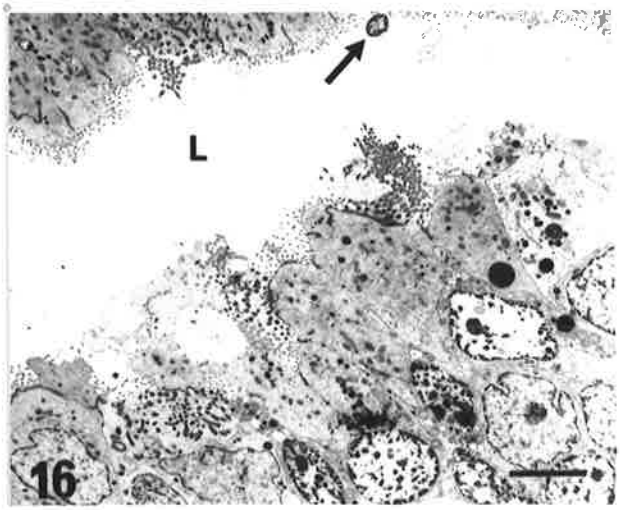
Figure 4.15 Ciliated cell from a gland in the utero-tubal junction has abundant polyribosomes, apical golgi complexes (GC), elongate electron-dense mitochondria with lamellar cristae (M) and a patch of electron-dense granular material (arrow). Bar = 1μm.

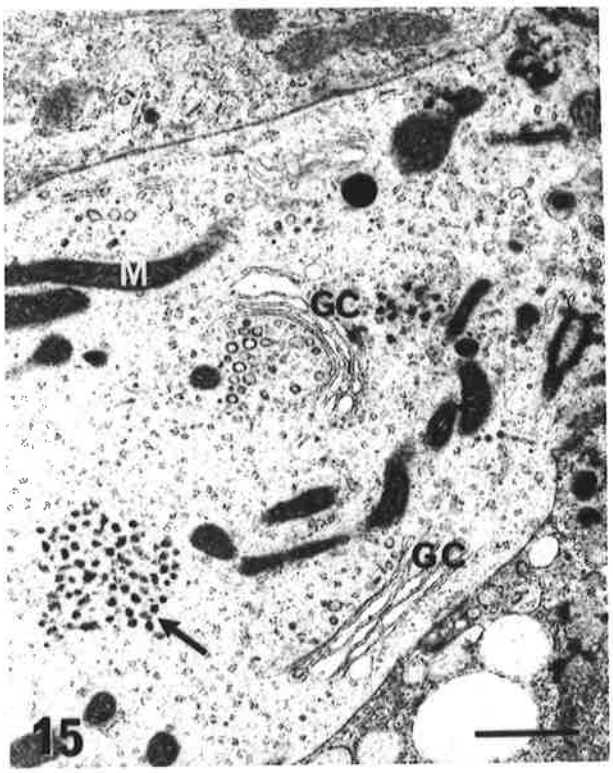
Figure 4.16 Luminal epithelium of the utero-tubal junction from a female which had one-cell uterine embryos shows that most cells are non-ciliated. Occasional sperm tails (arrow) are seen in the lumen at this stage. Bar = 5μm.

Figure 4.17 Higher magnification of the region shown in Fig. 4.15 shows that secretion (S) appears to have precipitated in the lumen (L) adjacent to the apical plasmalemma of a few ciliated cells (C); secretory cell (SC). Bar = 2.5μm.


SC
C
C
SC
SC
13


SG
14


L
16


M
GC
GC
15

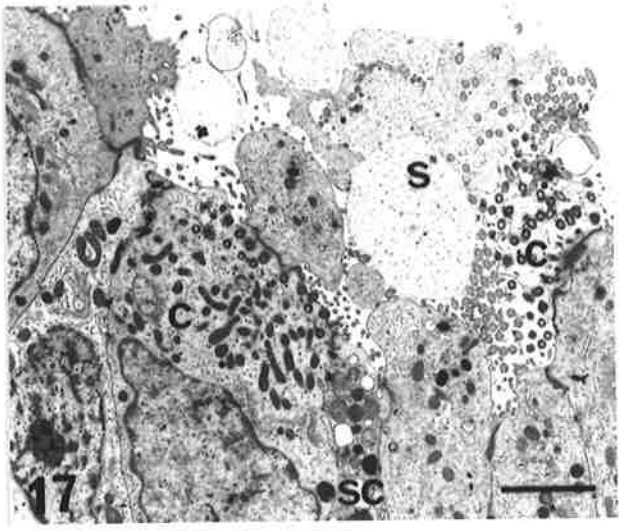
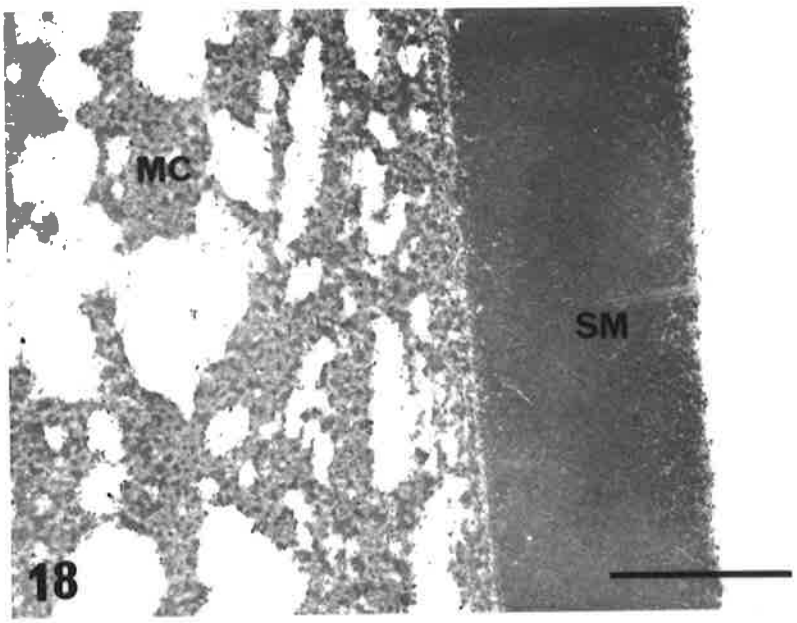
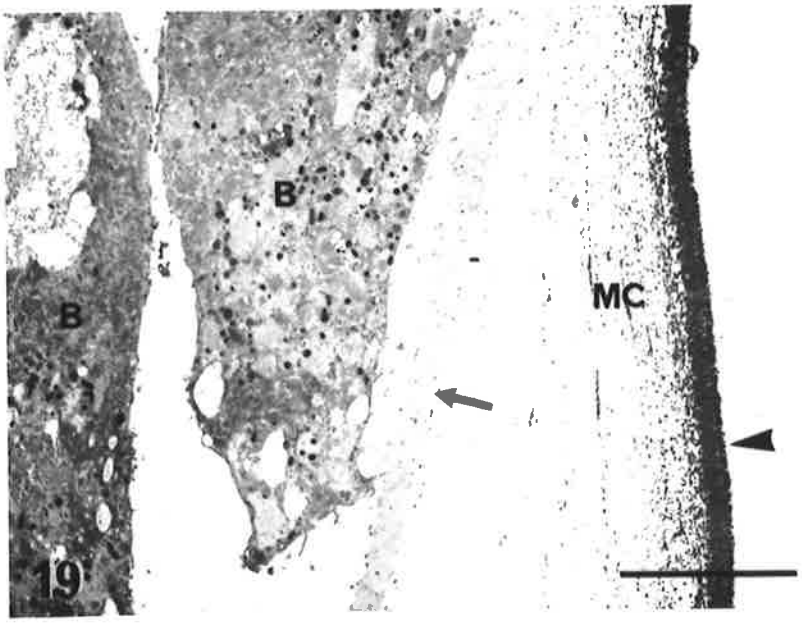

S
C
C
SC
17

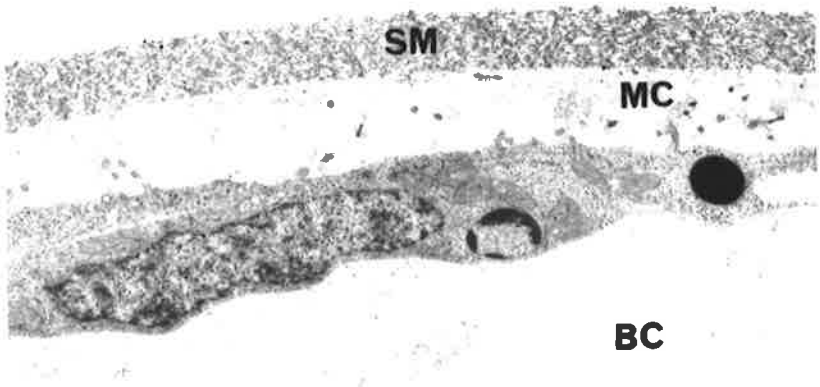
Figure 4.18 Shell membrane (SM) of one-cell uterine embryo is electron-dense, compact and granular and is 2μm thick; mucoid coat (MC). Bar = 1.6μm.

Figure 4.19 Cleaving uterine embryo with electron-dense compact shell membrane (arrowhead) and more diffuse mucoid coat (MC); blastomere (B), zona pellucida (arrow). Bar = 10μm.

Figure 4.20 Shell membrane (SM) surrounding unilaminar blastocyst is fibrous and in one layer; mucoid coat (MC) is present only in patches and the zona pellucida has disappeared; blastocoelic cavity (BC). Bar = 2.5μm.





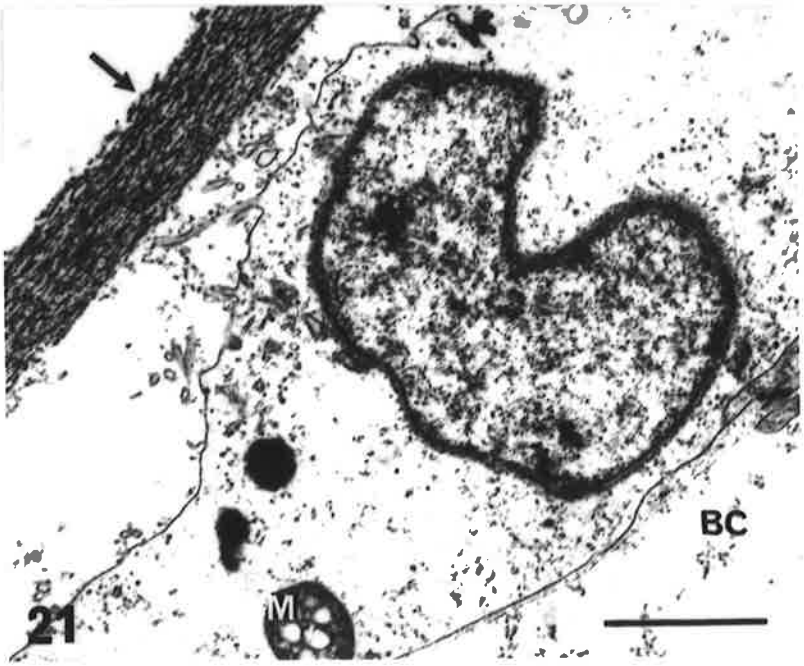


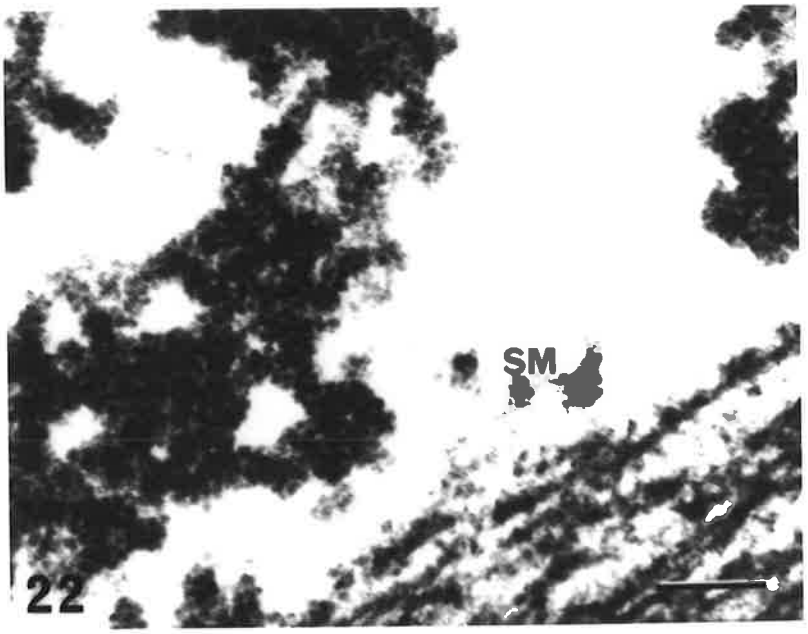
20

Figure 4.21 Bilaminar blastocysts are surronded by a fibrous shell membrane (arrow) and the mucoid coat has disappeared. Mitochondria (M) in the future trophectoderm cells are electron-dense with tubular cristae; blastocoelic cavity (BC). Bar = 2μm.

Figure 4.22 Higher magnification of shell membrane (SM) of blastocyst seen in Fig. 4.21 shows that in places it is granular on the outer surface but fibrous below. Bar = 200nm.

Figure 4.23 Immunogold labelling prior to embedding of an oocyte recovered from the uterus confirms that some of the polyclonal antibodies raised against the extra-cellular coats bind to shell membrane (SM) epitopes; colloidal gold particles (arrows). Bar = 200nm.


BC
M
21


SM
22

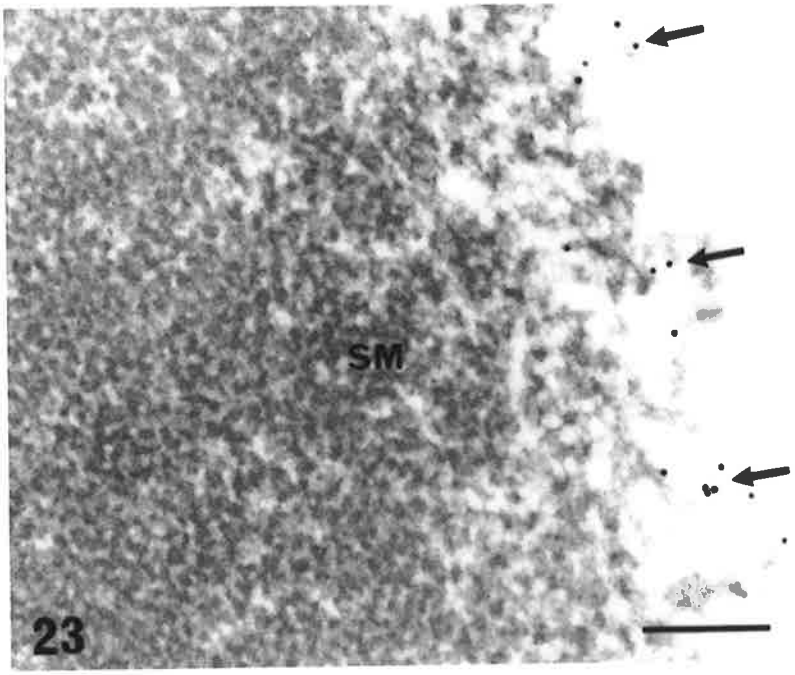

SM
23

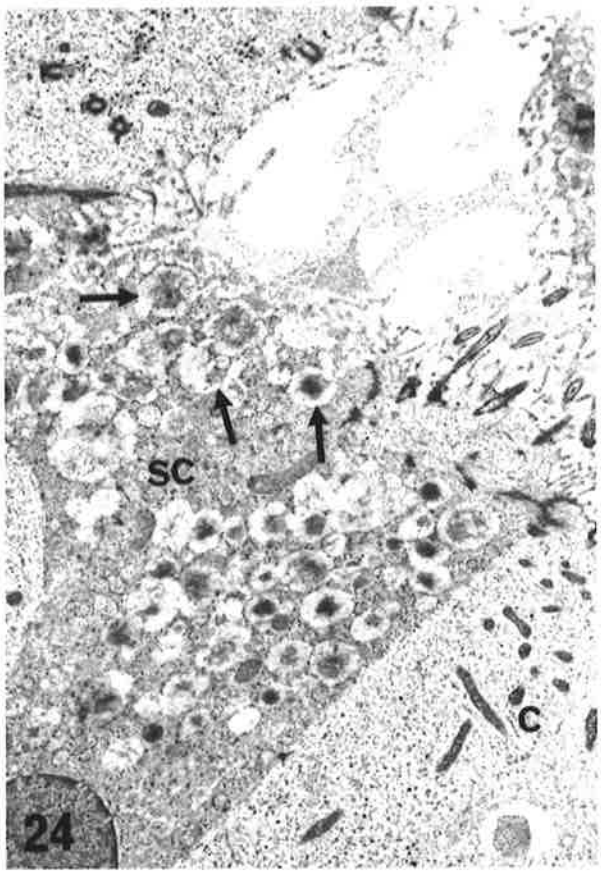
Figure 4.24 Low magnification of immunogold labelled epithelium of the ampulla of the oviduct of a female at oestrus for orientation purposes; ciliated cell (C), secretory cell (SC), secretory granules (arrows). Bar = 2μm.

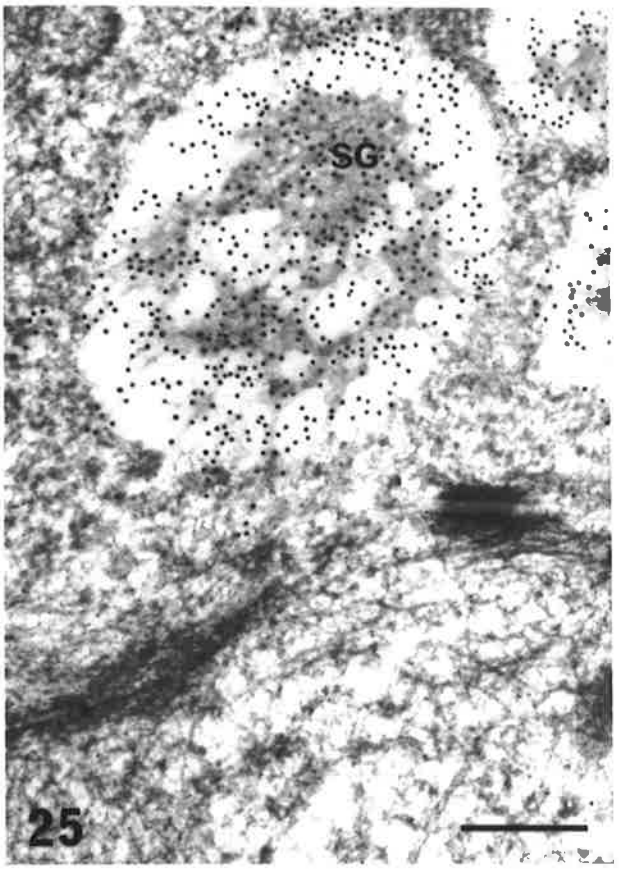
Figure 4.25 High magnification of part of the same region as shown in Fig. 4.24 shows that the heterogeneous secretory granule (SG) is immunoreactive with the antiserum to the extracellular coats of the oocyte. Bar = 250nm.

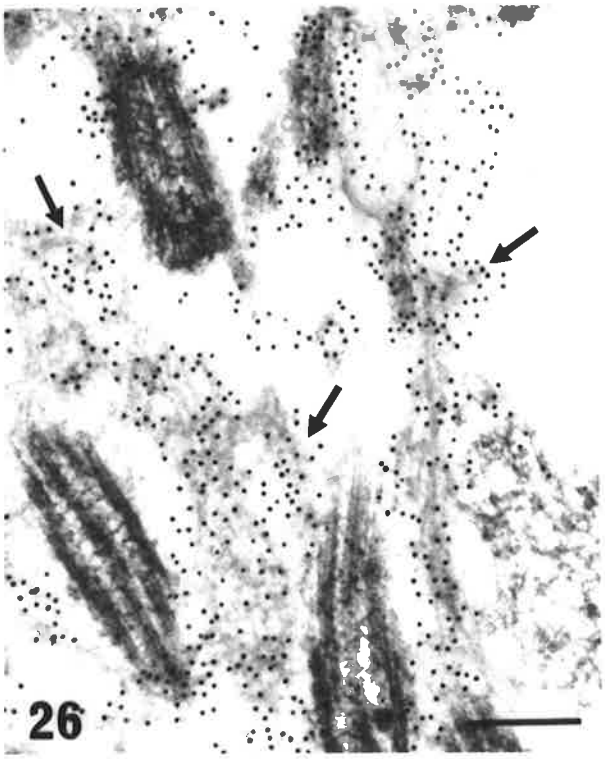
Figure 4.26 High magnification of part of the same region as shown in Fig. 4.24 shows that amorphous material (arrows) in the lumen of the ampulla between cilia is also immunoreactive. Bar = 250nm.

Figure 4.27 Immunolabelling of the ampulla of a female which had uterine embryos shows that most of the secretory granules have disappeared by this time. A large granule (arrow) appears to have just been shed by exocytosis. Bar = 1μm.

Figure 4.28 High magnification of the recently shed granule in Fig. 4.27 shows that it had contained immunoreactive material. Bar = 250nm.


SC
C
24


SG
25


26

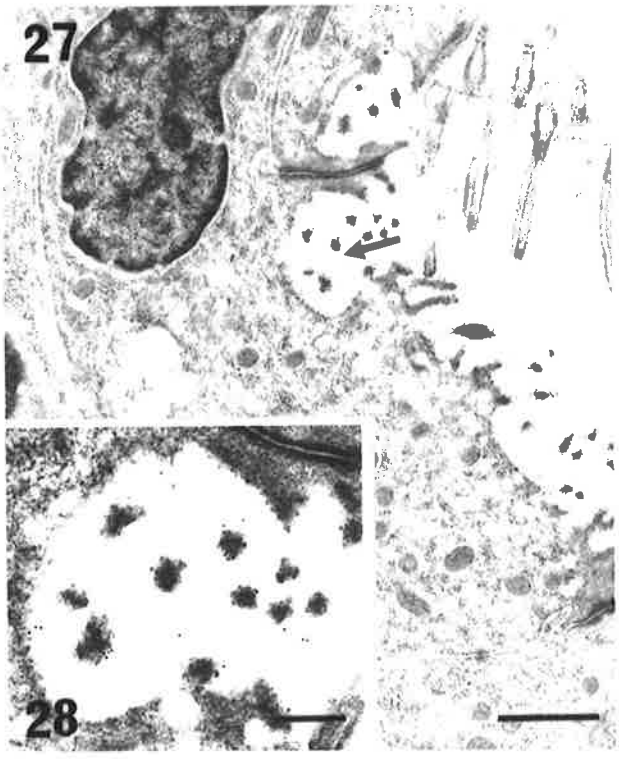

27
28

Figure 4.29 Low magnification of immunogold labelled epithelium of a crypt in the isthmus of the oviduct of a female at oestrus for orientation purposes; secretory granules (arrows). Bar = 2μm.

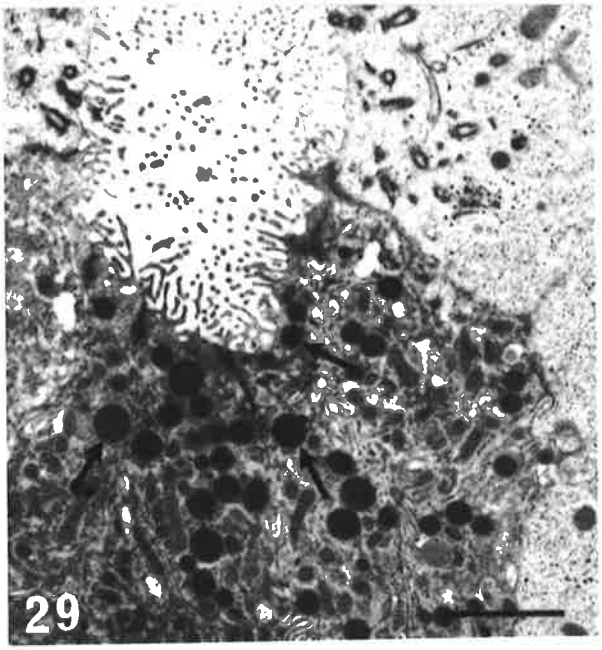
Figure 4.30 High magnification of a similar region of the isthmus to that in Fig. 4.29 shows that the electron-dense secretory granules (SG) in the isthmus are immunogold labelled with antiserum to the extracellular coats of the oocyte. Bar = 100nm.

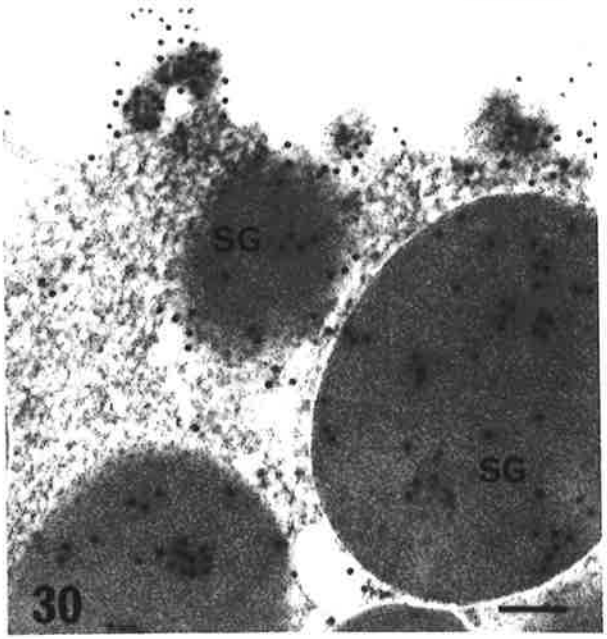
Figure 4.31 Amorphous material in the lumen of the crypt of the isthmus is also labelled with colloidal gold particles (arrows). Bar = 100nm.

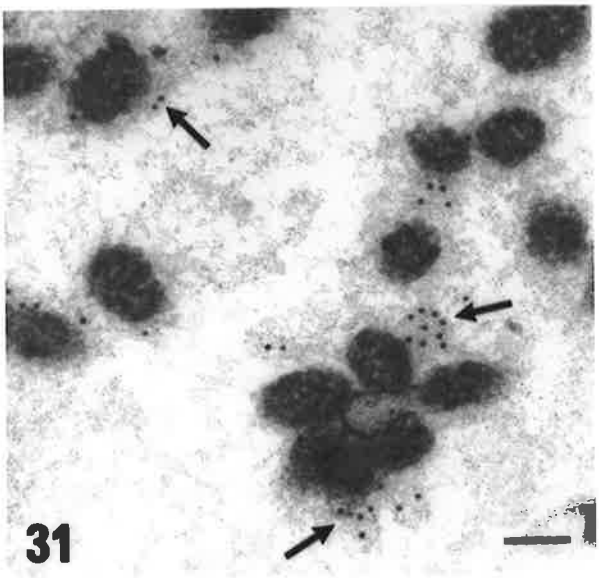
Figure 4.32 Low magnification of epithelium of a gland in the utero-tubal junction of a female with uterine embryos immunogold labelled with anti-EC polyclonal antibodies for orientation purposes; electron-lucent secretory granules (arrows). Gold particles are apparent in the lumen (arrowhead) even at this magnification. Bar = 1μm.

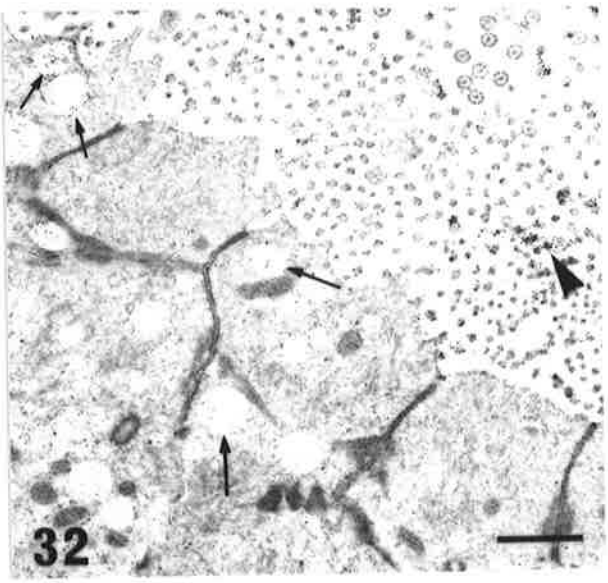
Figure 4.33 High magnification of the apex of a secretory cell seen in Fig. 4.32 shows that electron-lucent secretory granule (SG) is labelled with gold particles. Bar = 100nm.

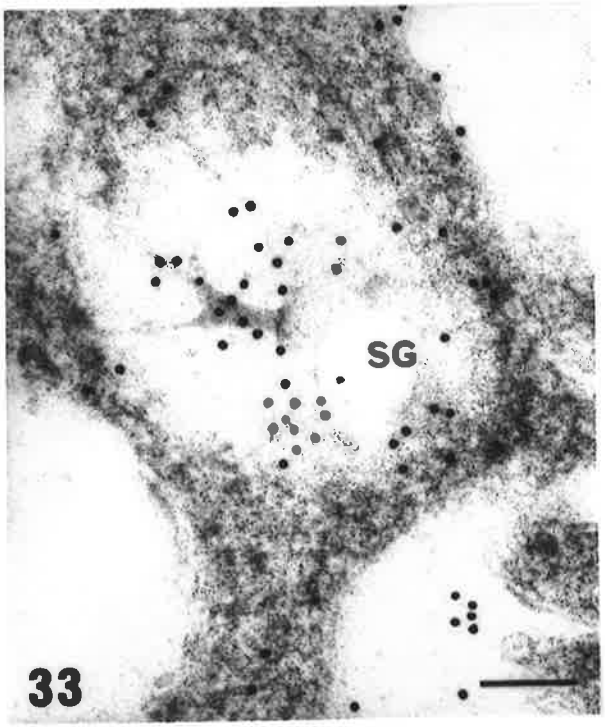
Figure 4.34 Glands in the utero-tubal junction from the same female with uterine embryos have abundant electron-lucent secretory granules (SG) which at higher magnification resemble those of Fig. 4.33 and are similarly immunoreactive. Bar = 2.5μm.

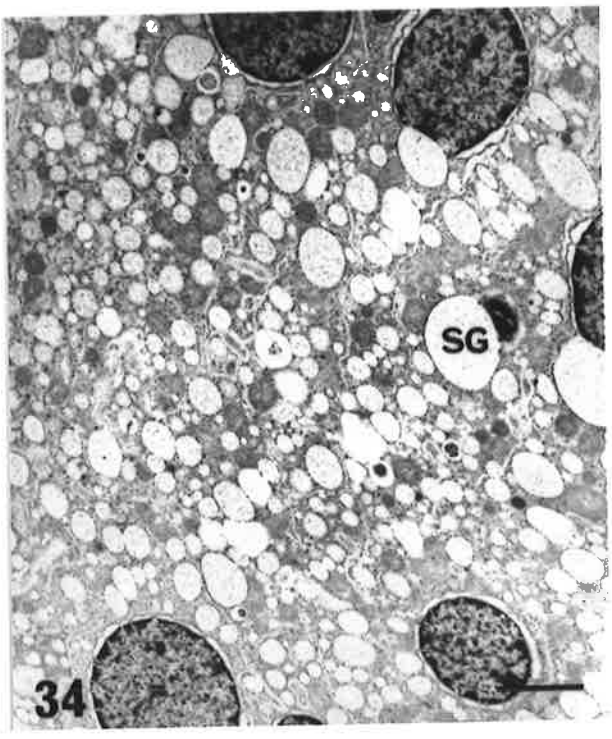

29


SG
SG
30


31


32


SG
33


SG
34

4.4 Discussion

Although these results have shown that the secretions of the ampulla and isthmus are histochemically similar, the ultrastructure of secretory granules in these two regions, which are both immunoreactive with the polyclonal anti-EC antibodies, is dissimilar. Those of the ampulla are more irregular in shape, have less homogeneous contents, and are much more variable in their electron density than those in the isthmus. Presumably these differences represent different glycoprotein/protein constituents of the secretory granules in the two regions and hence the mucoid coat. Alternatively, secretion from non-ciliated cells in the isthmus may be either a source of nutrition for the embryo, or a factor involved in its development, or even a material that binds the shell membrane to the mucoid layer. Differentiation by using monoclonal antibodies for further immunocytochemistry followed by Western blot analysis would identify the different molecular weight proteins in the mucoid coat and shell membrane and the precise cell populations responsible for their secretion.

Regional variations in electron density of secretory granules in oviducal non-ciliated cells is widespread amongst eutherian mammals (Nilsson and Reinius, 1969). In rats the secretory granules of the luminal epithelium of the ampulla are moderately electron-dense and homogeneous whereas in the isthmus these granules are of irregular electron-density (Abe, 1994). Similar ultrastructural findings have also been reported in the ampulla and isthmus of the golden hamster (Abe and Oikawa, 1991). In the luminal epithelium of the mouse oviduct secretory granules are electron-dense in the ampulla and more diffuse with filamentous material in the isthmus and monoclonal antibodies raised against oviducal glycoproteins only labelled secretory granules in the ampulla (Kapur and Johnson, 1988). In the bovine oviduct there are also regional variations in glycoprotein secretion (Abe *et al.*, 1993).

In the rabbit oviduct the ultrastructure of the secretory granules also varies with electron-dense homogeneous granules in the ampulla and electron-lucent heterogeneous granules in the isthmus (Jansen and Bajpai, 1982). However, it is the isthmus and not the ampulla which is responsible for mucin secretion in the rabbit. As marsupial and eutherian oviducts are both derived from the Müllerian ducts and as the ultrastructural features of the mucin secretory granules, their histochemistry and their mode of secretion are similar in rabbits (Jansen and Bajpai, 1982) and the dunnart the mucin coats may be homologous. Immunolabelling and fluorescence studies of the oviduct and early embryos of the domestic rabbit using the polyclonal antibodies raised against the dunnart extra-cellular coats would determine if there is conservation of mucoid epitopes between these two species. The fact that the secretory granules of the

epithelia of the ampulla and isthmus in the dunnart oviduct have similar histochemical properties, that is they are acidic glycoproteins, but they have dissimilar ultrastructure suggests that histochemistry alone is not necessarily sufficient to determine that secretions from different regions, or in different species, are the same.

Nilsson and Reinius (1969) suggested that electron-dense secretory granules of the oviduct are generally more proteinaceous, possibly rich in enzymes, while less dense granules are mucinous. In the dunnart the secretory granules of the ampulla contribute to the mucoid layer which has, as its name suggests, long been considered mucinous. The comparatively electron-lucent granules in the luminal and glandular epithelia of the utero-tubal junction and endometrium, which appear to be precursors of the shell membrane, are morphologically quite distinct from any of those of the oviduct. The granules containing the shell membrane precursors are comparatively electron-lucent, sometimes containing flocculent material. My histochemical work (see chapter 3, Roberts *et al.*, 1994) suggests that SM precursors are not mucinous. SDS-PAGE gel electrophoresis of the extra-cellular coats performed before raising the polyclonal antibodies stained with both coomassie blue and silver showed 12 protein bands suggesting that the shell membrane and mucoid layers are a mixture of proteins. Although there are varying electron densities in secretory granules in the different regions of the oviduct and utero-tubal junction, it is probably unwise to draw conclusions as to their contents from morphology alone (Fawcett, 1966).

Studies of recently fertilized embryos of the fat-tailed dunnart in this laboratory have suggested that the electron-dense secretory granules in the isthmic crypts only release their contents as zygotes pass through the oviduct (Breed *et al.*, 1989) as during the period of sperm storage the isthmic crypt epithelial cells contain electron-dense granules which are morphologically dissimilar to those of the ampulla (WG Breed, unpublished observations and this thesis). Hence, it was suggested that the former may be shell membrane precursors and the residual bodies within these cells after transport of zygotes to the uterus were possibly the remains of spermatozoa which had been phagocytosed by these cells. However, the secretory granules of epithelial cells in the utero-tubal junction and uterine glands which are particularly immunoreactive after embryos have entered the uterus, and undoubtedly contribute to the shell membrane, are electron-lucent and quite unlike those of the isthmus. Although it remains to be demonstrated exactly how the electron-lucent secretory material becomes an electron-dense shell membrane after secretion and deposition, the fact that embryos recovered from the lower isthmus do not have a shell membrane together with my light microscopical findings (see chapter 3; Roberts *et al.*, 1994) make it seem

unlikely that the isthmic granules could contribute to the shell membrane, despite their electron density.

These results suggest that in the dunnart, the shell membrane precursors come from the same cell populations as those of the grey short-tailed opossum (Baggott and Moore, 1990). A shell membrane in this species is only found surrounding uterine embryos and reaches its maximum thickness just prior to blastocyst expansion. In bandicoots, the shell membrane is also thickest in early to mid-unilaminar blastocysts prior to expansion (Lyne and Hollis, 1976). However, in the dunnart, it is thickest around cleaving embryos and then, as the blastocyst expands, it becomes fibrous in appearance and slightly thinner, but thicker again at the primitive streak stage when the embryonic vesicle is much larger.

Hollis and Lyne (1977) reported that partly bilaminar blastocysts of two species of bandicoots have additional granular material on their surface. This has a similar appearance to granular material found on the surface of dunnart bilaminar blastocysts which I suggest is newly deposited shell membrane material and indicates that SM synthesis and deposition are still occurring at this stage in development. The fact that the shell membrane is thickest around primitive streak embryos but completely fibrous suggests that its synthesis has only recently ceased at this late stage in pregnancy (about day 9). In the last chapter I argued that the shell membrane was necessary during cleavage and blastocyst development in marsupials to give structural integrity to the early embryo. In horses, a glycoprotein capsule, which is thought to have both protective and anti-adhesion properties (Oriol *et al.*, 1993), is secreted by the trophoblast from about day 6 of pregnancy and essentially replaces the zona pellucida after it is shed (Flood *et al.*, 1982). After the capsule is lost on day 22 implantation takes place (Enders and Liu, 1991a). In marsupials the zona pellucida has disappeared by the unilaminar blastocyst stage (Ward and Renfree, 1988; Bedford, 1991; Selwood, 1992). In the dunnart, trilaminar embryos are about 3mm across but for most of their surface are only two squamous cell layers thick and are most fragile. Given that implantation follows the loss of the shell membrane in all marsupials it may be that the shell membrane at late pre-implantation stages of pregnancy also protects the embryo.

In the North American opossum the ultrastructure of the shell membrane has only been examined in mid- to late-pregnancy just prior to its loss (Krause and Cutts, 1983) when it had the appearance of a fibrous mat. These observations were made in embryos on days 8 and 9 of their 12.5 day pregnancy. The shell membrane in the opossum is lost late on day 10 of pregnancy (Krause and Cutts, 1985b). In mid-

pregnancy in the dunnart when embryos are at the primitive streak stage the shell membrane is also fibrous after having been granular earlier in embryonic development.

In birds and lizards, the shell membrane precursors are extruded from the glands as fibres which subsequently are wrapped around the egg as a result of myometrial activity (Palmer *et al.*, 1993). In lizards the shell membrane consists of two layers, an inner fibrous and an outer layer of densely packed particles. However, in turtles shell membrane precursors are secreted as small particles which polymerize with each other into long fibres on the surface of the egg, (Palmer *et al.*, 1993). Perhaps this also happens to the shell membrane of the dunnart where it may first be deposited as granules which later coalesce, or possibly polymerise, into fibres as development proceeds. This may give it distensible properties as exemplified by its thinning as the blastocyst expands.

In birds (Hoffer, 1971) and tortoises (Palmer and Guillette, 1988) the shell membrane fibres appear to have an electron-dense medulla surrounded by an electron-lucent space which is in turn surrounded by an electron-dense cortex (Hoffer, 1971). In birds both layers appear to be a glycoprotein, with less than 6% carbohydrate and clusters of aspartic and glutamic acids. After experimental oxidation the layers appear to fuse which may suggest that they are different phases of the same material held separately *in vivo* by disulphide bonds (Candlish, 1972). In the tortoise the medulla consists of protein with an outer carbohydrate cortex (Palmer and Guillette, 1988). My previous histochemical studies have shown that the dunnart SM is neither rich in disulphide bonds nor sulfhydryl groups (see chap. 3 above, Roberts *et al.*, 1994) as has previously been suggested (Hughes, 1974a) but probably consists of basic proteins which bind acid dyes.

These results also show that the shell membrane of the dunnart is not a layered structure and therefore is different in appearance to that around the diapausing tammar wallaby blastocyst which Renfree (1993a, 1994) showed had three distinct layers. Walker and Hughes (1981) described a diapausing blastocyst of the red-necked wallaby but did not mention if there was layering of the shell membrane or not. Renfree (1993a, 1994) did not mention how long the tammar blastocyst had been in diapause. It may be that over a long period of embryonic diapause as occurs in the tammar, there may be sufficient oscillations in the hormonal milieu, particularly toward the end of suckling, that could cause an episodic stimulation of shell membrane synthesis and secretion producing the layering effect.

Hughes (1974a) found in the brush tailed possum that both mucoid and shell membrane precursors are secreted from three days before to three days after oestrus. My findings in the dunnart suggest that mucoid synthesis has begun at oestrus, as secreted mucoid surrounds ampullary oocytes, but when oocytes are not present mucoid does not appear in the tubal lumen. In the isthmus, too, secretory granules only appear to be released during the passage of oocytes/zygotes. Phillips and Fadem (1987) reported similar events in the grey short-tailed opossum and also speculated that the oocyte stimulates the epithelium to secrete the extra-cellular coats. It has been suggested that in rabbits, too, mucin secretion by the oviduct is most prevalent in the vicinity of the egg (Fredricsson, 1969). My results for the dunnart are in agreement with these findings and, in addition, suggest that shell membrane precursor synthesis at oestrus is minimal and is largely delayed until just before embryos enter the utero-tubal junction as immunoreactivity is strongest in this region and in the endometrial epithelia only after embryos have entered the uterus. Also mucoid secretion has all but ceased by the time that embryos have entered the uterus suggesting that there is a temporal difference in mucoid and shell membrane synthesis and secretion.

Many studies over the last twenty years have shown that the oviduct is not merely a corridor through which gametes and embryos travel on their way from the site of fertilization to the uterus. Secretions from oviducal epithelial cells may play an important role in early embryonic development in a variety of mammalian species as *in vitro* embryonic development and survival are greatly enhanced by co-culture with these cells (Ellington *et al.*, 1990; Xu *et al.*, 1992). In mice glycoproteins secreted by the ampulla are found in the matrix of the perivitelline space of ovulated oocytes and participate in the creation of a special microenvironment for fertilization and cleavage (Kapur and Johnson, 1988). In hamsters the glycoprotein, oviductin, binds to the zona pellucida as the embryo is transported down the oviduct (St-Jacques *et al.*, 1992). Oviduct specific glycoproteins in sheep, cows and pigs have been shown to have a similar molecular weight and immunolocalization using monoclonal antibodies but this was not the case for mice and rabbit oviducal glycoproteins which suggests conservation within ungulate species (Gandolfi *et al.*, 1993). These results suggest that there is probably some functional role for these proteins during the tubal residence of the eutherian embryo. It is clearly apparent that in marsupials, too, despite the short period of tubal life, oviducal epithelial cells play a significant role in early development.

Chapter 5 Implantation

5.1 Introduction

In marsupials implantation occurs about two-thirds the way through the short gestation before which time the embryo is surrounded by the permeable shell membrane (SM) which prevents physical contact between the trophoblast and uterine epithelium (Hughes, 1974a&b). In the tammar wallaby, the SM is shed, and implantation proceeds, after the action of alkaline proteinases at the interface between embryonic and maternal tissues which occurs on about day 18 of the 27 day pregnancy (Denker and Tyndale-Biscoe, 1986).

All marsupials, like most eutherians, develop a chorio-vitelline or yolk sac placenta which is composed of vascular and non-vascular regions; most marsupials do not subsequently develop a chorioallantoic placenta. Migration of mesoderm away from the embryo between the extra-embryonic trophectoderm and endoderm results in the formation of a yolk sac which has two regions: a vascular, or trilaminar, region (TYS) and an avascular , or bilaminar region (BYS) (Luckett, 1977).

The fetal-maternal cell associations at the time of implantation in marsupials have been little studied other than to determine whether the trophoblast is invasive (Hughes, 1974b). In some species the trophoblast erodes the uterine epithelium, eg. *Dasyurus viverrinus*, *Sminthopsis crassicaudata*, Dasyuridae (Hill, 1900; Hughes 1974b), *Philander opossum*, Didelphidae (Enders and Enders, 1969), *Phascolarctos cinereus*, Phascolarctidae (Caldwell, 1884; Hughes, 1984), and *Distoechurus pennatus*, Acrobatidae (Hughes *et al.*, 1987) although the depth of invasion and the area of uterine epithelium penetrated appear to be inconsistent between these species. In addition, the bandicoots (Peramelidae) develop an invasive chorio-allantoic placenta during the final 2 days of pregnancy (Padykula and Taylor, 1976, 1982). Although the trophoblast is invasive in these species, the ultrastructure of the embryonic-uterine cell interactions at the time of implantation has not been described for these or any other marsupial.

This part of the project sought to determine the stage of embryonic development of the loss of the shell membrane and the ultrastructure of implantation in *S. crassicaudata*. The results are compared with what is known of the cellular interactions at this time in eutherian mammals.

5.2 Materials and Methods

Female dunnarts were paired with fertile males and urine samples from each female were examined each morning for the presence of sperm by phase contrast microscopy. The last day that sperm were present in the smear was designated Day 1 of pregnancy.

Females were sacrificed as above (see page 44) 11-15 days after sperm had been found in the urine. Consequently a range of gestational ages was obtained. Immediately following death, uteri were dissected in routine TEM fixative and the stage of embryonic development and crown-rump length (CRL) of the embryos, when applicable, were recorded and photographs of the embryos were taken. Embryos from one female were processed for scanning electron microscopy (SEM) as follows: the tissue was fixed by immersion in routine TEM fixative (see page 45) for 24h, post-fixed in 1% OsO_4 for 1h, dehydrated in a graded series of acetones, critical point dried in CO_2 in a Balzers Union Critical Point Dryer, mounted on SEM stubs with double sided tape, coated with 20nm carbon/palladium and examined at 20kV with a Phillips 500 SEM. Some embryos and part of, or the whole, uterus from each animal were processed for light microscopy and embedded in paraffin as previously described (see page 45). Seven micron sections were cut and stained with haematoxylin and eosin (Drury and Wallington, 1980). Other uteri and embryos were fixed and processed as described previously for routine transmission electron microscopy (see page 45). Subsequently, 0.5µm thick plastic sections were cut and stained with toluidine blue (see page 45). Ultrathin sections were cut from selected blocks showing stages of implantation, stained with uranyl acetate and lead citrate, and viewed at 80kV in a JEOL 100S transmission electron microscope.

5.3 Results

By sacrificing animals at a variety of times after sperm had been found in the urine I have obtained a range of stages of implantation and development of the placenta in this species. The following is an ultrastructural description of implantation.

5.3.1 Preimplantation Stages

In early pregnancy the embryo was surrounded by the shell membrane and lay closely apposed to the simple, columnar, uterine luminal epithelium. At the unilaminar blastocyst stage embryos, enclosed by shell membranes, lay in depressions of the

endometrium in which the stroma was tightly packed with glands (Fig. 5.1). By the early bilaminar blastocyst stage embryos were enclosed by maternal epithelial folds although they could be flushed from the uteri with fixative. At this stage the surface of the uterine luminal epithelium had a smooth profile, the microvilli were short, and the cell apices contained abundant mitochondria. The lateral cell membranes below the apical junctional complex were also unfolded. The nuclei of the epithelial cells were euchromatic and often had a single prominent nucleolus (Fig. 5.2). The endometrial stroma had many glands whose epithelial cells had a well developed golgi complex, numerous mitochondria and large electron-lucent vacuoles in their apical cytoplasm. However, in some areas of the stroma, particularly below where blastocysts lay, the glands were further apart and there appeared to be an increased volume of extra-cellular matrix than at earlier stages.

At the bilaminar blastocyst stage the lumen of endometrial glands in the uterus contained abundant granular secretion. The glandular epithelial cells had numerous elongate electron-dense mitochondria below which dilated golgi complexes and basal nuclei were found. (Fig. 5.3) Apical secretory granules were not as plentiful as at earlier stages but contained a fine granular product (Fig. 5.4).

Trophoblast cells in early bilaminar blastocysts were squamous or cuboidal with sparse irregular microvilli and an electron-lucent cytoplasm containing few organelles which included mitochondria with tubular cristae, a little rough endoplasmic reticulum and occasional electron-dense vacuoles (Fig. 5.5). The newly differentiating endoderm had a similar appearance except that it was always more electron-dense than the adjacent trophoblast (Fig. 5.5).

Early trilaminar embryos which measured about 2.4mm with a round embryonic disc region and early primitive streak embryos 2.8mm in diameter, and primitive streak stage embryos from two different females which measured 3mm in diameter with an elongated embryonic disc region were recovered from four pregnant females killed on days 12, 11, 11 and 12 *pc* respectively. These embryos were still surrounded by a thin translucent shell membrane which was 1.5-1.8μm thick and could be flushed from the uterus with fixative. Despite this they were closely surrounded by endometrial folds and when they were displaced from their 'pockets' there was a distinct spherical depression in the luminal surfaces between which they had lain. These 'pockets' were clearly the future implantation chambers. At dissection there was no impression of there having been a distinct fluid filled uterine lumen but rather the endometrial epithelium appeared to be closed around the embryos.

Some uterine luminal epithelial cells closely associated with these early trilaminar /primitive streak embryos had a highly vacuolated cytoplasm. The mitochondria were as numerous as at unilaminar and bilaminar blastocyst stages and the microvilli were often branched. The terminal web region, which was devoid of organelles, was 0.8-1.0μm thick. Occasional lipid droplets were present.

Trophoblast in primitive streak embryos had sparse long irregular microvilli on its apical cell membrane which had not been seen at earlier stages in development (Fig. 5.6).

5.3.2 Epithelial Penetration

A single pregnant female was obtained on about day 10 of pregnancy with embryos at the early somite stage. The proamnion had begun to form (Fig. 5.7) and the ellipsoid yolk sac vesicle measured about 2 mm by 4 mm and the shell membrane had been lost. Up to six embryos within their yolk sacs, filled the uterine lumen whose epithelial lining had become very folded and had taken on a cobblestone appearance, that is, its cell apices had become dome shaped. Maternal epithelial cell microvilli were short, blunt and irregular. Lipid droplets and many mitochondria, which had an electron-dense matrix and lamellar cristae, had accumulated in the apical cytoplasm (Figs. 5.8 and 5.9). Rough endoplasmic reticulum was abundant throughout these cells but was particularly the dominant organelle of the perinuclear cytoplasm. Some maternal epithelial cells had numerous electron lucent vacuoles throughout their cytoplasm. Below the apical junctional complex the lateral membranes of these cells were extremely folded and there was extensive interdigitation of these processes between neighbouring cells (Fig. 5.9). Occasional maternal epithelial cells were very electron-dense but both light and dark cells had a similar ultrastructural appearance and appeared equally healthy (Fig. 5.9). Dark cells were only found in the uterine epithelium at this stage of pregnancy.

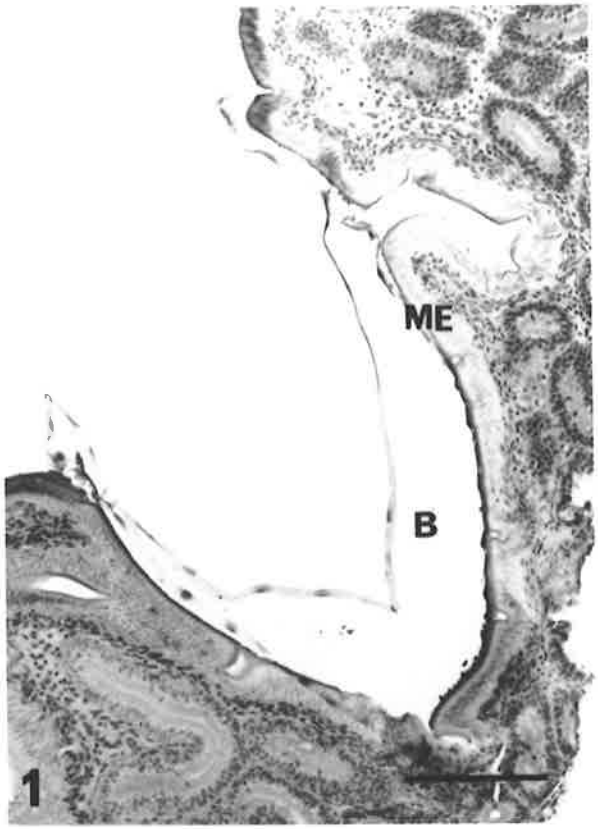
In this female the yolk sacs of the early somite embryos could be removed from the uterus using fine forceps but at least one of these appeared to be torn a little when viewed with the dissecting microscope. Some of these embryos were processed for scanning electron microscopy which showed that trophoblast cells adjacent to the embryo were large and had densely packed long regular microvilli (Fig. 5.10). The plasmalemma of the yolk sac cavity surface of the underlying endoderm had few microvilli and some vesicles (possibly secretory) which appeared to be budding off from the cell membrane (Fig. 5.11).

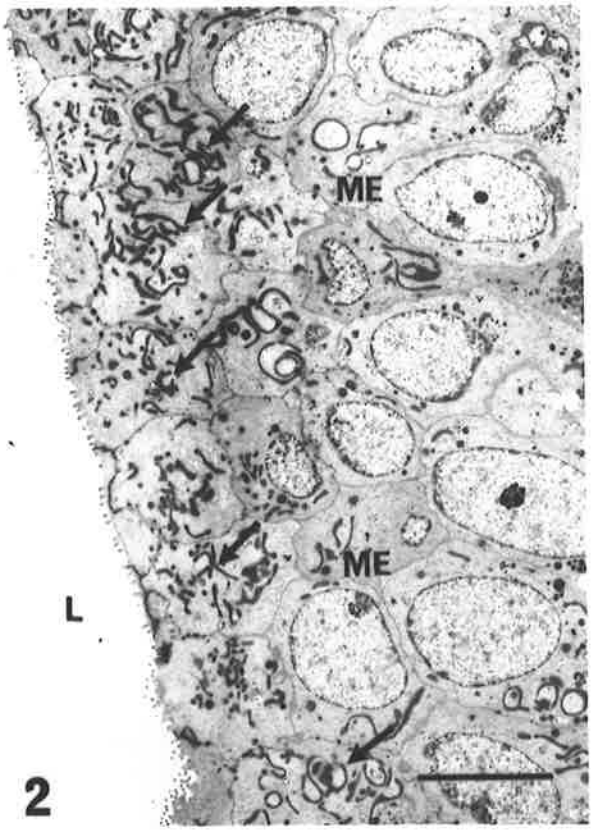
Figure 5.1 Unilaminar blastocyst (B) lying in a shallow depression of the endometrium is closely apposed to the uterine luminal epithelium (ME), gland (Gl). Bar = 50μm. H & E.

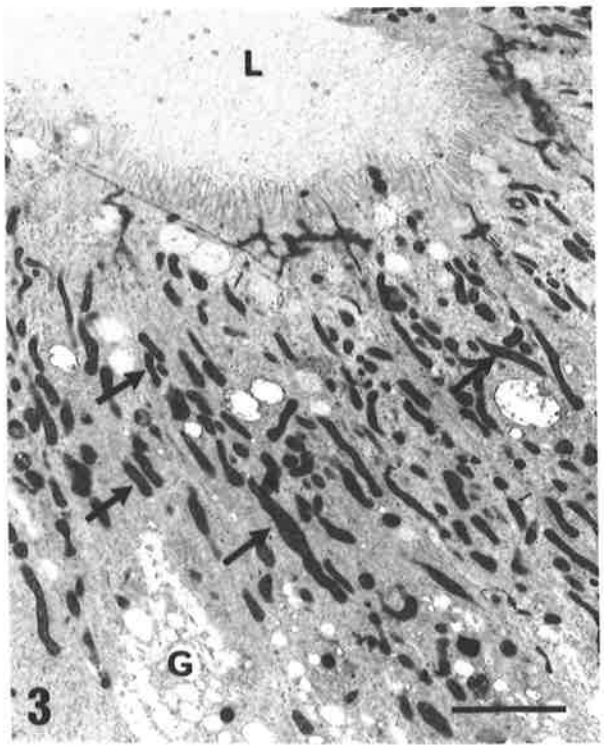
Figure 5.2 Uterine luminal epithelium (ME) at the early bilaminar blastocyst stage has a smooth profile with many apical mitochondria (arrows); lumen (L). Bar = 10μm.

Figure 5.3 Lumen (L) of uterine gland at bilaminar blastocyst stage contains abundant granular secretion. Epithelial cells have numerous elongate electron-dense mitochondria (arrows) below which dilated golgi complexes (G) are seen. Bar = 2.5μm.

Figure 5.4 Higher magnification of similar region of uterine gland at same stage as in Fig. 5.3 shows apical secretory granules (SG) are not as plentiful as at earlier stages but contain a fine granular product; lumen (L). Bar = 750nm.


ME
B
1


ME
ME
L
2


L
G
3

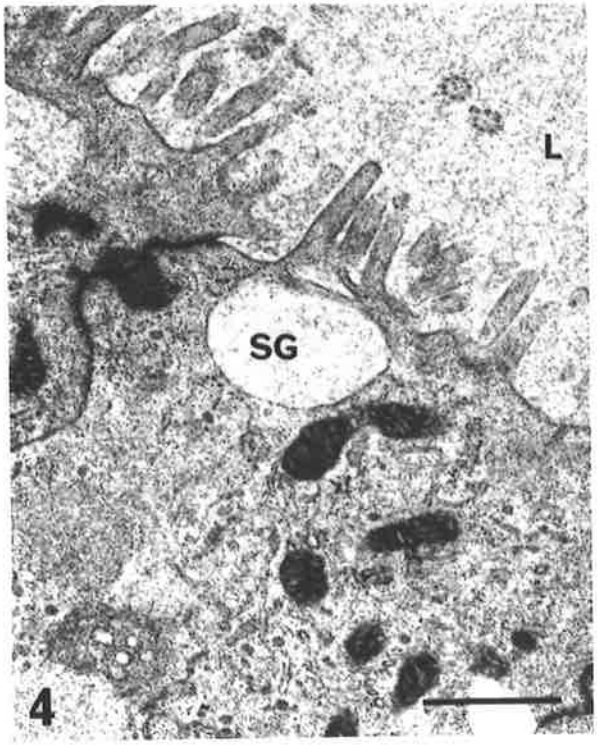
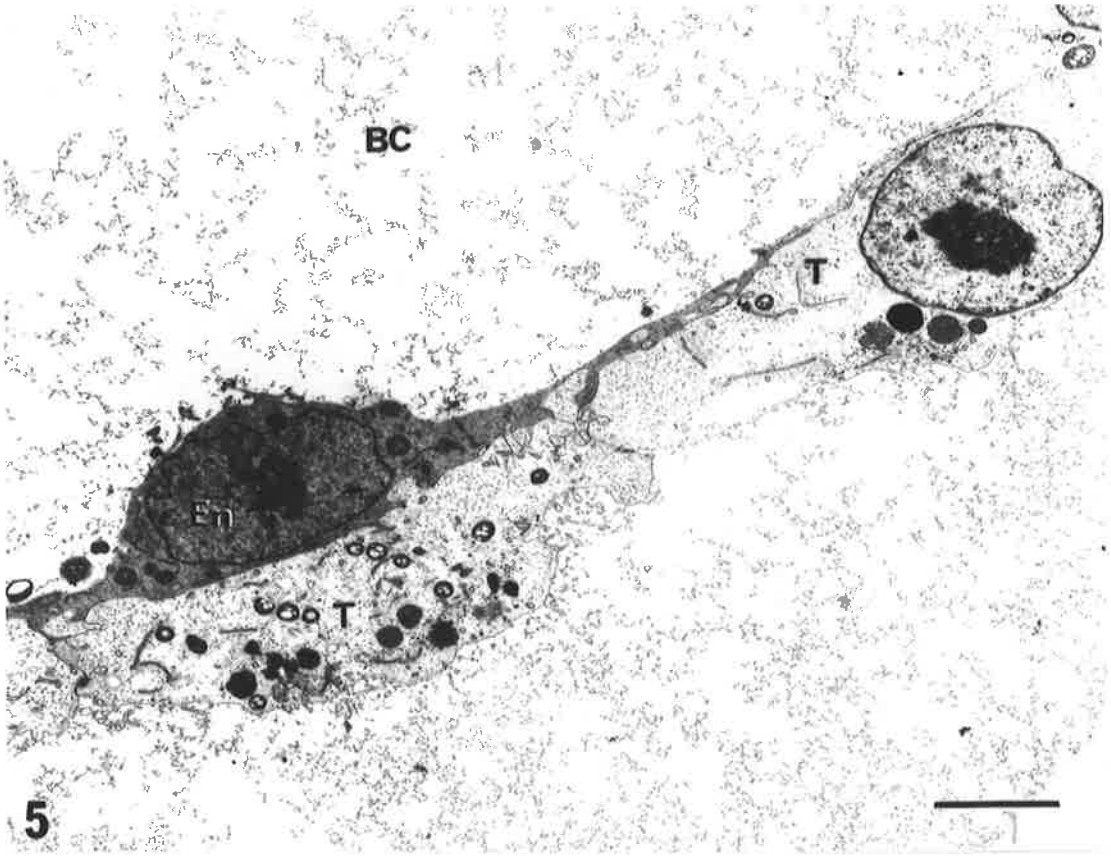
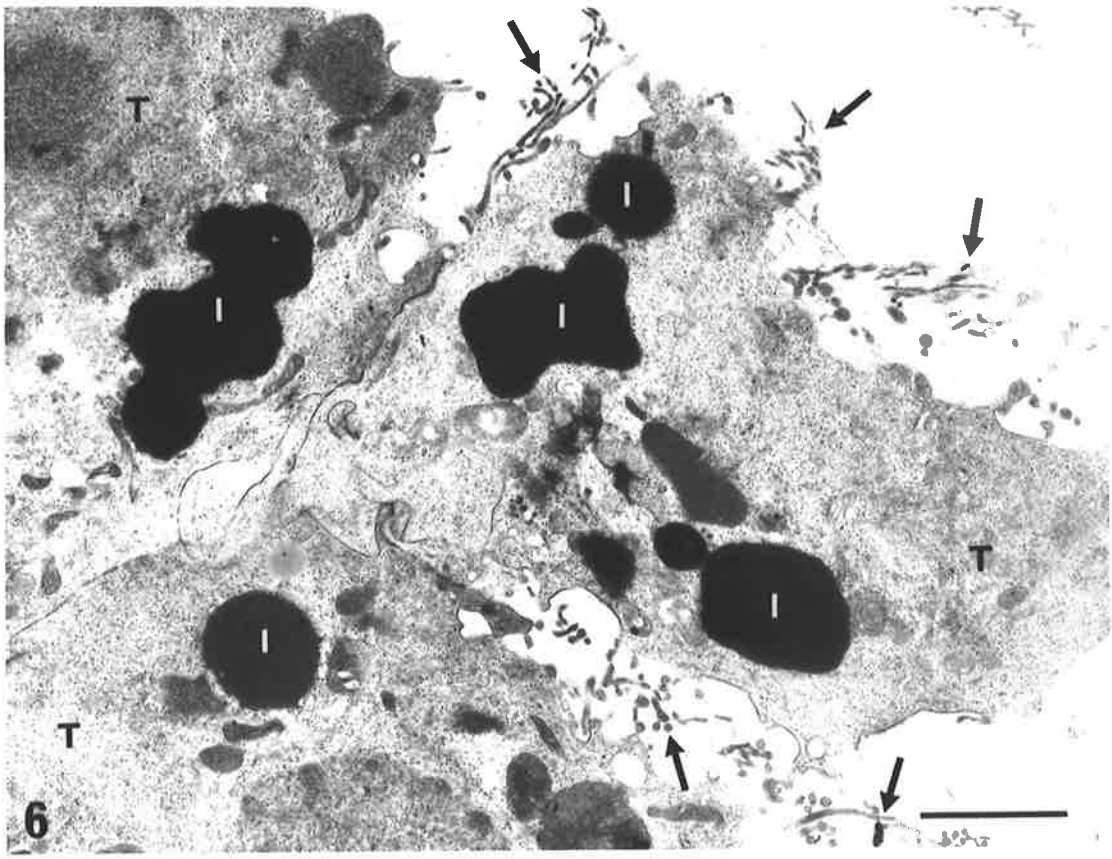

L
SG
4

Figure 5.5 Trophoblast cells (T) in early bilaminar blastocysts are very electron-lucent with few organelles; blastocoelic cavity (BC), endoderm (En). Bar = 5μm.

Figure 5.6 Trophoblast (T) from primitive streak embryo has sparse long irregular microvilli on its apical cell membrane (arrows) and several electron-dense inclusions (I). Bar = 2μm.


BC
T
En
T
5

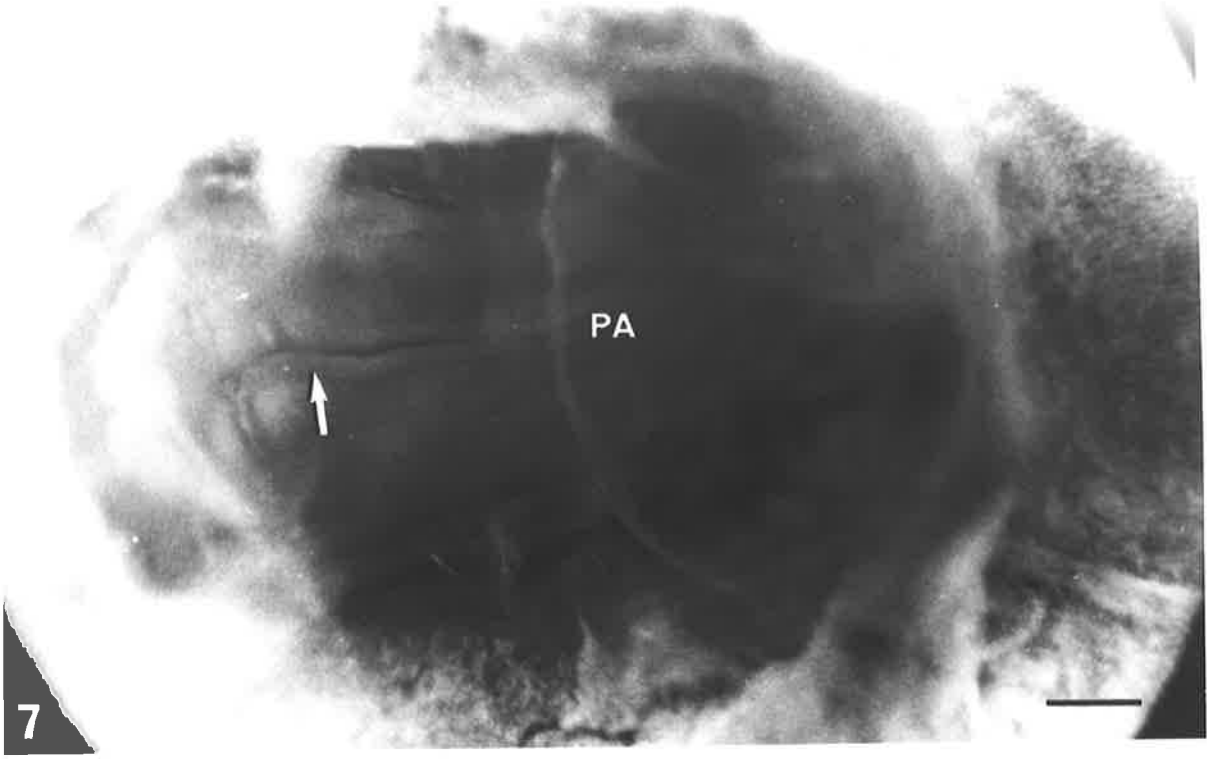

T
I
I
I
T
I
T
6

Figures 5.7-5.9 Early somite stage.

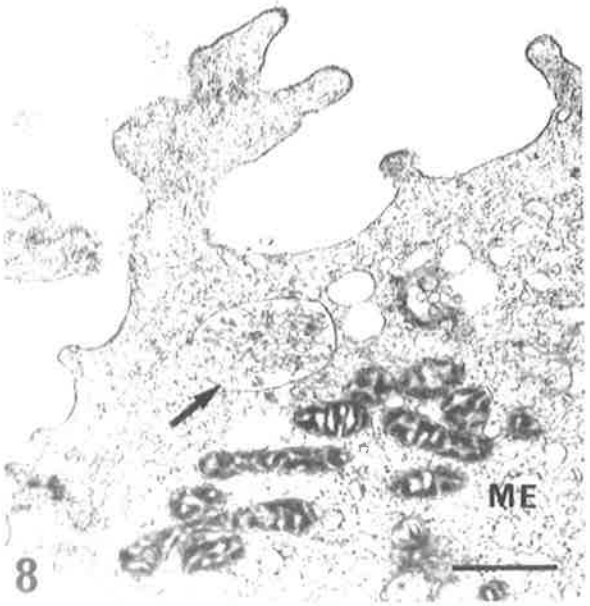
Figure 5.7 Embryonic disc region in the surface of part of the yolk sac vesicle. The proamnion (PA) is just forming above the cranial region of the embryo; neural groove (arrow). Bar = 200μm.

Figure 5.8 Microvilli of the uterine luminal epithelium (ME) are short, blunt and irregular and there are occasional multivesicular bodies (arrow). Many electron-dense mitochondria have accumulated apically. Bar = 500nm.

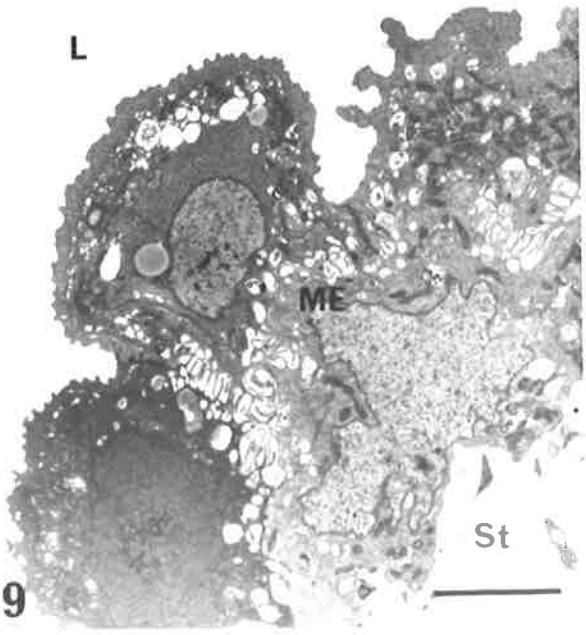
Figure 5.9 Apices of uterine luminal epithelial cells (ME) are dome shaped and there is abundant perinuclear RER. Some cells have many vacuoles. The intercellular membranes of the luminal epithelium are very folded and interdigitate with each other; lumen (L), stroma (St). Bar = 2μm.



7



8

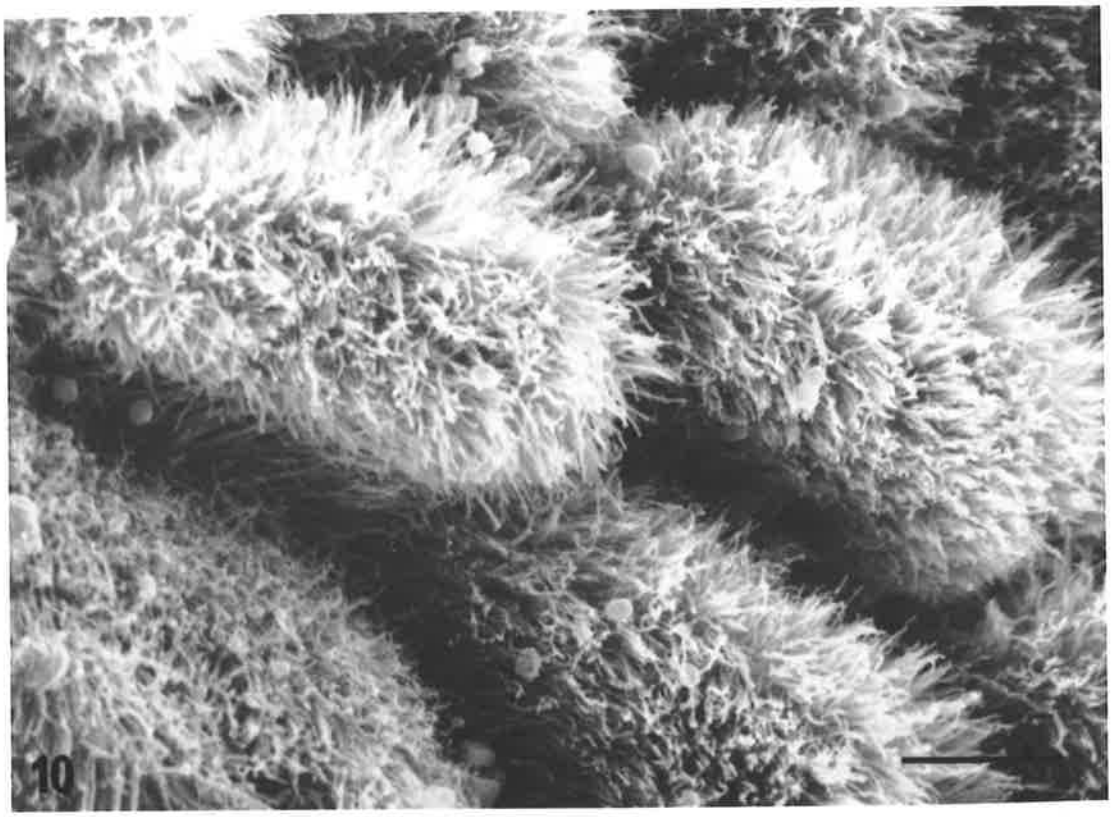


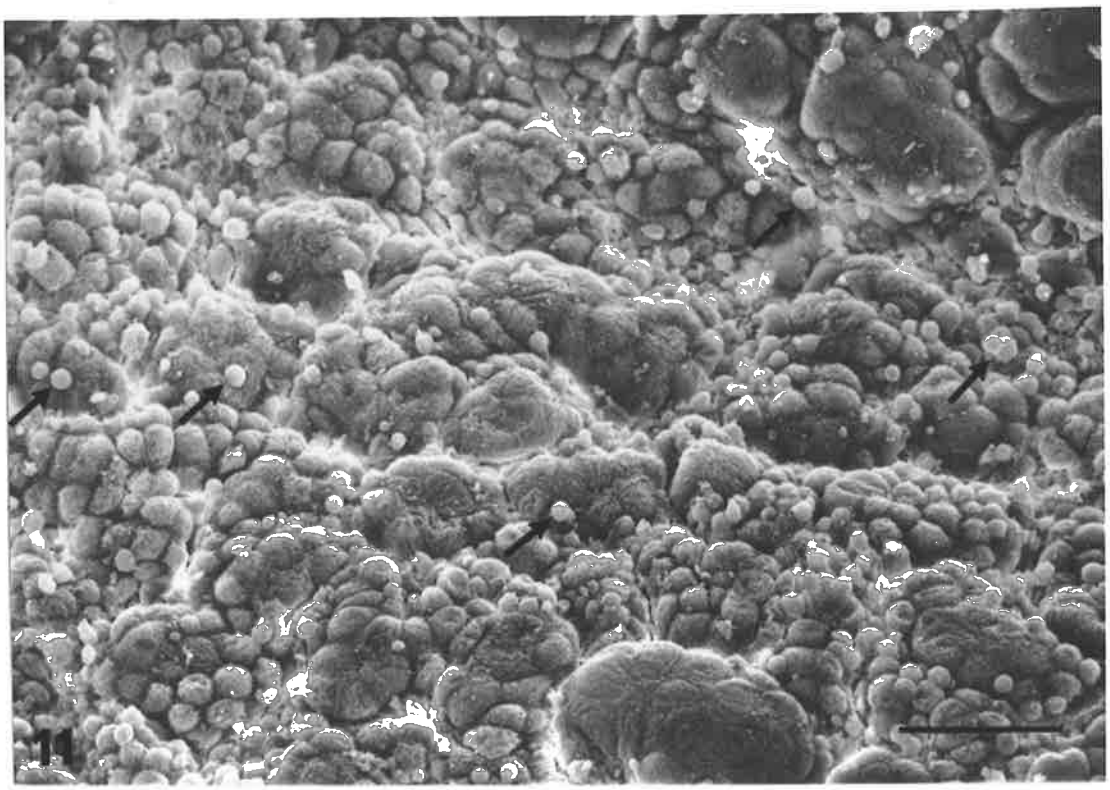
9

Figures 5.10-5.11 Early somite stage.

Figure 5.10 SEM of trophoblast adjacent to the embryo at early somite stage shows that trophoblast has many long microvilli. Bar = 5μm.

Figure 5.11 SEM of underlying endoderm of the same embryo shows that it has few microvilli with some vesicles (arrows) bulging from its plasmalemma. Bar = 50μm.


10


11

Trophoblast cells adjacent to the embryo processed for SEM (Fig. 5.10) were then reprocessed for TEM. These were extremely vacuolated, particularly in the perinuclear cytoplasm, while the peripheral cytoplasm was comparatively more electron-dense (Fig. 5.12). Most of the vacuoles were electron -lucent although there were a few that contained electron-dense material. The nuclei had numerous clumps of heterochromatin, particularly adjacent to the nuclear envelope. There was also a variation in the electron density of the nuclear matrix.

One complete uterus* from the female with early somite embryos was dissected into many pieces. These were all embedded and sections from both ends of 26 blocks examined, however, sections from only three of these 52 pieces of tissue (less than 6%) revealed cells that differed from maternal epithelial cells but which were adherent to the luminal epithelium (Fig. 5.13). These cells were highly vacuolated and resembled trophoblast adjacent to the embryo (Fig. 5.12) and they were, therefore, probably trophoblast cells which had migrated to the maternal epithelium. In oblique sections these cells appeared to have intruded between the uterine epithelial cells such that they appeared to be surrounded by maternal epithelium (Fig. 5.14). Long slender trophoblast cell processes appeared to be in contact with individual maternal epithelial cells (Fig. 5.15).

Some of these trophoblast cells had formed junctional complexes with maternal epithelial cells (Figs. 5.16 and 5.17). The numerous mitochondria were generally round with sparse lamellar cristae and granular matrix (Fig. 5.17). Some of these cells may have degenerated, possibly as a result of having been torn from the chorionic vesicle as the embryos were pulled from the uterus, although neighbouring maternal epithelial cells appeared healthy.

Apical junctional complexes were found between a few trophoblast and maternal epithelial cells and, below the intercellular junctions, areas of possible fusion between the two cell types were found (Figs. 5.18 and 5.19). Also a few trophoblast cells appeared to have fused with a neighbouring maternal epithelial cell and lay adjacent to the maternal epithelial basal lamina (Figs. 5.18 and 5.20). The resultant hybrid cells had the cytoplasmic characteristics of trophoblast while the apical cell membrane was morphologically similar to those of the neighbouring maternal epithelial cells (Fig. 5.21). Some of these hybrid cells appeared to be in different stages of transformation with

---

* Marsupials have two separate uteri rather than two uterine horns as occurs in many eutherian mammals. See Sharman, 1976 for details of their embryonic derivation.

variations in cytoplasmic electron density and numbers of vacuoles (Fig. 5.21). At this stage no trophoblast cells, or their processes, were found penetrating the maternal epithelial basal lamina.

5.3.3 Epithelial Penetration by the Bilaminar Yolk Sac

Epithelial penetration occurred in the bilaminar, or avascular, region of the yolk sac (BYS) on days 12 and 13 of pregnancy when long microvilli of trophoblast giant cells (TGCs) penetrated between the apical domes of maternal epithelial cells adjacent to the intercellular junctional complex (Fig. 5.22). At the same time, ectoplasmic processes, and often long regular microvilli, of TGCs surrounded processes of uterine luminal epithelial cells, particularly near the junctional complexes between maternal epithelial cells (Fig. 5.23). Trophoblast processes then pushed further down between these cells, invaginating the epithelium.

Maternal epithelial cells at this stage often contained large lipid droplets although occasionally several small lipid droplets appeared to be coalescing (Fig. 5.22). The epithelial cytoplasm was comparatively electron-dense with many mitochondria and abundant RER. Processes of TGCs also penetrated into the lumina of endometrial glands (Fig. 5.24).

Subsequently, TGCs intruded between maternal epithelial cells, presumably having broken down their intercellular junctions and replaced them with hybrid junctions between themselves and the disrupted epithelium. This occurred at discrete sites along the epithelium with only a single invading TGC at each site of penetration which was separated by a number of intact maternal epithelial cells (Fig. 5.25). In some places epithelial penetration occurred above a maternal sub-epithelial capillary and in others no maternal blood vessels were present in close proximity. Occasionally, the uterine epithelial cells appeared to have been pushed aside and into the endometrial stroma by the invading TGCs and the latter had begun to spread laterally below the epithelium but above its basal lamina (Fig. 5.26).

Invasive TGCs were less electron-dense than the adjacent maternal uterine epithelial cells. They had two, or more, huge, presumably polyploid, euchromatic nuclei with highly invaginated nuclear envelopes. In some sections a prominent nucleolus was seen in each nucleus. Mitoses were never found. Electron-lucent nuclei, which were rounded and regular in outline and different from the larger nuclei, were found in the apical cytoplasm of these trophoblast peg-like structures at about the same level as the nuclei of the adjacent maternal epithelial cells (Figs. 5.26 and 5.27).

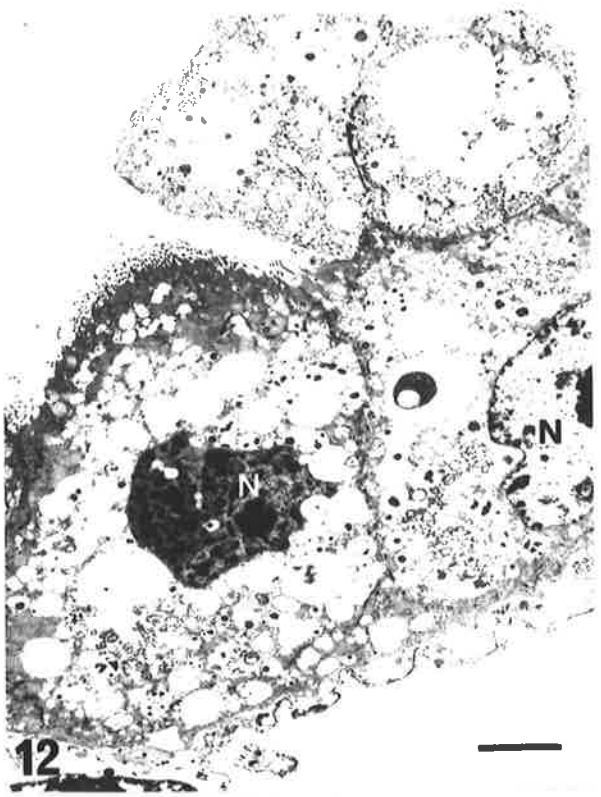
Figures 5.12-5.15 Early somite stage.

Figure 5.12 Trophoblast cells of the yolk sac adjacent to the embryo are highly vacuolated. Nuclei (N) are of varying electron density. Bar = 5μm.

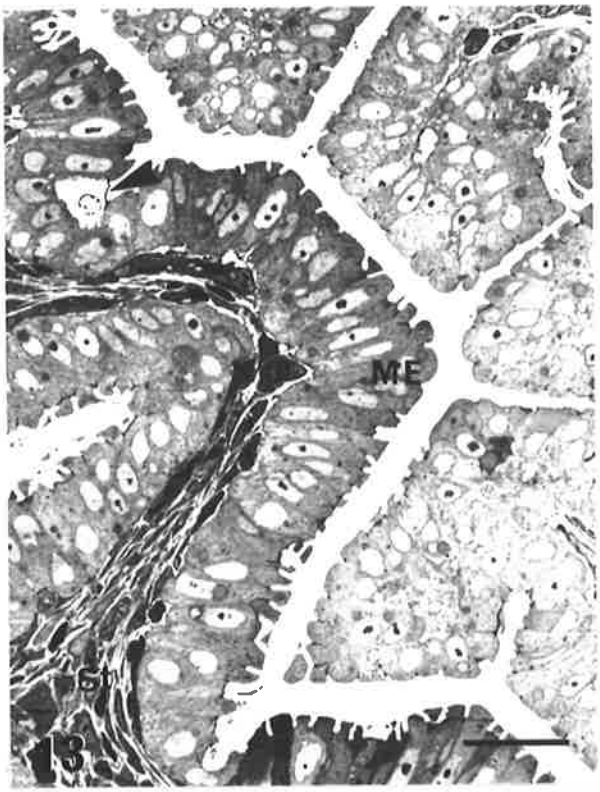
Figure 5.13 When embryos were pulled from the uterus occasional trophoblast cells (arrow) were left behind attached to the uterine epithelium (ME); stroma (St). Bar = 10μm. Tol. Blue.

Figure 5.14 Trophoblast (T) of embryo has intruded between maternal epithelial cells (ME). Bar = 5μm.

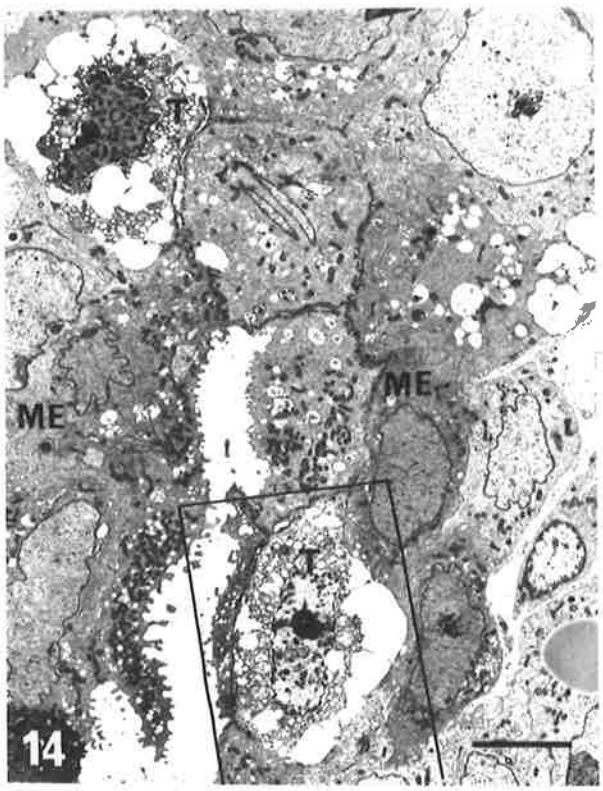
Figure 5.15 Higher magnification of the lower trophoblast cell in enclosed area in Fig. 5.14. Long slender processes (arrows) of vacuolated trophoblast (T) have made contact with uterine epithelial cells (ME). Bar = 2μm.



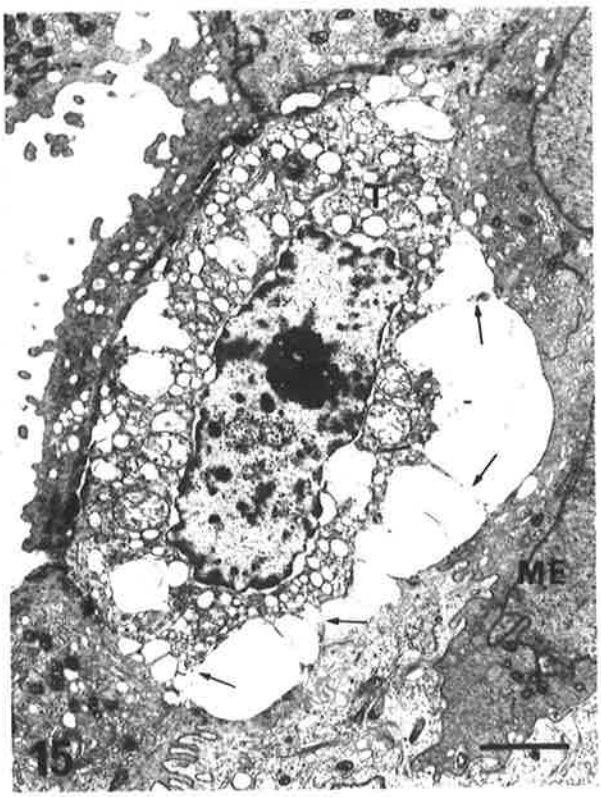
12



13



14



15

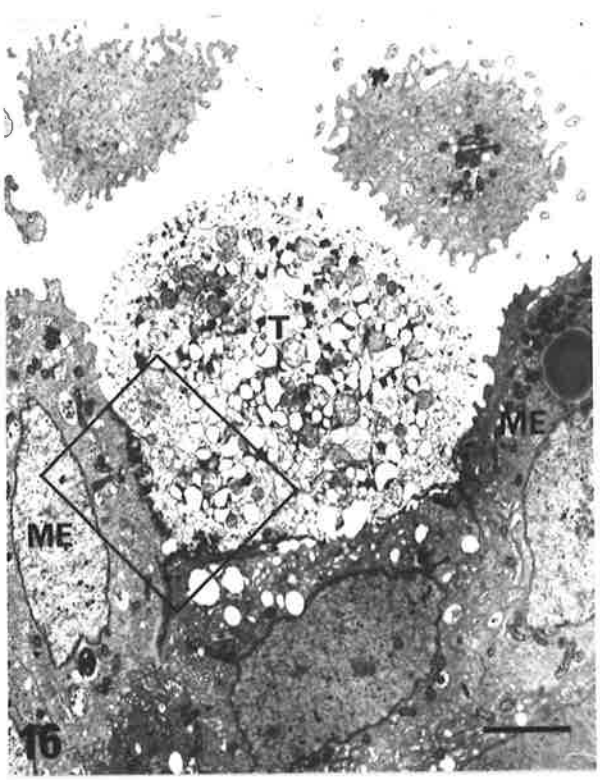
Figures 5.16-5.19 Early somite stage.

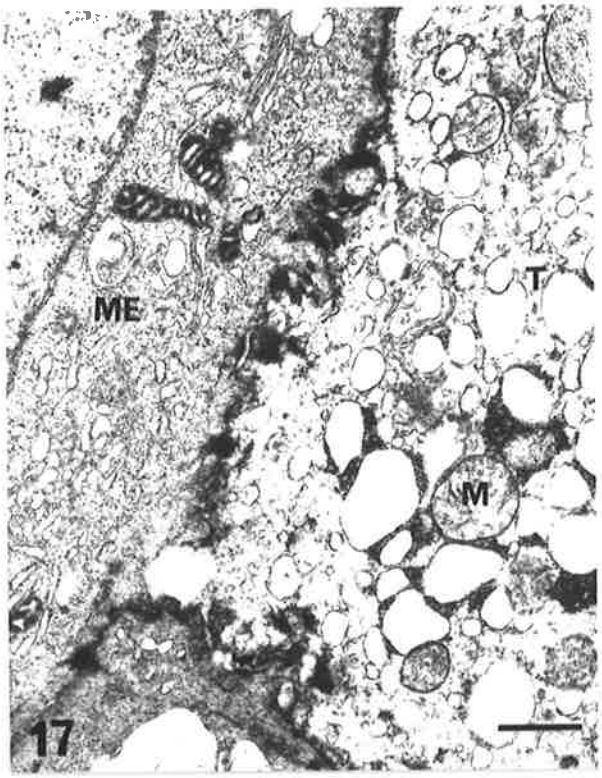
Figure 5.16 Trophoblast cells (T), which are highly vacuolated, subsequently form intercellular junctions with maternal epithelial cells (ME). Bar = 5μm.

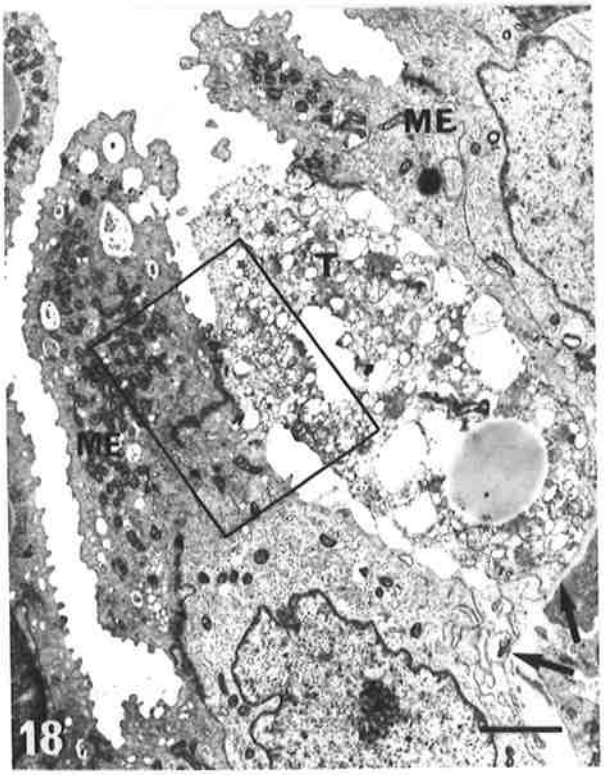
Figure 5.17 Higher magnification of the junctions between fetal and maternal cells in the box in Fig. 5.10 shows that there is an apical junctional complex including several desmosomes; mitochondrion (M). Bar = 500nm.

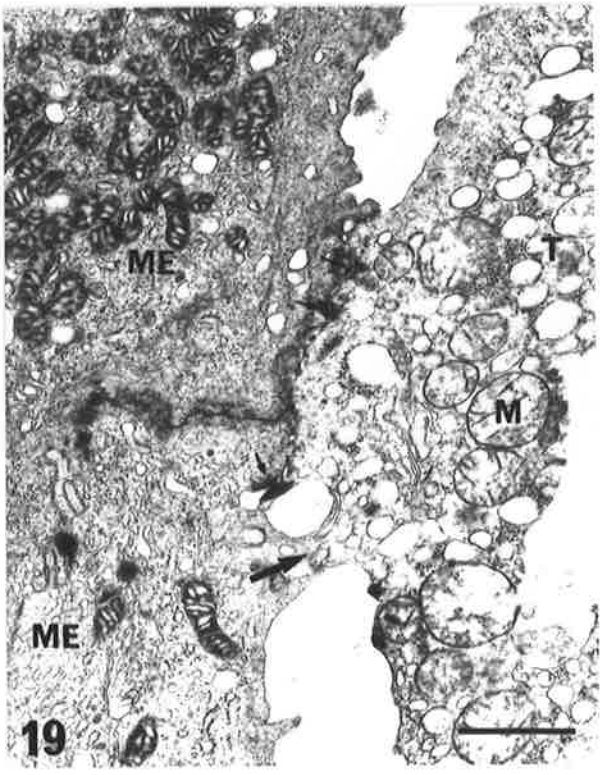
Figure 5.18 Trophoblast cells (T) have intruded between maternal epithelial cells (ME), formed an apical junctional complex with them, and appear to have fused with their lateral cell membranes below the junction; arrows show the epithelial basal lamina. Bar = 2μm.

Figure 5.19 Higher magnification of the intercellular junctional area in the box in Fig. 5.18. Trophoblast (T) and uterine epithelium (ME) appear to have fused over a small area below the junction (large arrow); desmosomes (small arrows), mitochondrion (M). Bar = 1μm.


T
ME
ME
16


ME
T
M
17

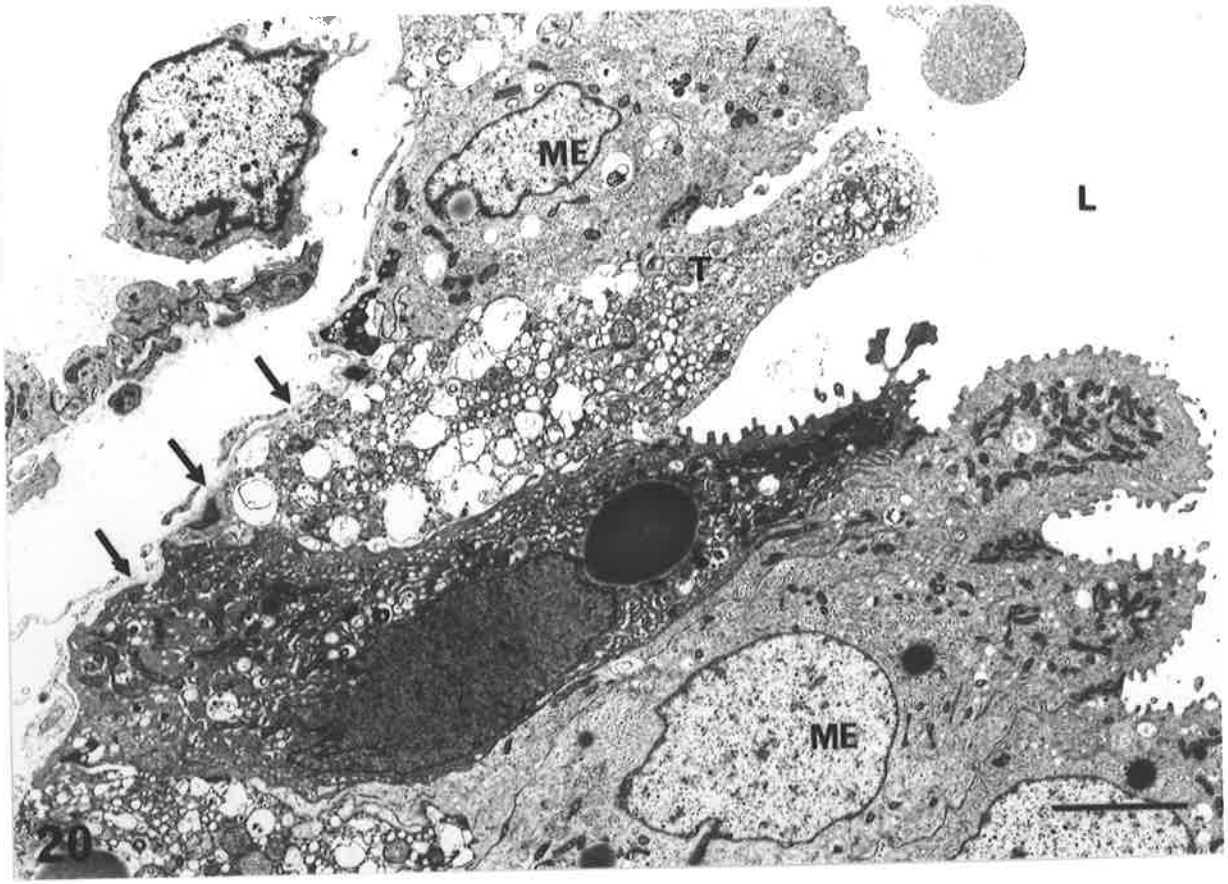

ME
T
ME
18

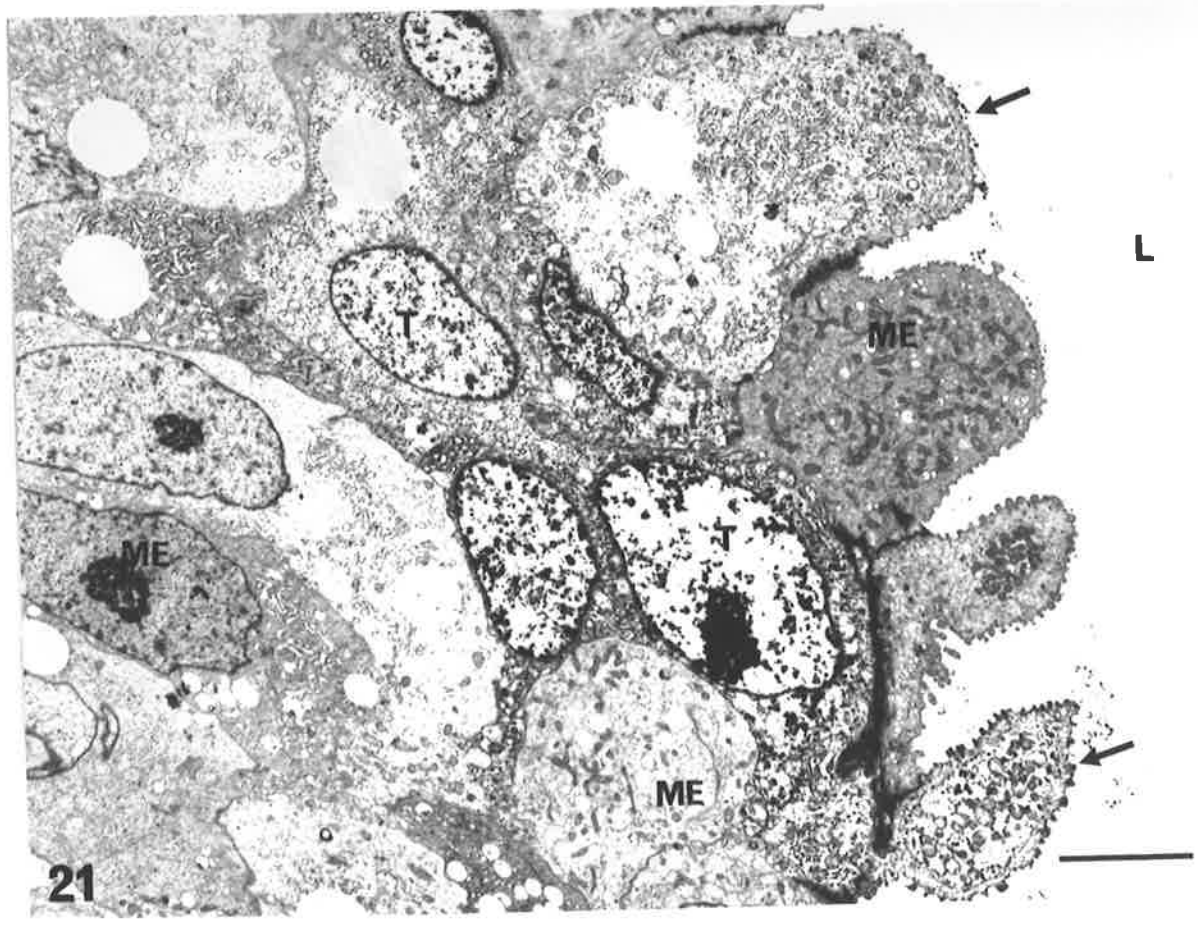

ME
T
M
ME
19

Figures 5.20-5.21 Early somite stage.

Figure 5.20 Trophoblast (T) is adjacent to the maternal epithelial basal lamina (arrows). A comparatively electron-dense maternal epithelial cell may have been undergoing transformation as a result of contact with trophoblast; maternal epithelium (ME). Bar = 5μm.

Figure 5.21 Oblique section through maternal luminal epithelium shows that some trophoblast cells (T) appear to have fused with the maternal epithelium (ME) producing hybrid cells with cytoplasmic features that resembled trophoblast and an apical plasma membrane that resembles that of the maternal epithelium (arrows); lumen (L). Bar = 5μm.

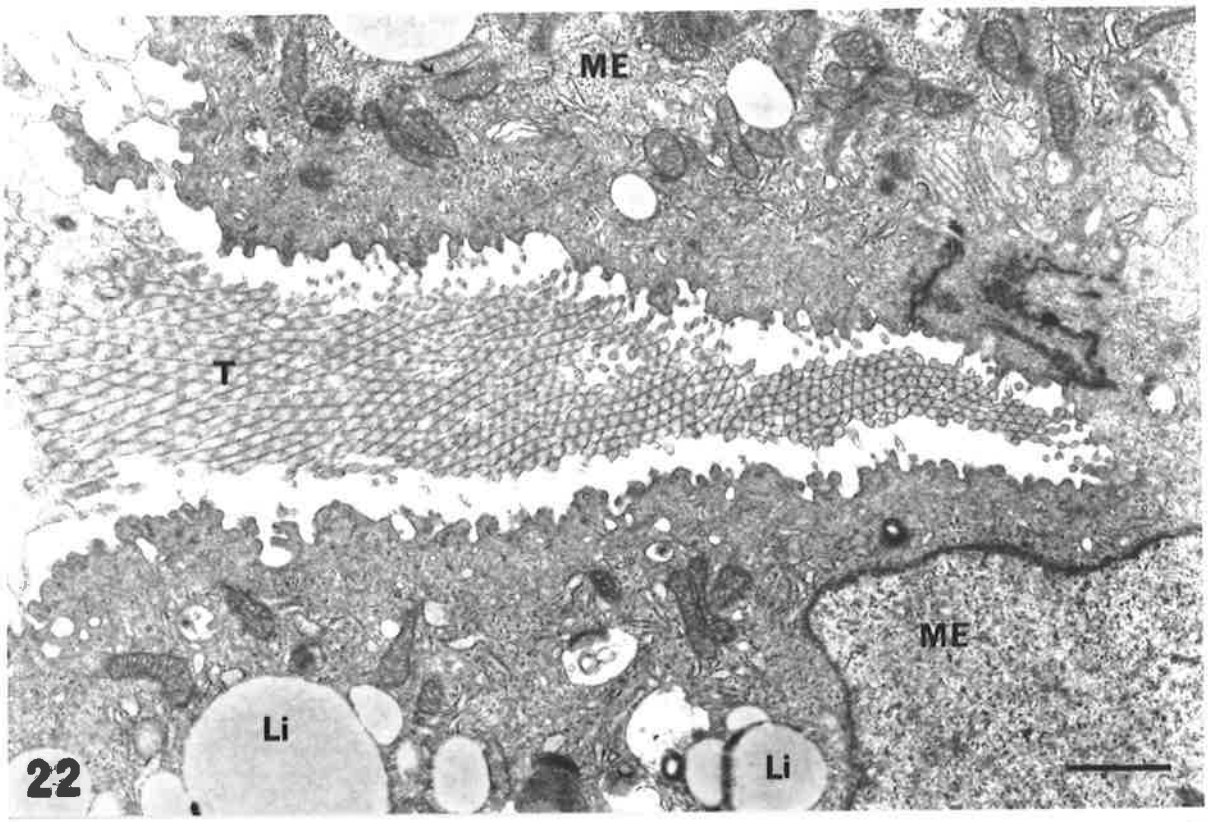

ME
T
L
ME
20


T
ME
L
ME
T
ME
21

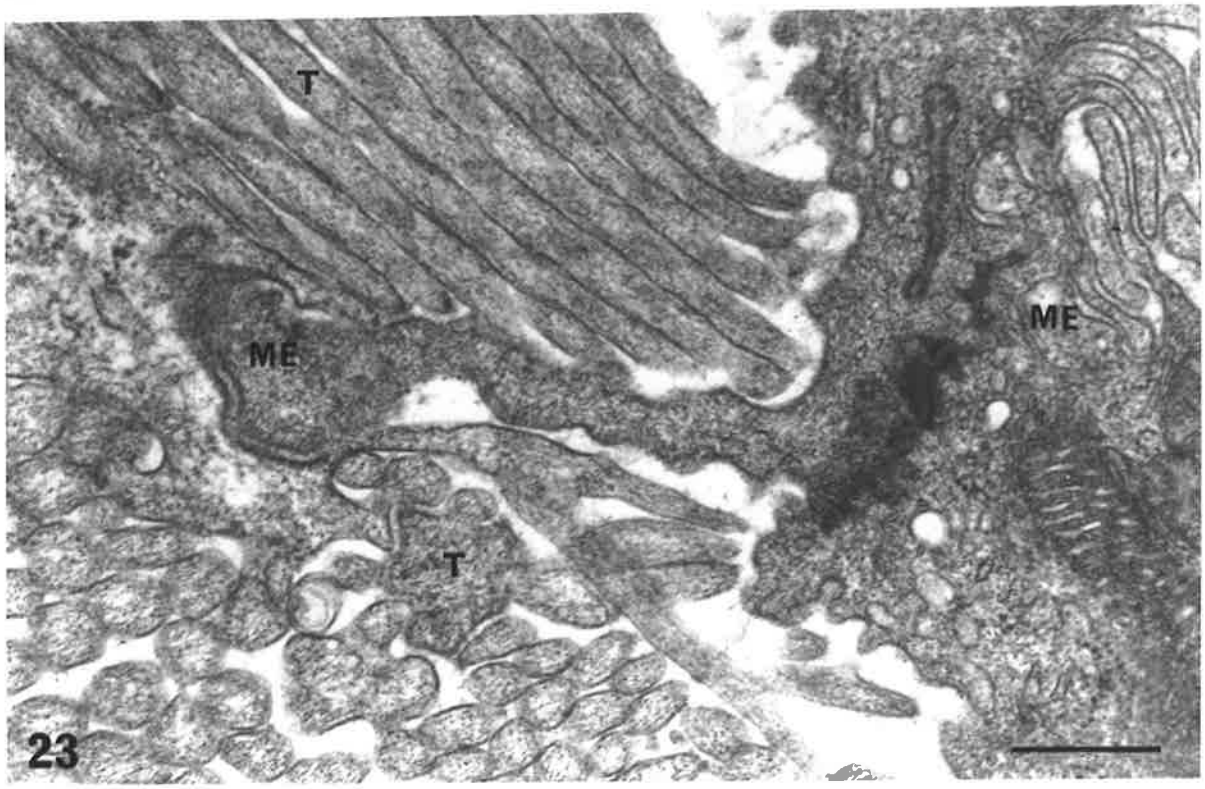
Figures 5.22-5.23 Bilaminar yolk sac (BYS) in late pregnancy.

Figure 5.22 Long microvillous processes of trophoblast (T) have penetrated between apical domes of uterine epithelial cells (ME) towards their intercellular junctional complexes; lipid (Li). Bar = 1μm.

Figure 5.23 Long regular microvilli of trophoblast (T) surround a narrow apical process of a maternal epithelial cell (ME). Bar = 500nm.

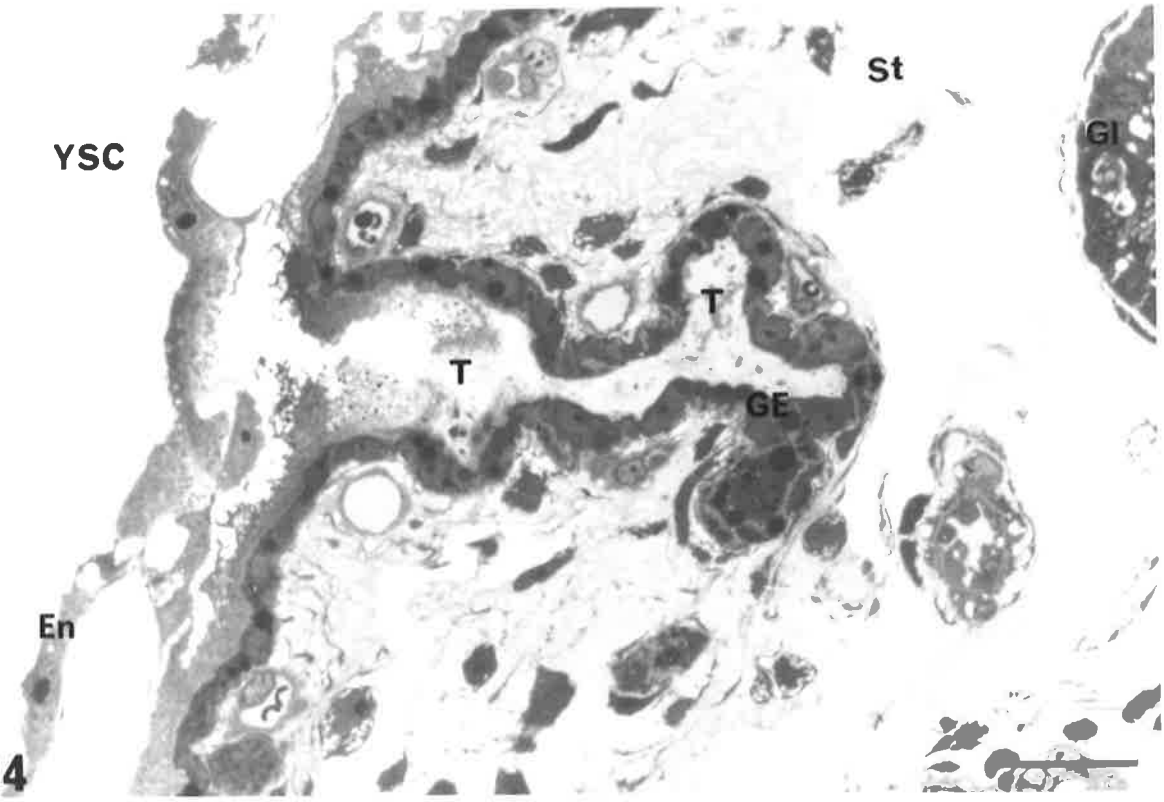


22



23

Figure 5.24 Trophoblast (T) in the bilaminar yolk sac has penetrated into the lumen of an endometrial gland (Gl); endoderm (En), gland epithelium (GE), uterine stroma (St), yolk sac cavity (YSC). Bar = 10μm. Tol. Blue.



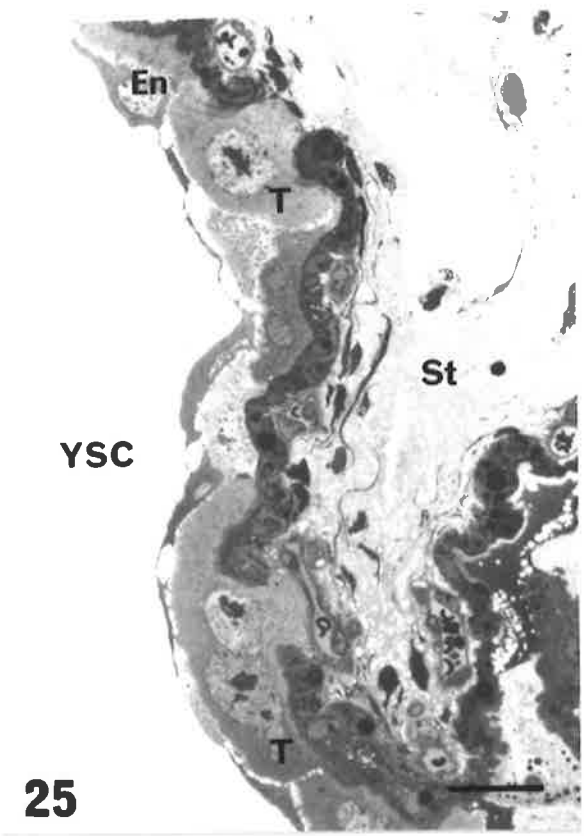
24

Figures 5.25-5.27 Bilaminar yolk sac (BYS) late in pregnancy.

Figure 5.25 Single multinucleate trophoblast giant cells (T) have penetrated the uterine epithelium at discrete sites with many apparently healthy maternal epithelial cells between them. Sometimes there was an endometrial capillary present at the site of invasion; endoderm (En), uterine stroma (St), yolk sac cavity (YSC); Bar = 20μm. Tol. Blue.

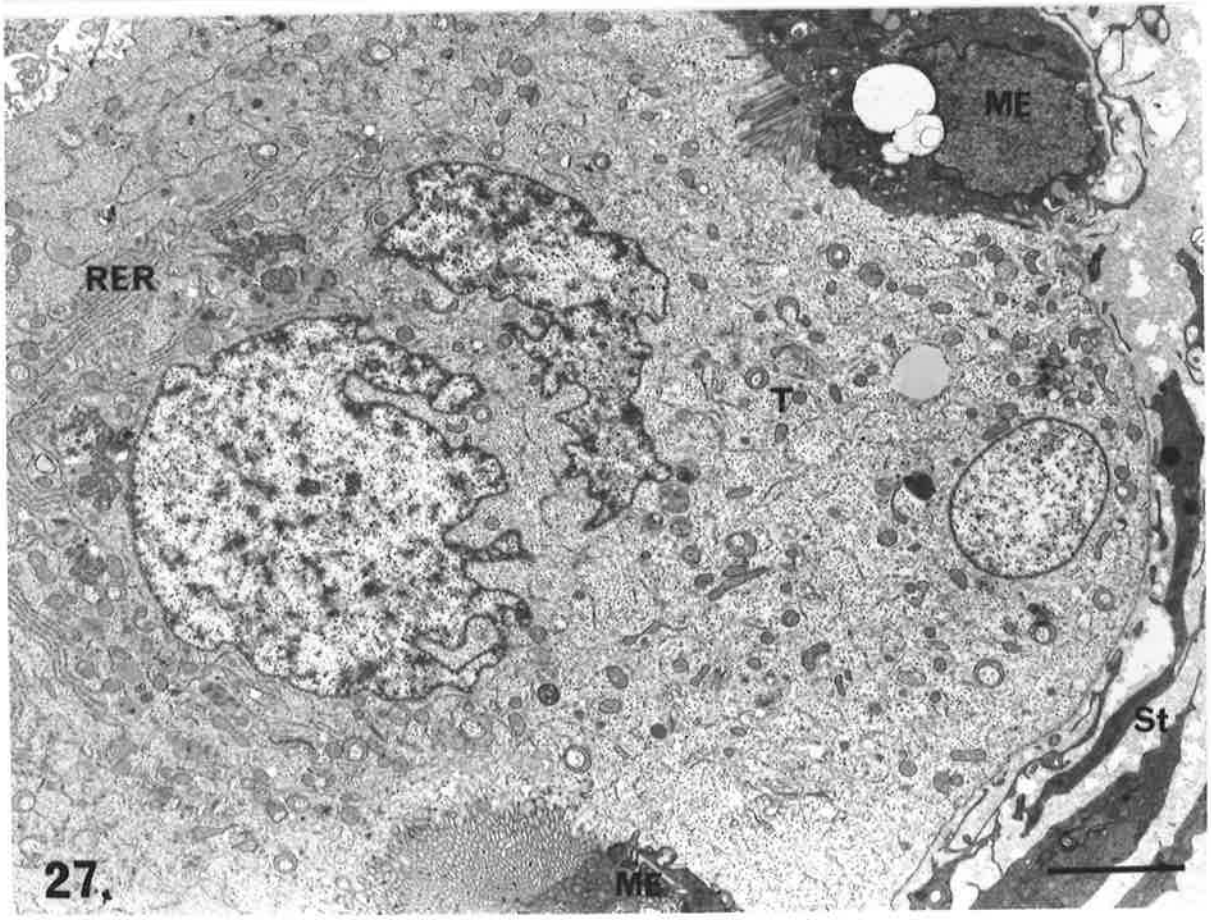
Figure 5.26 Occasionally trophoblast giant cells (T) appear to have pushed the maternal epithelial cells (ME) aside and into the stroma and then spread laterally before penetrating the basal lamina (arrow). The pale stained nucleus in the apical cytoplasm of the trophoblast may have been maternal in origin; endoderm (En). Bar = 10μm. Tol. Blue.

Figure 5.27 Trophoblast (T), which was comparatively electron-lucent, formed peg-like structures as it invaded the maternal epithelium. Two nuclei with irregular nuclear envelopes were found toward the base of the cell and another, with a smooth outline, at the same level as the maternal epithelial nuclei. There was abundant rough endoplasmic reticulum (RER) below the basal nuclei. Bar = 5μm.



25

26



27.

TGCs apposed to the maternal epithelium in this region had punctate foci of very long and regular microvilli interspersed with areas of plasma membrane that had fewer irregular short microvilli. Each trophoblast microvillous region was closely associated with blunt maternal microvilli (Figs. 5.28 and 5.29).

Many invading TGCs were found adjacent to the intact maternal epithelial basal lamina and sent processes between it and the adjacent epithelial cells (Figs. 5.26 and 5.30). The lateral cell membranes of invading trophoblast were either smooth or interdigitated with maternal epithelial lateral membranes (Fig. 5.31). TGCs occasionally surrounded processes of maternal stromal cells which had penetrated the uterine epithelial basal lamina and may have formed desmosomes with them (Fig. 5.32).

Numerous perinuclear golgi complexes were found in the apical cytoplasm of invasive TGCs as well as a little sparsely granular rough endoplasmic reticulum (RER) and many rosettes of polyribosomes (Fig. 5.33). Mitochondria were numerous throughout the cytoplasm and had irregular cristae, many of which were tubular (Fig. 5.33). Below the nucleus RER was considerably more abundant (Fig. 5.27).

As the placenta developed, groups of uterine epithelial cells were seen completely surrounded by trophoblast although they did not appear to have been phagocytosed by the TGCs (Fig. 5.34). The nuclear envelopes of these surrounded maternal epithelial cells were invaginated (Fig. 5.34). Organelles in the cytoplasm of these cells appeared quite healthy although many had large vacuoles (Fig. 5.35). However, occasionally maternal epithelial cells, found between trophoblast and endoderm, appeared to be undergoing apoptosis as cytoplasmic vesicles were found with or without pyknotic nuclei (Fig. 5.36).

Intrusive stages in the bilaminar yolk sac placenta were seen in each of two dunnarts during the last day or so of pregnancy, particularly in the periphery of the invasive annular region. That is, in this species, implantation and placentation are compressed into the last three days of gestation such that epithelial penetration is still proceeding on the day before, and probably on the day of, birth concurrent with a functioning yolk sac placenta.

5.3.4 Fetal Maternal Interactions in the Trilaminar Yolk Sac

As the placenta developed it was found that in the trilaminar, or vascular, region of the yolk sac (TYS), trophoblast was in intimate contact with the highly folded endometrium. Long microvilli of the trophoblast interdigitated with shorter irregular

microvilli as well as cell processes of the maternal uterine epithelium (Figs. 5.37 and 5.38). In some places cytoplasmic and microvillous processes of trophoblast significantly indented maternal epithelial cells (Fig. 5.39). However, epithelial penetration was never seen in the TYS on the last two days of pregnancy. Thus, an intimate epitheliochorial association occurred between the uterine epithelium and TYS.

5.4 Discussion

In eutherian mammals the ultrastructure of implantation has been described in many species. Schlafke and Enders (1975) reviewed the cell-cell interactions at implantation in a range of species and showed that the process always begins with the apposition, followed by adhesion, of trophoblast to maternal uterine epithelial cells. In species which develop an epitheliochorial placenta this is as far as implantation proceeds. However, in species that have an invasive placenta there are three methods by which trophoblast may penetrate the epithelium: fusion with, intrusion into, or displacement of, cells of the uterine luminal epithelium.

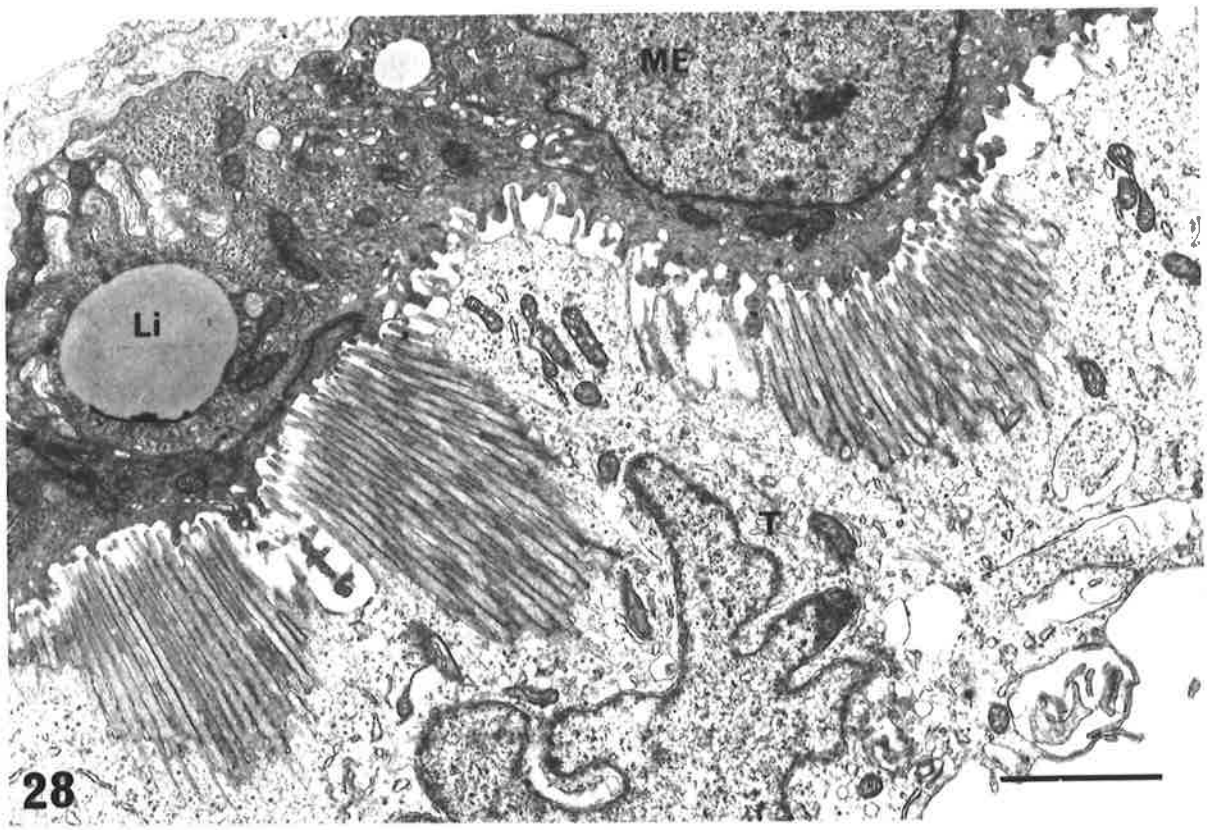
In the dunnart, the appositional stage of implantation may occur for several days prior to adhesion as in one female unilaminar blastocysts in depressions of the endometrium were found (Roberts and Breed, 1994b). Figure 5.1 resembles a micrograph of early North American opossum embryos published in 1915 by Spurgeon and Brooks which these authors interpreted as implantation having already taken place. In another female, early bilaminar blastocysts which could still be flushed from the uteri but were completely surrounded by folds of the endometrium. At this later time it appeared that these embryos were fixed in their final position, despite the presence of a shell membrane. This apparent fixation of position was maintained in the females which carried early embryonic disc and primitive streak embryos and the endometrium appeared to have closed around individual embryos. In the pregnant mare, prolonged apposition of embryos has been shown to occur as embryonic fixation occurs from day 16 of gestation, despite the persistence of an extracellular embryonic capsule (Flood *et al.*, 1982), until day 22 and the development of the yolk sac placenta (Enders and Liu, 1991a).

Although timing pregnancy in the fat-tailed dunnart is difficult because of the occurrence of sperm storage prior to ovulation in some females, trophoblast begins to intrude into, and possibly fuse with, the maternal epithelium about day 10 after fertilization. At this time the embryo is at the early somite stage of development. Consequently, in the dunnart implantation is centric (established terminology of

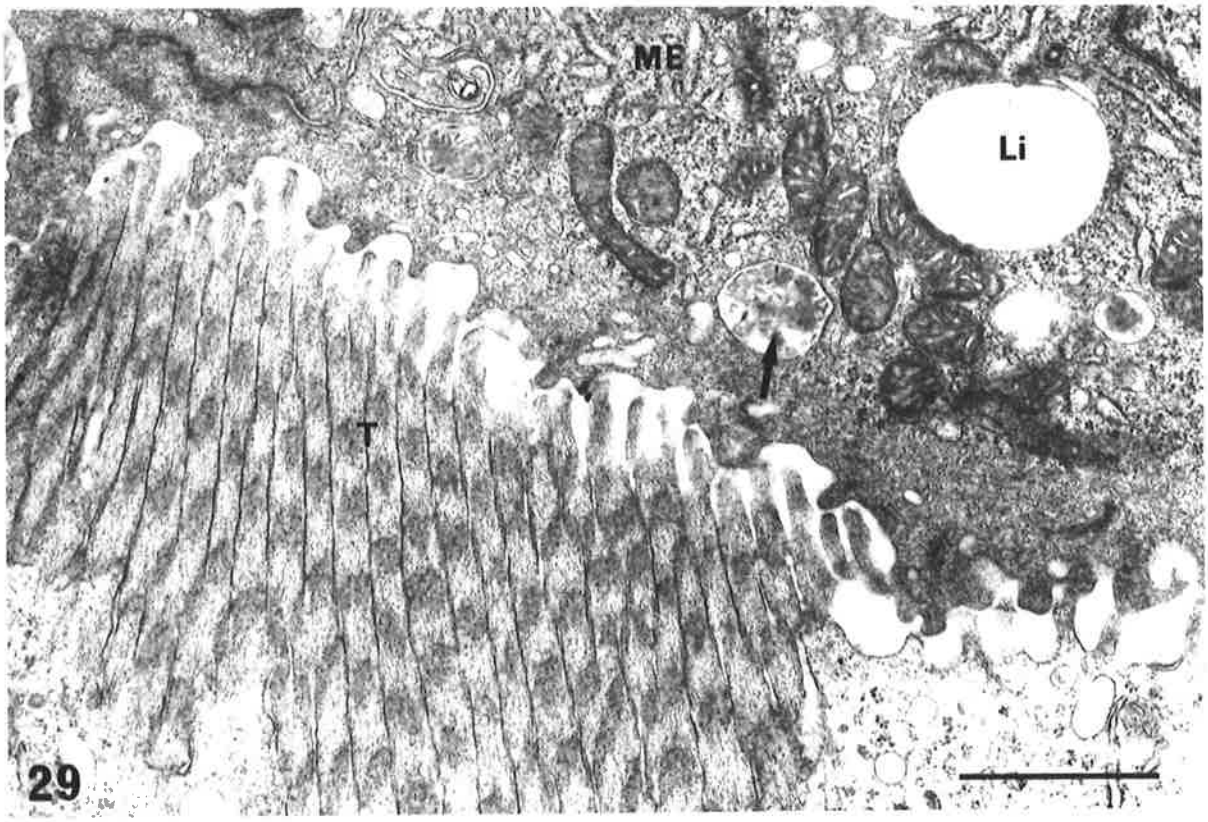
Figures 5.28-5.29 Bilaminar yolk sac (BYS) late in pregnancy.

Figure 5.28 Plasma membrane of invasive trophoblast (T) is apposed to the apex of adjacent maternal epithelium (ME) and has punctate foci of long regular microvilli interspersed with areas of shorter and fewer microvilli. Bar = 2μm.

Figure 5.29 Higher magnification of a similar area to that in figure 5.22 showing interdigitation between maternal and trophoblast microvilli (T); lipid (Li), multivesicular body (arrow) in maternal epithelium (ME). Bar = 500nm.



28



29

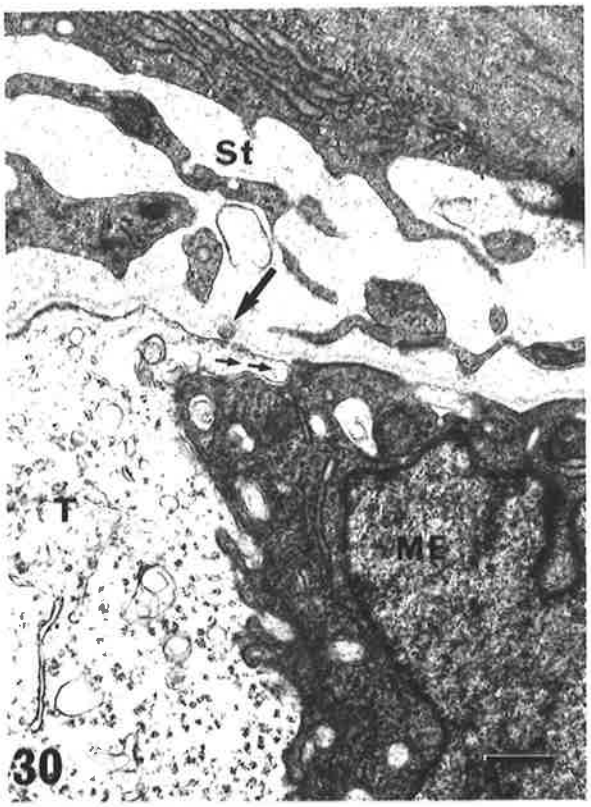
Figures 5.30-5.33 Invasive peg-like trophoblast of bilaminar yolk sac (BYS) late in pregnancy.

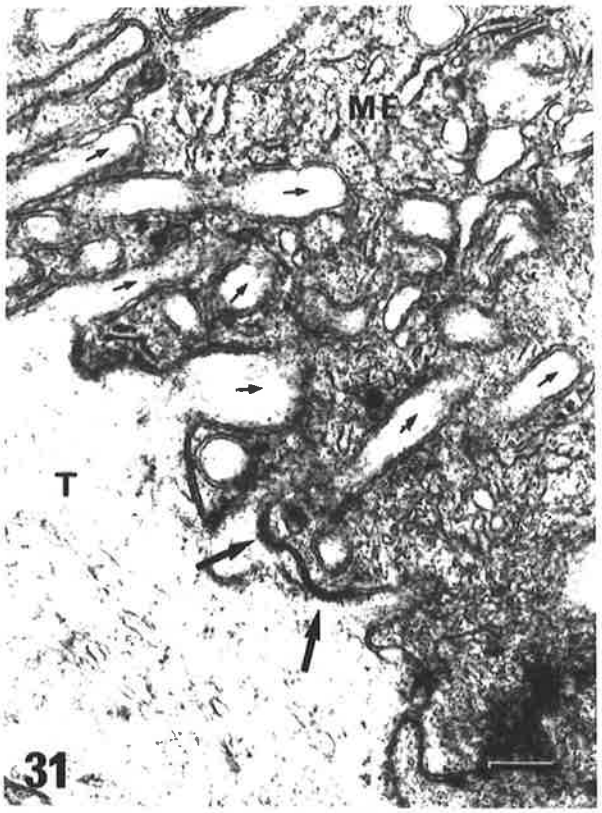
Figure 5.30 Trophoblast (T) spread laterally (small arrows) above the maternal epithelial basal lamina (arrow) before migration into the endometrial stroma (St); adjacent maternal epithelium (ME). Bar = 500nm.

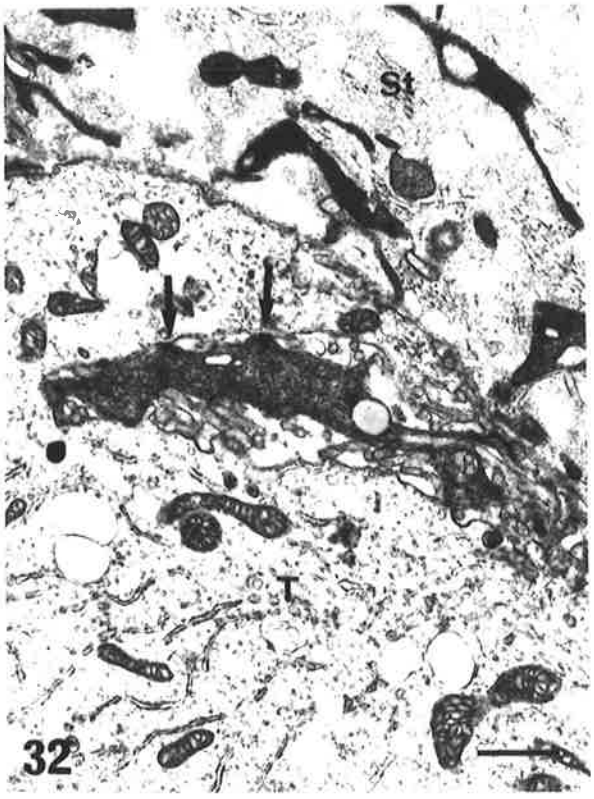
Figure 5.31 Trophoblast (T) lateral plasma membrane (small arrows) sometimes interdigitated with that of maternal epithelium (ME). Occasionally coated pits were found along the trophoblast lateral (large arrows) and apical plasma membranes. Bar = 250nm.

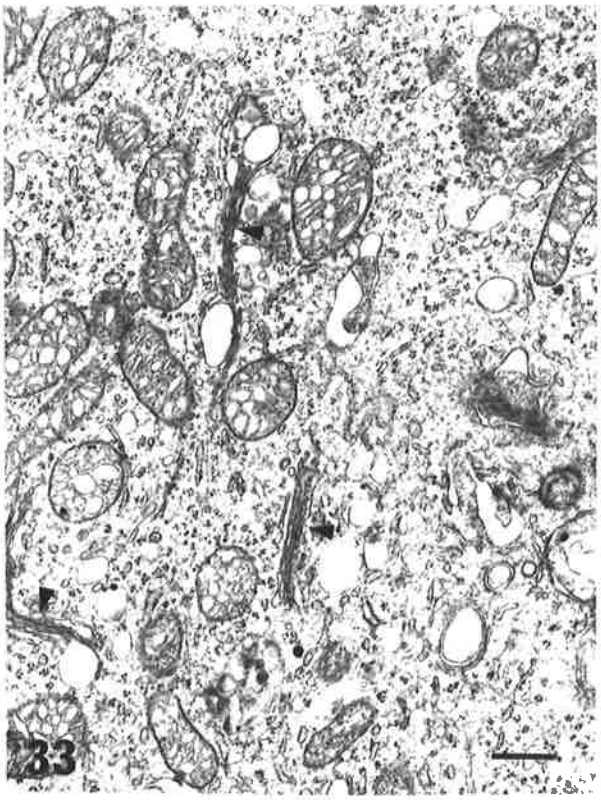
Figure 5.32 A process of a maternal stromal cell, presumably a fibroblast, was occasionally found penetrating the maternal epithelial basal lamina and may have made desmosomes (arrows) with trophoblast (T) which surrounded it. Bar = 1μm.

Figure 5.33 Invasive TGC cytoplasm has many rosettes of ribosomes, perinuclear stacks of cisternae of golgi complexes (arrowheads) and mitochondria with both irregular and tubular cristae. Bar = 500nm.


St
T
ME
30


ME
T
31


St
T
32

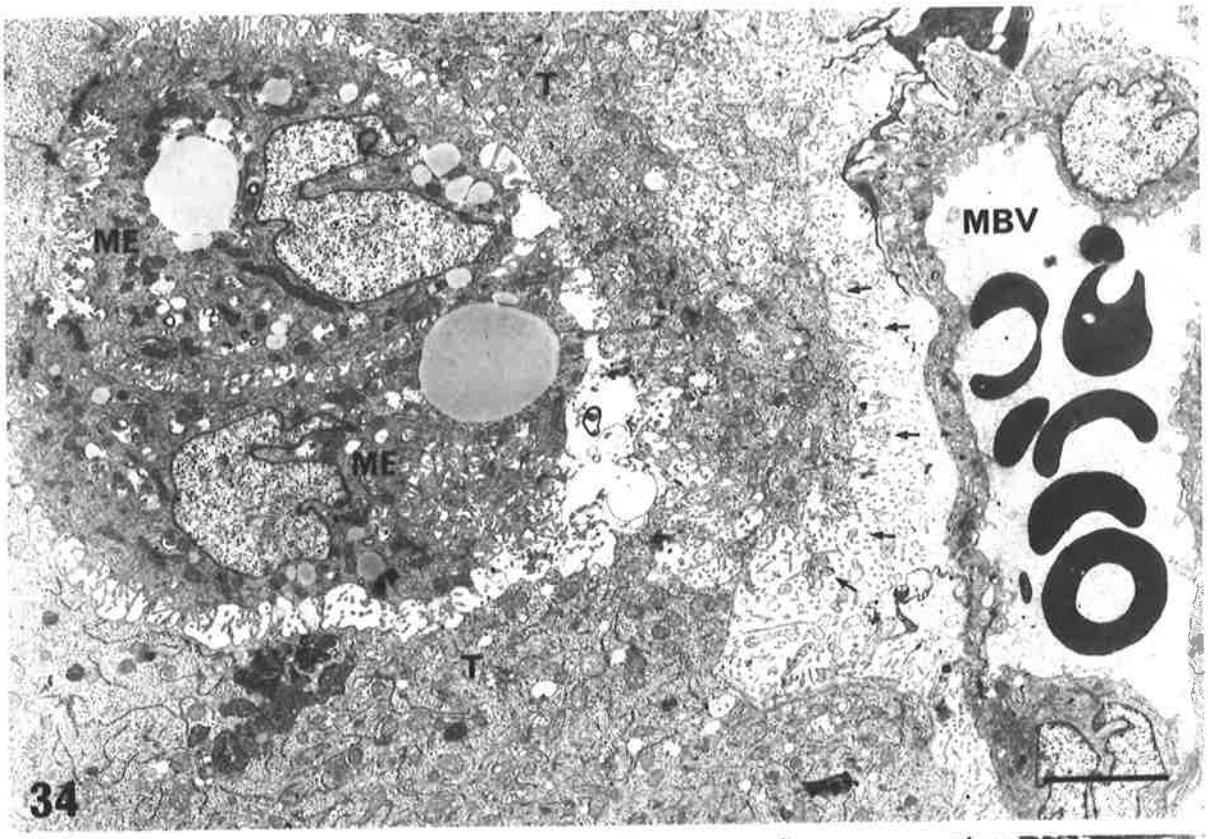

33

Figures 5.34-5.36 Bilaminar yolk sac (BYS) late in pregnancy.

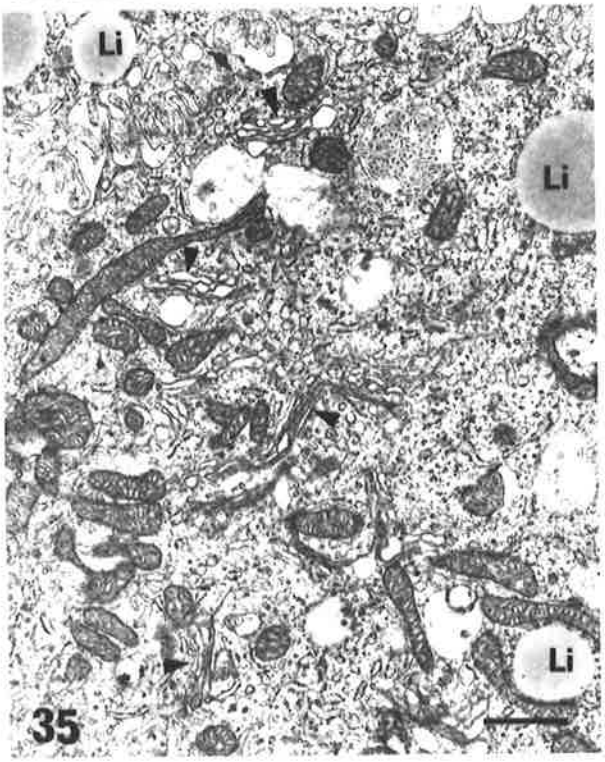
Figure 5.34 Late in pregnancy in the BYS groups of maternal epithelial cells (ME) are seen surrounded by trophoblast giant cells (T); processes of trophoblast have penetrated the residual maternal epithelial basal lamina (arrows); sometimes a maternal blood vessel is visible (MBV). Bar = 5μm.

Figure 5.35 Organelles in surrounded maternal epithelial cells appeared healthy; golgi complexes (arrowheads); lipid (Li). Bar = 1μm.

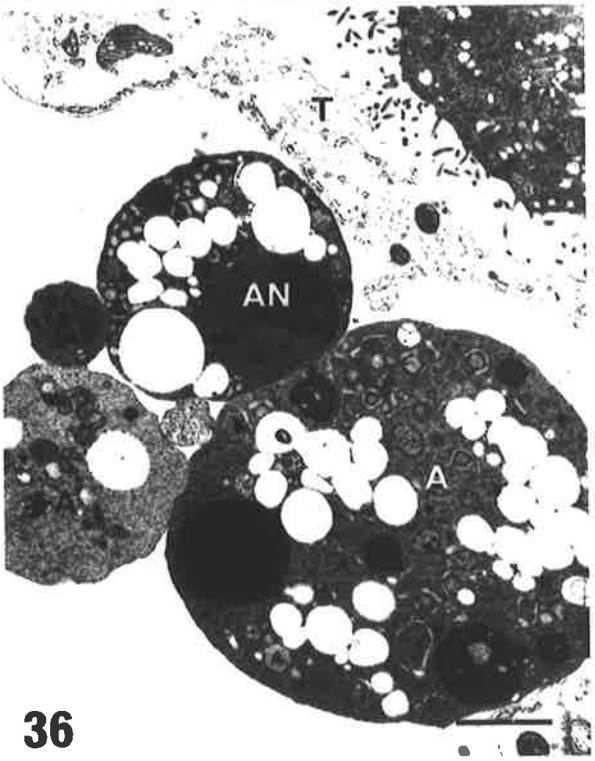
Figure 5.36 Some maternal epithelial cells appear to be undergoing apoptosis (A), and are fragmented and have pyknotic nuclei (AN). Bar = 2μm.



34



35



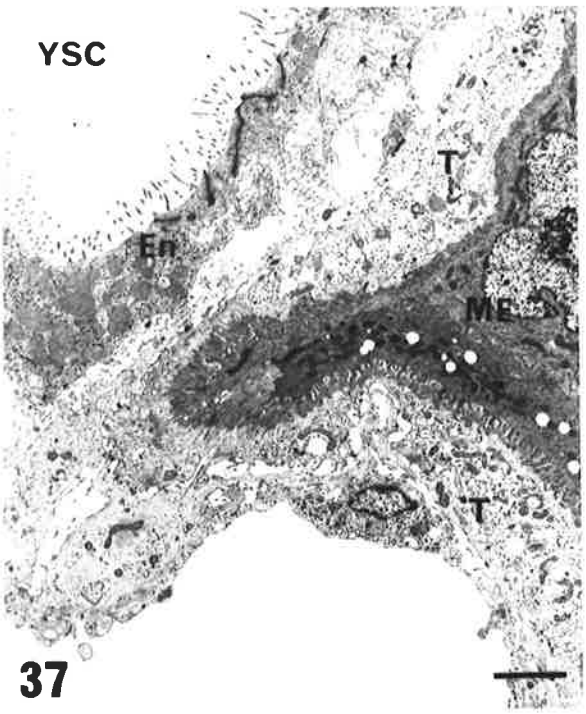
36

Figures 5.37-5.39 Trilaminar yolk sac (TYS) late in pregnancy.

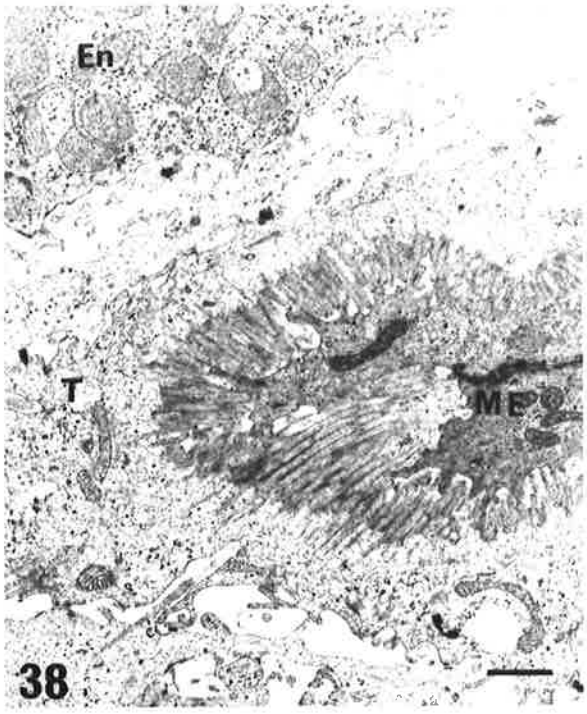
Figure 5.37 The TYS closely follows the contours of the folded maternal epithelium (ME); endoderm (En), trophoblast (T), yolk sac cavity (YSC). Bar = 3μm.

Figure 5.38 Higher magnification of area in Fig. 5.37 showing extensive interdigitation of long regular trophoblast microvilli with the irregular microvilli and apical processes of maternal epithelium (ME); endoderm (En), trophoblast (T). Bar = 1μm.

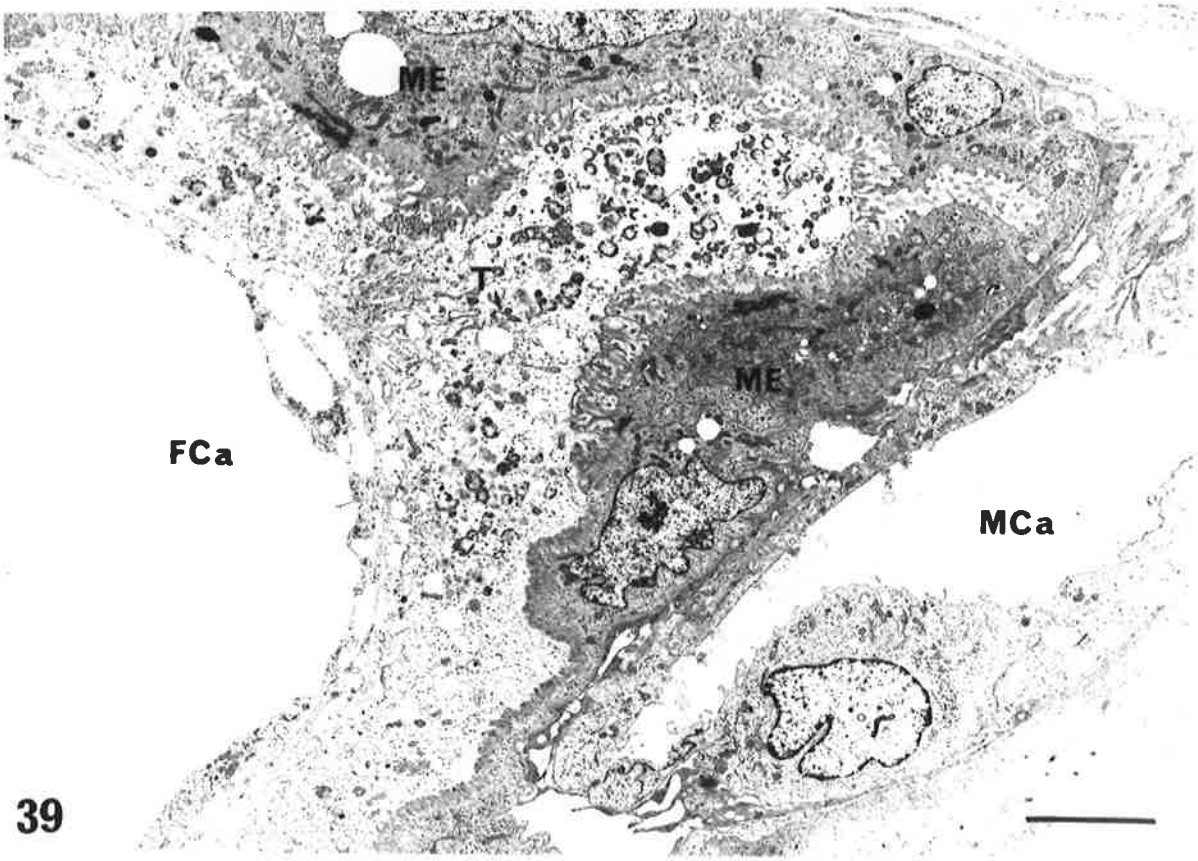
Figure 5.39 Processes of trophoblast (T) invaginate the thin maternal epithelium (ME); fetal capillary (FCa), maternal capillary (MCa). Note the similarity of trophoblast cytoplasm with that seen earlier adjacent to the early somite embryo seen in figure 5.12. Bar = 5μm.



37



38



39

Wimsatt, 1975) and subsequently invasive. This also occurs in the rhesus monkey (Enders and Hendrickx, 1980; Enders *et al.*, 1983) and in the marmoset (Smith *et al.*, 1987). In the latter two species trophoblast subsequently erodes the maternal endothelium forming a haemochorial placenta.

In the dunnart at the early somite stage maternal uterine epithelial cell apices are dome shaped and have short, blunt and irregular microvilli. In eutherians these features characterize uterine epithelial cells at early implantation stages e.g. the rat (Tachi *et al.*, 1970), mouse (Enders and Schlafke, 1979), pig (King *et al.*, 1982) and some other species (Murphy, 1992).

Although no resin sections of early somite embryos *in situ* were cut, cells that were probably trophoblast cells appeared to invade, and possibly fuse with, uterine epithelial cells. The former resembled trophoblast cells adjacent to the embryonic disc in tissue from the yolk sacs embedded separately. Uterine epithelial cells with an electron-dense cytoplasm were found at this stage in pregnancy. They were present throughout the uterus and may have developed as the result of the close proximity of embryos. They were not observed at any other stage of pregnancy.

Also, since only a small percentage of blocks from the whole uterus at the early somite stage appeared to have trophoblast cells penetrating the epithelium, it seems likely that the trophoblast of only one embryo was penetrating the endometrium at this time. The fact that the shell membrane was still present around an early primitive streak stage embryo (Hughes, 1974, also made the same finding) suggests that the shell membrane is indeed lost by the early somite stage in this species and that the early somite stage appears to mark the beginning of epithelial penetration. However, these observations were made from only one female so further investigations of females at a similar stage in pregnancy need to be conducted to confirm this observation.

Interdigitation of the lateral membranes of maternal epithelial cells was extensive from the early somite stage of development and has also been reported to occur in the pig on the day of attachment of the epitheliochorial placenta (King *et al.*, 1982). Such interdigitation, along with the large numbers of mitochondria and apical vesicles seen in these cells at the early implantation stage, suggests transport of water and solutes across the epithelium. Electron-lucent apical vesicles have also been found in the uterine epithelium of the rat in early pregnancy at the time of trophoblast attachment (Parr and Parr, 1978) which were thought to be associated with the removal of uterine luminal fluid prior to implantation.

Darkly stained uterine luminal epithelial cells, which did not appear to be degenerating, were found only at the time of early epithelial penetration so they may also be associated with this process but in what way is unclear. Dark maternal epithelial cells were found at the late attachment stage in the rat by Tachi *et al.* (1970) the mitochondria of which were shrunken and lacked well defined cristae which these authors thought to be a sign of their degeneration. Also, during the invasive stage in the rat, lipid droplets were found in the uterine epithelium (Tachi *et al.*, 1970). In the dunnart lipid droplets were present in abundance from the early somite stage and presumably play a role in trophoblast nutrition.

The cells that appeared to be invading at the early somite stage were adjacent to the embryo in the chorionic vesicles and were therefore possibly part of the trilaminar, or vascularized, yolk sac at this time in development. However, in females late in pregnancy epithelial penetration in this region of the yolk sac has not been found. It may be that attachment and epithelial penetration first occur in this area but later, as the placenta develops, these invading trophoblast cells may degenerate and an epitheliochorial relationship ensues. By contrast in the bilaminar region of the yolk sac, adjacent to the sinus terminalis, further epithelial penetration then takes place by intrusion, and possibly fusion. In cats (Leiser, 1982) and ungulates (Wathes and Wooding, 1980; King *et al.*, 1982) attachment first occurs adjacent to the embryo and proceeds towards the periphery of the chorionic vesicle. Perhaps this is also the case in the dunnart.

In the South American didelphid, *Philander opossum*, erosion of the uterine epithelium adjacent to the sinus terminalis occurs in an annular region at the junction between the TYS and the BYS by large trophoblast cells from the TYS which are multinucleate (Enders and Enders, 1969). In the dunnart the invasive trophoblast takes place in an annular region outside the sinus terminalis in the avascular, or bilaminar, yolk sac placenta. This may be a different pattern of invasiveness between American and Australian marsupial yolk sac placentae.

In the dunnart bilaminar yolk sac placenta adjacent to the sinus terminalis, trophoblast giant cells (TGCs), eroded the maternal epithelium on the last day or two of pregnancy by a process that resembles intrusive implantation seen in the ferret (Enders and Schlafke, 1971b), guinea pig (Schlafke and Enders, 1975), rhesus monkey (Enders *et al.*, 1983) and marmoset (Smith *et al.*, 1987). In some sections, the maternal epithelium remained intact on either side of where a TGC had penetrated and the trophoblast itself resembled the syncytial pegs seen in implantation in the rabbit after fusion of syncytiotrophoblast with maternal epithelium prior to the erosion of its basal lamina

(Enders and Schlafke, 1971a). In the rabbit trophoblast pegs were seen much more frequently than actual cell fusion of apical cell membranes of maternal and embryonic cells.

The electron-lucent round nuclei found in the apical cytoplasm of invading TGCs, at the same level as those of the maternal epithelium, resembled the nuclei of maternal epithelial cells that had been surrounded by TGCs. It is possible that fusion had in fact occurred in this region and that the nuclei of the surrounded maternal epithelial cells had remained intact for sometime after cell-cell contact and fusion. In ruminant placentae the so-called maternal syncytial plaques, which form as the result of the fusion of one or more trophoblast binucleate cells with a single maternal epithelial cell, produce hybrid cells in which there is an odd number of nuclei, that is the maternal nucleus remains within the resultant hybrid syncytium (Wooding, 1992). Fusion between trophoblast and maternal epithelial syncytium also occurs in the chorioallantoic placenta of bandicoots (Padykula and Taylor, 1976, 1982) producing heterokaryons i.e., cells with two types of apparently healthy nuclei. However, in the dunnart no trophoblast and maternal cells at the point of fusion were found in the bilaminar yolk sac but further work is needed to clarify whether the trophoblast peg-like structures are formed after fusion.

Larsen (1970) reported from observations of light microscopical sections that, in day 7 human blastocysts, fusion between syncytiotrophoblast and syncytial maternal epithelial cells occurs. However, there has been no electron microscopic evidence for fusion at this time in human implantation sites *in vivo* but by day 11 intrusion into the uterine epithelium has been shown to occur (Knoth and Larsen, 1972). Implantation sites from other primate species have shown that epithelial penetration is by intrusion. However, in macaques, Larsen (1970) has suggested that the initial contact between trophoblast and maternal epithelium involves fusion, but again this was based on light microscopic evidence. Perhaps fusion may occur at implantation in some species which, a little later, exhibit intrusion. It is possible that in the dunnart a combination of mechanisms of epithelial penetration takes place as the present study has shown that intrusion and probably fusion appear to have occurred.

Punctate areas of trophoblast that had very long microvilli were often found apposed to maternal epithelial plasmalemma. These may aid in anchoring the invading trophoblast cells, as well as the developing placenta, to the endometrium and allow the trophoblast to flow into the endometrial stroma. It has also been suggested that the desmosomes found between the lateral membranes of these two cell types have a similar function in a variety of species (Enders and Schlafke, 1969).

As many TGCs adjacent to the residual maternal epithelial basal lamina were found in the dunnart this may represent trophoblast hesitation at the basal lamina prior to invasion of the endometrial stroma, a phenomenon that has been reported in eutherian species such as the rat (Tachi *et al.*, 1970), rabbit (Enders and Schlafke, 1971a), ferret (Enders and Schlafke, 1971b), rhesus monkey (Enders *et al.*, 1983) and mouse (Blankenship and Given, 1992). In rats (Schlafke *et al.*, 1985) and mice (Blankenship and Given, 1992) decidual cell processes penetrate the basal lamina before trophoblast cell processes. Similarly in the dunnart stromal cells may penetrate the epithelial basal lamina before the trophoblast but further clarification of this is required.

As the uterine epithelium is eroded, TGCs surround groups of maternal epithelial cells which appear to undergo apoptosis. This was observed in full term placentae but there were some areas where no uterine epithelial cells remained. However, Welsh and Enders (1991, 1993) suggested that in rat implantation sites epithelial cell death occurs by more than one type of apoptosis, that is there is more variation in the morphological features of cell death than those established for apoptosis and necrosis. The morphological criteria for programmed cell death need to be further elucidated before firm conclusions can be made about the nature of uterine epithelial cell death in the dunnart.

As the trophoblast in marsupials is homologous with that of eutherian mammals (Padykula and Taylor, 1982; Blackburn *et al.*, 1988) it is not surprising that, in the dunnart, the cell associations at the time of implantation are similar to those seen in some eutherian species. The fact that the chorion is associated with the yolk sac as opposed to the allantois does not appear to have resulted in divergent processes of trophoblastic penetration of the uterine epithelium. Implantation in many eutherian species begins prior to the development of the allantois and often occurs in the region of the yolk sac such that the first placenta formed is choriovitelline. For example, in the rabbit epithelial penetration first occurs on day 7 above the yolk sac on the antimesometrial pole of the uterus followed the next day by syncytiotrophoblast invasion of the mesometrial uterine epithelium above where the future chorioallantoic placenta forms (Enders and Schlafke, 1971a). Trophoblast activity during penetration is morphologically similar in both regions. Also in the mare the trophoblast cells that migrate from the chorionic girdle into the endometrium to form the endometrial cups, the source of equine chorionic gonadotrophin, are derived from a band of the chorion that is neither apposed to the yolk sac nor the allantois (Enders and Liu, 1991b).

From these results, and those on the variety of cell interactions found at implantation in eutherian mammals, it appears that implantation in the fat-tailed dunnart, a

marsupial, is a variation of the mammalian theme and is a means to access maternal oxygen and nutrients for an extremely fast-growing embryo (Roberts and Breed, 1994b) even though the placenta formed functions for a maximum of only three days.

Chapter 6 Placentation - Light Microscopical Structure

6.1 Introduction

In chapter 5 (and in Roberts and Breed, 1994a) it was established that in the dunnart trophoblast invades the endometrium at implantation by the process of intrusion and possibly fusion with the uterine luminal epithelium. Hughes (1974b) found that the allantois in this species does not fuse with the chorion and that the yolk sac (with the chorion) is the only fetal membrane to take part in the formation of a placenta with the endometrium. However, in Hughes' study the light microscopic structure of only one post-implantation female was examined and its gestational age was not accurately known. Consequently, it was decided to further examine the ontogeny of placental development in this species by studying a range of gestational ages and to determine the extent of trophoblast invasion and, therefore, the type of definitive placenta that is formed.

In the present study of the fat-tailed dunnart, *Sminthopsis crassicaudata*, the histochemistry and histological structure of the yolk sac placenta at the light microscopical level of resolution are described. Some comparative notes on the structure of the placenta of the South American didelphid marsupial, *Monodelphis domestica*, are also presented.

6.2 Materials and Methods

Female dunnarts were paired with fertile males and every morning urine samples from each female were placed on glass slides and examined for the presence of spermatozoa. The first day that spermatozoa were present in the smear was designated day 1 *post-coitum* (*pc*) and the first day of pregnancy was assumed to be the last day that spermatozoa were present in the smear, although sperm storage may have occurred after this time in some females (Selwood, 1987).

In the first study a total of 69 females were smeared daily for between 4 and 29 days over a period of 4 months. As the successful pregnancy rate was not as high as expected early in the study it was decided to also check females for signs of pregnancy by abdominal palpation. In the second study 32 females were maintained in pairs for up to two weeks and urine samples were examined daily as above.

Females were sacrificed as described above (see page 43) 9-15 days *pc* and gave a range of gestational ages with an additional female on day 4 *pc* which had cleaving embryos. Five non-pregnant females 11-15 days *pc* were also sacrificed. In addition uteri containing bilaminar blastocysts of one animal were obtained at an unknown gestational age from one animal used in a separate study.

Immediately following death uteri were dissected in routine TEM fixative (see page 45). Tissues from a few females were photographed with the dissecting microscope using Kodak Tech Pan ASA 50 film. The stage of embryonic development and crown-rump length (CRL), when applicable, were recorded. The selected uteri for light microscopy were slit to allow fixative to enter the lumina and either processed whole or dissected into pieces between separate sites of embryonic attachment. Uteri and embryos were processed for light and electron microscopy as described above (see page 45).

For histochemical examination 7µm serial paraffin sections were cut and stained with either haematoxylin and eosin, periodic acid Schiff (PAS), PAS with amylase digestion, or alcian blue at pH 1.0 or 2.5 (Drury and Wallington, 1980). Also 0.5µm thick plastic sections were cut and stained with 0.025% toluidine blue in 0.5% sodium tetraborate.

Uteri of a single pregnant grey short-tailed opossum (*Monodelphis domestica*) were obtained fortuitously by Dr. W.G. Breed during a study of cytoskeletal elements in early embryos in Dr. G. Schatten's laboratory at the University of Wisconsin, Madison. This individual had previously been obtained from Dr. J. Vander Berg at the Southwest Foundation for Medical Research, San Antonio and was estimated to be on day 13 of the 14.5 day pregnancy. Immediately after death the uteri were removed and fixed in 10% phosphate buffered formalin. After returning the material to Adelaide the tissue was processed and stained as above for light microscopy.

6.3 Results

6.3.1 Placental Structure in *Sminthopsis crassicaudata*

6.3.1.1 Selection of Animals

In the first study, of the 69 females from which vaginal cell samples were obtained, only 25 were found to have spermatozoa in the urine. Fourteen females were sacrificed 9-15 days *pc*, nine of which were found to be pregnant at death, and the other 11

females that had sperm-positive smears were not killed as no evidence of pregnancy was found upon abdominal palpation. Histological sections showed that two of the pregnant animals had degenerating blastocysts. Therefore, out of 25 females with spermatozoa in the urine the successful pregnancy rate (to the time of death) was only 28%.

In the second study 32 females were paired with males and of these 18 had sperm in the urine, 11 became pregnant, nine of which had viable embryos *in utero* at death. Uteri of the other two females contained degenerating embryos. Those in one female had apparently died at the unilaminar blastocyst stage and measured about 1.4mm in diameter while those in the other measured 900μm. Two of the pregnant females had both viable and apparently dead embryos *in utero*. In one of these there were 2.4mm primitive streak embryos in one uterus and, in the other, seven degenerate cleavage stage embryos were found. The second female had seven, apparently viable, 3mm primitive streak embryos, with four collapsed large embryos which appeared to have only recently died. The successful pregnancy rate for this group of females was 50%.

6.3.1.2 Non-pregnant Animals

At the time of death, 9-15 days after spermatozoa had been found in the urine, the uteri of five animals were about 2mm in diameter. Histology revealed that the luminal epithelium was simple, columnar and unfolded and overlay a stroma with little extracellular matrix in which many glands occurred. The endometrial glandular epithelium generally had a similar appearance to that lining the lumen although occasionally glands were found that were widely dilated with a simple squamous epithelium. The endometrium of non-pregnant females was similar to that of early pregnant animals (see Fig. 5.1).

Secretion from a few of the glands was PAS-positive and amylase resistant. In addition a different secretion, which was PAS-negative and alcian blue positive at pH 2.5 but negative at pH 1.0, was found at the surface of part of the uterine lumen and in a few of the glands.

6.3.1.3 Pregnant Animals

Results from the studies have been pooled and the embryonic developmental stages found in pregnant animals killed 9 - 15 days *pc* is shown (Table 6.1). The apparent inconsistency of the number of days *pc* and the stage of embryonic development was due to the variable length of time that spermatozoa were stored in the isthmus of the female oviduct before ovulation and fertilization.

Table 6.1 Embryonic developmental stages obtained of *Sminthopsis crassicaudata.*

Sp in smear	Days *pc*	Embryonic Development
day 1 *pc*	4	4-cell Embryo, SM present.
days 1&2 *pc*	14.5	Unilaminar Blastocyst[a] 750µm diam. SM present.
day 1 *pc*	9	Unilaminar Blastocyst, 900µm diam. SM present.
unknown	unknown	Early Bilaminar Blastocyst, 1.8mm diam. SM present.
day 1 *pc*	9	Early Bilaminar Blastocyst, 2mm diam. SM present.
day 1 *pc*	12	Early Trilaminar Embryo 2.4mm diam. SM present.
day 1 *pc*	11	Early Embryonic Disc, 2.8mm diam. SM present.
day 1 *pc*	12	Primitive Streak, 3mm diam. SM present.
day 1 *pc*	11	Primitive Streak, 3mm diam. SM present
days 1&2 *pc*	14	Early Somite, SM absent, Implanting.
days 1&3 *pc*	13	2.9-3.2mm CRL, manus paddle-shaped.
day 1 *pc*	11	3.6mm CRL, manus with digital ray, no hindlimb bud.
day 1 *pc*	12	3.0-3.5mm CRL, manus with digital rays, hindlimb bud.
day 1 *pc*	12	3.2-3.8mm CRL (only 1 @ 4mm CRL embryo was found in one uterus), manus with digital rays, hindlimb bud.
day 1 *pc*	12	3.2mm CRL manus - distinct digits, hindlimb paddle.
day 1 *pc*	12	3.3mm CRL (only 1 @ 4.5mm CRL embryo was found in one uterus), manus - distinct digits, hindlimb paddle.
day 1 *pc*	11	3.5mm CRL (2 @ 4mm CRL), manus - distinct digits, hindlimb paddle.
day 1 *pc*	13.8	Neonates <7h old, 4.0-4.2mm CRL manus with claws, hindlimb paddle.

Sp - Spermatozoa; *pc - post coitum*; CRL - Crown Rump Length; SM - Shell Membrane.
[a]In this species birth may occur between 13.5 and 16 days *pc*; this variation is largely due to storage of spermatozoa in the isthmus for up to three days between mating and ovulation.

6.3.1.4 Pre-implantation Embryos

Four expanded unilaminar blastocysts of approximately 750μm in diameter, surrounded by a thin mucoid layer and a shell membrane, were flushed from one uterus. Paraffin sections of the other uterus from the same female, showed five collapsed embryos lying in shallow depressions of the simple columnar epithelium (see Fig. 5.1). The underlying endometrial stroma was highly glandular.

Eight early bilaminar blastocysts (approximately 1.8mm in diameter) were found in 'pockets' of the endometrium of one uterus completely enveloped by folds of intact luminal epithelium. These blastocysts, still surrounded by a SM, could also be flushed out of the uteri with fixative.

The endometrial stroma close to the blastocysts was oedematous. The simple columnar luminal epithelium had a thin coat of PAS-positive, amylase resistant, material which was also found in the basal cytoplasm of many luminal epithelial cells. The secretion of the glands was generally eosinophilic with a few glands having PAS-positive, amylase resistant, secretion. All secretions were alcian blue negative.

No histochemical staining was performed on uteri which had contained trilaminar / primitive streak embryos. The ultrastructure of the uterine epithelium at these stages in development was described in chapter 5.

6.3.1.5 Early Somite Embryos

The uteri in one animal were dorso-ventrally flattened and measured about 13mm in diameter and 8mm thick. Superficially, the endometrium was folded into aglandular thin tongues of tissue composed of epithelium with a thin layer of vascularized stroma, although below these folds the endometrium was very glandular (Fig. 6.1). The epithelium was of a simple cuboidal type and its cell apices bulged into the lumen giving its surface a cobblestone appearance (Figs. 6.2 and 6.3). Many capillaries were found immediately below the basement membrane of the epithelium. The myometrium was very thin (Fig. 6.1).

Chorionic vesicles flushed from the uteri had lost their shell membranes which lay in clumps, visible with the dissecting microscope, in the uterine lumen. The yolk sac had an irregular ellipsoid shape and measured about 4mm by 2mm, after sectioning (Fig. 6.1). Embryos were at the early somite stage (Fig. 6.4 and see Fig. 5.6, chapter 5). Convoluted ribbon-like fragments of shell membrane lay adjacent to the endometrium (Fig. 6.1). At this time the embryo was still a part of the wall of the chorionic vesicle

(Fig. 6.1 and 6.4) and had a pro-amniotic head fold such that the future head was submerged beneath the surface of the vesicle. Blood islands, with primitive erythroblasts, had formed in the extra-embryonic mesoderm in an annular region adjacent to the embryo (Fig. 6.5).

In the yolk sac, trophoblast cells showed regional differences in size. In the region of the bilaminar yolk sac they were large and cuboidal (Fig. 6.2). In the trilaminar region, near to the maternal epithelium, they were large and vacuolated above the extraembryonic mesoderm (Fig. 6.3) but when trophoblast cells, which were closely apposed to the mesoderm below, were some distance away from the endometrium they were tall and columnar (Fig. 6.5). Trophoblast cells at the abembryonic pole of the yolk sac were attenuated and resembled the squamous endoderm.

6.3.1.6 Late Pregnant Stages - 2.9-4.5mm CRL

Organogenesis was well under way in embryos of five females sacrificed on days 11-13 *pc*. In different females the placental site was either mesometrial, anti-mesometrial or in one female a combination of the two. That is, embryos (3.2-3.8mm CRL), each closely enclosed within a thin amniotic membrane and enshrouded by their yolk sacs, were closely surrounded by the endometrium and the discoid placenta, visible with the dissecting microscope, was attached to both walls of the endometrium (Fig. 6.6). The allantois of each embryo approached, but did not fuse with, the chorion (Fig. 6.7) and thus did not take part in placentation. In the second uterus of the female whose right uterus is shown in figures 6.6 and 6.7, only one large (4mm CRL) embryo was found in one uterus and the placental site was also large and very vascular and the sinus terminalis could be clearly seen (Figs. 6.8 and 6.9).

Uteri which contained larger numbers of embryos were invariably bigger than those with smaller numbers although this could not be completely explained by the larger volume of embryos. The endometrial surface area and volume also appeared larger although these were not quantified.

After the development of the placenta, the simple, cuboidal, uterine, luminal epithelium had lost its cobblestone appearance. The yolk sac was composed of three regions: 1, the unattached, bilaminar omphalopleure (yolk sac); 2, the attached, bilaminar, avascular, yolk sac placenta (BYS); and 3, the attached trilaminar, vascular, yolk sac placenta (TYS). In region 1 the lateral walls of yolk sacs of neighbouring embryos in three females fused and were lost late in pregnancy such that up to five embryos occupied the same chorionic cavity. In two females embryos were attached further apart such that no fusion between yolk sacs occurred. In one uterus of one

Figures 6.1 - 6.5 Early somite embryo *Sminthopsis crassicaudata.*

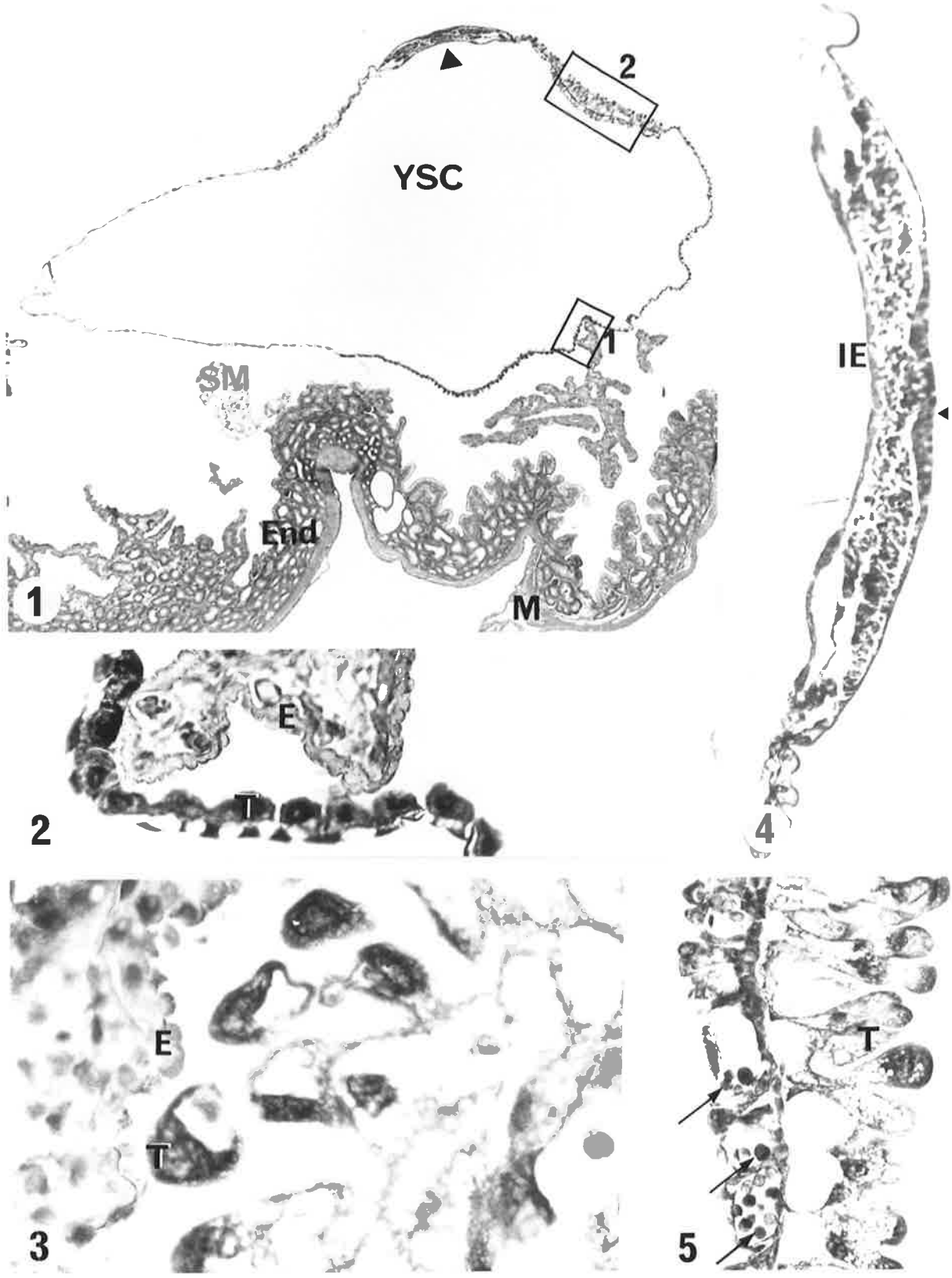
Figure 6.1 Yolk sac of early somite embryo which is still a part of the wall of the vesicle; embryo (arrowhead), shell membrane (SM), endometrium (End), myometrium (My), yolk sac cavity (YSC) Bar = 500μm. H & E.

Figure 6.2 Higher magnification of bilaminar yolk sac (box 1 in Fig. 6.1) shows that the uterine luminal epithelium (E) has a cobblestone appearance and trophoblast cells (T) are large and cuboidal. Bar = 50μm. H & E.

Figure 6.3 Trophoblast cells (T) are large and vacuolated and adjacent to the maternal epithelium (ME). Bar = 25μm. H & E.

Figure 6.4 Enlargement of embryo shown in Fig. 6.1 at the early somite stage, neuroectoderm (arrowhead), embryonic endoderm (IE). Bar = 100μm. H & E.

Figure 6.5 Higher magnification of the extra-embryonic trilaminar region (box 2 in Fig. 6.1); blood islands with primitive erythroblasts (arrows) are present in the mesoderm adjacent to the embryo; trophoblast above them (T) is tall and columnar. Bar = 50μm. H & E.


2
YSC
1
SM
End
M
1
IE
4
E
T
2
E
T
3
T
5

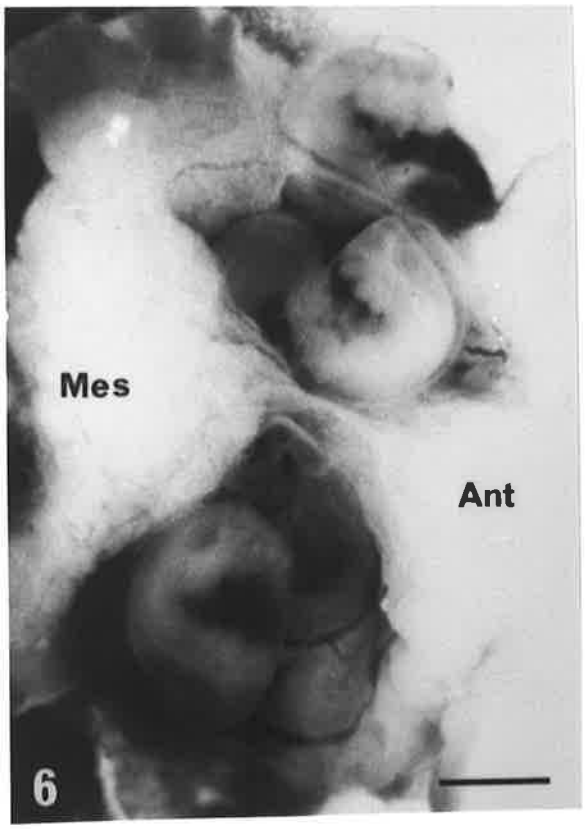
Figures 6.6 - 6.9 Day 12 embryos, CRL 3.2-4mm, *Sminthopsis crassicaudata.*

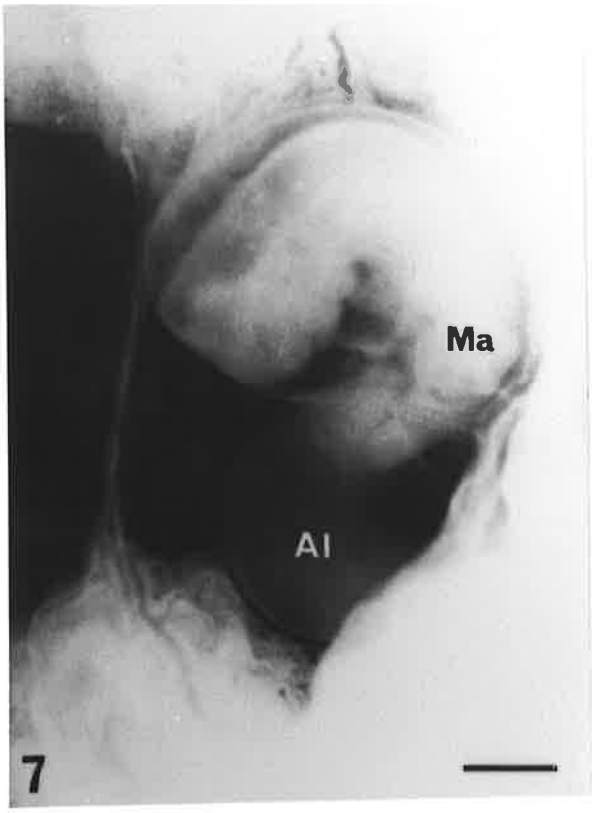
Figure 6.6 Attachment of the placental discs in this female was to both mesometrial (Mes) and anti-mesometrial (Ant) walls of the endometrium. Bar = 2mm.

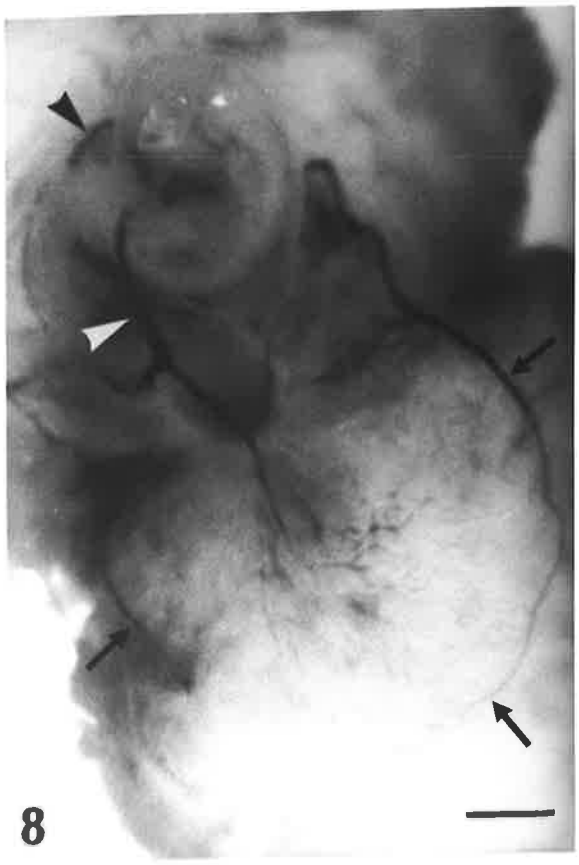
Figure 6.7 The allantois (Al) was enclosed within folds of the yolk sac but did not fuse with the chorion; Manus (Ma) had digital rays. Bar = 1mm.

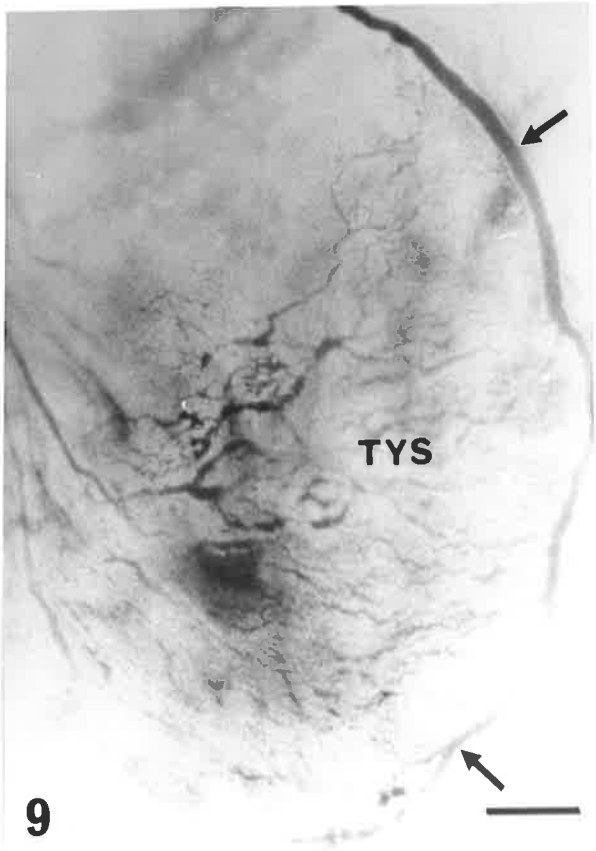
Figure 6.8 Only one large embryo (CRL 4mm) was found in the second uterus of the same female as that of Figs. 6.6 and 6.7. The placental site was larger than those of the smaller embryos in the other uterus; vitelline vessels (arrowheads), sinus terminalis (arrows). Bar = 2mm.

Figure 6.9 Higher magnification of the placental site seen in Fig. 6.8 shows a very vascular trilaminar yolk sac (TYS) bounded by the sinus terminalis (arrows). Bar = 1mm.


Mes
Ant
6


Ma
Al
7


8


TYS
9

female two embryos were attached separately and the yolk sacs of three embryos had fused.

Region 1 was composed of two simple epithelial cell layers where squamous trophoblast overlay a squamous endoderm which lined the yolk sac cavity. This part of the chorionic vesicle was not closely apposed to the uterine epithelium.

In region 2 (the bilaminar yolk sac placenta) epithelial penetration continued even on the last day of pregnancy and uterine epithelial cells became surrounded by trophoblast giant cells (TGCs) (Figs. 6.10 and 6.11). Nuclei of TGCs were up to 70µm in diameter and presumably polyploid. The uterine epithelium was replaced on one side of many endometrial folds by TGCs of the avascular yolk sac but it remained intact on the other side (Fig. 6.10). The cytoplasm of the TGCs was pale and extremely vacuolated.

In the BYS, TGCs of up to 160µm in diameter replaced the maternal epithelium (Fig. 6.12) but did not appear to have invaded the maternal capillaries. An endotheliochorial placenta had apparently formed, although the boundary between fetal and maternal tissue in paraffin sections was not easily discernible.

In region 3 the convoluted chorion followed the contours of the endometrium forming a discoid trilaminar yolk sac placenta. Trophoblast cells in the TYS were usually, but not always, much smaller (15µm - 30µm) than the TGCs of the BYS and were not invasive, thus an epitheliochorial placenta had developed (Figs. 6.13 and 6.14). The distance between fetal and maternal blood in the TYS was less than 10µm as both maternal stromal, fetal mesodermal, and trophoblast nuclei near the placental capillaries were displaced. At the junction between the TYS and BYS a large blood vessel, the sinus terminalis, occurred. Below the placenta in the endometrial stroma there were scattered neutrophils, lymphocytes and macrophages, particularly below the invasive TGCs.

Late in development (embryonic manus paddle-shaped) the allantois formed an evaginated diverticulum which approached, but did not reach, the chorion. The wall of the allantois was composed of vascular mesenchyme. At the forelimb digit stage the allantois had become smaller, and remained within folds of the yolk sac.

TGCs in the BYS and TYS contained PAS-positive, amylase resistant, and alcian blue positive at pH 2.5 (and at pH 1.0 in one animal) granules in their apical cytoplasm (Figs. 6.15 - 6.17). Similar material was found on the cell surface of TGCs and on the apical surface of the uterine epithelium, especially at the interface between fetal and

maternal tissues, although this material was found on the surface of the luminal epithelium even away from sites of intimate contact. The cytoplasm of small trophoblast cells (about 15μm in diameter) in the unattached bilaminar yolk sac had the same staining properties as the TGCs (Fig. 6.18). The gland lumina were both PAS and alcian blue negative at this stage (Fig. 6.17).

6.3.1.7 Post-parturition

One animal had given birth between three and seven hours before the time of death and had 10 neonates (4.2mm CRL; 15mg) attached to the 10 teats. Drops of milk in the stomachs of the neonates could be seen through the still moist skin of their abdomens. The young had gaping mouths and were firmly attached to the teats. The digits of the neonatal manus had claws while the pes was still paddle-shaped, although scalloped where the future digits would develop, and the eyespot was pigmented.

The mother's uterine epithelium, where present, was simple and cuboidal and its luminal surface was coated with a PAS-positive, amylase resistant, alcian blue positive (at both pH 1.0 and pH 2.5) material. The secretion within the endometrial glands was PAS-positive but alcian blue negative. The nuclei of TGCs had become pyknotic and there was a massive infiltration of granular leukocytes and lymphocytes into the endometrial stroma. It was unclear whether the remains of the placenta would subsequently have been shed or resorbed as there was an additional embryo found in the median pseudo-vagina.

6.3.2 Placenta of *Monodelphis domestica* - 7mm CRL

The most mature embryo found in the uteri of the grey short-tailed opossum on day 13 of the 14.5 day pregnancy had a CRL of 7mm, the manus had 5 distinct digits, the pes was paddle-shaped and the eyespot was pigmented (Fig. 6.19). The endometrium was very folded and glandular with a simple columnar epithelium which had a smooth luminal surface (Fig. 6.20). Remnants of the shell membrane (SM) lay between maternal and fetal tissues in patches across the surface of the yolk sac, especially in the avascular region (Fig. 6.20), but in many places it had already been shed.

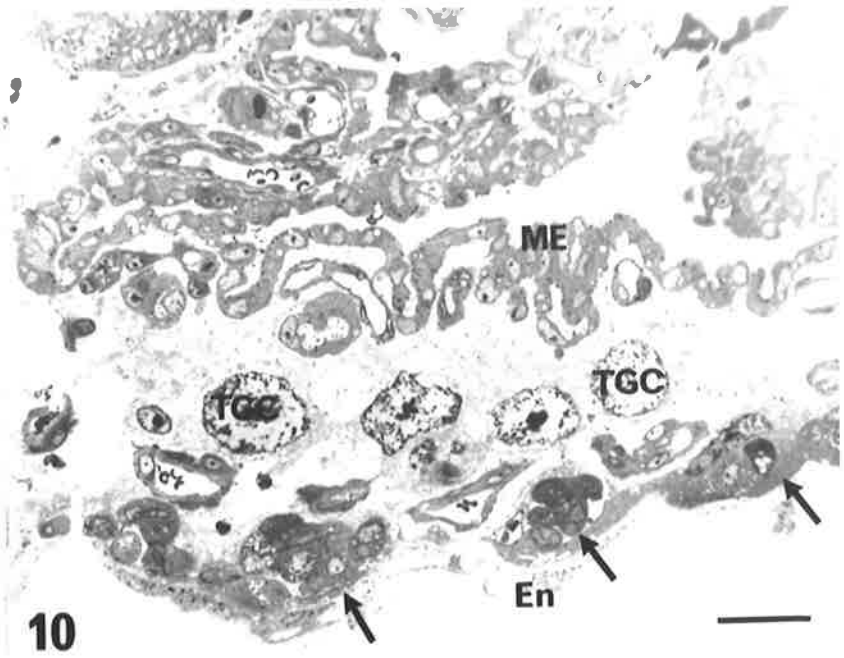
The placenta of *Monodelphis domestica* was also choriovitelline. There were three regions of the yolk sac at this stage of gestation: 1, an unattached bilaminar, avascular yolk sac which occupied the uterine lumen enveloping the embryo and which, over much of its surface, followed the contours of the folded endometrium; 2, a very attenuated avascular yolk sac placenta closely apposed to the uterine luminal

Figures 6.10-6.12 Bilaminar Yolk Sac Placenta (BYS) of *Sminthopsis crassicaudata.*

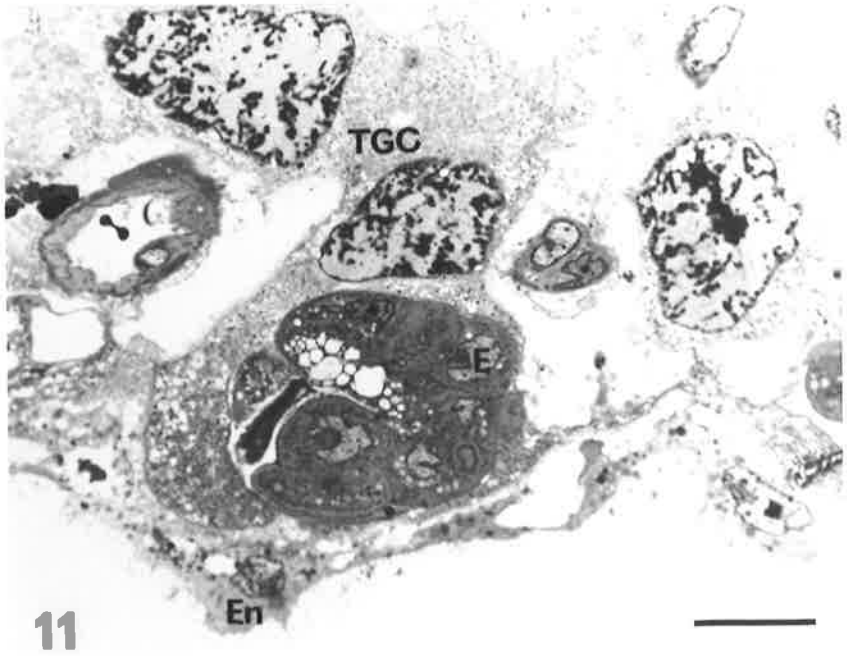
Figure 6.10 Epithelial penetration has taken place and groups of maternal epithelial cells (arrows) on one side of an endometrial fold are surrounded by trophoblast giant cells (TGC), while the epithelium on the other side of the fold remains intact (ME), extra-embryonic endoderm (En). Bar = 25μm. Tol. Blue.

Figure 6.11 Vacuolated uterine epithelial cells (E) surrounded by TGCs are degenerate; maternal capillaries (arrowhead) are also found between the trophoblast and extra-embryonic endoderm (En). Bar = 50μm. Tol. Blue.

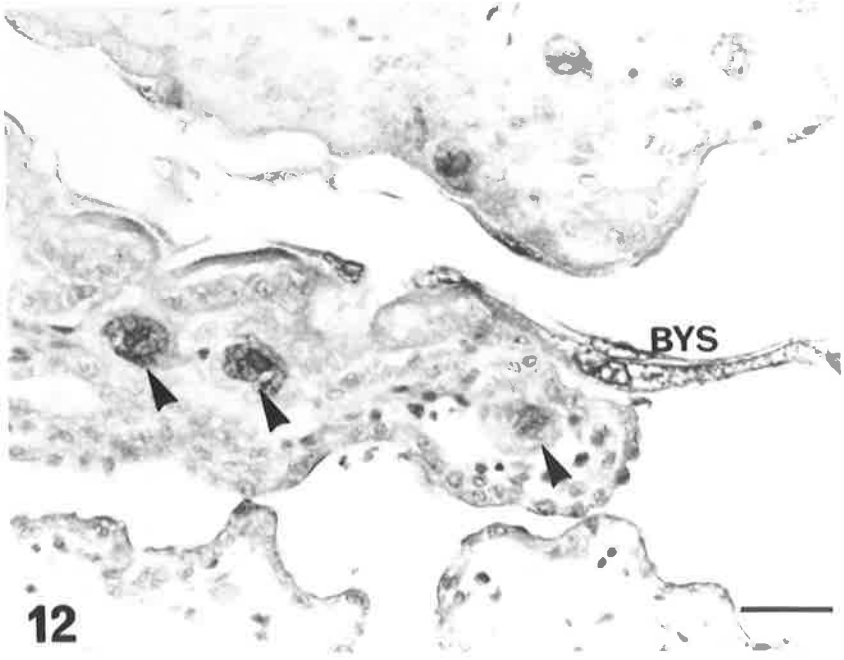
Figure 6.12 Bilaminar yolk sac placenta (BYS) appears to merge with folds of endometrium; TGCs (arrowheads) have invaded the endometrial stroma. Bar = 50μm. H & E.



10



11

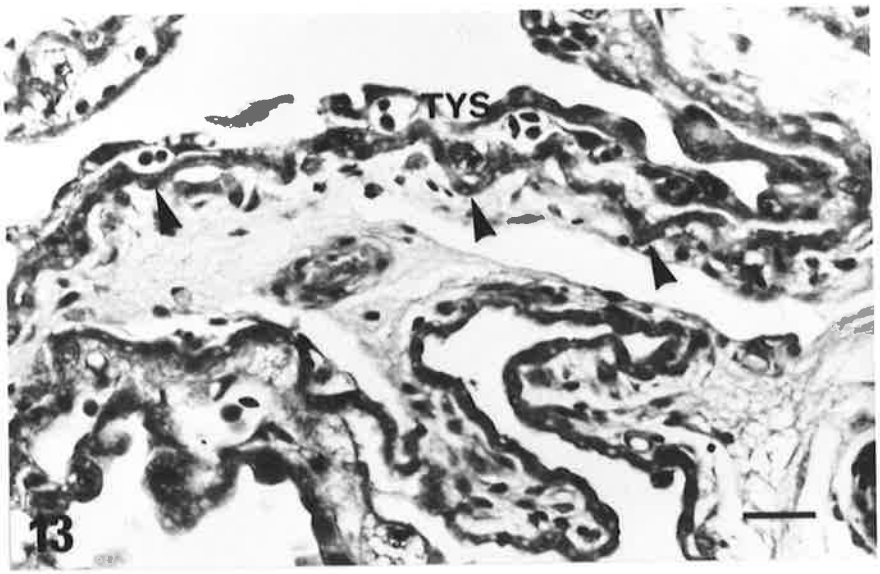


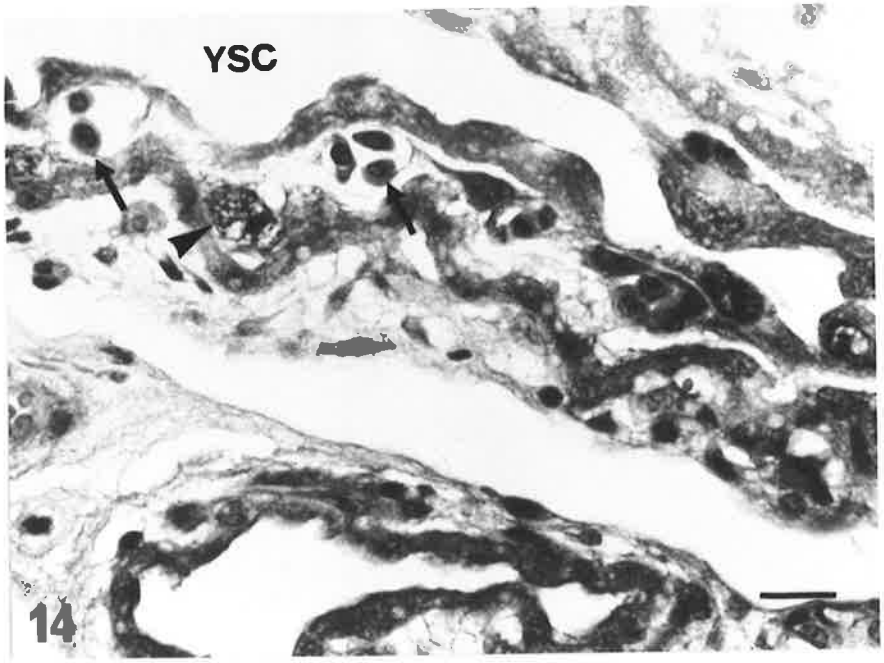
12

Figures 6.13 and 6.14 Trilaminar Yolk Sac Placenta (TYS) of *Sminthopsis crassicaudata.*

Figure 6.13 Trilaminar yolk sac (TYS) follows contours of folded endometrium but does not erode the epithelium (arrowheads). Bar = 50μm. H & E.

Figure 6.14 Trophoblast cells are usually smaller in the TYS than in the BYS although occasional TGCs (arrowhead) are found adjacent to fetal capillaries. Nucleated fetal erythrocytes (arrows) fill the extensive capillary network within the TYS, yolk sac cavity (YSC). Bar = 25μm. H & E.

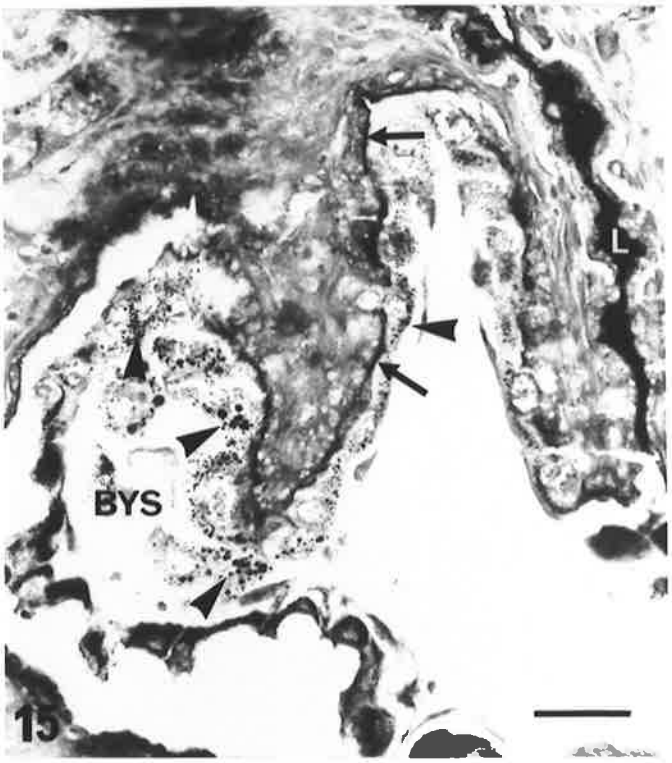

TYS
13

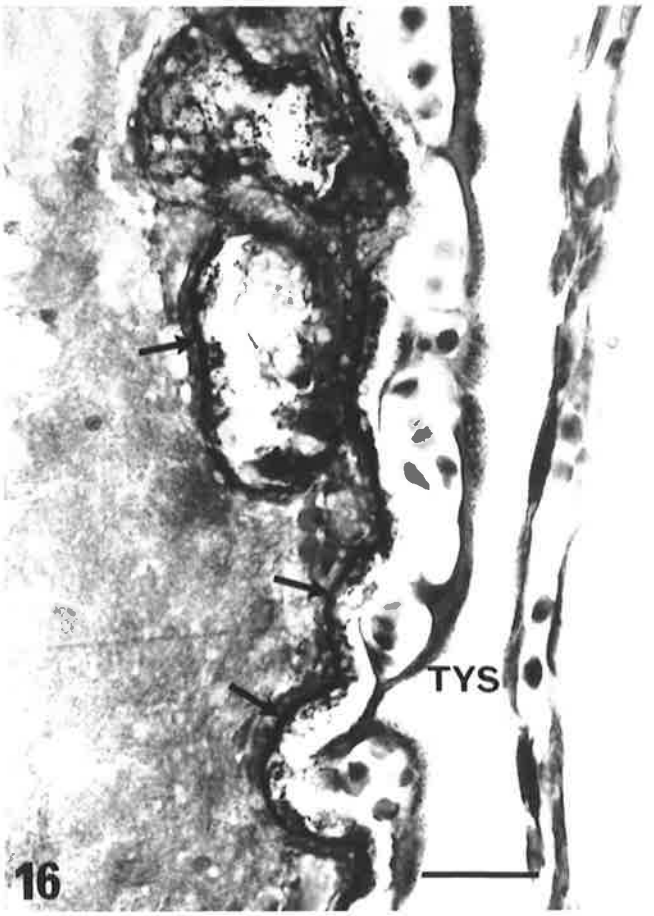

YSC
14

Figures 6.15 and 6.16 PAS staining of placenta of *Sminthopsis crassicaudata*.

Figure 6.15 Cytoplasm of TGCs in the BYS (arrowheads) contains PAS-positive and amylase resistant granules. Similar material coats uterine luminal epithelial cells (arrows) and fills the lumen (L) between closely adjacent folds of endometrium. Bar = 50μm.

Figure 6.16 The plasmalemma of TGCs is coated with PAS-positive material which is particularly evident at the interface between fetal and maternal tissues in the TYS (arrows). Bar = 25μm.

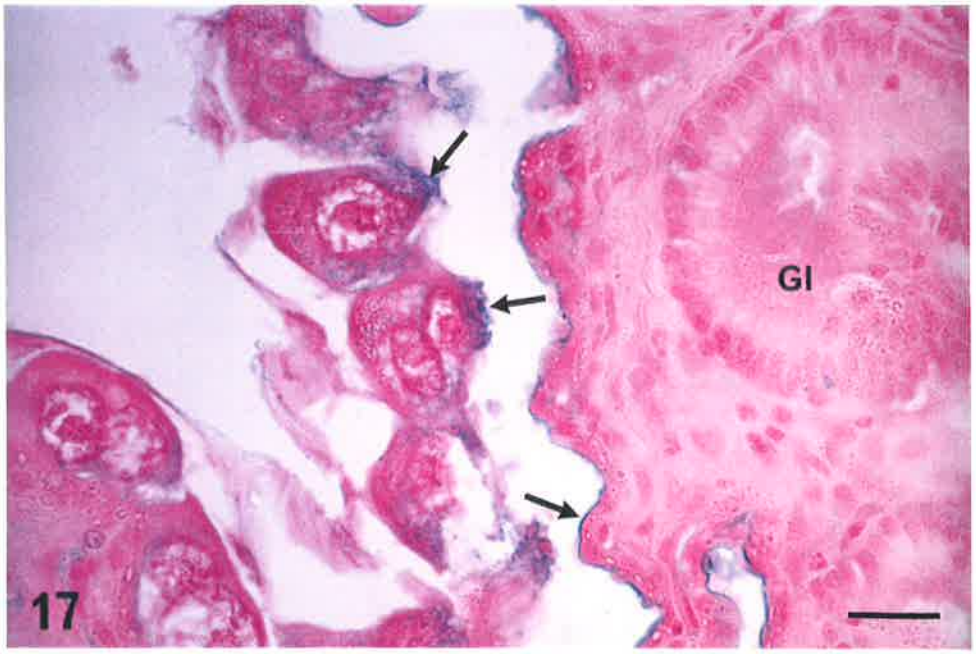

L
BYS
15


TYS
16

Figures 6.17 and 6.18 Alcian Blue (pH 2.5) staining of placenta of *Sminthopsis crassicaudata*.

Figure 6.17 Secretion at the interface between fetal and maternal tissues in the BYS is Alcian Blue (pH 2.5) positive as are granules within trophblast giant cells (arrows). The endometrium including the gland (Gl) lumen is negative. The gap between trophoblast and uterine luminal epithelium is an artefact of processing. Bar = 25μm.

Figure 6.18 Trophoblast of the unattached bilaminar yolk sac (BYS) also contains Alcian Blue (pH 2.5) positive granules (arrows). Bar = 50μm.


GI
17

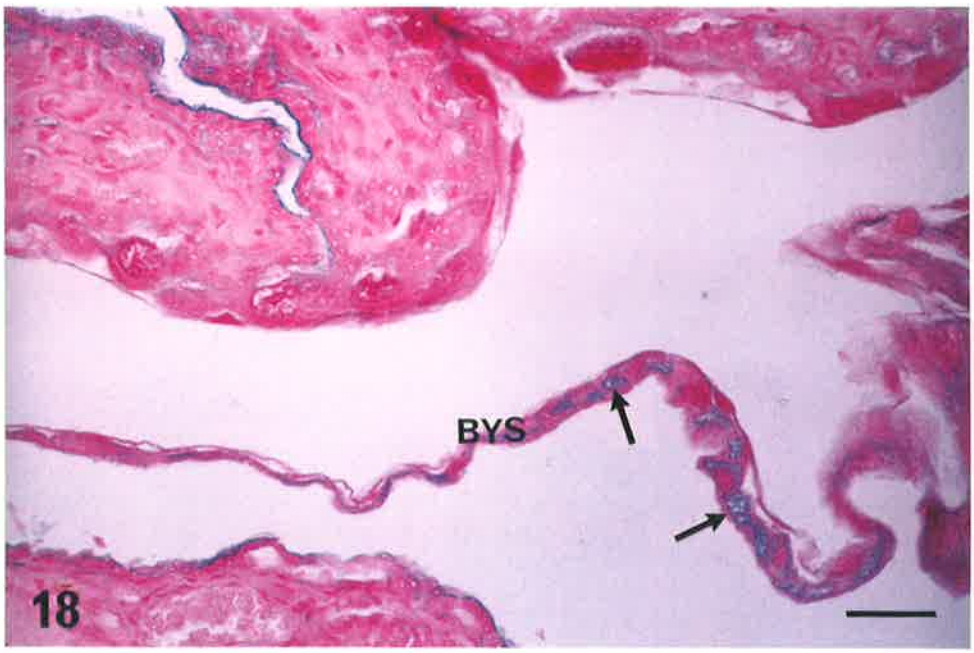
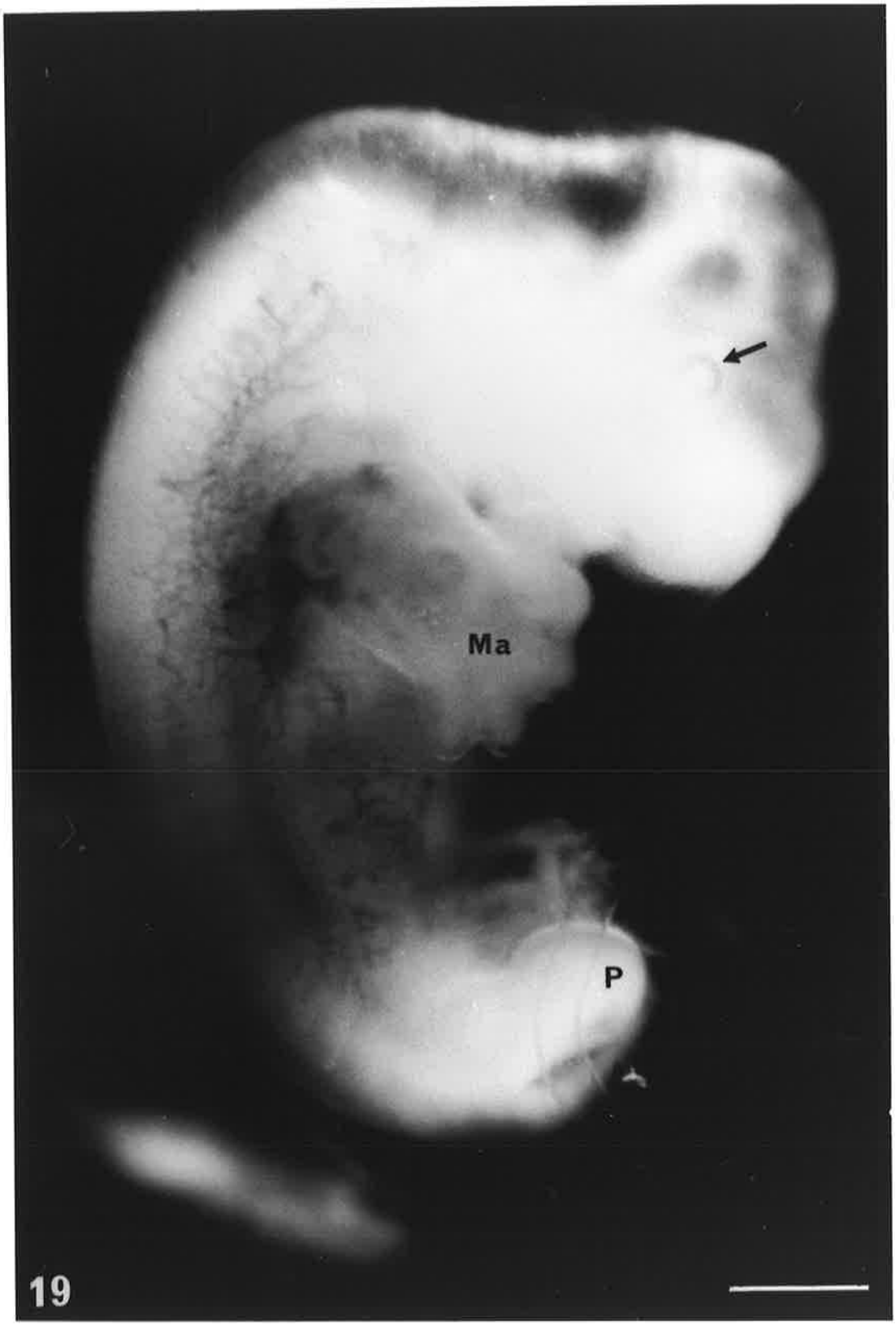

BYS
18

Figure 6.19 Embryo (7mm CRL) of *Monodelphis domestica* on day 13 *pc*. The manus (Ma) had 5 distinct digits, the pes (P) was paddle-shaped and the eyespot was pigmented (arrow). Bar = 2mm.


Ma
P
19

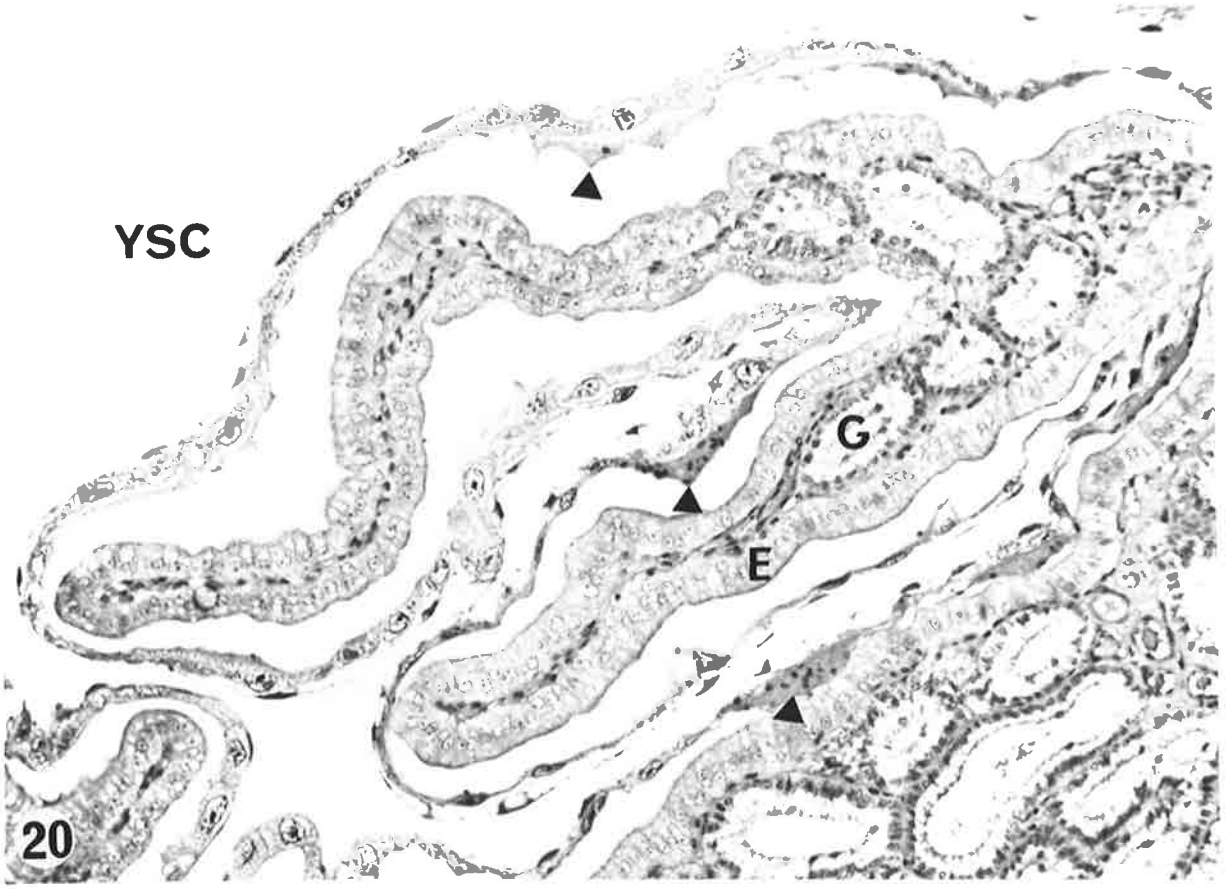
Figures 6.20 - 6.23 Placenta of *Monodelphis domestica* on day 13 *pc.*

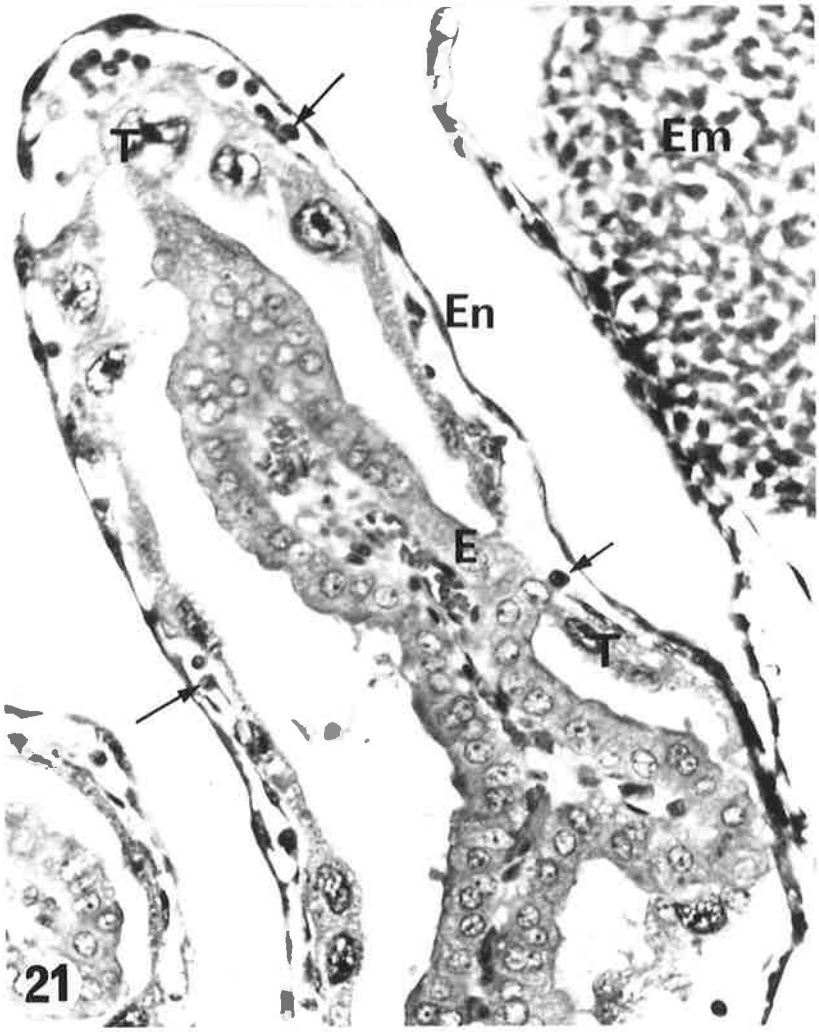
Figure 6.20 The yolk sac follows highly folded contours of the simple columnar endometrial epithelium (E) beneath which many glands are found, gland (Gl), yolk sac cavity (YSC), shell membrane fragments (arrowheads). Bar = 100μm. H & E.

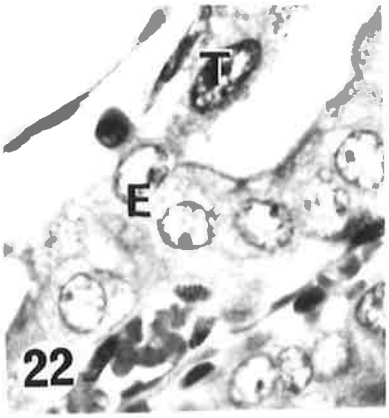
Figure 6.21 In the TYS, trophoblast (T) is attached to the uterine luminal epithelium (E) in discontinuous areas; extra-embryonic endoderm (En), fetal erythrocytes (arrows), embryo (Em). Bar = 50μm. H & E.

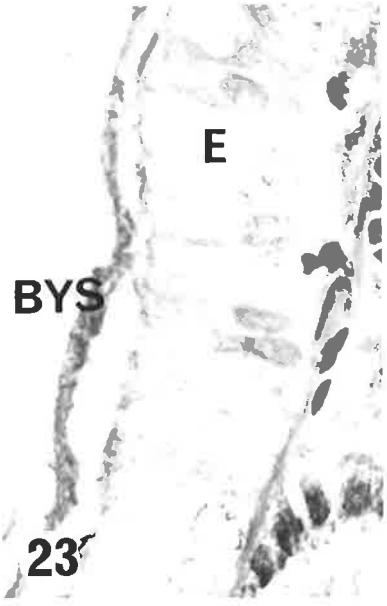
Figure 6.22 Uterine luminal epithelium (E) is continuous with trophoblast (T) at sites of attachment to TYS. Bar = 25μm. H & E.

Figure 6.23 The BYS is closely apposed to maternal epithelium (E) and is very attenuated; both trophoblast and endoderm are squamous and indistinguishable. Bar = 25μm. H & E.


YSC
G
E
20


T
Em
En
E
T
21


T
E
22


E
BYS
23

epithelium (BYS); and 3, a trilaminar, vascular, yolk sac placenta (Fig. 6.21 and 6.22). Each embryo lay separately in its own yolk sac some distance from the other embryos.

The unattached bilaminar, or avascular, yolk sac had occasional large trophoblast cells with a squamous endoderm lining its inner surface. A more attenuated region of the bilaminar yolk sac was found closely apposed to the uterine luminal epithelium (Fig. 6.23). The trophoblast in this region was squamous and its cytoplasm contained eosinophilic granules. Similar material was found in layers between the trophoblast and endoderm in some parts of this region.

The trilaminar, or vascular, yolk sac placenta was composed of an inner attenuated endodermal layer, an overlying vascular mesodermal layer and an outer layer of trophoblast giant cells (TGCs) which, in discontinuous areas, appeared to be attached to the uterine epithelium (Figs. 6.21 and 6.22). The largest TGCs were cuboidal and measured about 50μm in diameter and those near the sinus terminalis were columnar and measured about 50 by 25μm. The cytoplasm of the TGCs had many vacuoles, some of which contained eosinophilic granules.

The allantois was present at this stage although it remained close to the embryo and was enveloped by folds of the yolk sac. It had thin walls and was comparatively avascular.

Histochemical staining showed that the uterine epithelium and the endometrial glands were PAS and alcian blue negative. The layer between the endoderm and trophoblast in the BYS was weakly PAS-positive and amylase resistant but alcian blue negative.

6.4 Discussion

This study has extended the previous findings on the placental histology of the fat-tailed dunnart, *Sminthopsis crassicaudata*, which were based on a single pregnant female (Hughes, 1974b). For my study 17 pregnant females at a variety of developmental stages were investigated. Seven of these were sacrificed late in gestation with a fully developed placenta. By comparing placental structure in the dunnart with that of a didelphid marsupial, the grey short-tailed opossum, *Monodelphis domestica*, this investigation has emphasised the variation in choriovitelline placental structure between representatives of two primitive marsupial families, particularly in regard to the presence and location of TGCs and the likely extent of invasion of fetal tissue into the endometrium.

The endometrium in pregnant dunnarts, like that in eutherian mammals and bandicoots (Padykula and Taylor, 1977), has a simple columnar epithelium. Padykula and Taylor (1971) and Krause and Cutts (1985b) reported that there is a pseudostratified, columnar, luminal epithelium in the pregnant uterus of *Didelphis virginiana*. However, this conclusion may be based on observations of tangential sections of the epithelium which could give the impression that it is pseudostratified when, in fact, it is of a simple columnar type. Flynn (1930) found that early in pregnancy in *Bettongia cuniculus* the uterine epithelial cells, although composed of a single layer, appeared very "crowded" after having undergone extensive proliferation at pro-oestrus and oestrus. As pregnancy progressed the endometrium became highly folded with a largely increased surface area and a simple columnar epithelium. My observations of the dunnart uterine luminal epithelium were similar to those of Flynn.

It was not possible to obtain accurately timed pregnancies in the dunnart because of the lack of synchrony between female receptivity, mating and ovulation. Females may copulate with a male for up to 3 days or for only one day before ovulation. Also storage of spermatozoa in the isthmus of the female occurs for up to 3 days prior to ovulation (Selwood, 1987; Breed *et al.*, 1989) and, consequently, estimations of the stage of pregnancy from the time of mating of these animals can be erroneous. However, this is not the case for the opossum in which accurately timed pregnancies can be obtained as ovulation follows 14-16 h after the onset of oestrous behaviour (Baggott *et al.*, 1987).

By the early somite stage (about Day 10) the dunnart embryo has completely shed its shell membrane (SM) and implantation, which is centric, has begun. Hughes (1974b) found the SM to be present in *S. crassicaudata* embryos at the primitive streak stage which suggests that in our study the SM had just broken down in the early somite embryo. The stage of embryonic development at the time of the loss of the shell membrane has been recorded as 13-15 somite stage in *Sminthopsis macroura* (Selwood and Woolley, 1991) and 25 somite stage in *Schoinobates volans* (Bancroft, 1973) and *Macropus rufogriseus* (Walker and Rose, 1981). My study shows that the SM of the opossum is lost later in development than in the dunnart as patches of SM were found between the chorionic vesicle and the maternal uterine epithelium when the embryo had paddle-shaped hind limbs. In the tammar wallaby, the action of acid and alkaline proteinases secreted by the fetal endoderm and trophoblast, as well as by endometrial gland and stromal cells, erodes the SM allowing the trophoblast to make contact with the uterine luminal epithelium (Denker and Tyndale-Biscoe, 1986). Presumably these proteinases are also secreted at implantation in the dunnart and the opossum. Krause and Cutts (1985b) found that *Didelphis* embryos have many somites and that brain

differentiation has begun prior to implantation. Presumably in these species trophoblast is able to take up and transport to the embryo the required nutrients and oxygen, despite the presence of the shell membrane.

It is unusual that in the dunnart there is no consistent region of the endometrium where attachment occurs as found in most eutherian species (Wimsatt, 1975). In this study embryos were found attached to the anti-mesometrial, mesometrial, or a combination of both, regions of the endometrium. Also in a few females embryos were found attached to the endometrium close together such that the lateral walls of their yolk sacs had fused and subsequently degenerated resulting in their appearing to share one voluminous yolk sac. This also occurs in the four-eyed opossum (*Philander opossum*) (Enders and Enders, 1969). Embryos in polytocous eutherian species are usually equally spaced at implantation so that there is no overlap of the already large fetal membranes of litter mates in pigs (Anderson, 1978) and no competition for implantation sites in rodents (Martin, 1979). In domestic cats the chorioallantoic sacs of neighbouring fetuses become apposed to each other but they do not fuse, instead there is interdigitation of trophoblast microvilli or, when these are absent, the smooth plasma membranes of trophoblast are in contact with each other (Leiser and Enders, 1980a). Spacing of embryos is thought to be induced by the inhibitory effect of progesterone on the longitudinal smooth muscle of the myometrium and the increased sensitivity and activity of the circular muscle layer which closes the uterine lumen, increasing contact between the embryo and the luminal epithelium (Martin, 1979). More recent research has suggested that relaxin has a significant effect in the antimesometrial positioning of the rat blastocyst because of the differential effect it has on the circular and longitudinal muscle layers (Rogers *et al.*, 1983; Massa and Martin, 1994).

In the dunnart the fetal trophoblast erodes the maternal epithelium in the region of the avascular, bilaminar, yolk sac placenta (BYS) and comes to surround the maternal endothelium. The groups of degenerating maternal uterine epithelial cells found surrounded by trophoblast in late pregnancy in the present study indicate that the trophoblast replaces, and presumably phagocytoses, them. Enlarged trophoblast cells of the BYS of the only other dasyurid species investigated, *Dasyurus viverrinus*, apparently eroded the maternal epithelium and surrounded "maternal blood sinuses" (Hill, 1900) although it was not clear whether the maternal endothelial cells were actually engulfed by trophoblast or not.

In the trilaminar yolk sac placenta (TYS) the maternal epithelium remains intact, although the nuclei of all cell layers between the fetal and maternal capillaries are

displaced immediately above maternal capillaries such that the two bloodstreams come to lie in close proximity with only thin cytoplasmic processes of intervening cell layers between them. This is also seen in the TYS of the petaurids, *Pseudocheirus peregrinus*, (Sharman, 1961; Hughes *et al.*, 1965) and *Schoinobates volans* (Bancroft, 1973), and in the macropod, *Bettongia cuniculus* (Flynn, 1930). In the chorioallantoic placenta of the pig fetal and maternal capillaries indent the epithelia above them, thus reducing the distance for diffusion of oxygen and carbon dioxide (Enders, 1982). The same situation in the marsupial yolk sac placenta presumably also facilitates exchange.

The results of the PAS and alcian blue staining suggest that for the first 2/3 of pregnancy the endometrial glands are actively secreting neutral glycoproteins which presumably comprise some of the histotrophic nutrition for the early embryo. After the development of the placenta, the trophoblast cells of all regions of the yolk sac produce a PAS and alcian blue positive (pH 2.5), amylase resistant secretion which coats them as well as the maternal epithelium. This is presumably a carboxylated glycoprotein, possibly like the sialomucins (fibrinoid) that coat the trophoblast in the mouse placenta (Bradbury *et al.*, 1965). Fleming and Harder (1981) found a similar trophoblastic secretion between the yolk sac and uterine epithelium in *Didelphis virginiana* and suggested that it may play a role in antigenic isolation of the fetal trophoblast. However, this remains to be established.

The results for *Monodelphis domestica* suggest that the trophoblast does not erode the maternal epithelium. However, as this observation is based on only one pregnant individual killed at least 24h before parturition, the possibility that trophoblast invasion of the maternal epithelium occurs on the final day of pregnancy cannot be ruled out.

I have shown that implantation in the dunnart is not "rudimentary" as has been suggested by Hughes (1974b, p. 184) despite his own description of the placenta of a late pregnant female of this species. Clearly, from the present study epithelial penetration takes place over an extensive area. In their research on *Philander opossum* Enders and Enders (1969) found that trophoblast penetrates the uterine epithelium in discontinuous areas and forms, what these authors considered to be, a series of holdfasts, keeping the choriovitelline placenta of individual fetuses attached to the endometrium. In the dunnart invasion is so extensive in the BYS that it presumably has a placental function rather than merely being an anchor.

The results suggest that implantation occurs as late as 3 days before birth in the dunnart, as has been found in*Sminthopsis macroura* (Selwood and Woolley, 1991) and in *Didelphis virginiana* (Krause and Cutts, 1985b), from which time amniogenesis, organogenesis and embryonic/fetal growth must proceed at an astonishing rate. It appears that, in the dunnart, in the space of 3.5 days the embryo develops from the early somite stage to a neonate that, although extremely small, can haul itself up to the pouch unaided, digest milk, excrete urine via mesonephric kidneys and breathe air (Tyndale-Biscoe and Renfree, 1987). This rapid development is presumably supported by the ability of the yolk sac placenta (bilaminar and trilaminar) to deliver nutrients and oxygen.

There is extensive erosion of the maternal epithelium in the acrobatid, *Distoechurus pennatus*, as invasive TGCs are present over the whole BYS and replace the uterine epithelium over the entire region obliterating the uterine lumen (Hughes *et al.*, 1987). In the koala, *Phascolarctos cinereus*, only occasional enlarged ectodermal cells penetrate the uterine epithelium in an annular zone of the BYS (Hughes, 1974b). However, in *Philander opossum*, a South American didelphid, there is a small annular region of invasion at the junction between the TYS and the BYS by large trophoblast cells from the TYS which are multinucleate (Enders and Enders, 1969). Perhaps this difference in the amount of trophoblast invasiveness will be extended in the future as a result of more comparative work on the placentae of the Australian and American marsupials. Or, on the contrary, more thorough investigations in a variety of marsupial species may reveal that there are fewer differences than are apparent at present.

The results of this study show that in the dunnart the trilaminar yolk sac late in pregnancy is non-invasive and forms an epitheliochorial placenta while the bilaminar yolk sac is invasive. However, it is clear that the light microscope does not achieve sufficient resolution to determine the extent of trophoblast invasion of the endometrium in the region of the BYS during placental development. Ultrastructural studies are required to accurately determine the maternal cell type in contact with trophoblast and indicate the functional morphology of the cell types involved. These are the subject of the next chapter.

Chapter 7 Placentation - Ultrastructure

7.1 Introduction

There have been few ultrastructural descriptions of the marsupial placenta and none on that of a dasyurid. Detailed studies at the resolution of the transmission electron microscope (TEM) have so far only been those on the didelphids *Philander opossum* (Enders and Enders, 1969) and *Didelphis virginiana* (Krause and Cutts, 1985b) and the bandicoot species, *Isoodon macrourus* and *Perameles gunnii* (Padykula and Taylor, 1976, 1982).

In chapter 6 it was established that in the dunnart there are three regions of the yolk sac placenta. The trilaminar yolk sac (TYS) which is discoid, and non-invasive, forms an epitheliochorial placenta. The bilaminar yolk sac (BYS) adjacent to the sinus terminalis is invasive and appears to erode the epithelium and stroma of the endometrium and progresses as far as the endothelium, forming an endotheliochorial placenta, whereas the remainder of the BYS trophoblast is non-invasive.

In this chapter an ultrastructural description of the placenta of the dunnart is presented. First some structural details on the trophoblast of pre-implantation embryos are described in an attempt to gain some insight into how embryos might derive nutrients prior to the development of the placenta.

7.2 Materials and Methods

Tissues from the same pregnant fat-tailed dunnarts at the range of gestational ages as given in chapter 6 (Table 6.1) were used. They were fixed and processed for routine transmission electron microscopy (TEM) using the methods described on page 45 of this thesis. Thick plastic sections (0.5μm) were cut and stained with toluidine blue and ultrathin sections were cut, stained and viewed as described on page 46.

7.3 Results

Table 6.1 (chapter 6) shows the developmental stages of fat-tailed dunnart embryos obtained for this study.

7.3.1 Morphological Features of Pre-implantation Embryos

During the course of this study extensive yolk material was only evident in oocytes, zygotes, and, after the first cleavage division, between the blastomeres within the perivitelline space at one pole of the embryo (see also Breed and Leigh, 1990).

In the 4-cell embryos, recovered from a female sacrificed on day 4 *pc*, coated pits were seen along the plasmalemma adjacent to the shell membrane of blastomeres (Fig. 7.1). The numerous mitochondria were electron-dense with lamellar cristae and patches of electron-dense granular material, vacuoles and lipid droplets were also seen.

In unilaminar blastocysts (750μm in diameter) the squamous protoderm had coated pits on the plasma membrane adjacent to the blastocoelic cavity (Fig. 7.2) as well as on the apical plasmalemma adjacent to the remnants of the mucoid coat. Coated vesicles were also seen in the apical cytoplasm of protoderm (Fig. 7.3). The mitochondria of these cells had become less electron-dense than in cleaving embryos and the cristae were more irregular. Occasionally electron-lucent and electron-dense lipid droplets were found in protoderm adjacent to the blastocoelic cavity which appeared to be undergoing transport to the blastocoelic fluid (Figs. 7.3 and 7.4).

In bilaminar blastocysts (1.8mm in diameter) trophectoderm cells also had coated pits on their apical plasma membrane and coated vesicles just below it (Fig. 7.5). The cytoplasm of these cells was extremely electron-lucent and contained a few electron-dense mitochondria with tubular cristae. Occasional cisternae of rough endoplasmic reticulum and lysosomes were also found (Fig. 7.5).

Trophoblast of primitive streak embryos had long but sparse microvilli along the apical plasma membrane (see Fig. 6.6). Coated pits and coated vesicles which were larger than in younger embryos were seen on the apical and lateral cell membranes of these cells (Fig. 7.6). A portion of the lateral cell membranes was exposed to the space between trophoblast and the shell membrane which had previously been occupied by the mucoid coat. Large irregular electron-dense inclusions were a prominent feature of these cells.

Figure 7.1 Blastomere of a 4-cell embryo has coated pits (arrowhead) on its apical plasma membrane. There are numerous electron-dense mitochondria with lamellar cristae (arrows), apical vacuoles and lipid droplets (Li). Bar = 1μm.

Figures 7.2 -7.4 Unilaminar Blastocysts.

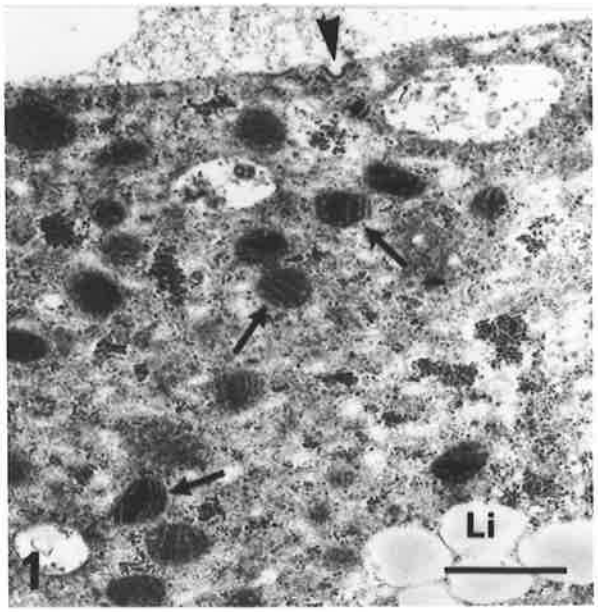
Figure 7.2 Protoderm has coated pits (arrowhead) on the plasmamembrane adjacent to the blastocoelic cavity as well as coated vesicles just below the cell membrane adjacent to remnants of the mucoid coat (MC); shell membrane (SM). Bar = 500nm.

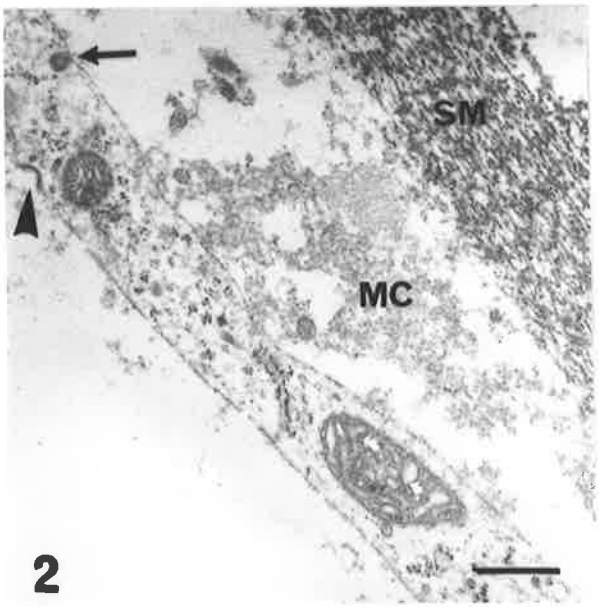
Figure 7.3 Protoderm has coated vesicles (arrows) in the apical cytoplasm. Mitochondria are less electron-dense than in cleaving embryos and lipid droplets (Li) are seen distending the cell membrane adjacent to the blastocoelic cavity (BC) and appear to be about to be shed into the blastocoelic fluid. Bar = 500nm.

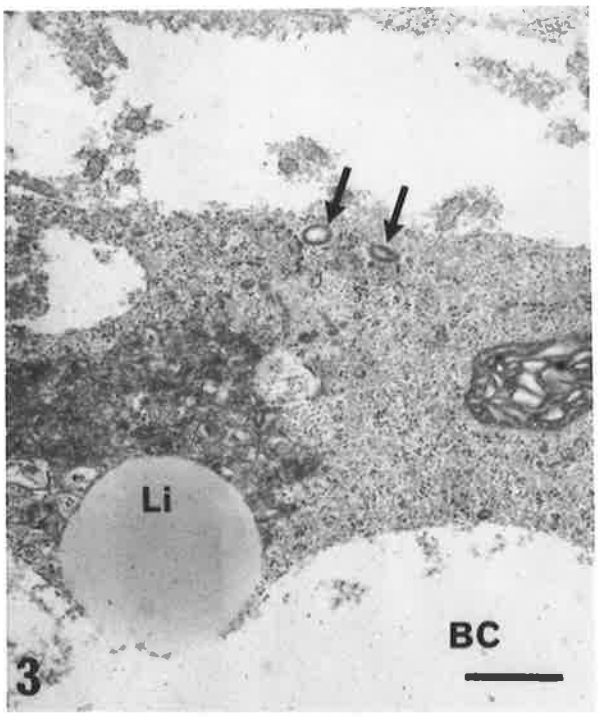
Figure 7.4 Lipid droplets (Li) are sometimes electron-dense and also appear to be in the process of transport to the blastocoelic cavity (BC). Bar = 500nm.

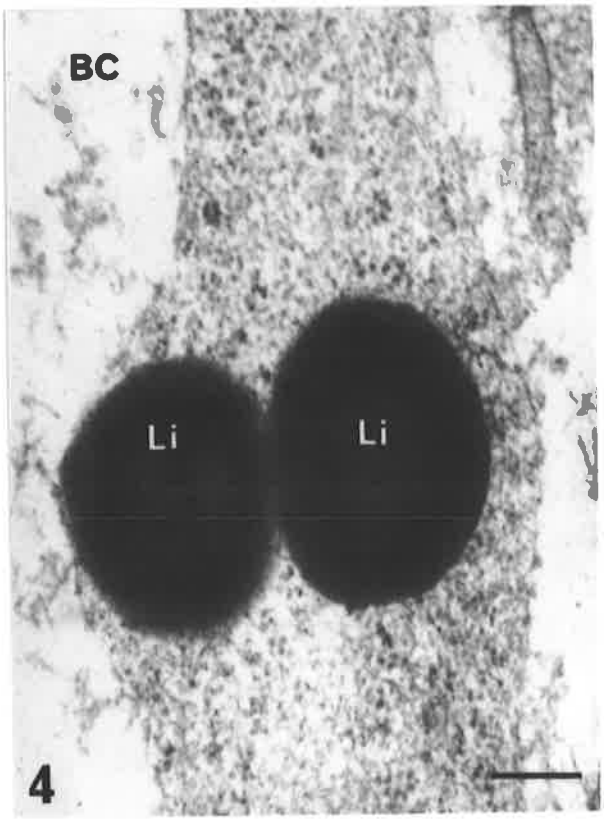
Figure 7.5 Trophectoderm of bilaminar blastocysts has coated pits on the apical cell membrane (arrowhead) and coated vesicles (arrows) in its apical cytoplasm. Mitochondria are electron-dense with tubular cristae (M) and occasional lysosomes (Ly) and rough endoplasmic reticulum (RER) are present. The nucleus (N) is euchromatic. Bar = 1μm.

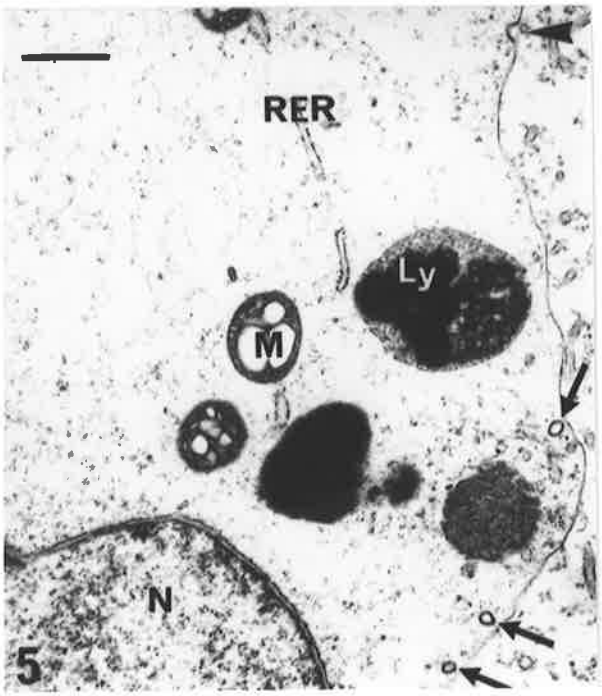
Figure 7.6 Trophoblast of primitive streak embryos has large coated pits on its apical cell membrane (arrowheads), long sparse microvilli, and electron-dense inclusions (I). Bar = 250nm.

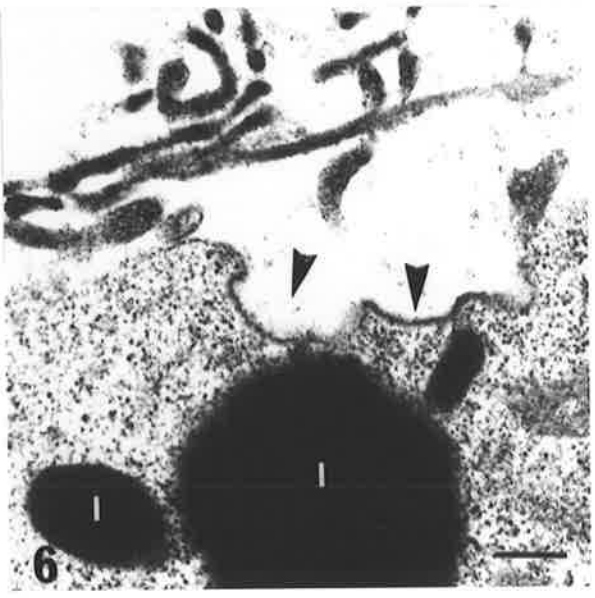

Li
1


SM
MC
2


Li
BC
3


BC
Li
Li
4


RER
Ly
M
N
5


I
I
6

The endometrial glands during preimplantation development of the embryo had a similar ultrastructural appearance to those of non-pregnant females. At the unilaminar blastocyst stage there were numerous gland profiles in each thick section and the secretion in the lumen was relatively pale when stained with toluidine blue (Fig. 7.7). The glands of females which had early somite embryos had changed as compared with earlier stages. Low power electron micrographs showed that many of the glands appeared 'full of holes' (Fig. 7.8) but higher magnification revealed that the holes were dilated golgi complexes with abundant adjacent vesicles (Fig. 7.9) as well as large intercellular spaces, particularly at the bases of the cells (Fig. 7.10). Nuclei of the glandular epithelium were moderately electron-dense with patches of heterochromatin (Fig. 7.10). There was little visible secretion in the gland lumina at this stage.

7.3.2 Sinus Terminalis

The sinus terminalis is a large blood vessel of about 45 by 20μm (Fig. 7.11) which marks the boundary between the trilaminar and bilaminar regions of the yolk sac. It contains the characteristic nucleated erythrocytes of prenatal marsupials. The attachment of the yolk sac to the endometrium in this region was convoluted with profuse interdigitation of fetal and maternal tissues (Fig. 7.11). In thick plastic sections incubated with toluidine blue the maternal epithelium stained strongly, as did the extra-embryonic endoderm, but the intervening trophoblast stained very lightly. There was a little endometrial stroma at the base of the thin fold of endometrium to which the sinus terminalis was attached. Trophoblast markedly invaginated the uterine epithelium on all sides of this fold producing a highly convoluted profile and the endoderm that lined the yolk sac in this region was cuboidal and uninucleate. The apical cytoplasm was vacuolated and the pale-staining nucleus had a prominent nucleolus (Fig. 7.11).

Electron micrographs revealed that uterine epithelial cells in this region were comparatively electron-dense, uninucleate and attenuated. They were squamous, about 1.5-5μm tall and comparatively few nuclei were seen in each section (Fig. 7.12). Nuclei were elongate with abundant euchromatin and had patches of heterochromatin. The cytoplasm contained lipid droplets, numerous mitochondria and abundant rough endoplasmic reticulum. Capillaries were seen immediately below the uterine luminal epithelium in this (Figs. 7.12 and 7.13), and all, regions of the endometrium.

By contrast, trophoblast was extremely electron-lucent, mitochondria were the dominant organelle of the cytoplasm, and occasional small lysosomes were present (Figs. 7.12 and 7.13). Regular trophoblast microvilli closely interdigitated with those

of uterine epithelial cells forming a very intimate attachment (Fig. 7.13). Sections showed that the arrangement of embryonic and maternal cells was very convoluted with small patches of trophoblast microvilli appearing adjacent to stretches of lateral membranes (Fig. 7.12).

Electron micrographs revealed that the endoderm had numerous apical mitochondria which had tubular cristae and a moderately electron-dense matrix. There was also abundant rough endoplasmic reticulum (Fig. 7.14).

7.3.3 Bilaminar Yolk Sac Placenta

Early in the development of the bilaminar yolk sac (BYS) placenta trophoblast surrounded groups of maternal epithelial cells (see Fig. 6.10, chapter 6). Also in one female (embryos 3.0-3.5mm CRL) large multinucleate trophoblast cells were seen that appeared to have two types of nuclei (Fig. 7.15). These trophoblast cells were possibly a little further in development than those seen in figure 5.34 (chapter 5). These trophoblast giant cells (TGCs), although multinucleate, were not syncitial. TGCs had begun to surround the endometrial capillaries (Fig. 7.16). Nuclei of trophoblast origin had abundant euchromatin, a little heterochromatin, a prominent nucleolus and had a very irregular outline while the paler nuclei, which may have been maternal in origin, had a more regular curved profile and were euchromatic (Fig. 7.17) There were many mitochondria with tubular cristae and large amounts of rough endoplasmic reticulum in the apical cytoplasm (Fig. 7.17).

Occasionally, on the last day of pregnancy, groups of uterine epithelial cells were still found surrounded by trophoblast in some parts of the BYS but trophoblast had migrated into the stroma around and beyond the endometrial capillaries (Fig. 7.18). Figures 7.18-7.25 come from a similar region of the BYS seen in figures 6.10-6.12 (chapter 6). Huge multinucleate TGCs maintained contact with the extra-embryonic endoderm, surrounded maternal epithelial cells (Figs. 7.19 and 7.20), and dominated the endometrial stroma (Fig. 7.18). Many of the surrounded epithelial cells appeared healthy although some were clearly degenerate (Fig. 7.20). Trophoblast did not appear to have phagocytosed these cells as their microvilli remained intact (Figs. 7.20 and 7.21). TGCs in the stroma appeared extremely vacuolated at low magnification (Fig. 7.22). Nuclei had abundant euchromatin with patches of heterochromatin and a prominent nucleolus. In some regions trophoblast did not surround the maternal stromal endothelial cells closely (Fig. 7.22).

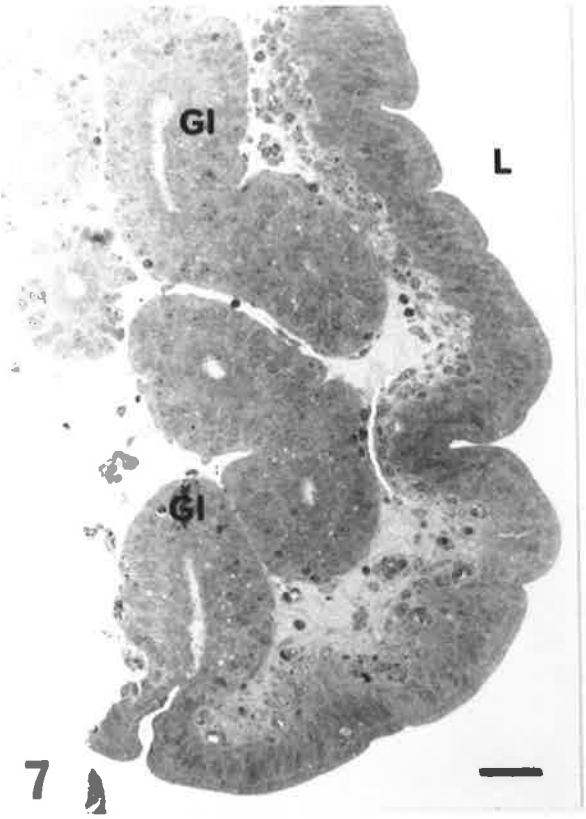
At higher magnification it was clear that there was no intervening cell membrane between the trophoblast nuclei, and the cytoplasm was full of vesicles of endoplasmic

Figure 7.7 Endometrium at unilaminar blastocyst stage. The luminal epithelium has a relatively smooth contour, there are many gland profiles present and the secretion in the gland (Gl) lumina does not stain strongly. Bar = 10μm. Tol. Blue.

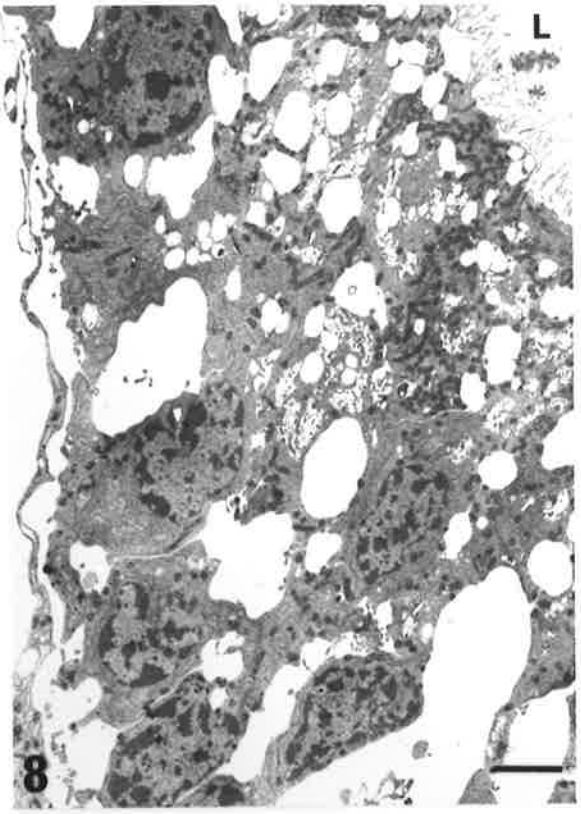
Figure 7.8 Part of a gland at the early somite stage appears 'full of holes'; lumen (L). Bar = 250nm.

Figure 7.9 Higher magnification of part of Fig. 7.8 shows that cisternae of the golgi complex (G) at this stage are widely dilated. Bar = 250nm.

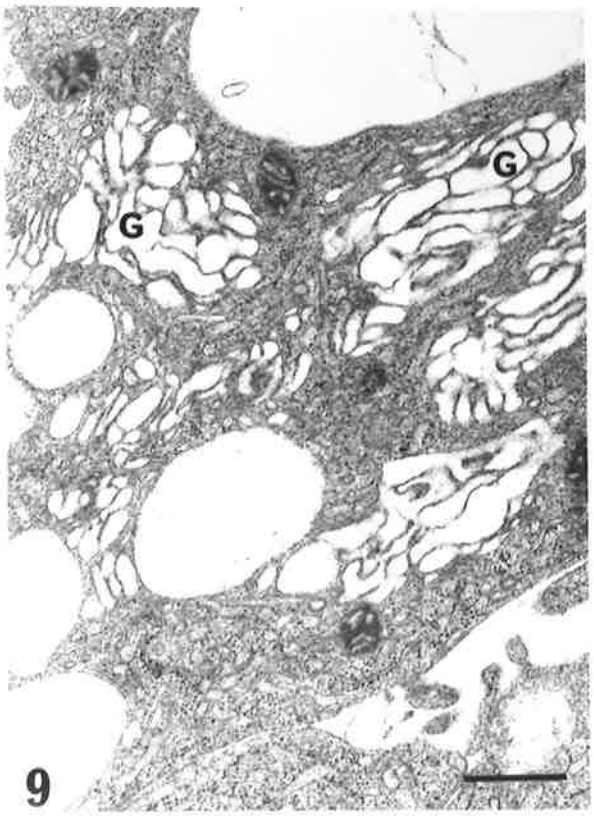
Figure 7.10 Higher magnification of basal part of gland shown in Fig. 7.8 shows that there are large intercellular spaces (IS) in the glandular epithelium. Bar = 2μm.



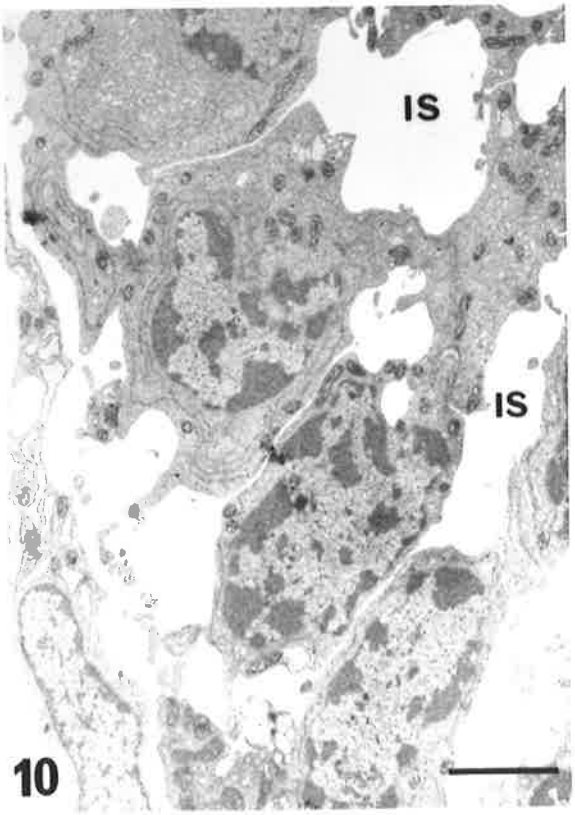
7



8

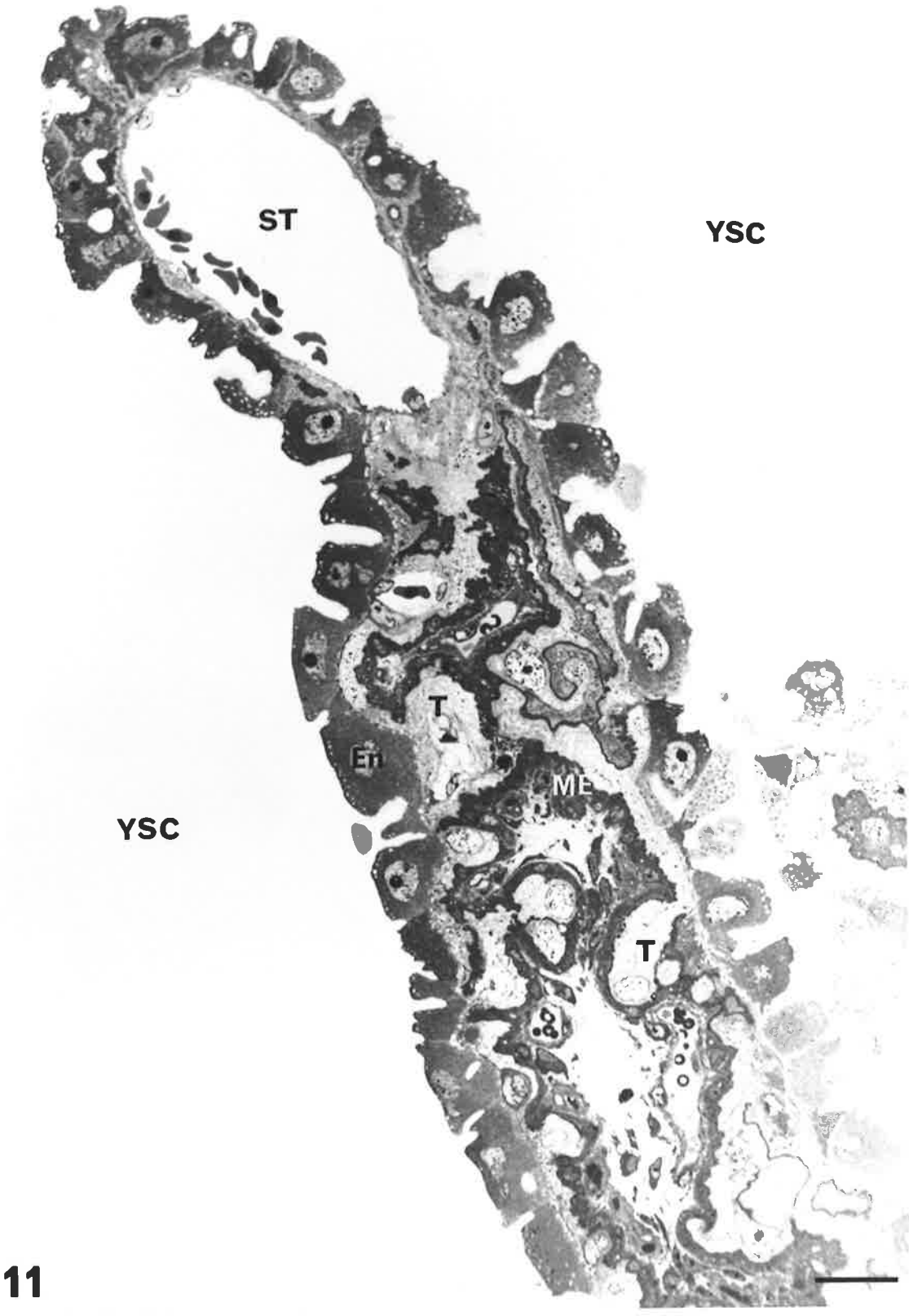


9



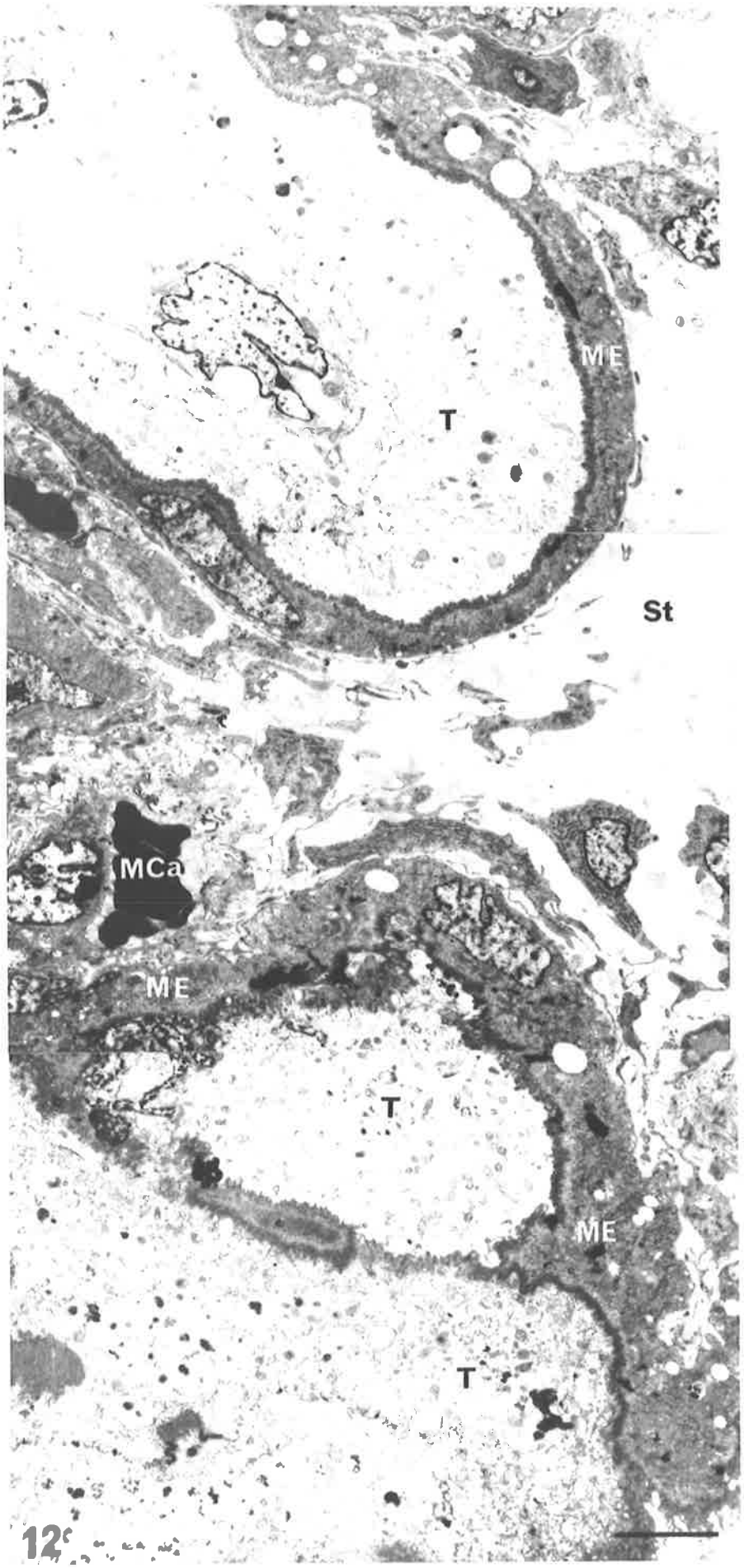
10

Figure 7.11 Thick plastic section of the sinus terminalis (ST) late in pregnancy shows that attachment in this region is highly convoluted. Trophoblast (T) invaginates the maternal epithelium (ME) and the endoderm (En) is cuboidal. The whole structure protrudes into the yolk sac cavity (YSC). Bar = 10μm. Tol. Blue.



11

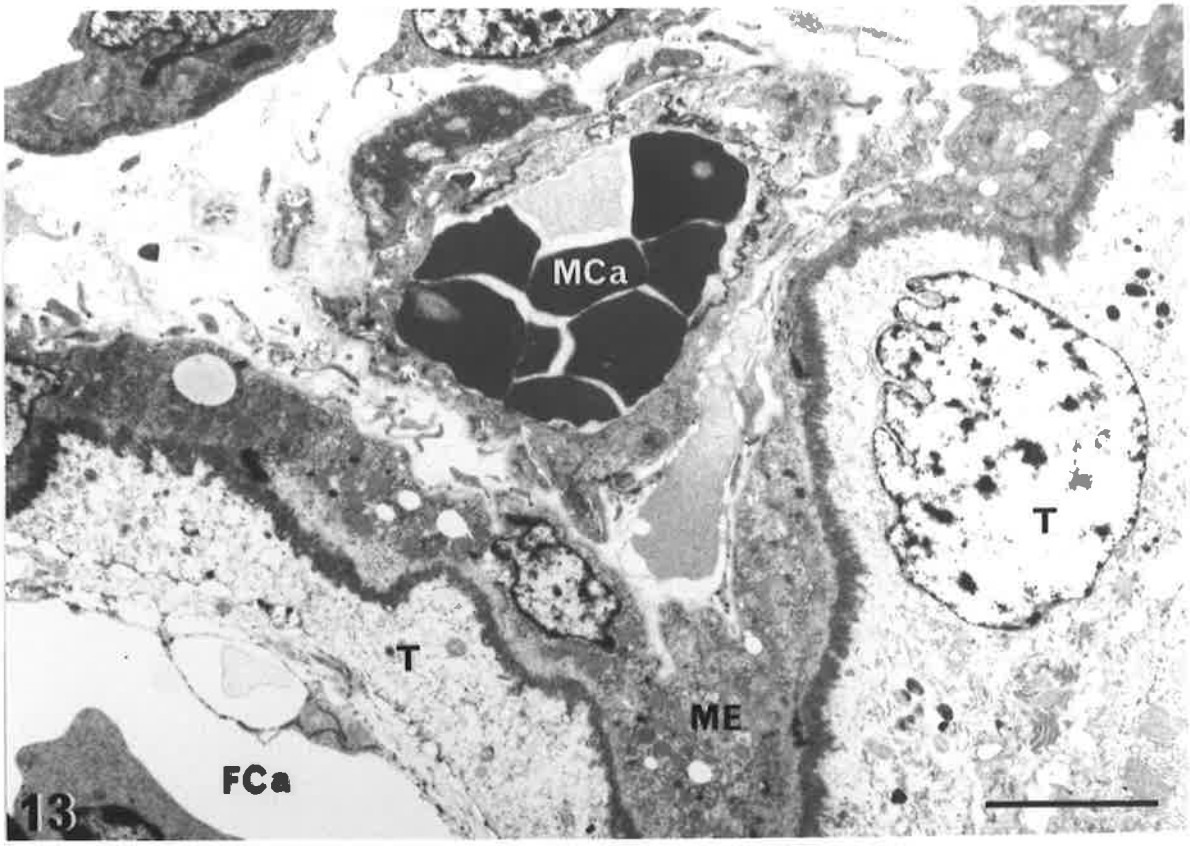
Figure 7.12 Electron micrograph of the feto-maternal relationship immediately below the sinus terminalis shows that trophoblast (T) is electron-lucent in contrast to the more electron-dense maternal epithelium (ME) which is attenuated and squamous, with a few, predominantly euchromatic, nuclei. The maternal capillaries (MCa) are directly below the epithelium in the endometrial stroma (St). Bar = 5μm.

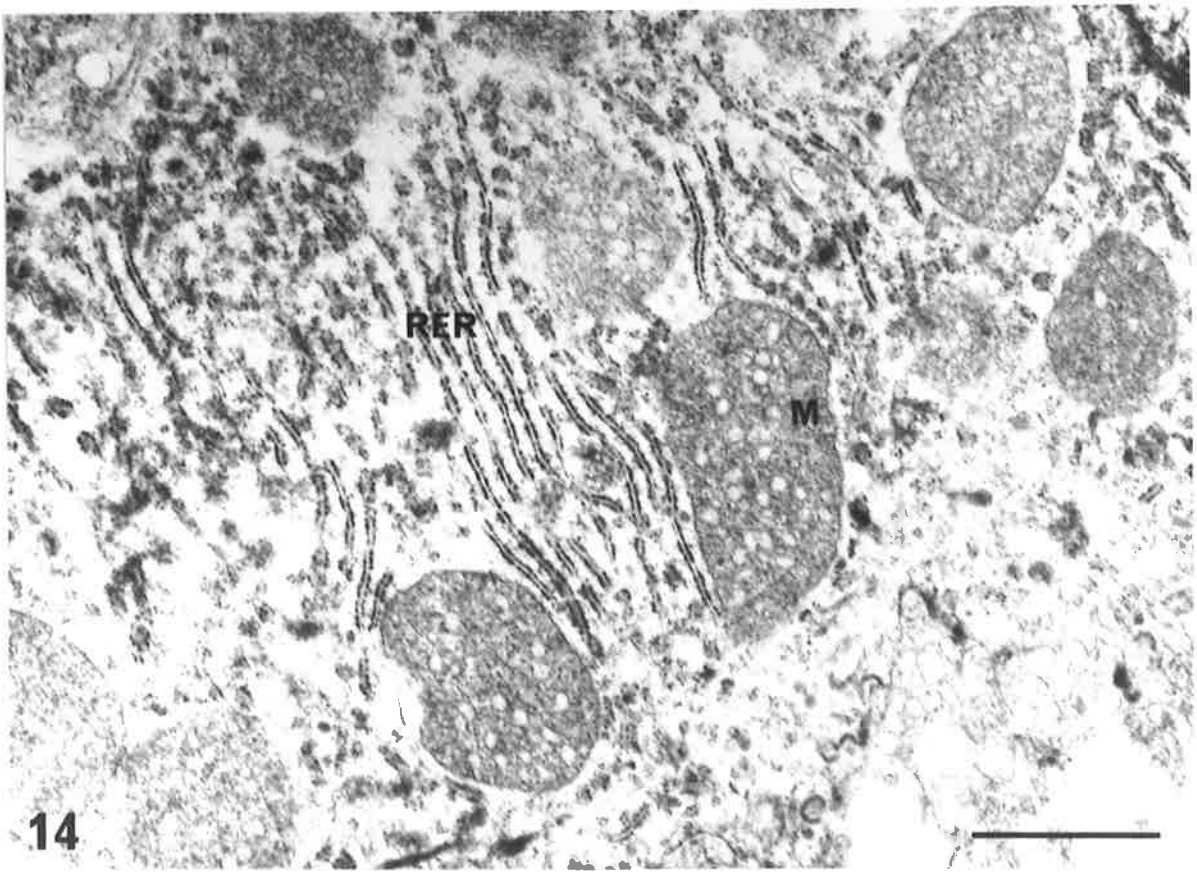


12

Figure 7.13 Higher magnification of a similar region to that in Fig. 7.12 shows that microvilli of trophoblast (T) and maternal epithelium (ME) interdigitate and where fetal (FCa) and maternal capillaries (MCa) are present the intervening cell layers are attenuated. Bar = 5μm.

Figure 7.14 Endoderm which lines the yolk sac cavity in the region of the sinus terminalis has numerous mitochondria (M) with tubular cristae and abundant rough endoplasmic reticulum (RER). Bar = 1μm.


MCa
T
T
ME
FCa
13

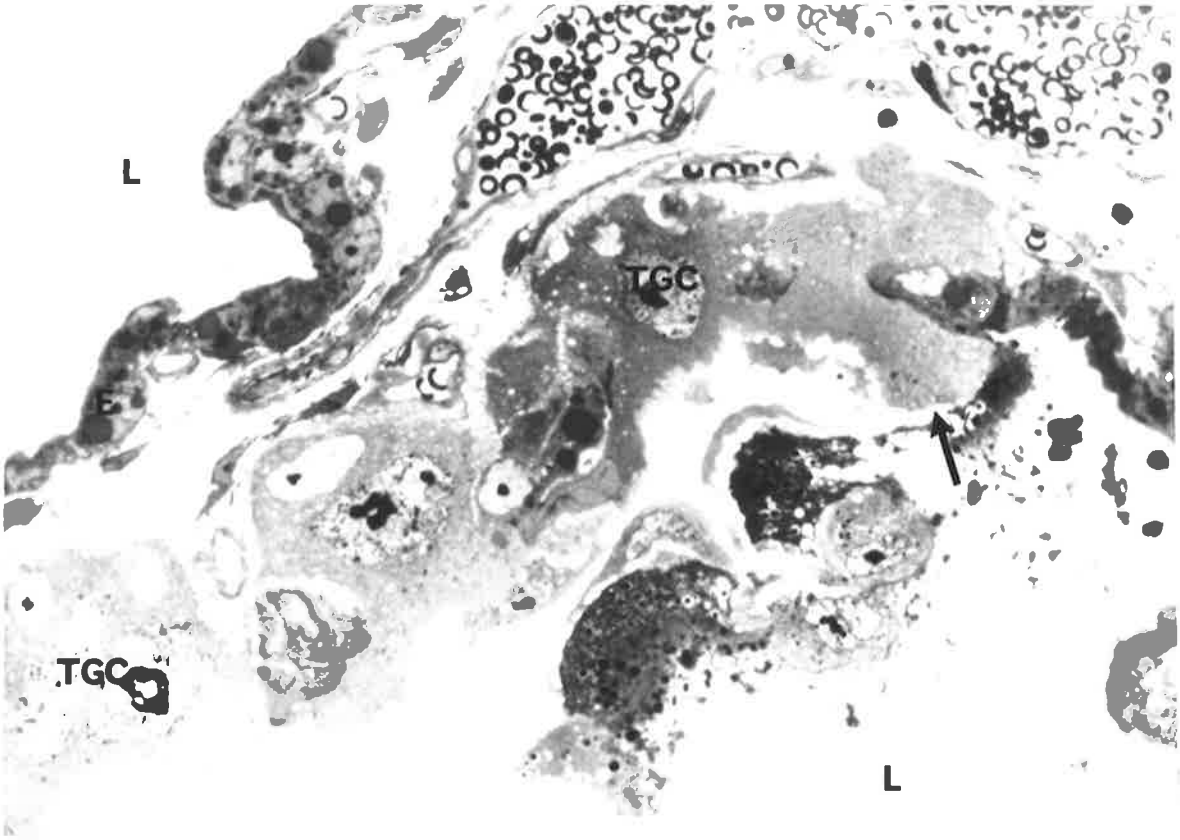

RER
M
14

Figures 7.15-7.17 Bilaminar Yolk Sac Placenta.

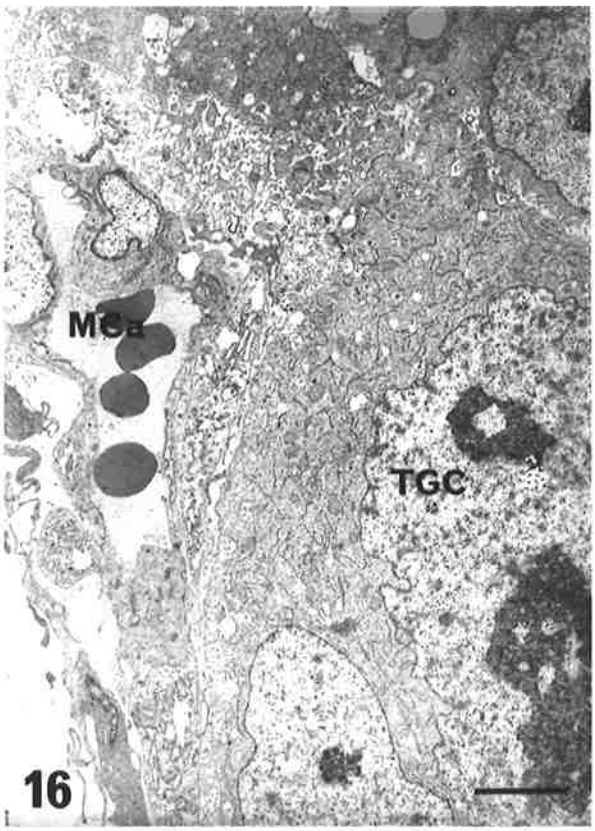
Figure 7.15 Thick plastic section shows that in some regions the BYS (arrow) merges with the uterine epithelium (E) of a thin endometrial fold and where trophoblast has invaded the endometrial stroma large trophoblast giant cells (TGC) have two kinds of nuclei; lumen (L). Bar = 20μm. Tol. Blue.

Figure 7.16 Trophoblast giant cells (TGC) are cellular and multinucleate and are adjacent to maternal capillaries (MCa) at this stage. Bar = 5μm.

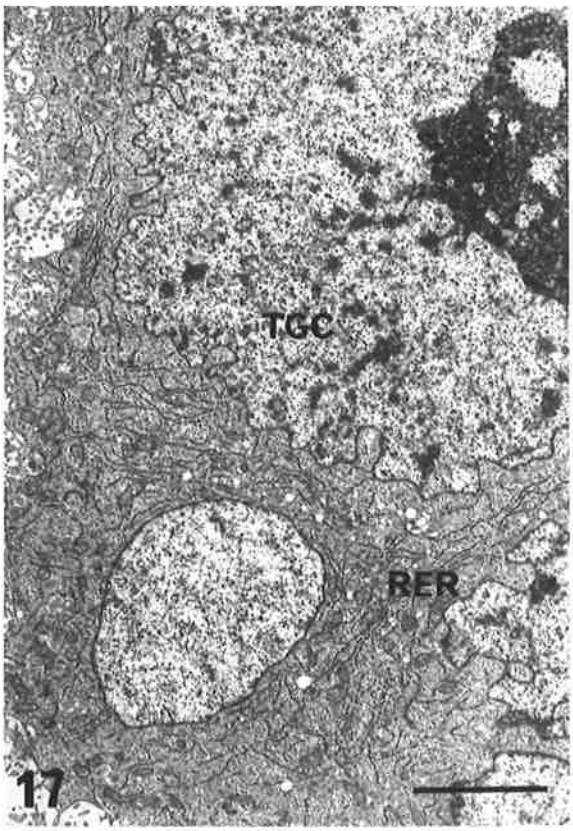
Figure 7.17 Trophoblast giant cells (TGC) adjacent to those seen in Fig. 7.16 have abundant rough endoplasmic reticulum (RER). Trophoblast nuclei have an invaginated nuclear envelope, abundant euchromatin and a prominent nucleolus. Bar = 5μm.



15



16



17

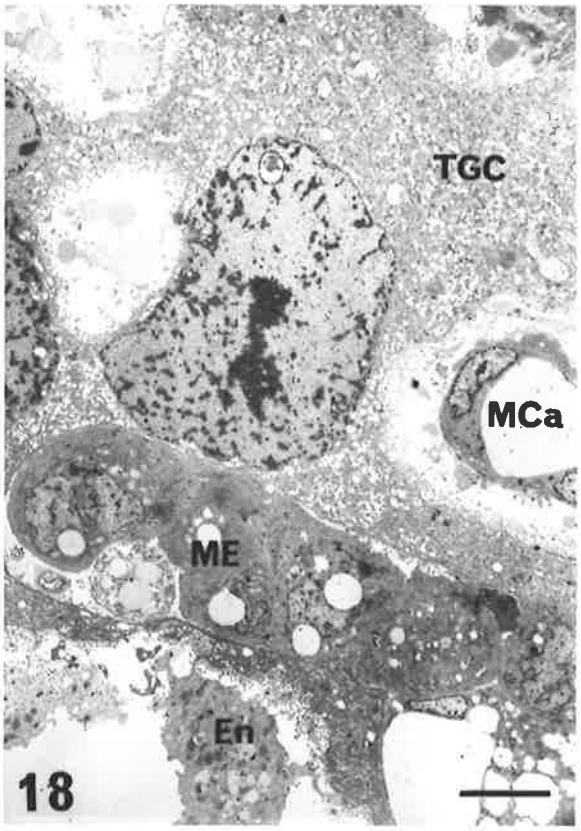
Figures 7.18-7.21 Bilaminar Yolk Sac Placenta.

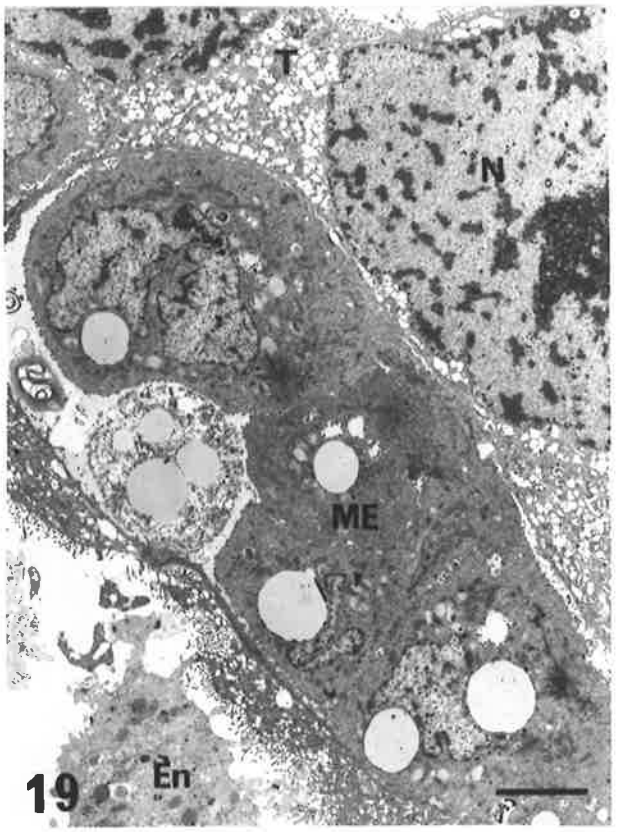
Figure 7.18 Electron micrograph of similar region of the BYS to that seen in Fig. 6.10 shows that trophoblast giant cells (TGC) surround maternal epithelial cells (ME) and have invaded the endometrial stroma and surrounded maternal capillaries (MCa). The endoderm (En) is below. Bar = 10μm.

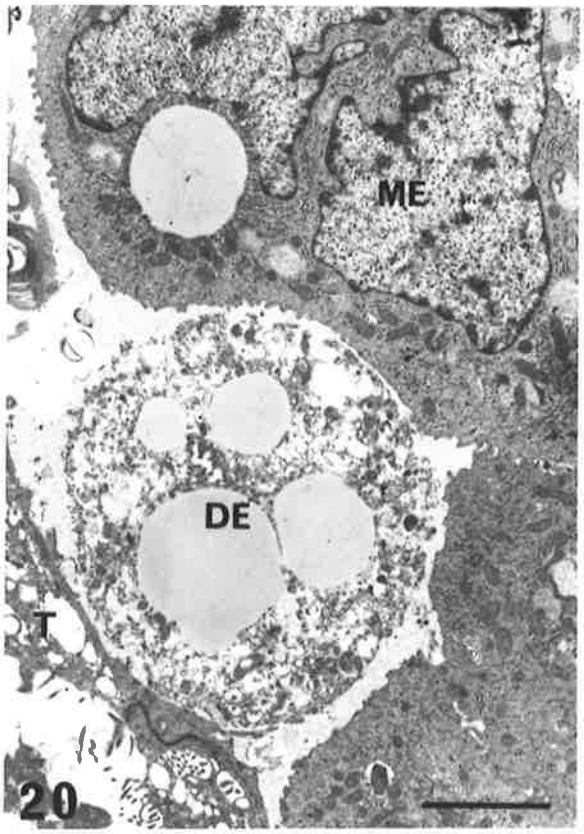
Figure 7.19 Slightly higher magnification of Fig. 7.18 shows that trophoblast (T) surrounds the maternal epithelium (ME) and still maintains contact with the endoderm (En); trophoblast nucleus (N). Bar = 5μm.

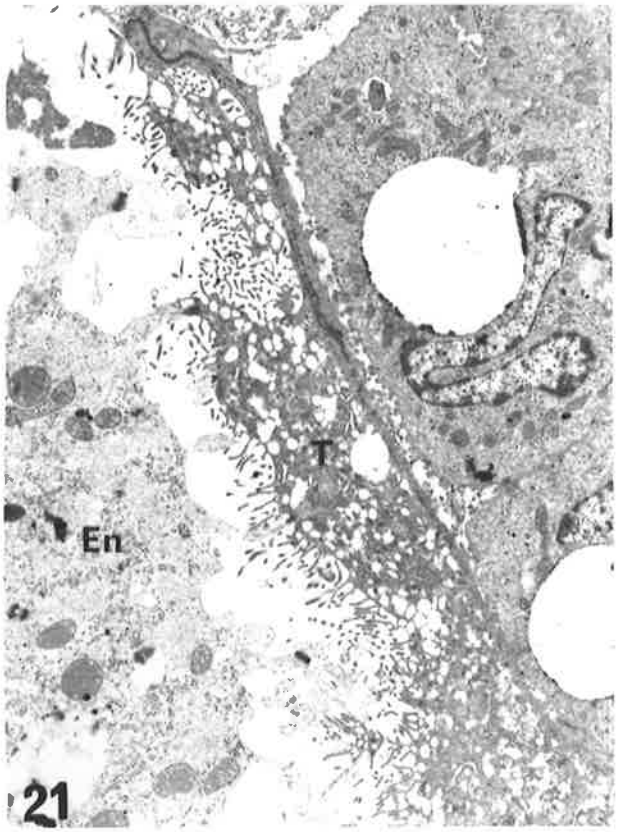
Figure 7.20 Higher magnification of Fig. 7.19 shows that some maternal epithelial cells (ME) apparently remain healthy but some are clearly degenerate (DE). Bar = 2.5μm.

Figure 7.21 Trophoblast (T) has surrounded but does not appear to have phagocytosed maternal epithelial cells as there is a space between the maternal cell membranes and trophoblast, and maternal microvilli remain intact. Trophoblast also remains in contact with the endoderm (En). Bar = 2μm.


TGC
MCa
ME
En
18


T
N
ME
En
19


ME
DE
T
20


T
En
21

reticulum, some of which had ribosomes attached (Fig. 7.23). Mitochondria had thin lamellar cristae which had a similar appearance to those seen in trophoblast at early epithelial penetration by early somite embryos (see Figs. 5.15-5.19). In a few places bundles of fibrous material were completely surrounded by trophoblast in which both smooth and rough endoplasmic reticulae were found (Fig. 7.24). Higher magnification showed that these fibres were striated with a periodicity of approximately 65nm and therefore were probably collagen fibrils (Fig. 7.25).

In some regions of the BYS trophoblast made an even more intimate contact with the endometrial capillaries, closely surrounding them as well as nearby, apparently healthy, glands (Fig. 7.26). Occasionally trophoblast surrounded glands that appeared to be degenerating. The folded trophoblast cell membrane interdigitated with folds of the endothelial plasmalemma such that only a thin layer of moderately electron-dense material, the endothelial basal lamina, could be seen between the two (Fig. 7.27).

Trophoblast giant cells adjacent, but below, the intact epithelium distal to the invaded epithelial surface of an endometrial fold which formed the maternal side of the placenta (see Fig. 5.15) approached, but did not contact, the basal lamina of the uterine epithelium (Fig. 7.28). In some sections the uterine luminal epithelium had a very distinct basal lamina and a folded basal cell membrane (Fig. 7.29), although the latter was not as marked as that at early somite stages (see Fig. 5.9).

In some regions of the BYS late in pregnancy trophoblast appeared to have completely surrounded many endometrial stromal cells (Fig. 7.30). Below the intact uterine epithelium the stroma appeared to be almost entirely composed of trophoblast (Fig. 7.31). These TGCs contained a variety of inclusions, many of which were lysosomes which had the appearance of degenerating cellular remnants (Fig. 7.32). In other sections remnants of, presumably, phagocytosed cells or portions of cells, were found in a TGC near to the plasma membrane (Fig. 7.33). In the adjacent extracellular matrix part of a degenerating cell, which may have been a leukocyte, was seen. Coated pits were also found on the cell membrane of the TGC (Fig. 7.33).

Sometimes, rather unusual stromal blood vessels which appeared partly occluded were found closely surrounded by trophoblast but they had not apparently been phagocytosed (Fig. 7.34). Higher magnification revealed that the endothelial basal lamina was markedly, but irregularly thickened measuring between 150 and 750nm thick (Fig. 7.35).

In some parts of the BYS, particularly below intact epithelium, fibroblasts with dilated rough endoplasmic reticulum were present and abundant bundles of collagen fibrils were found in the adjacent extra-cellular matrix (Fig. 7.36). Maternal neutrophils were found occasionally adjacent to trophoblast and fibroblasts (Fig. 7.37).

At the periphery of this annular region of attachment a non-invasive BYS closely followed the contours of the uterine epithelium (Fig. 7.38). However, even on the last day of pregnancy, occasional trophoblast cells had penetrated the uterine epithelium (Fig. 7.39) and extended into the stroma below the non-invasive region above it (Fig. 7.40). Thick plastic sections showed that the cytoplasmic features of invasive trophoblast were quite different than those of non-invasive trophoblast (Fig. 7.40). Toluidine blue stained the latter strongly while the former were much paler. In electron micrographs the invasive TGCs had similar features to those described earlier (see Figs. 7.16 and 7.17) while non-invasive trophoblast was more electron-dense than the uterine luminal epithelium with large numbers of vesicles with heterogeneous electron-density (Fig. 7.41). Microvilli were long and there were intercellular junctional complexes.

7.3.4 Unattached Bilaminar Yolk Sac

The morphology of trophoblast in the unattached region of the bilaminar yolk sac was similar to that in the non-invasive region (Fig. 7.42). It had a large number of granules with varying electron-density in the cytoplasm and long microvilli. Coated pits were found on the apical plasma membrane (Fig. 7.43). Between neighbouring trophoblast cells, which were uninucleate, there was a distinct intercellular space below an apical junction (Fig. 7.43). Higher magnification showed that the intercellular junction was similar in appearance to an adhering junction (Fig. 7.44). Trophoblast was attached to the underlying endoderm by a series of desmosomes (Fig. 7.45).

7.3.5 Trilaminar Yolk Sac Placenta

In the trilaminar yolk sac placenta thick plastic sections showed a convoluted attachment between trophoblast and uterine epithelium. Fetal and maternal capillaries could be seen adjacent to each other with attenuated intervening cell layers (Fig. 7.46). Electron micrographs revealed that the distance for diffusion between maternal and fetal blood was very small (about 4μm) because of extreme attenuation of the six cell layers present between the two (Figs. 7.47 and 7.48). Consequently, in this region the placenta is epitheliochorial. In some sections nuclei of all cell layers were not apparent between the two bloodstreams (Fig. 7.48). Higher magnification showed that the microvilli of trophoblast interdigitated with those of the uterine epithelium and that

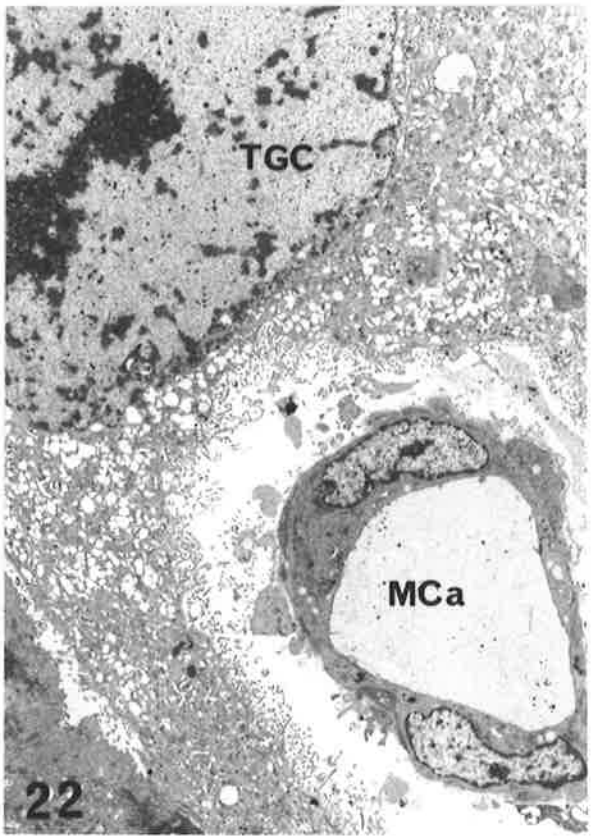
Figures 7.22-7.25 Bilaminar Yolk Sac Placenta.

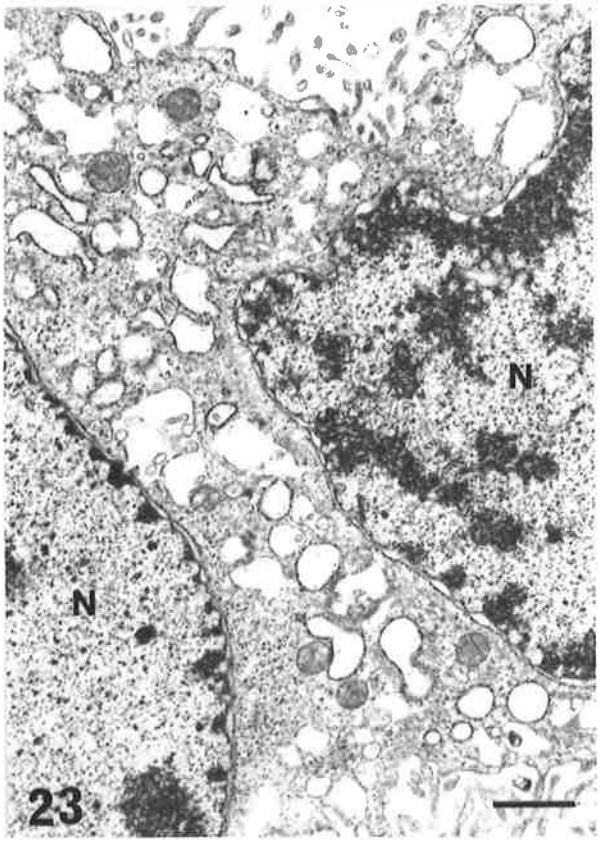
Figure 7.22 Higher magnification of Fig. 7.18 shows that a trophoblast giant cell (TGC) surrounds a maternal capillary (MCa) but there is a large space between trophoblast and the endothelium. Bar = 5μm.

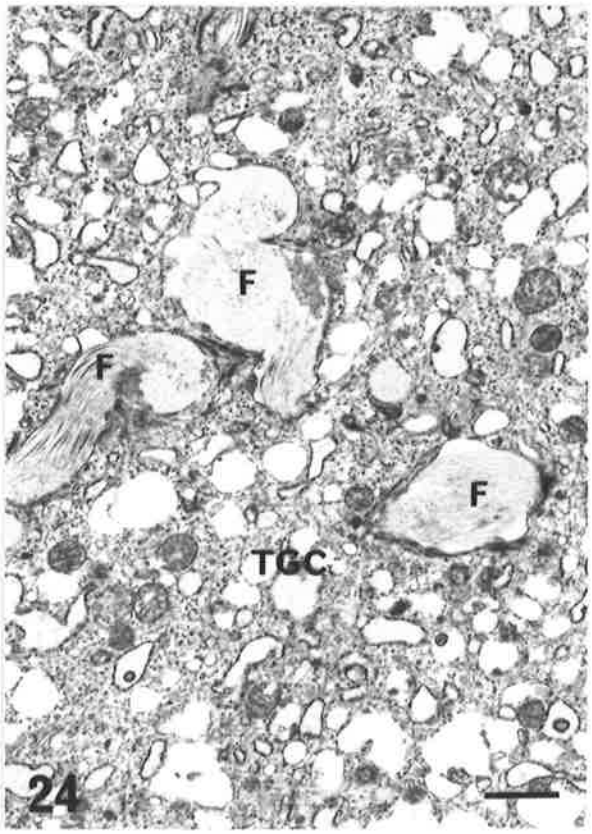
Figure 7.23 Higher magnification shows that there is no intervening cell membrane between nuclei of TGCs in the endometrial stroma. Thus they are multinucleate, possibly syncytial. Dilated vesicles of endoplasmic reticulum, some of which have ribosomes attached, dominate the cytoplasm. Nuclei (N) are predominantly euchromatic with patches of heterochromatin. Bar = 1μm.

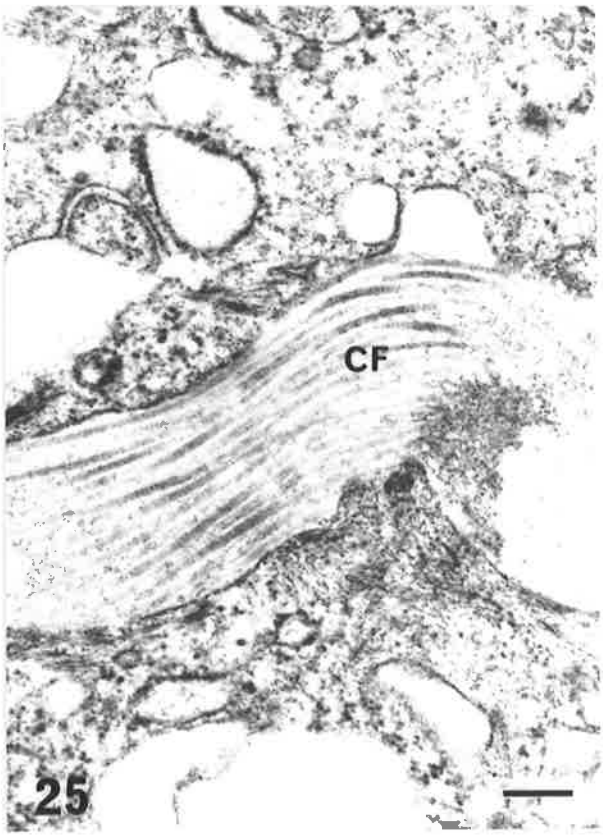
Figure 7.24 Cytoplasm of a trophoblast giant cell shows that there are bundles of fibrous material (F) which trophoblast appears to have engulfed. Bar = 1μm.

Figure 7.25 Higher magnification of the fibrous material in Fig. 7.24 shows that the fibrils are striated and are probably collagen fibrils (CF) with similar appearance to collagen in the endometrial stroma. Bar = 250nm.


TGC
MCa
22


N
N
23


F
F
F
TGC
24


CF
25

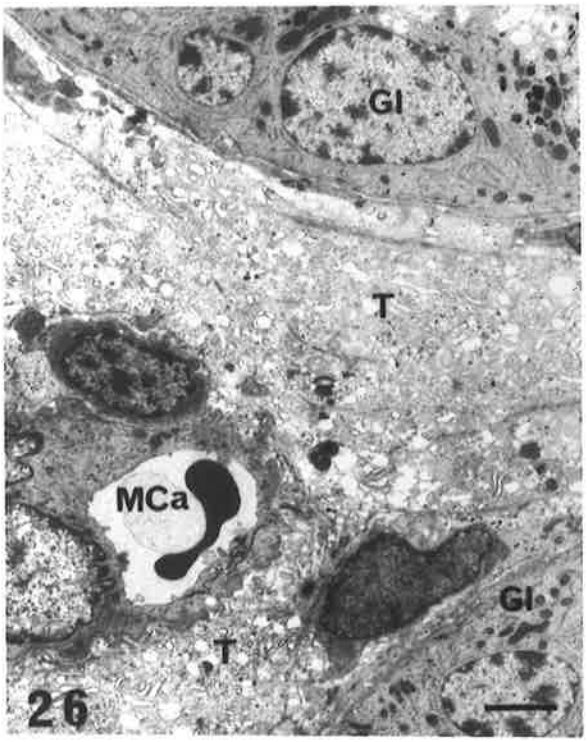
Figures 7.26-7.29 Bilaminar Yolk Sac Placenta.

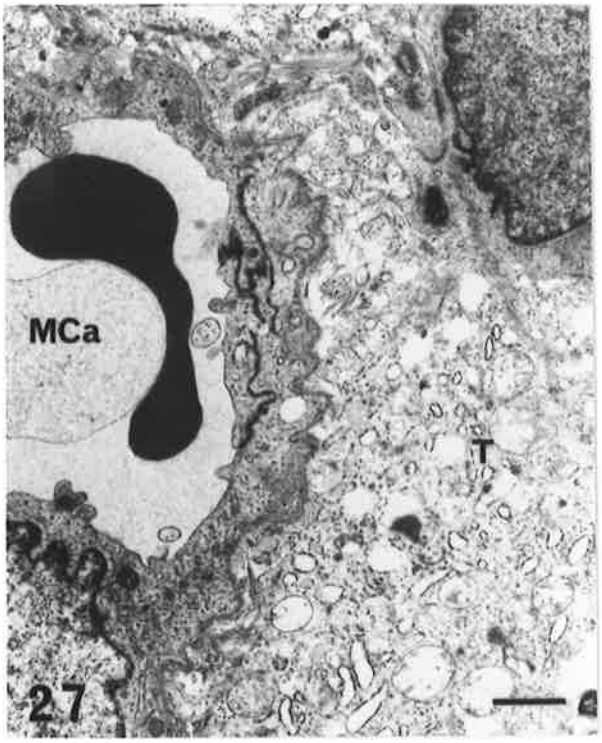
Figure 7.26 In this region trophoblast has surrounded a maternal capillary (MCa) and endometrial glands (Gl) more intimately than seen in previous micrographs. Bar = 2.5μm.

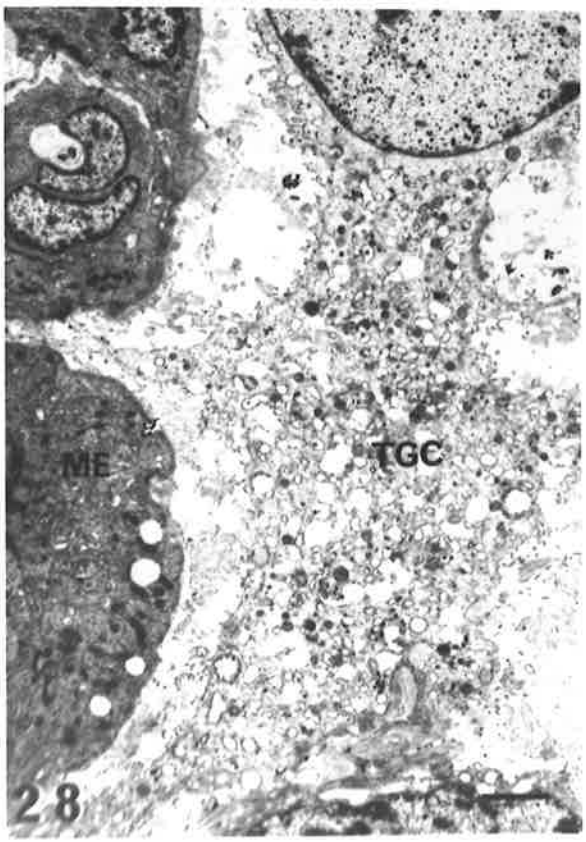
Figure 7.27 Higher magnification of Fig. 7.26 shows that trophoblast (T) approaches the endothelium of the maternal capillary (MCa) and is often in contact with the endothelial basal lamina. Bar = 1μm.

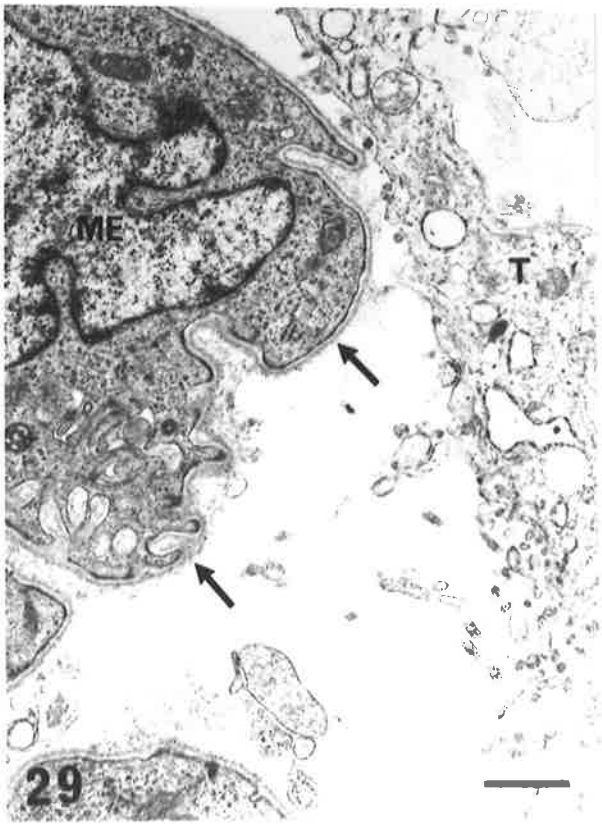
Figure 7.28 A trophoblast giant cell approaches the maternal epithelium (ME) on the other side of an endometrial fold in which it dominates the stroma. Bar = 2.5μm.

Figure 7.29 A similar region to that of Fig. 7.28 shows that the basal lamina of the maternal epithelium is prominent and the basal and lateral cell membranes are folded; trophoblast (T). Bar = 1μm.


Gl
T
MCa
Gl
T
26


MCa
T
27


ME
TGC
28


ME
T
29

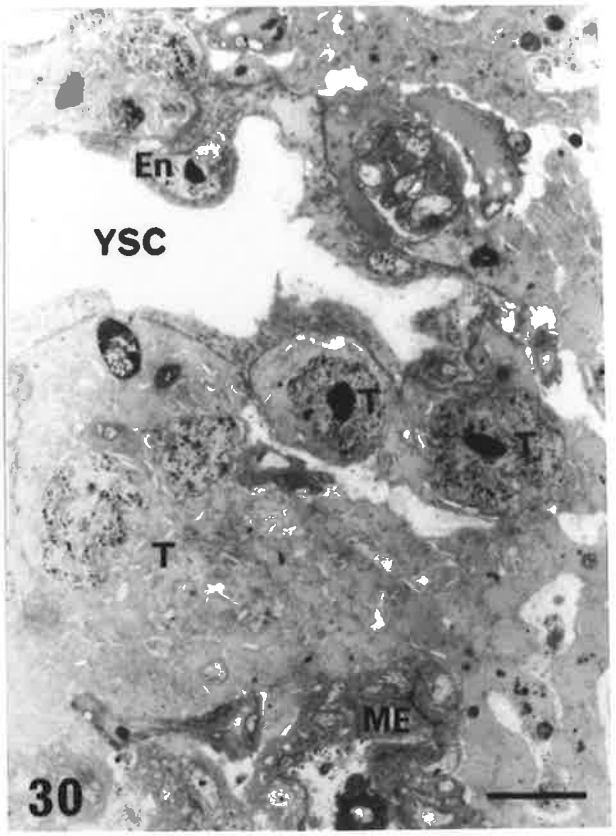
Figures 7.30-7.33 Bilaminar Yolk Sac Placenta.

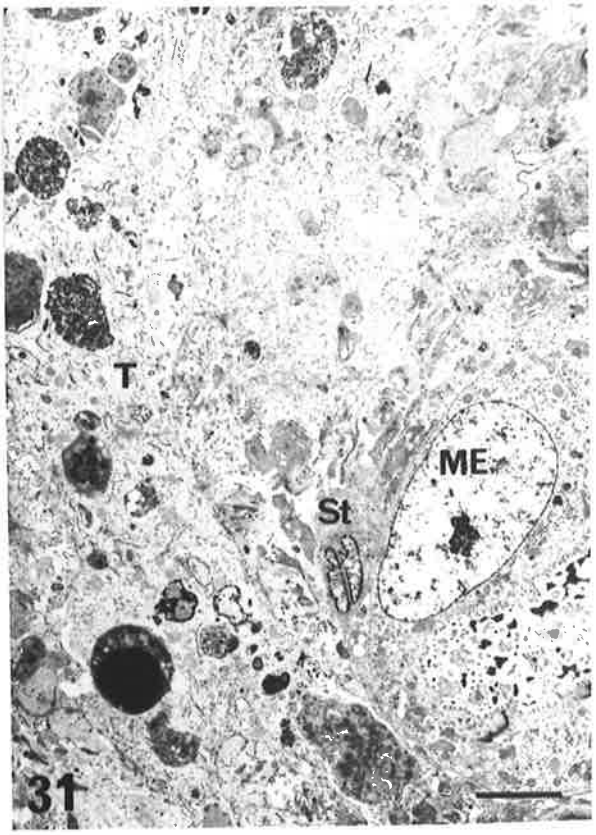
Figure 7.30 Thick plastic section shows that trophoblast (T) of the BYS dominates the endometrial stroma; maternal epithelium, endoderm (En), yolk sac cavity (YSC). Bar = 25μm. Tol. Blue.

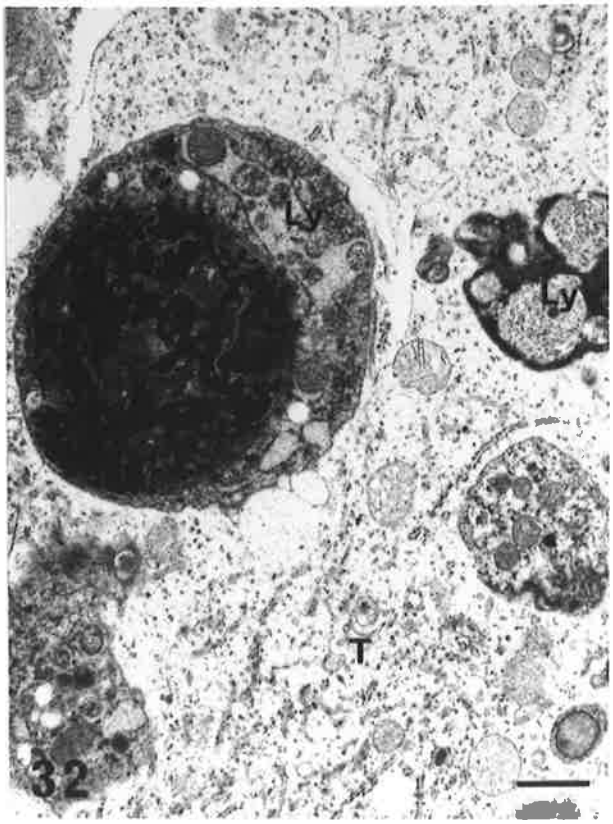
Figure 7.31 Electron micrograph of a similar region of the endometrial stroma shown in Fig. 7.30 shows that trophoblast (T) appears to have phagocytosed stromal cells; stroma (St). Bar = 5μm.

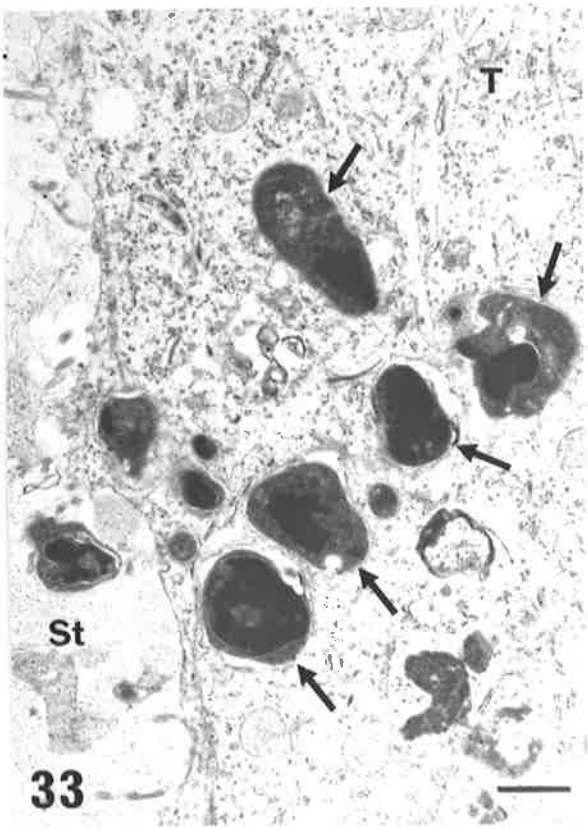
Figure 7.32 Higher magnification of part of trophoblast in Fig. 7.31 shows lysosomes (Ly) in trophoblast (T) cytoplasm which have the appearance of cellular remains. Bar = 1μm.

Figure 7.33 Trophoblast (T) near to that seen in Fig. 7.32 also appears to have phagocytosed maternal leukocytes (arrows) which may be in the initial stages of breakdown by lysosomal enzymes. Another degenarate cell is in the fibrous stroma (St) adjacent. Bar = 1μm.


En
YSC
T
T
T
ME
30


T
ME
St
31


Ly
Ly
T
32


T
St
33

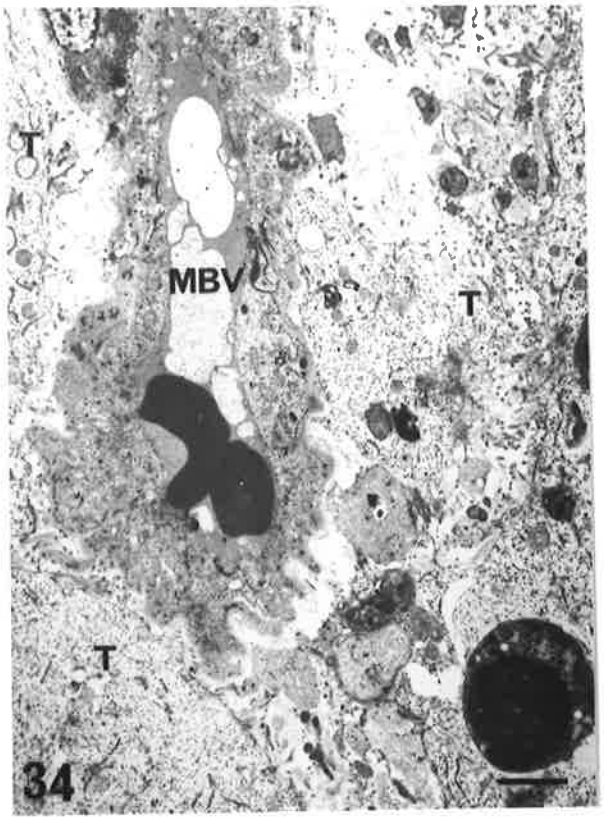
Figures 7.34-7.37 Bilaminar Yolk Sac Placenta.

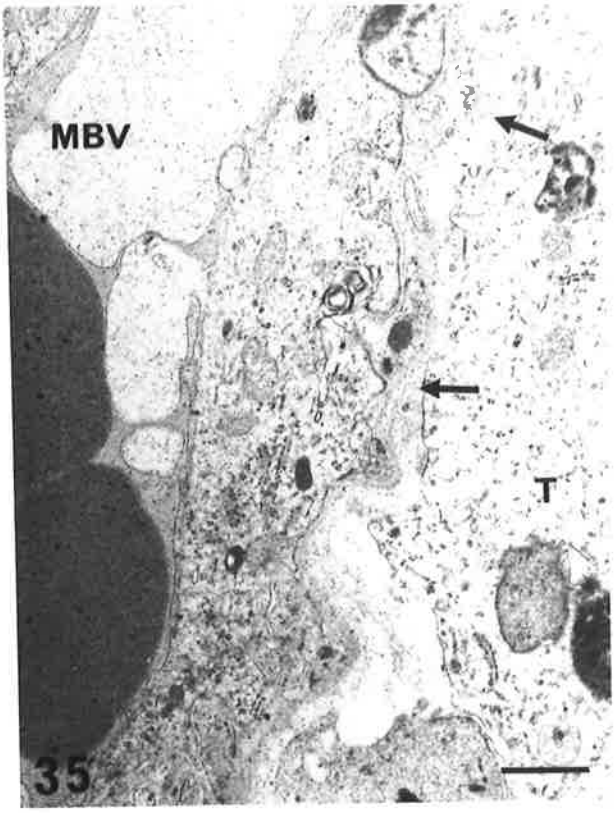
Figure 7.34 Stroma adjacent to that seen in Fig. 31 shows that trophoblast (T) also surrounds an odd looking maternal blood vessel (MBV). Bar = 2.5μm.

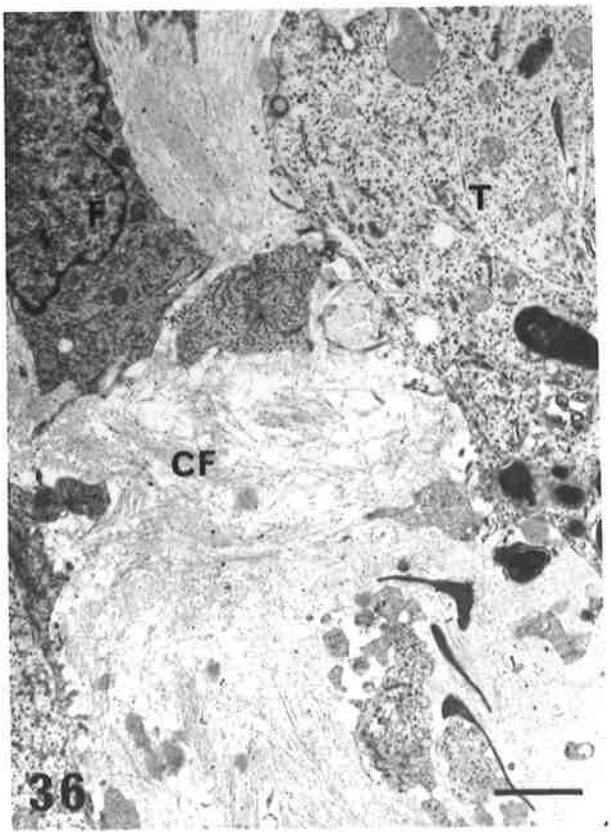
Figure 7.35 Higher magnification of Fig. 7.34 shows that the maternal endothelial basal lamina is markedly thickened where trophoblast (T) approaches (arrows); maternal blood vessel (MBV). Bar = 1μm.

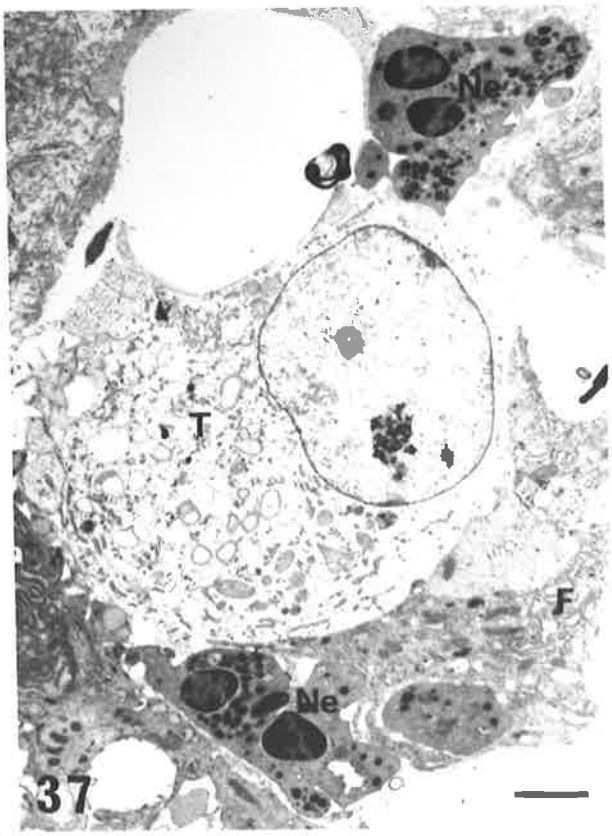
Figure 7.36 Below intact maternal epithelium maternal fibroblasts (F) are present and have secreted abundant collagen fibrils (CF) into the stroma, some of which are adjacent to trophoblast (T). Bar = 2μm.

Figure 7.37 Occasional isolated trophoblast cells (T) in the stroma are seen alongside maternal neutrophils (Ne) and fibroblasts (F) in which the rough endoplasmic reticulum is dilated. Bar = 2.5μm.


T
MBV
T
T
34


MBV
T
35


F
T
CF
36


Ne
T
F
Ne
37

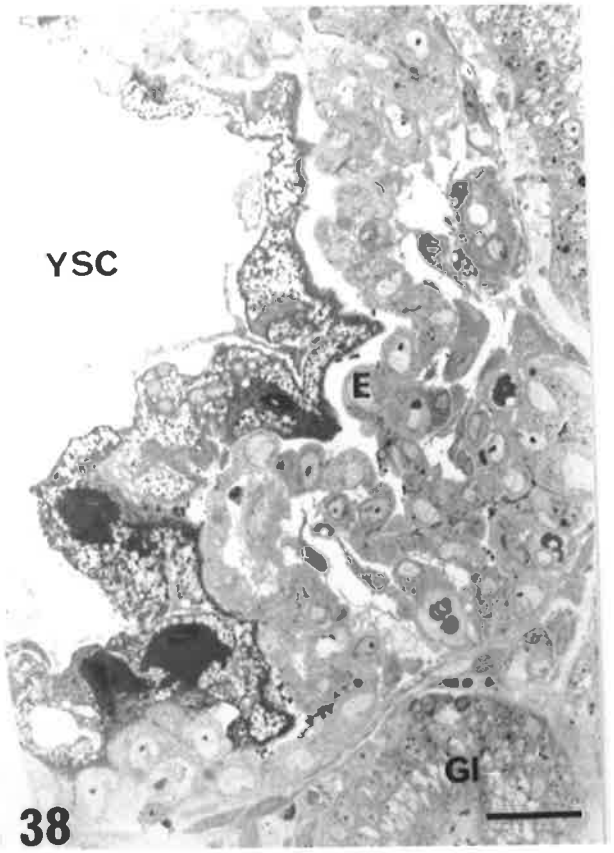
Figures 7.38-7.41 Non-invasive Bilaminar Yolk Sac.

Figure 7.38 At the periphery of the BYS a non-invasive region follows the contours of the uterine epithelium (E); yolk sac cavity (YSC), gland (GL). The apparent separation between the yolk sac and uterine epithelium is an artefact of tissue preparation. Bar = 25μm. Tol. Blue.

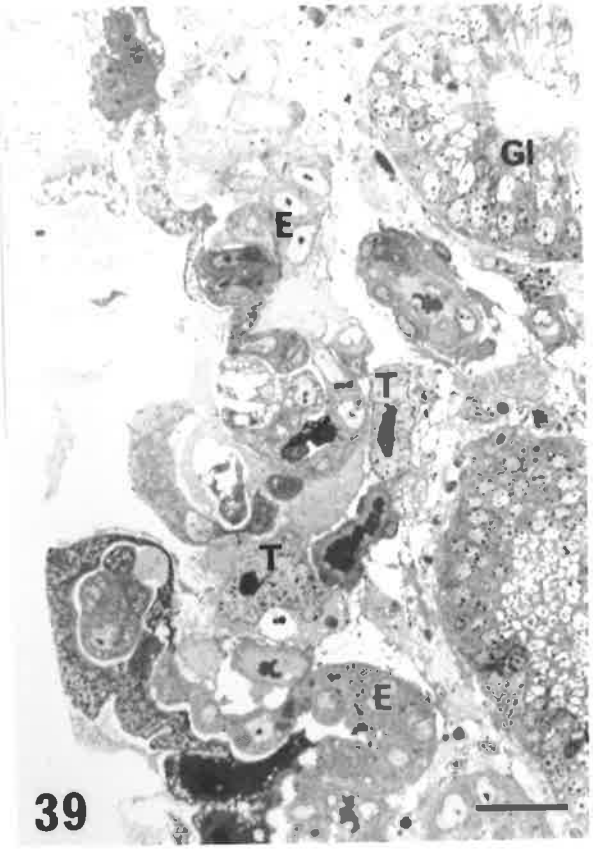
Figure 7.39 On the last day of pregnancy even in this non-invasive region occasional trophoblast cells (T) penetrate the uterine epithelium (E); endometrial gland (Gl). Bar = 25μm. Tol. Blue.

Figure 7.40 In some sections trophoblast appears to have migrated into the stroma below intact uterine epithelium (E) in this non-invasive region. Invasive trophoblast (T) stains much more weakly with toluidine blue than does the non-invasive trophoblast which is vacuolated. Bar = 25μm. Tol. Blue.

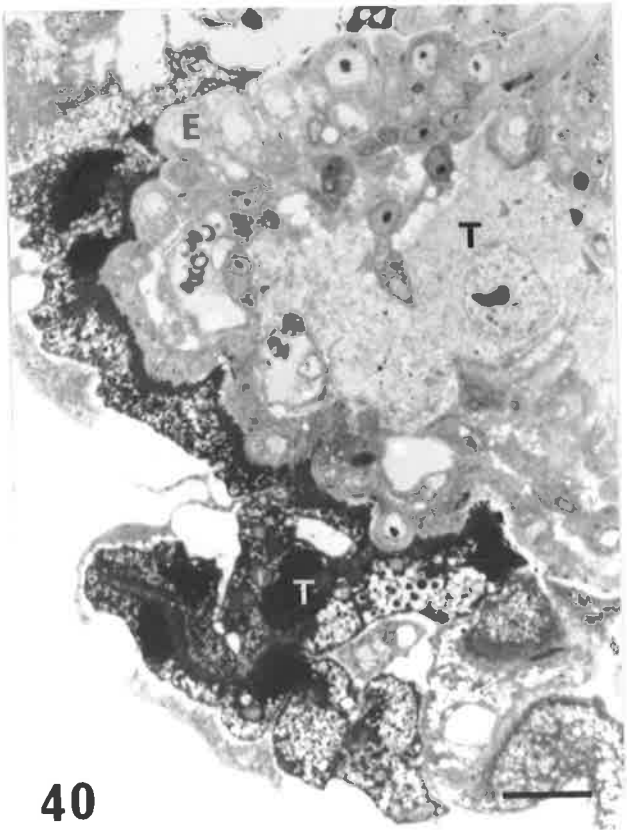
Figure 7.41 Electron micrograph shows that the vacuoles in non-invasive trophoblast (T) are heterogeneous with comparatively electron-lucent granules as well as electron-dense material; uterine epithelium (E), endoderm (En). Bar = 25μm.



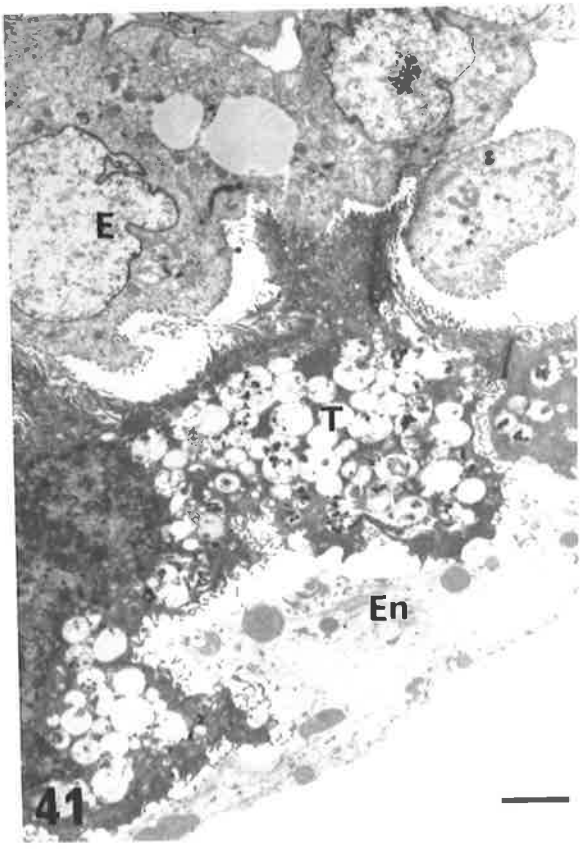
38



39



40



41

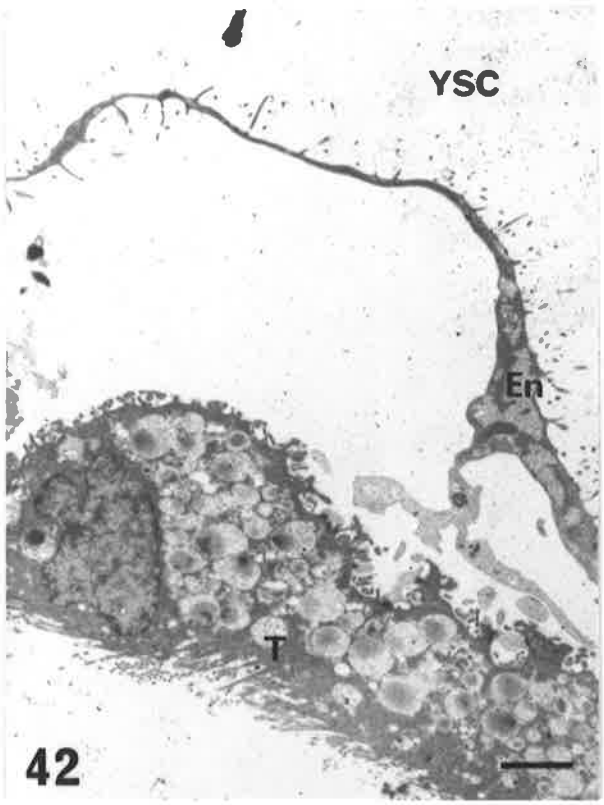
Figures 7.42-7.45 Unattached Bilaminar Yolk Sac.

Figure 7.42 Trophoblast (T) in the unattached BYS is similar to that of the non-invasive BYS with heterogeneous vesicles and electron-dense cytoplasm. Endoderm (En) is extremely attenuated and comparatively electron-dense; yolk sac cavity (YSC). Bar = 2.5μm.

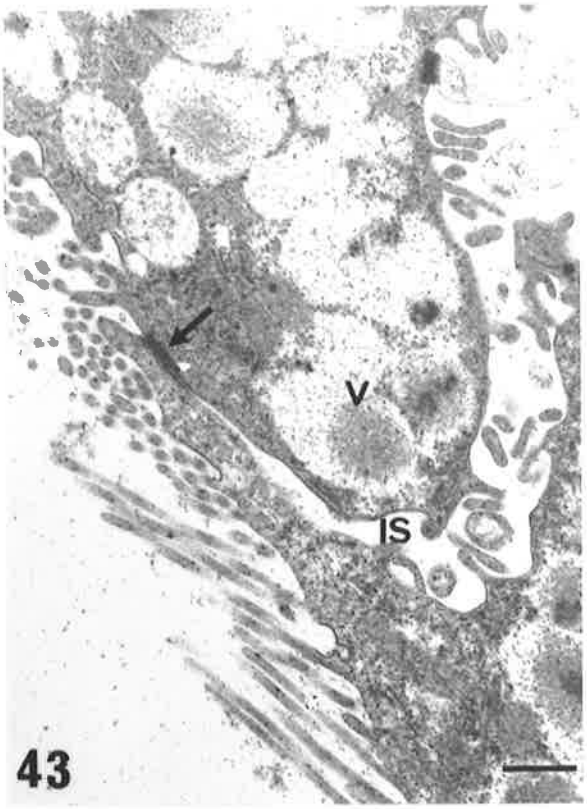
Figure 7.43 Trophoblast cells have many heterogeneous granular vesicles (V). An apical junction between trophoblast is present above a large intercellular space (IS). Bar = 250nm.

Figure 7.44 Higher magnification of Fig. 7.43 shows that the junction between trophoblast resembles an adhering junction. Bar = 100nm.

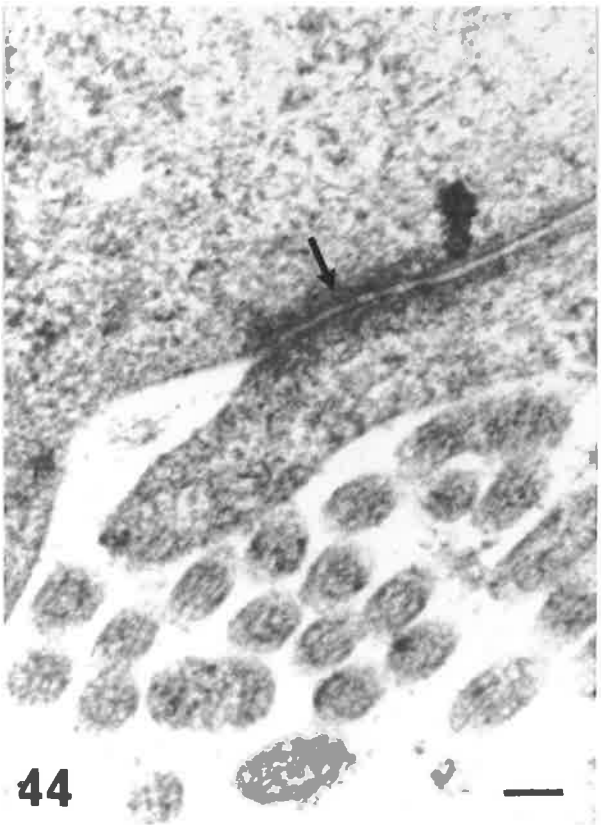
Figure 7.45 Trophoblast is bound to endoderm by desmosomes (arrows); vesicle (V). Bar = 250nm.



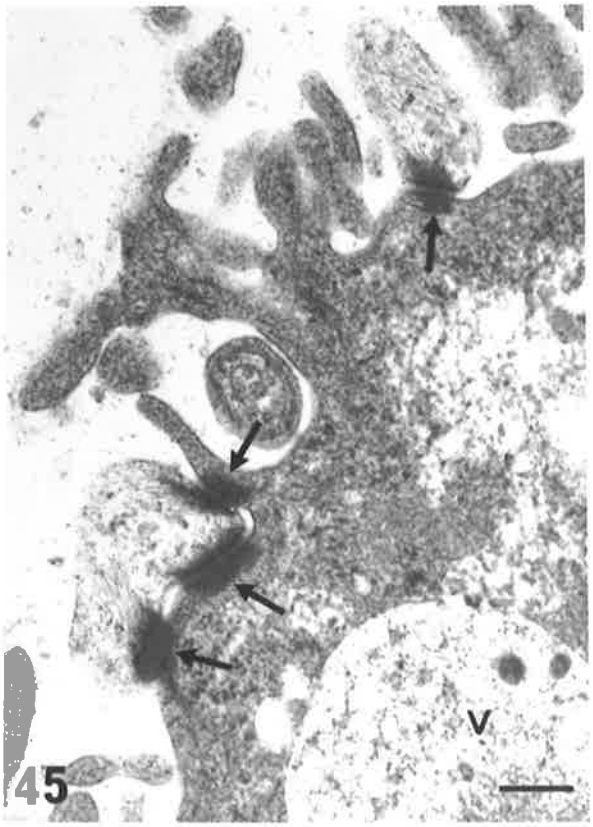
42



43



44



45

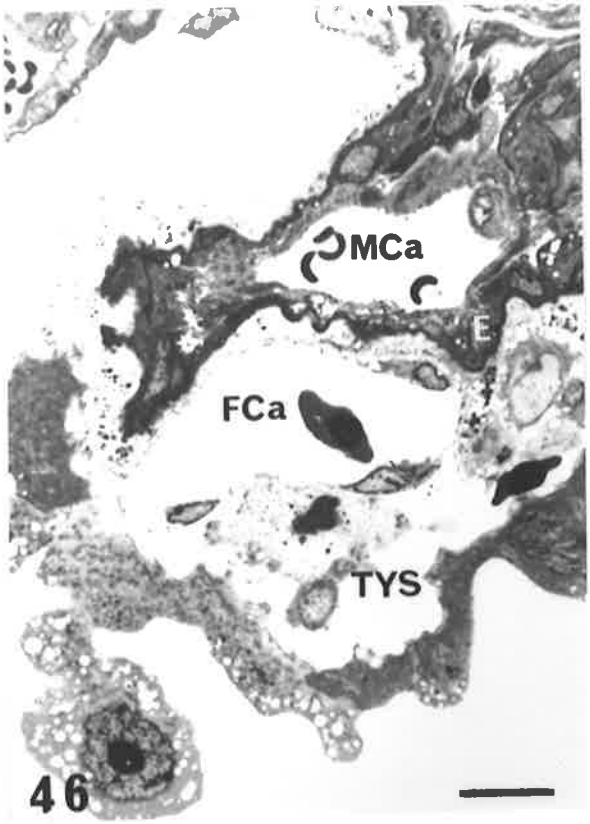
Figures 7.46-7.49 Trilaminar Yolk Sac Placenta.

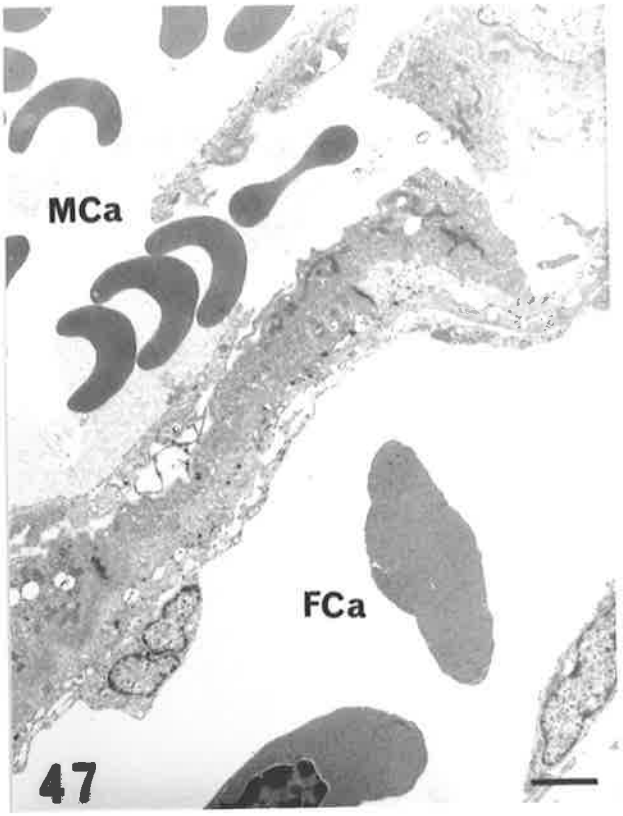
Figure 7.46 Thick plastic section shows that attachment between trophoblast and maternal epithelium is convoluted in the trilaminar yolk sac (TYS) and maternal (MCa) and fetal capillaries (FCa) are in close proximity; trilaminar yolk sac (TYS). Bar = 10μm. Tol. Blue.

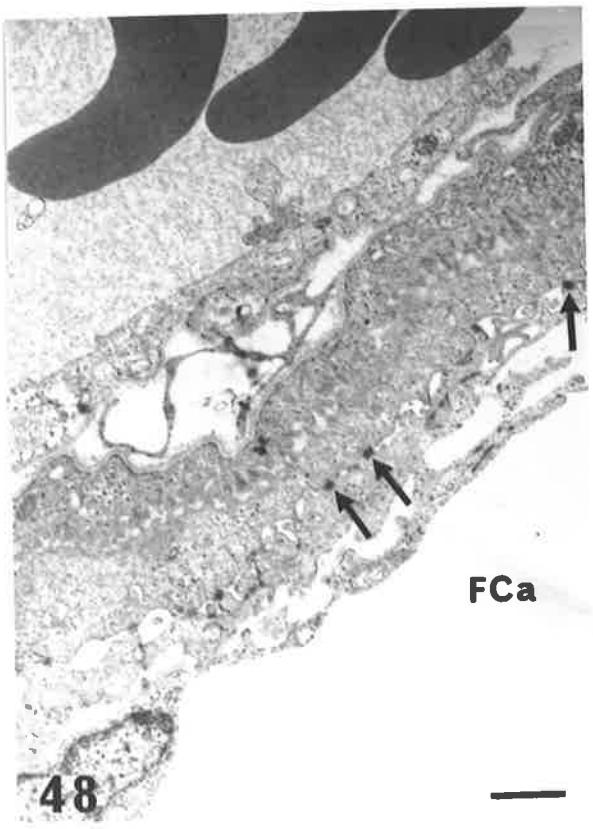
Figure 7.47 Electron micrograph shows that the intervening cell layers between fetal and maternal blood are extremely attenuated with no nuclei present for some distance between the two; maternal capillary (MCa), fetal capillary (FCa). Bar = 2.5μm.

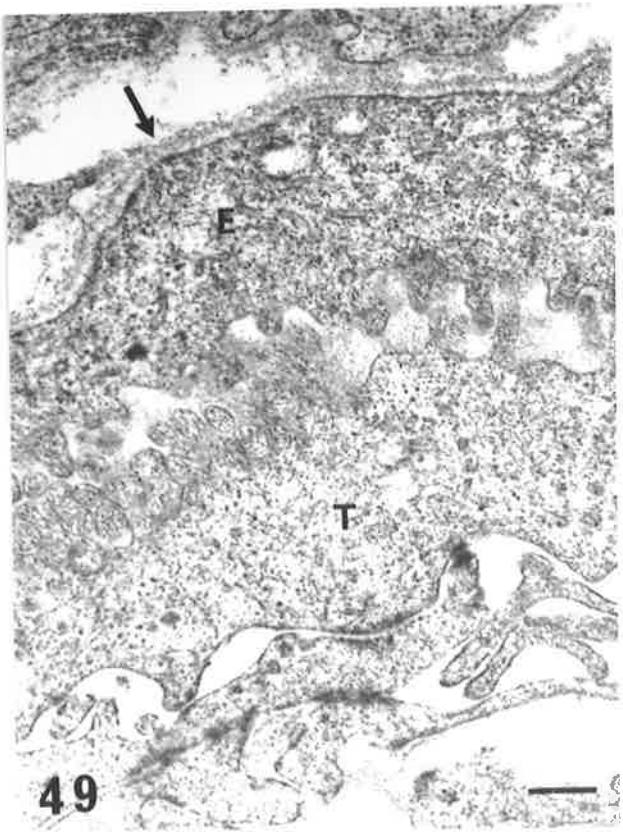
Figure 7.48 Higher magnification shows that the distance for diffusion between maternal and fetal blood is as little as 4μm; fetal capillary (FCa). Desmosomes are present between trophoblast and fetal mesoderm. Bar = 1μm.

Figure 7.49 Higher magnification of the same region as in Fig. 7.48 shows that there is interdigitation of microvilli between trophoblast (T) and uterine luminal epithelium (E) and some electron-dense material, probably glycocalyx, is present between the two. There is a prominent basal lamina beneath the uterine epithelium (arrow) but none beneath trophoblast. Bar = 250nm.


MCa
E
FCa
TYS
46


MCa
FCa
47


FCa
48


E
T
49

there was also a little intervening material which may be glycocalyx (Fig. 7.49). A distinct basal lamina was present below the uterine luminal epithelium but no basal lamina was found below trophoblast. Desmosomes were found between trophoblast and the mesoderm below (Figs. 7.48 and 7.49).

Trophoblast was usually more electron-lucent than the uterine epithelium and invaginated the latter such that in places only a thin strip of its cytoplasm (0.2μm) remained adjacent to the capillaries (Fig. 7.50). Small vesicles could sometimes be seen in the maternal endothelium, epithelium and fetal endothelium in these regions (Fig. 7.51) which may be an indication of macromolecular transport between the two bloodstreams. Also stromal cell processes were absent between maternal endothelium and epithelium in many places (Fig. 7.51).

In some parts of the TYS trophoblast invaginated the epithelium but the endoderm did not closely follow it. The resultant space in between was occupied by fetal capillaries (Fig. 7.52). Only occasional trophoblast nuclei were seen such that in this region trophoblast was usually squamous.

In some parts of the TYS trophoblast had patches of long regular microvilli interspersed with short blunt microvilli (Fig. 7.53) on its apical plasma membrane which were similar to those seen in implantation stages (see Fig. 5.28, chapter 5). In some regions there was a space of 0.5-1.0μm between trophoblast and uterine luminal epithelium (Fig. 7.54). Electron-lucent vesicles were seen in the cytoplasm of trophoblast between the long microvilli (Fig. 7.54) and other similar vesicles were seen budding off golgi complexes deeper in the cell which may indicate that these vesicles were leaving trophoblast rather than being taken up by endocytosis.

At this late stage in pregnancy in a similar region of the TYS lipid droplets were still found in the uterine epithelium, often with mitochondria and rough endoplasmic reticulum nearby (Figs. 7.55 and 7.56). In a part of the uterine epithelium near to the TYS seen in figures 7.55 and 7.56, but where there was no close attachment, a lipid droplet was seen covered by a very thin remnant of cytoplasm and may have been about to be shed into the uterine lumen (Fig 7.57). In this, and all other regions examined, the uterine luminal epithelium were always non-ciliated with comparatively blunt microvilli.

In the TYS trophoblast intercellular junctional complexes were present (Fig. 7.55). However, sometimes no lateral cell membranes were seen below the intercellular junctions (Fig. 7.58).

7.3.6 Endoderm

In each region of the yolk sac the profile of endoderm cells differed but ultrastructural features of the cytoplasm were similar. Endoderm in the BYS was squamous to cuboidal and had long sparse microvilli and coated pits on the apical cell membrane, that is, the membrane that lines the yolk sac cavity (Fig. 7.59). Mitochondria had tubular cristae and an electron-dense matrix. Rough endoplasmic reticulum was found in the cytoplasm. Endoderm overlying the sinus terminalis was cuboidal and had long thin microvilli (Fig. 7.60). The nucleus had an invaginated envelope and was predominantly euchromatic with a large nucleolus. The cytoplasm was comparatively electron-dense. (A higher magnification of endoderm in this region is seen in figure 7.14). Endoderm associated with the TYS was squamous to cuboidal. There were many microvilli and coated pits on the apical plasma membrane and large vacuoles in the apical cytoplasm which contained amorphous and membranous material (Fig. 7.61). Higher magnification showed irregular shaped mitochondria, rough endoplasmic reticulum and rosettes of ribosomes in the cytoplasm (Fig. 7.62).

7.3.7 Electron-dense Material at the Feto-Maternal Interface

Electron-dense material was found at the interface between the uterine luminal epithelium and trophoblast in the TYS as well as in the BYS, especially where trophoblast was non-invasive, and adjacent to invasive regions (Figs. 7.63-7.64). This material completely filled the space between trophoblast and maternal epithelium, but was particularly evident between long trophoblast microvilli (Fig. 7.64). This material was less electron-dense closer to the uterine luminal epithelium and in some places was absent immediately adjacent to the short blunt maternal microvilli (Fig. 7.64). There was no similar material within the maternal epithelial cells.

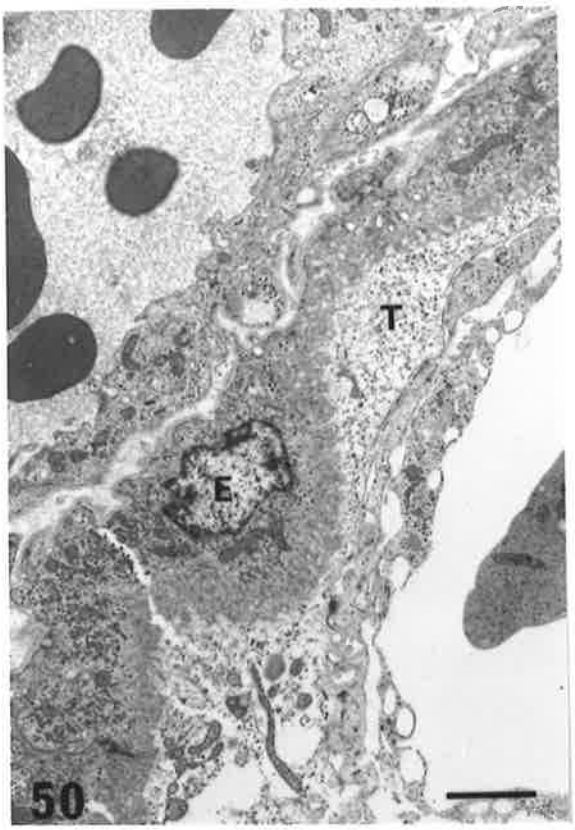
In some sections electron-dense material was found in patches at the interface between fetal and maternal cells and within vesicles in the adjacent trophoblast cytoplasm (Fig. 7.65). Electron-dense vesicles in trophoblast were often seen near to golgi complexes alongside dilated electron-lucent golgi vesicles (Fig. 7.66). In some cells large membrane-bound vesicles, adjacent to dilated golgi complexes, were found which contained moderately electron-dense flocculent material (Fig. 7.67). In other cells these were present as well as the electron-dense vesicles (Fig. 7.68). These may have been the result of condensation of the contents of vesicles like those in figure 7.67. The electron-dense vesicles were surrounded by only one limiting membrane which suggests that they were of trophoblast origin and therefore were possibly secretory granules.

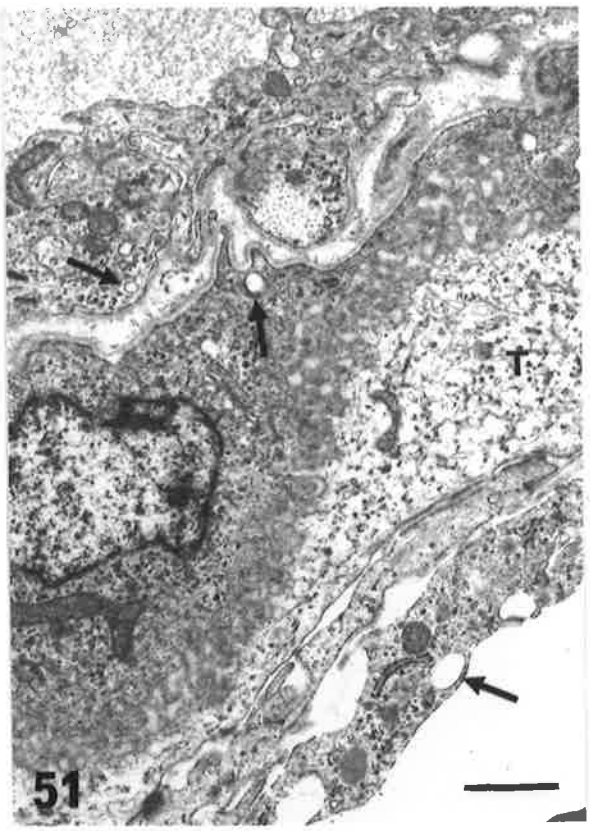
Figures 7.50-7.52 Trilaminar Yolk Sac Placenta.

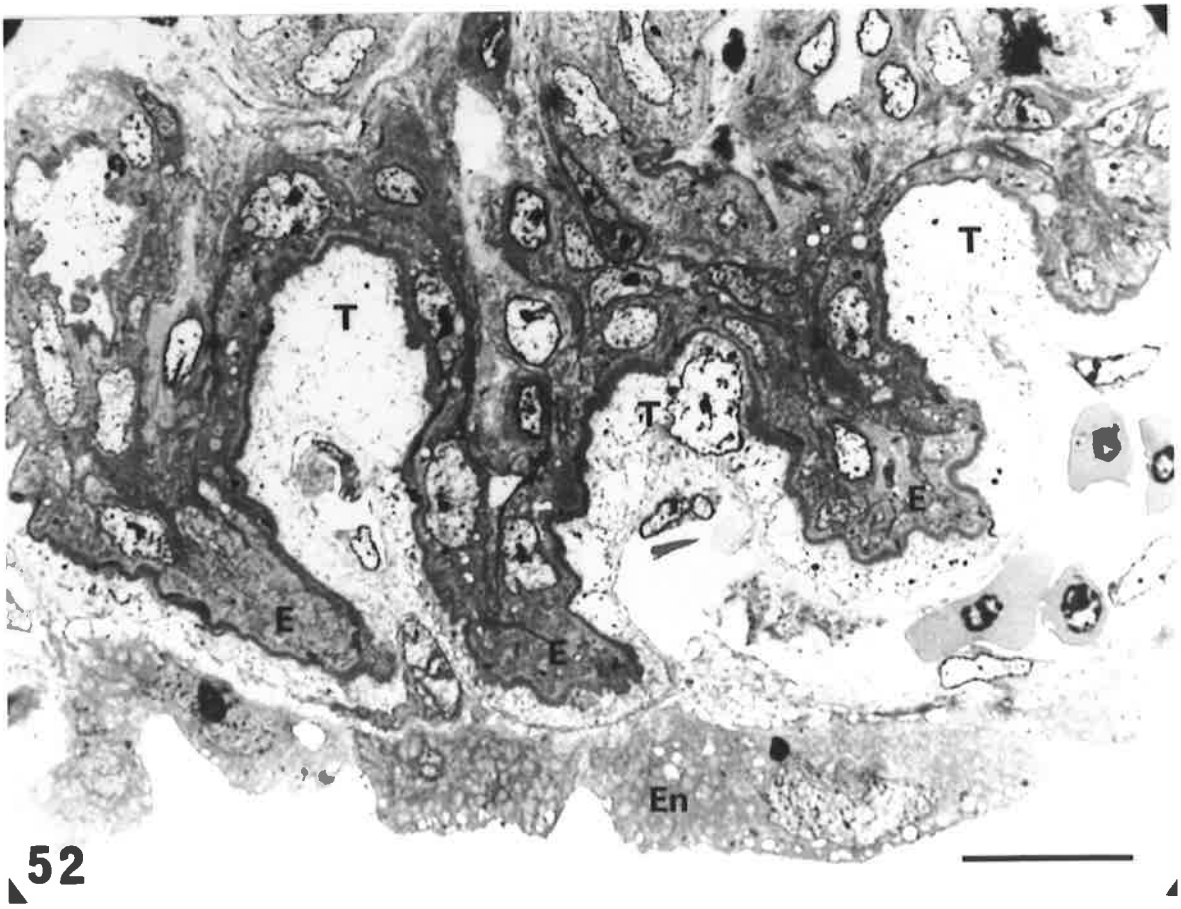
Figure 7.50 Trophoblast (T) is generally more electron-lucent than the uterine epithelium (E) and often only a thin trace of its cytoplasm is present adjacent to capillaries. Bar = 2μm.

Figure 7.51 Higher magnification of Fig. 7.50 shows that vesicles (arrows) are often present in each of the intervening cell layers and often no stromal cell processes are seen between maternal endothelium and epithelium; trophoblast (T). Bar = 1μm.

Figure 7.52 Thick plastic section shows that trophoblast (T) invaginates the uterine epithelium (E) but endoderm (En) does not closely follow it leaving a space which is occupied by fetal capillaries. Bar = 10μm. Tol. Blue.


T
E
50


T
51


T
T
T
E
E
E
En
52

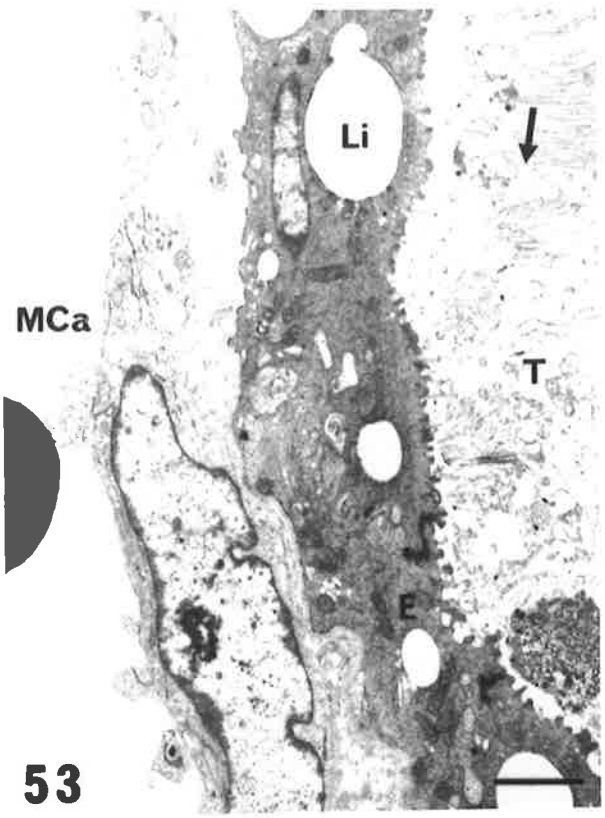
Figures 7.53-7.56 Trilaminar Yolk Sac Placenta.

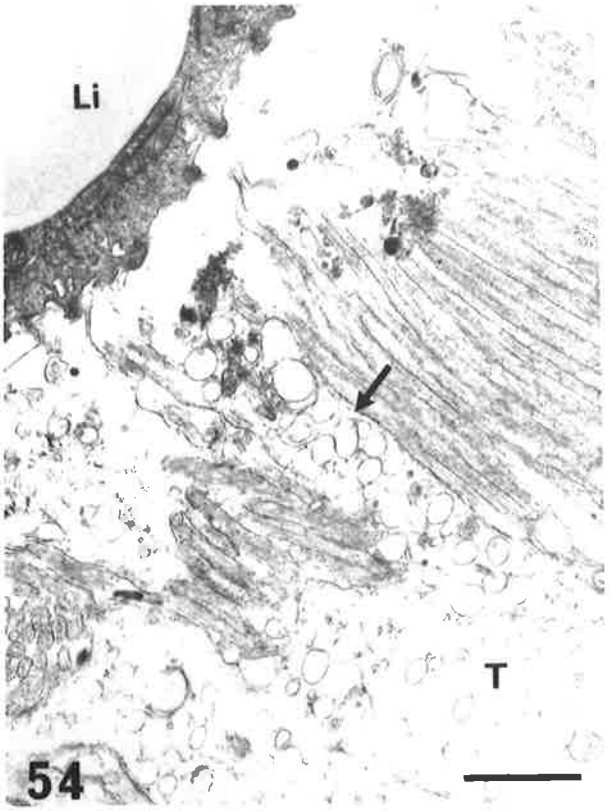
Figure 7.53 In some parts of the TYS there is not the close interdigitation between fetal and maternal microvilli seen in previous electron micrographs, but rather there is a space present between the two cell types, particularly in regions where there are long trophoblast microvilli. In these regions the uterine epithelium (E) has many lipid droplets (Li) and is more electron-dense than the adjacent trophoblast (T) which contains many electron-lucent vesicles (arrow); maternal capillary (MCa). Bar = 2μm.

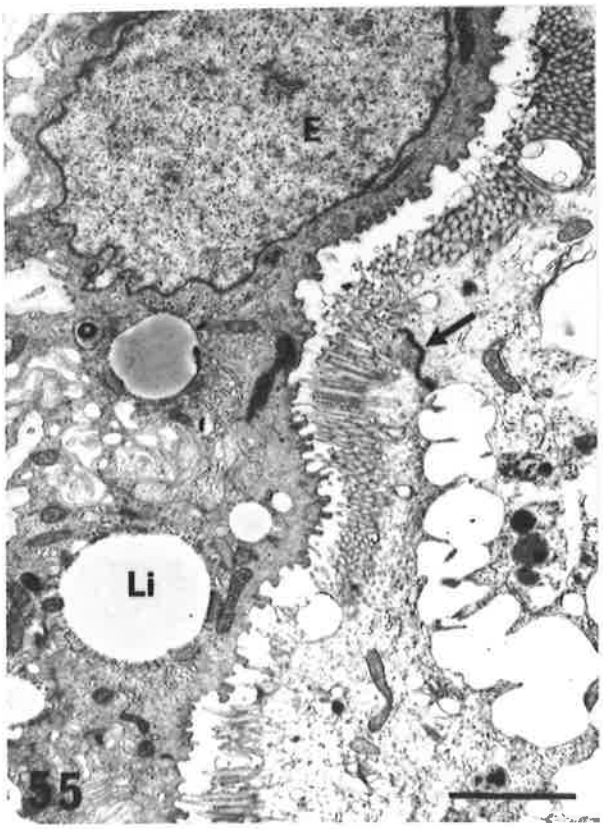
Figure 7.54 Higher magnification of Fig. 7.53 shows that electron-lucent vesicles are between long trophoblast microvilli; lipid (Li). Bar = 1μm.

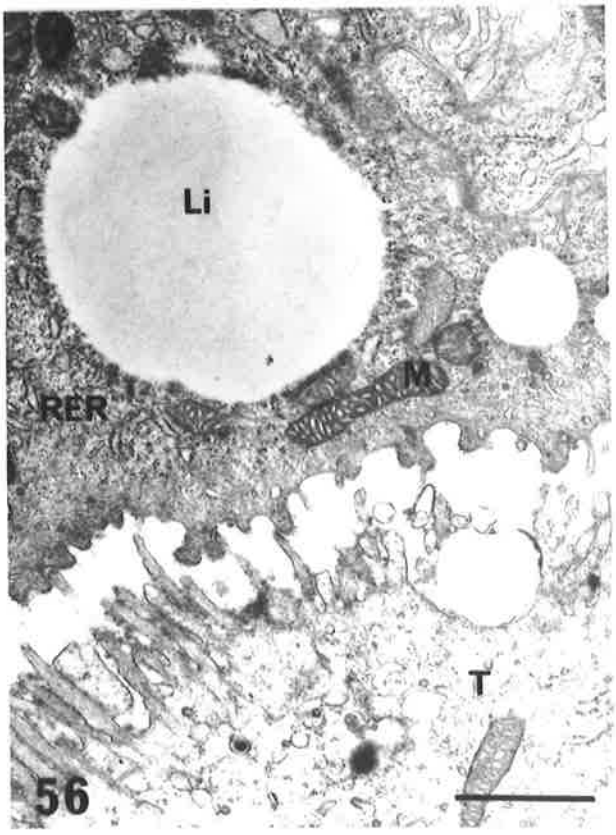
Figure 7.55 In this region of the TYS trophoblast is cellular, as opposed to syncitial, as there is an apical junctional complex present between neighbouring cells. Lipid droplets (Li) are present in the uterine epithelium (E). Bar = 2μm.

Figure 7.56 Higher magnification shows that the lipid droplet (Li) in the uterine epithelium is associated with mitochondria (M), and rough endoplasmic reticulum (RER) is nearby. Bar = 1μm.


Li
MCa
T
E
53


Li
T
54

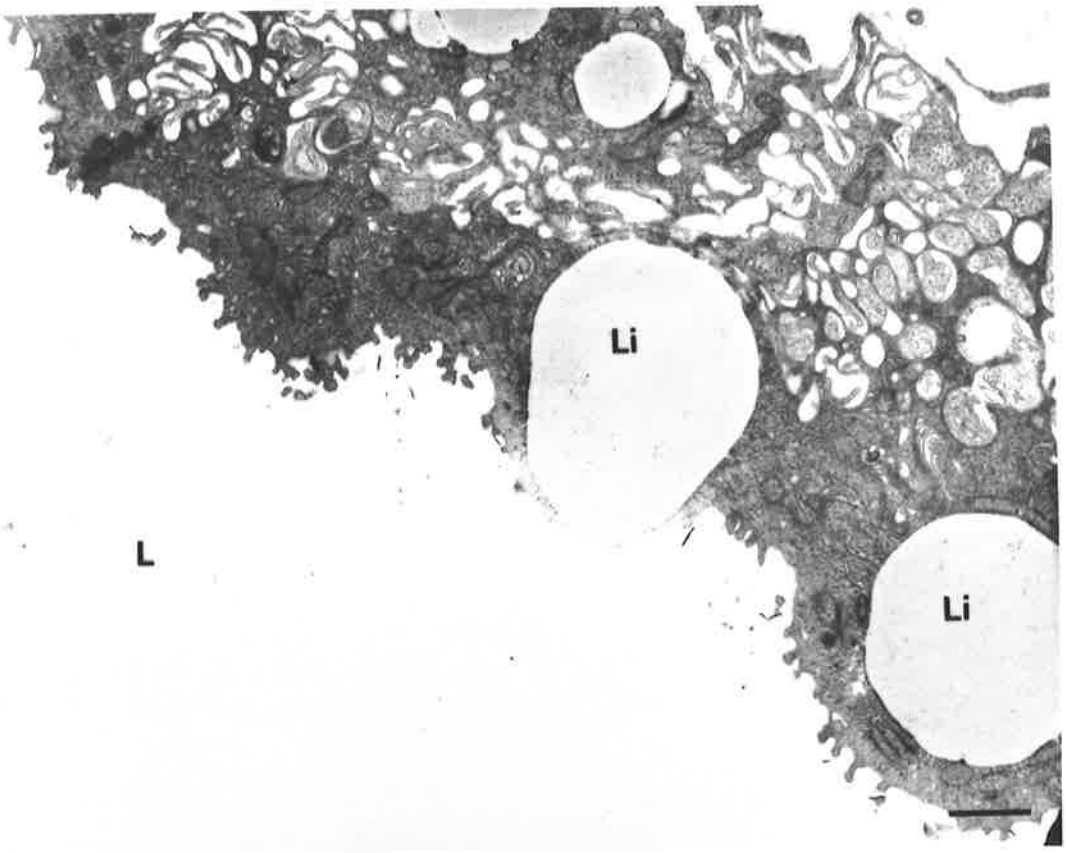

E
Li
55


Li
RER
M
T
56

Figures 7.57 and 7.58 Trilaminar Yolk Sac Placenta.

Figure 7.57 In a region adjacent to the area seen in Fig. 7.55 where the TYS had been displaced from the uterine epithelium lipid droplets are seen, one of which has a thin protrusion of cytoplasm above it and appears to be about to be transported to the uterine lumen (L). Bar = 1μm.

Figure 7.58 Intercellular junctional complex (arrow) between trophoblast cells appears to have no intercellular mambrane below suggesting that perhaps trophoblast cells have fused. Bar = 250nm.



57

58

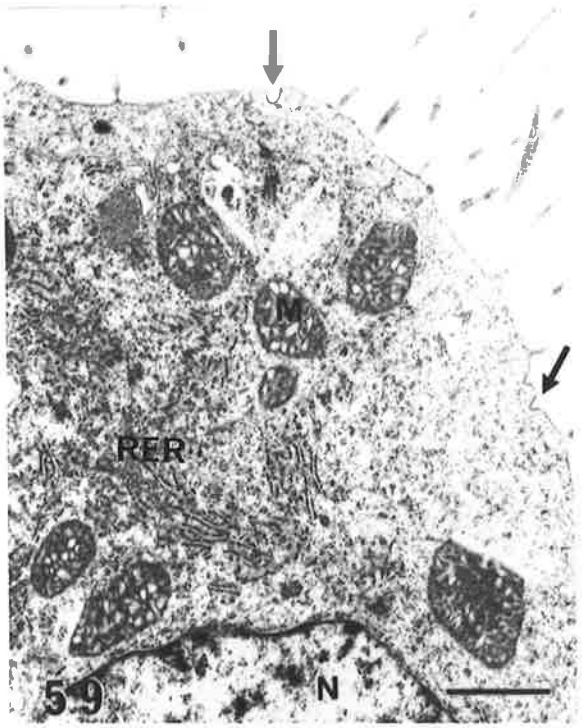
Figures 7.59-7.62 Endoderm.

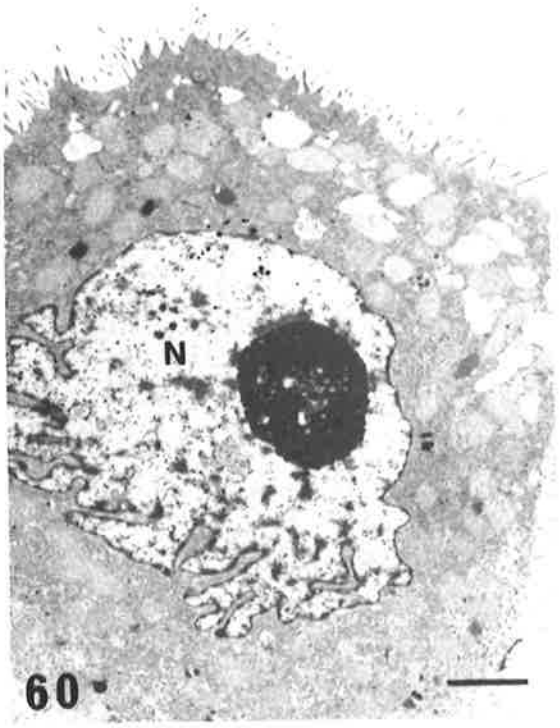
Figure 7.59 Endoderm in the bilaminar yolk sac is cuboidal, has long sparse microvilli and coated pits (arrows) on the cell membrane adjacent to the yolk sac cavity. Mitochondria (M) are electron dense with tubular cristae and there is abundant rough endoplasmic reticulum (RER). Bar = 1μm.

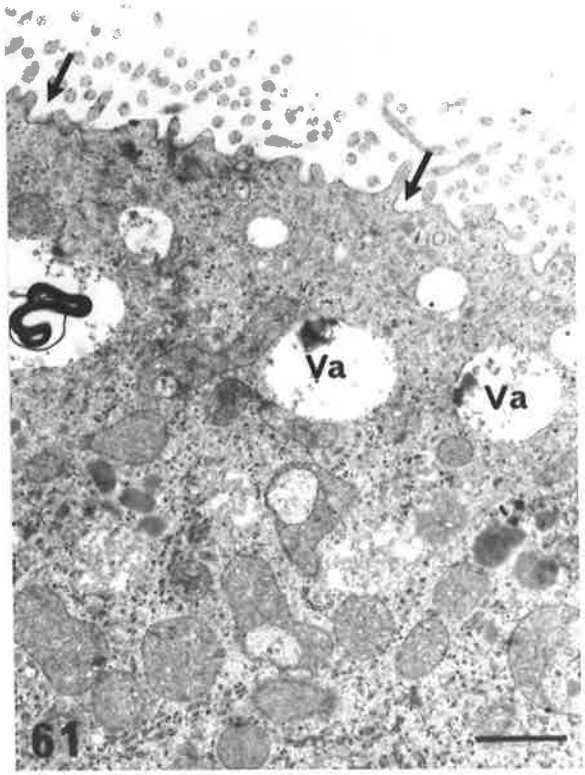
Figure 7.60 Endoderm overlying the sinus terminalis is cuboidal with long sparse microvilli. The nucleus (N) is invaginated, predominantly euchromatic with a prominent nucleolus. Bar = 2.5μm.

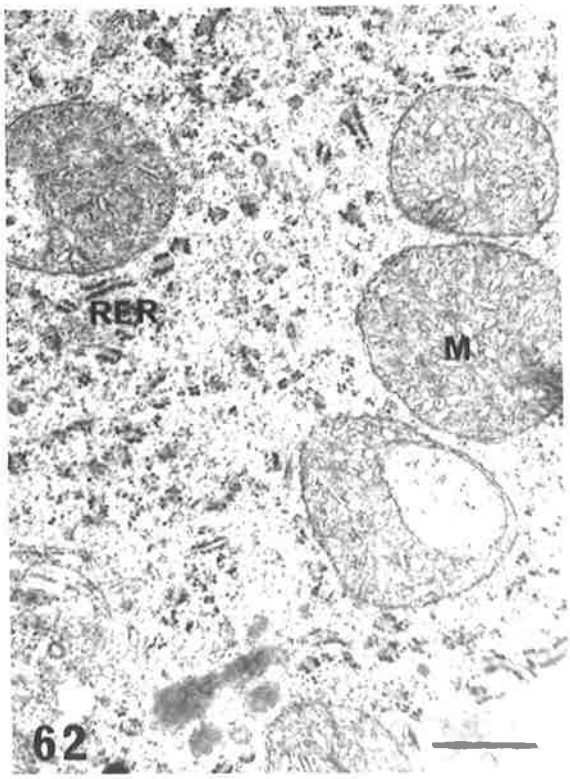
Figure 7.61 Endoderm in the trilaminar yolk sac has coated pits on the cell membrane adjacent to the yolk sac cavity (arrows) and apical vacuoles (Va) which contain flocculent, and sometimes membranous, material. Bar = 1μm.

Figure 7.62 Higher magnification of the same endoderm cell in Fig. 7.61 shows that there are irregular mitochondria (M), rough endoplasmic reticulum and rosettes of polyribosomes in the cytoplasm. Bar = 500nm.


M
RER
N
59


N
60


Va
Va
61


RER
M
62

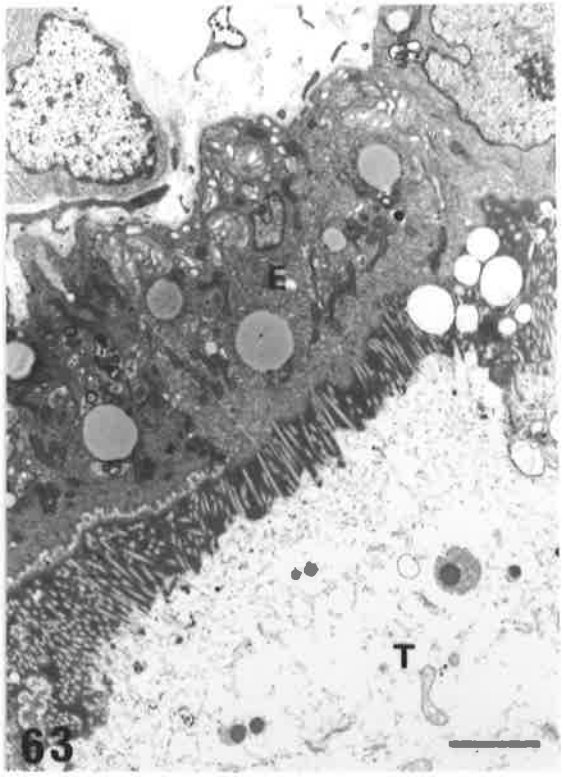
Figures 7.63-7.66 Electron-dense material at the Feto-Maternal Interface.

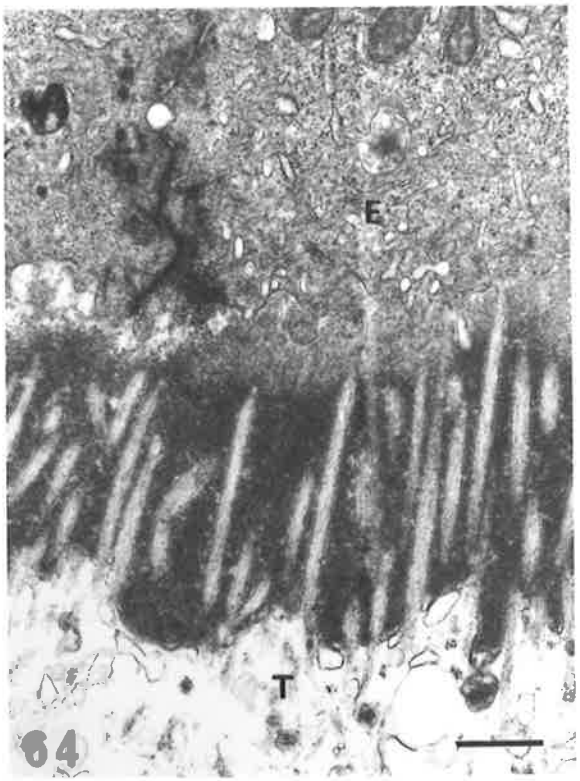
Figure 7.63 Electron-dense material is present between non-invasive trophoblast (T) and uterine epithelium (E) adjacent to regions of invasive trophoblast in the BYS. Bar = 2.5μm.

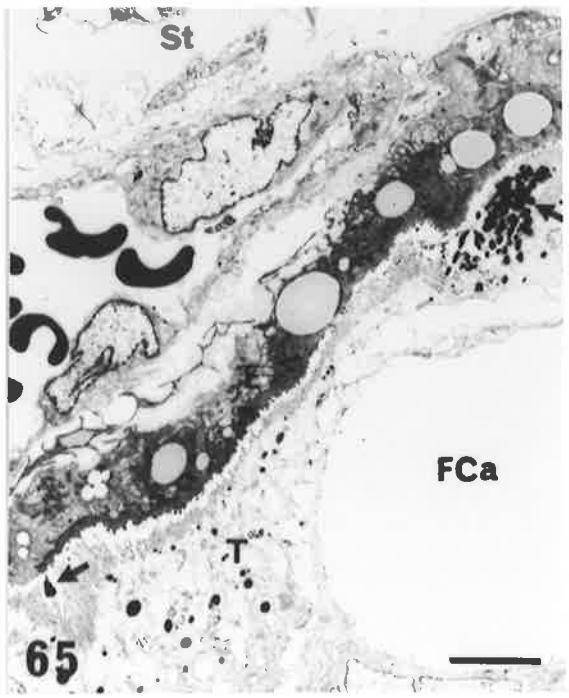
Figure 7.64 Higher magnification of Fig. 7.63 shows that the material is most electron-dense between the long trophoblast (T) microvilli and is less dense or absent near the blunt uterine epithelial (E) microvilli. Bar = 500nm.

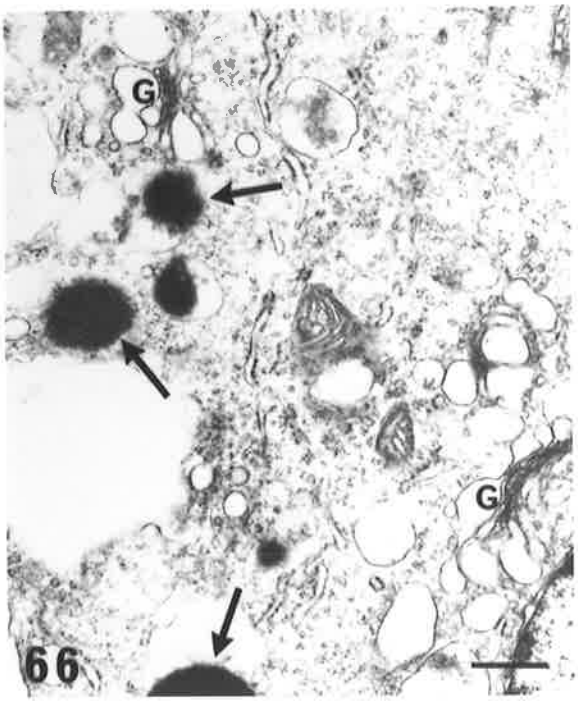
Figure 7.65 Sometimes electron-dense material is seen in patches (arrows) at the feto-maternal interface in the TYS. Electron-dense vesicles are also present in trophoblast (T) cytoplasm, fetal capillary (FCa), endometrial stroma (St). Bar = 5μm.

Figure 7.66 Electron-dense vesicles (arrows) in trophoblast are adjacent to dilated golgi complexes (G). Bar = 500nm.


E
T
63


E
T
64


St
FCa
T
65


G
G
66

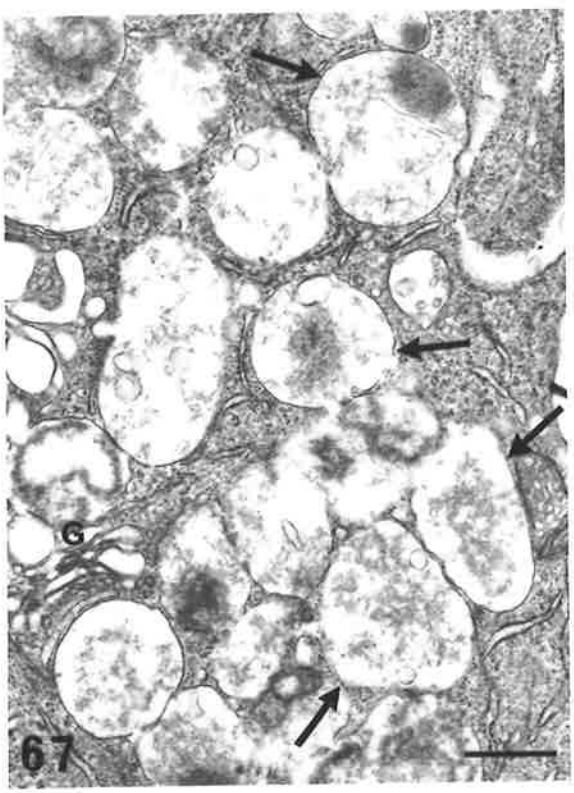
Figures 7.67-7.70 Electron-dense material at the Feto-Maternal Interface.

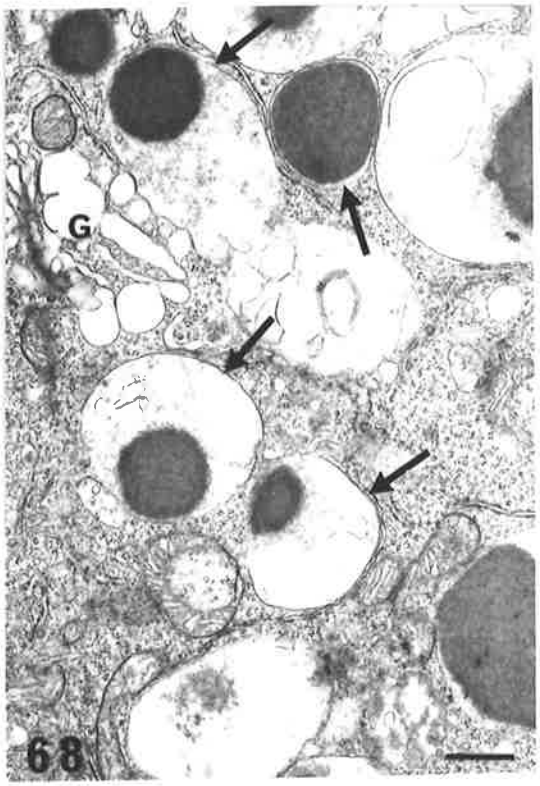
Figure 7.67 Membrane-bound vesicles (arrows) adjacent to dilated golgi complexes (G) contain flocculent material some of which appears more electron-dense than the rest. Bar = 500nm.

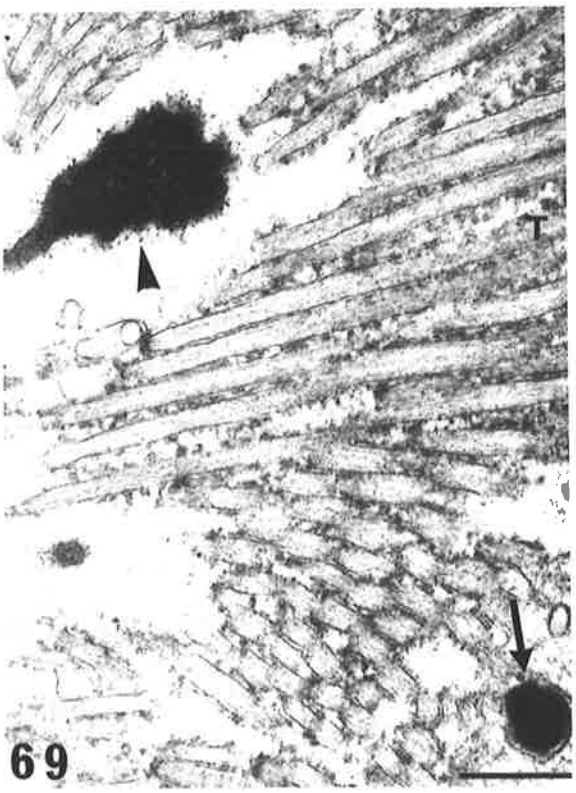
Figure 7.68 In some trophoblast there appeared to be a mixture of electron-dense and less dense membrane bound vesicles. Some of these appeared to be condensing vesicles. Bar = 500nm.

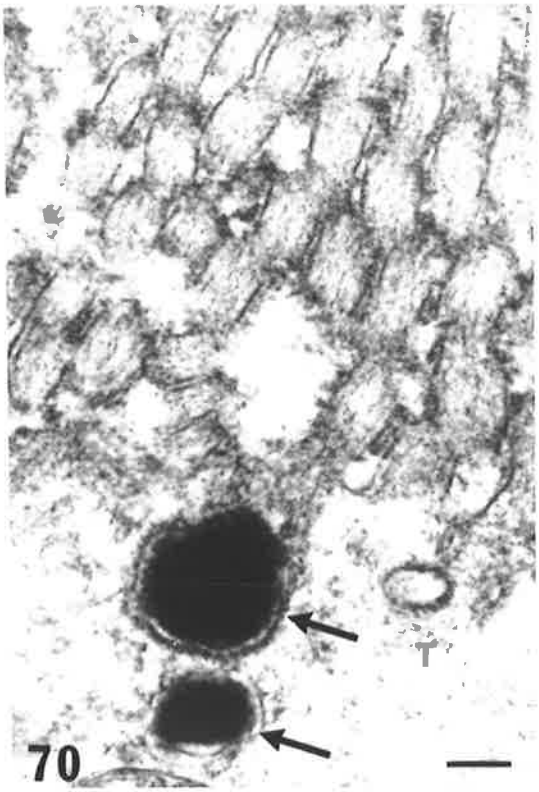
Figure 7.69 Higher magnification of the apical microvillous region of trophoblast (T) seen in Fig. 7.65 shows a clump of electron-dense material (arrowhead) in the lumen and a coated electron-dense vesicle (arrow) in the apical cytoplasm. Bar = 500nm.

Figure 7.70 Higher magnification shows that the apical electron-dense coated vesicle in trophoblast (T) had another immediately below it (arrows). Bar = 250nm.


G
67


G
68


T
69


T
70

Some of this electron-dense material was seen in small clumps between trophoblast and maternal epithelium. A higher magnification of part of the plasmalemma of trophoblast seen in figure 7.65 shows such a clump in the lumen as well as an electron-dense vesicle in the apical cytoplasm immediately below the long microvilli (Fig. 7.69). This vesicle, and another immediately below it, (Fig. 7.70) appear to be coated, a phenomenon commonly associated with endocytosis rather than exocytosis (see discussion).

7.4 Discussion

This electron microscopical study has revealed the most extensive invasion described to date in a marsupial yolk sac placenta. In the BYS adjacent to the sinus terminalis trophoblast erodes the endometrium to the maternal endothelium which, in some areas, shows marked thickening of its basal lamina. Trophoblast appears to behave in a similar way to that found in endotheliochorial chorioallantoic eutherian placentae.

The coated pits and vesicles of protoderm and trophoblast in preimplantation embryos suggest that macromolecular uptake takes place. This may occur from both directions, since coated pits were found on cell membranes adjacent to both the shell membrane and blastocoelic cavity, a finding which is consistent with the observations of Selwood and Sathananthan (1988) who found pinocytotic vesicles on all plasma membranes of *Antechinus* embryos at 2- and 4-cell stages whether facing the SM or yolk. Amino acids, proteins and glucose are present in the blastocoelic fluid (Renfree, 1973) and may serve as a nutrient reserve. Lipids may also be present in the blastocoele as both electron-dense and electron-lucent lipid droplets were seen in protoderm of unilaminar blastocysts and appeared to be in the process of transport to the blastocoelic cavity.

In primitive streak embryos of the dunnart the long microvilli, and increased number and size of coated pits and vesicles, suggest that macromolecular uptake by trophoblast at this stage increases. Renfree (1973) found in tammar wallabies that the electrophoretic pattern of yolk sac fluid of preimplantation embryos resembled that of uterine flushings and concluded that proteins were transferred unchanged from the uterine fluid to the yolk sac fluid. Presumably endocytosis by coated pits plays a role in this transfer. In preimplantation pig embryos pinocytosis by all blastomeres during cleavage, and then by trophoblast in subsequent stages, takes place, and the frequency of coated pits increases from blastocyst formation (Stroband and Van der Lende, 1990). Preimplantation domestic cat embryos also have numerous coated pits and

long, sparse microvilli on the apical cell membranes of trophoblast (Leiser, 1982) and in all species examined coated pits are characteristic at this stage (Enders, 1971).

Blastocysts at a variety of developmental stages were found closely associated with the uterine luminal epithelium. At dissection a semi-spherical cavity was always seen on either side of the embryo at these appositional stages giving the impression that there was a distinct implantation chamber and an absence of copious volumes of uterine luminal fluid. It appears that there is little uterine luminal fluid present at implantation in many eutherian species (Martin (1984). Hormonally induced uterine closure at implantation allows the embryo close contact with the uterine luminal epithelium for the transfer of gases nutrients and wastes. Growth factors from, and embryonic signals to, the mother may also be exchanged as occurs in various eutherian chorioallantoic placentae (Hodgen and Itskovitz, 1988). Oestradiol secretion by preimplantation pig embryos stimulates secretion of protein from the uterine glandular epithelium which becomes part of the histotrophic nutrition for the embryo (Geisert *et al.*, 1982). Work is needed to ascertain whether marsupial preimplantation embryos secrete any signals, growth factors, steroid or peptide hormones.

The cellular interactions at the attachment site between trophoblast and maternal epithelium at the sinus terminalis was convoluted. It has been suggested that in the horse, where the choriovitelline placenta (TYS) is important early in pregnancy, the sinus terminalis holds the embryo in place and forms a secure attachment (Enders and Liu, 1991a). It would be reasonable to conclude that in the dunnart, too, the interdigitation of fetal and maternal tissues in this region could provide such an intimate attachment that would resist displacement. However, the fact that at the same time there is extensive erosion of the endometrium in the BYS suggests that the sinus terminalis would not be required for anchorage.

In the dunnart, trophoblast was invasive over an extensive area in an annular region of the BYS adjacent to the sinus terminalis. Trophoblast either fused with, or phagocytosed, endometrial cells which presumably became a nutrient source. TGCs then migrated into the stroma, phagocytosed stromal cells, and came to surround maternal blood vessels and glands.

The ultrastructural findings show that in the BYS region an endotheliochorial placenta develops. In some parts of the BYS trophoblast became apposed to the basal lamina of the maternal endothelium, but at no stage did it appear to penetrate it. In some areas the endothelial basal lamina was markedly thickened where trophoblast approached which is similar to that which occurs in the endotheliochorial placenta of

the domestic cat (Leiser and Koob, 1993), the ferret (Lawn and Chiquoine, 1965), a variety of bats, insectivores, tupaiids, seals and elephants (Wynn, 1971; Rasweiler, 1993). Maternal endothelial hypertrophy occurs in many species of bats, especially in the vascular tuft region where the discoidal haemochorial placenta develops (Rasweiler, 1991, 1993). The endothelial basal lamina thickens and possibly incorporates factors secreted by the endothelium which play an important role in controlling trophoblast growth (Rasweiler, 1993). It is interesting that this thickening occurs in a variety of species which develop endotheliochorial placentae, as well as some bats which develop haemochorial placentae.

In the non-invasive dunnart BYS, trophoblast was uninucleate, cellular, and had a comparatively electron-dense cytoplasm with heterogeneous vesicles. However, invasive trophoblast found within the endometrial stroma was comparatively electron-lucent with abundant smooth and rough endoplasmic reticulae and was multinucleate. Trophoblast seen at the point of epithelial penetration was binucleate with abundant rough endoplasmic reticulum (see chapter 5). In eutherians both cytotrophoblast and syncytiotrophoblast are found in endotheliochorial and haemochorial placentae (Luckett, 1977).

Is the multinucleate trophoblast of the dunnart syncytial? Its ultrastructural features, particularly the vesicular rough and smooth endoplasmic reticulae, the invaginated nuclear membrane and predominantly euchromatic nucleus, resemble those of human syncytiotrophoblast (Boyd and Hamilton, 1970; Knoth and Larsen, 1972; Wynn, 1973; Dearden *et al.*, 1983; Benirschke and Kaufmann, 1990). However, nuclei in TGCs of the dunnart are much larger than those in human syncytiotrophoblast and are of similar size to the nuclei in polyploid uninucleate TGCs in rodents (Hoffman and Wooding, 1993). The syncytiotrophoblast is thought to form as a result of fusion of cytotrophoblast as mitoses are never seen in the former and occasionally, the remains of intercellular junctions are seen in intrasyncytial clefts (Boyd and Hamilton, 1970, Enders, 1971; Leiser and Enders, 1980a; Pijnenborg *et al.*, 1981; Benirschke and Kaufmann, 1990; Hoffman and Wooding, 1993). In the dunnart TGCs were never seen in mitosis and occasionally intercellular junctions were found which had no underlying lateral cell membranes (Fig. 7.58). Perhaps TGCs are the result of fusion of cytotrophoblast cells in the dunnart. It cannot be emphatically concluded that the multinucleate TGCs were syncytial as there is not enough evidence to suggest syncytiotrophoblast covered the invasive surface of the yolk sac, but the ultrastructural features described above suggest that this may be the case.

In the trilaminar yolk sac (TYS) of the dunnart trophoblast closely followed the contours of the uterine luminal epithelium which was folded and formed an epitheliochorial placenta. Although there were six cell layers present between fetal and maternal bloodstreams (fetal endothelium, mesoderm, trophoblast, uterine epithelium, mesodermal cells and endothelium) there was often great attenuation of these intervening cells. In many sections nuclei were not apparent adjacent to capillaries, only thin layers of cytoplasm were present, and sometimes there were only thin cytoplasmic processes of maternal stromal cells present which did not always form a continuous layer. The minimum distance found between fetal and maternal blood in the TYS was 4μm. In epitheliochorial placentae of eutherian mammals there is always a smaller distance between maternal and fetal red blood cells than would be expected given the presence of six intervening cell layers (Enders, 1982). Even though the pig placenta is strictly epitheliochorial the reduction is achieved by indentation of both trophoblast and maternal epithelium. It seems that the dunnart trilaminar yolk sac placenta achieves a similar relationship, presumably also facilitating exchange, particularly gaseous.

Vesicles were often found in the cytoplasm of cells intervening between the fetal and maternal bloodstreams in the TYS which may have been involved in the transfer of non-diffusible substances from the maternal to fetal bloodstreams. Curiously, trophoblast was never seen with a basal lamina. Epithelial cells normally secrete a basal lamina which forms an adhesion interface and permeability barrier with the connective tissue below (Carr and Toner, 1982; Stevens and Lowe, 1992; Alberts *et al.*, 1994). Trophoblast in the eutherian chorioallantoic placenta, including the endotheliochorial placenta of the ferret (Lawn and Chiquoine, 1965), also normally has a basal lamina and so too does trophoblast in the TYS of the four-eyed opossum, a South American didelphid marsupial (Enders and Enders, 1969). It may be that in the dunnart the absence of a basal lamina around trophoblast facilitates uptake by this cell type.

Alternatively, the lack of a trophoblast basal lamina may be a reflection of its state of differentiation. During development cells can express two major phenotypes and may switch between these: 1. mesenchymal/fibroblastoid phenotype compatible with cells moving individually, 2. epithelioid phenotype characterized by cells expressing apico-basal polarity in which apical junctional complexes, composed of the zonula occludens, zonula adherens and desmosomes, develop between cells such that they are found, and move, in sheets rather than individually (Denker, 1993). At implantation both the trophoblast and uterine luminal epithelium appear to be involved in epithelial/mesenchymal transformations to some degree (Denker, 1993). As epithelial

cells do not normally adhere to each other at their apical plasma membranes both cell types must lose this property in order to allow implantation. In those species with invasive placentae the epithelial/mesenchymal switch allows trophoblast to migrate deep into the endometrial stroma. Perhaps a part of this phenotypic switch in the dunnart trophoblast may involve the loss of its ability or need to secrete a basal lamina.

In the dunnart the uterine luminal epithelium, unlike the glandular epithelium, remained non-ciliated and cellular which is similar to that in the acrobatid New Guinea pen-tailed possum (Hughes *et al.*, 1987). In the bandicoot during blastocyst development there is extensive remodelling of the uterine luminal epithelium which transforms from a simple columnar cell layer to a series of syncytial masses called homokaryons. These are multinucleate cells with one type of nucleus, derived from the fusion of non-ciliated uterine epithelial cells and are interspersed with occasional ciliated cells (Padykula and Taylor, 1976, 1977, 1982). At the same time maternal endometrial capillaries extend up between the homokaryons, thus forming what will be the maternal component of the future yolk sac and chorioallantoic placentae. In the dunnart, too, endometrial capillaries were much closer to the uterine lumen than when preimplantation embryos were present but no evidence of multinucleated maternal epithelial cells was found. This indentation of the basal part of the uterine luminal epithelium by maternal capillaries has also been seen in the development of the chorioallantoic placenta of the pig (Enders, 1982; King *et al.*, 1982; Dantzer and Leiser, 1994).

The ultrastructure of endoderm in all regions of the yolk sac suggested that it was actively involved in biosynthesis since abundant rough endoplasmic reticulum, numerous mitochondria and a prominent nucleolus were present (Fawcett, 1966; Carr and Toner, 1982). Mitochondria generally had tubular cristae which is a feature often associated with steroid synthesis. Multinucleate trophoblast had abundant rough and smooth endoplasmic reticulum, and some mitochondria had tubular cristae. Smooth endoplasmic reticulum is the dominant organelle associated with steroid synthesis in the testis and other steroid secreting tissues in a large number of mammalian species (Christensen and Gillim, 1969; Christensen, 1975; Ewing and Zirkin, 1983; deKretser and Kerr, 1988) and in syncytiotrophoblast in the placenta (Allen, 1975).

Lipid droplets are also commonly found in steroid synthesizing cells as they supply cholesterol esters which are substrates for steroid synthesis (Allen, 1975; Ewing and Zirkin, 1983). In the dunnart lipid droplets were commonly found in the uterine epithelium closely associated with trophoblast and occasionally appeared to be

undergoing transport to the uterine lumen (Fig. 7.57). In rats, by contrast, Boshier (1976) found that at implantation sites lipid droplets had disappeared from uterine luminal epithelial cells, and so too had non-specific esterase activity, whereas between implantation sites both features remained at pre-implantation levels. It was concluded that trophoblast induced catabolism of intra-epithelial neutral lipids at implantation, possibly by releasing oestradiol, which releases precursors for metabolism or re-synthesis (Boshier, 1976).

In the domestic cat endoderm has been implicated in steroid metabolism because of its ultrastructural features and the presence of 17ß hydroxy steroid dehydrogenase, the enzyme which catalizes oestradiol synthesis (Tiedemann, 1976). Perhaps in the dunnart, uterine epithelium, trophoblast and endoderm co-operate in steroid biosynthesis or at least metabolism. To test this these three cell types need to be screened immunocytochemically or histochemically for the presence of enzymes of the steroid biosynthetic pathway, particularly 3ß hydroxy steroid dehydrogenase which converts pregnenolone to progesterone (Galil and Deane, 1966), and 17ß hydroxy steroid dehydrogenase.

Studies on endocrine function in marsupial pregnancy have shown that, in some species at least, the yolk sac placenta does secrete steroid hormones (Renfree, 1977; Bradshaw *et al.*, 1975; Heap *et al.*, 1980) which may have a local morphogenetic effect on the uterine tissue (Renfree, 1970, 1972, 1980). In *Antechinus stuartii*, there are histological differences in the uteri of pregnant and non-pregnant females which correlate with plasma progesterone, differentiation of the corpus luteum, and the stage of embryonic development (Cruz and Selwood, 1993). There is evidence, then, to suggest that the yolk sac placenta of marsupials is indeed an endocrine organ but that perhaps it has only local effects on the endometrium without necessarily raising peripheral plasma levels of progesterone. This occurs in the pregnant mare in which a significant amount of progesterone is produced by the placenta but it is not transported systemically but is metabolised by the uterus (Heap *et al.*, 1973). There have not been any studies to localise the embryonic cell population responsible for this local morphogenetic effect on the pregnant marsupial uterus.

Electron-dense material found within trophoblast and at the feto-maternal interface appears to be trophoblastic in origin. Its occurrence appears to coincide with the PAS positive, amylase resistant and alcian blue positive material described in chapter 6. This material in paraffin sections was not seen in the endometrial gland lumina which suggests that it was not a uterine glandular secretion. It was also never seen within uterine luminal epithelial cells, nor was there electron-dense material in this cell layer

which could account for the volume of secreted material. This contrasts with a similarly toluidine blue positive and electron-dense material at the feto-maternal interface in the interareolar part of the porcine placenta which, although of similar appearance in electron micrographs, is also present in both the endometrial luminal and glandular epithelia and was considered to comprise the histiotrophe (Dantzer *et al.*, 1981).

In the dunnart there was a variation in the electron density of vesicles within different regions of the yolk sac trophoblast such that vesicles in the unattached BYS were more heterogeneous and less dense than those in the attached BYS adjacent to invasive trophoblast. This may indicate differences in protein composition of vesicles between these two regions.

Although it is well known that clathrin coated vesicles are associated with endocytosis there are cases where clathrin coated secretory vesicles are found (Carr and Toner, 1982; Marsh and Quinn, 1991; Alberts *et al*, 1994). In some cases the protein coating of secretory granules is composed of coatamer, not clathrin. Coatamer is a large protein complex consisting of seven individual coat protein subunits (Alberts *et al.*, 1994). Coatamer coated vesicles are also found in transit between various organelles (RER and golgi complexes) and the plasma membrane and it has been suggested that protein coating of vesicles is important in intracellular transport (Alberts *et al.*, 1994). Consequently, sometimes it is difficult to ascertain whether exocytosis or endocytosis is taking place (Plattner, 1991). Routine TEM cannot discriminate between the proteins which coat vesicles so immunogold labelling of these vesicles with monoclonal antibodies to clathrin and coatamer is needed to distinguish the two.

During the 1960s research on the nature of the immunological barrier between the fetus and maternal tissues showed that a secretion coated the trophoblast cells which was PAS positive, amylase resistant and rich in hyaluronic and sialic acids. This was called 'placental fibrinoid' and it was thought to prevent the passage of fetal antigens from the surface of the trophoblast and, therefore, prevents immunological rejection of the fetus by the mother (Kirby *et al.*, 1964; Bradbury *et al.*, 1965; Jones and Kemp, 1969; Behrman (1971). Bulmer and Dickson (1960) had previously described this secretion and found that it was particularly evident in the TGCs of the rat placenta. Wynn (1971) also found a secretion in the epitheliochorial placentae of ungulates that coated the trophoblast and referred to it as a sialomucin coat rather than fibrinoid, and also thought that it may contribute to the immunological protection of trophoblast. It was later suggested that this sialomucin coat may even be a chorionic gonadotrophin (CG) and prevents immunological rejection because it preferentially binds to

lymphocytes such that they are unable to bind to trophoblast cells (Amoroso and Perry, 1975). However, when the placentae of other eutherians were examined no such coating of trophoblast was found (Wynn, 1971; Amoroso and Perry, 1975) but this does not exclude the possibility that this is a mechanism to prevent immunological rejection in some species.

Endometrial cup cells of the horse placenta secrete huge quantities of a chorionic gonadotrophin, pregnant mare's serum gonadotrophin (PMSG) (Steven, 1982). In the past it was thought that this was secreted by maternal epithelial cells in this region but ultrastuctural observations have confirmed that these cells are, in fact, trophoblast cells that migrate into the endometrial cup tissue (Allen *et al.*, 1973). PMSG, like other CGs, is a PAS positive glycoprotein (Steven, 1982) and has a very high sialic acid content (Amoroso and Perry, 1975). Chorionic gonadotrophins have been found in a variety of eutherian species (Heap and Flint, 1984).

Renfree and Hobson (unpublished observations cited in Tyndale-Biscoe and Renfree, 1987) found that, using the mouse uterine weight assay for chorionic gonadotrophin activity of Hobson (1983), CG biological activity is present in the placenta of the tammar wallaby. To determine whether the trophoblast secretion found in the dunnart is a CG, immunogold labelling at the TEM level is required.

Yolk sac placentation is extremely common and functionally significant in many eutherian species, particularly the domestic cat (Tiedemann, 1976, 1977, 1979), horse (Steven, 1982; Enders and Liu, 1991a), strepsirhine primates (King, 1993), bats (Wimsatt and Enders, 1980; Rasweiler, 1993), mouse (Poelmann and Mentink, 1982), rat (Padykula *et al.*, 1966; Deren *et al.*, 1966; Williams *et al.*, 1975a&b; Freeman *et al.*, 1981; Gupta *et al.*, 1982; Freeman and Lloyd , 1983; Hartfield *et al.*, 1989; Grub *et al.*, 1991; Beckman *et al.*, 1994) and guinea-pig (King and Enders, 1970; Schröder *et al.*, 1991).

In the domestic cat the vascular yolk sac placenta is present from days 13-24 *pc* during which time it carries out various functions, including fetal nutrition, primitive haemopoiesis, hepatic (metabolic) functions and steroid metabolism (Tiedemann, 1976, 1977). The nutritional functions are taken over after the broad attachment of the choriovitelline placenta is lost with the expansion of the allantois and the formation of the chorioallantoic placenta from day 24 (Tiedemann, 1976). However, near term trophoblast cells in the paraplacenta, polar zone and haematoma regions of the chorion are also involved in phagocytosis of the histolytic products formed by invasion into the endometrial stroma (Leiser and Enders, 1980a&b). These regions are thought

to form an important nutritional adjunct to the villous chorioallantoic placenta (Leiser and Enders, 1980a&b). Consequently, nutrition in the fetal cat relies on a combination of both haemotrophe and histotrophe from the entire surface of the chorion and is by no means confined to the traditionally defined chorioallantoic placenta.

In most strepsirhine primates the yolk sac is large early in gestation and a transient choriovitelline placenta develops (King, 1993). The yolk sac increases in size 8,000-fold between the attachment at the early blastocyst stage and the 18 somites stage when both vascular and avascular trilaminar regions are present as well as a bilaminar omphalopleure (avascular yolk sac, BYS). The yolk sac continues to increase in size until it is displaced by the growing allantois (King, 1993) whereas in haplorhine primates the secondary yolk sac does not form a placental attachment (Luckett, 1977; King, 1993). Even in haplorhines in which no choriovitelline placenta occurs the yolk sac has considerable functional significance including haemopoiesis, synthesis of serum and other proteins, and it is the site of origin of the primordial germ cells (Fujimoto *et al.*, 1977; King, 1993). In humans the yolk sac endoderm is the site of the first haemopoiesis (Takashina, 1987). In addition, mesothelial and endodermal cells of the macaque yolk sac have the ability to absorb exogenous proteins such that this may be an additional pathway for exchange, even in haplorhines.

Strepsirhine primates also develop a large allantoic sac from which a diffuse epitheliochorial placenta forms, although there may be small areas of trophoblast invasion (King, 1993). Additionally, the smooth chorion is absorptive and areolae develop above openings of uterine glands as occurs in a variety of ungulate species (Dantzer *et al.*, 1981; King *et al.*, 1982; Steven, 1982). In the black mastiff bat a choriovitelline placenta develops at early somite stages, a chorioallantoic diffuse labyrinthine endotheliochorial placenta in mid-gestation, and finally a discoidal haemochorial placenta late in pregnancy at the site of the endometrial vascular tuft (Rasweiler, 1990).

In rodents the yolk sac is thought to be important for fetal nutrition, both prior to the development of the chorioallantoic placenta (Freeman *et al.*, 1981) and through to term (Beckman *et al.*, 1994). However, the yolk sac is inverted such that the endoderm is in contact with the endometrium. Endoderm cells absorb proteins by pinocytosis which are digested by lysosomal enzymes (Williams *et al.*, 1975a&b; Freeman *et al.*, 1981; Beckman *et al.*, 1994). Amino acid products of digestion are incorporated into new proteins in the endoderm and secreted into the yolk sac cavity and the vitelline vessels for transport to the embryo (Deren *et al.*, 1966; King and Enders, 1970; Williams *et al.*, 1975a&b; Thomas *et al.*, 1990). The rat chorioallantoic placenta cannot synthesize and

deliver the required volume of protein at the necessary rate for fetal development late in pregnancy and so the yolk sac is probably essential for fetal nutrition (Beckman *et al.*, 1994).

The variety of placental associations in eutherian mammals, which are not confined to the definitive chorioallantoic placenta, places the marsupial yolk sac placenta in true perspective. Marsupial placentae are part of the continuum of placental evolution.

Epitheliochorial placenta may be the most advanced because it represents maximal resistance to placental invasion and low level invasion as occurs in the horse and ruminants, may be a means to deliver hormones. (Porter *et al.*, 1982). It has been suggested that highly invasive placentae are the most primitive as the eutherian groups which exhibit haemochorial placentation, insectivores, most rodents, lagomorphs, primates, many chiropterans and the armadillos, are most like the primitive mammalian stock because they have retained many ancestral anatomical characters (Pijnenborg *et al.*, 1981). Similarly, species with the least invasive placentae tend to belong to groups which also have a large number of highly derived anatomical characters like the artiodactyls and perissodactyls (Pijnenborg *et al.*, 1981). This has some plausibility when one considers invasiveness in marsupial placentae. The two dasyurids studied to date, the eastern quoll and the fat-tailed dunnart, develop endotheliochorial placentae (Hill, 1900; Roberts and Breed, 1994b; this thesis), and the peramelids form an invasive chorioallantoic placenta (Padykula and Taylor, 1976, 1982; Hughes *et al.*, 1990). These two marsupial families both retain primitive characters (Clemens *et al.*, 1979). By contrast the macropods which are considered to be a more recently evolved marsupial family with a suite of highly derived anatomical characters somewhat like the eutherian ungulates, develop a non-invasive yolk sac placenta. However, of the three American didelphids of which the placentae have been examined, two have non-invasive placentae, the North American opossum (Krause and Cutts, 1985b) and the grey short-tailed opossum (Harder *et al.*, *1993*) and the other, the four-eyed opossum, has discontinuous sites of trophoblast invasion (Enders and Enders, 1969). Perhaps at this stage it would be premature to speculate as to which is the more primitive.

In conclusion, I have found that, despite the presence of the shell membrane around embryos for more than 2/3 of pregnancy, an invasive placenta develops in the dunnart which, although associated with the yolk sac, has feto-maternal cell interactions similar to those of the carnivore chorioallantoic placenta which suggests nutrient uptake and possibly hormone production.

Further work is required on selected marsupial species to investigate the variations of implantation and placentation in this mammalian group. As marsupial reproduction is characterized by a shorter gestation followed by a longer period of lactation than in eutherians it may be that there has not been strong selective pressure to develop a chorioallantoic placenta in most marsupial families as the yolk sac forms the definitive placenta. Clearly, to imply that these animals are "aplacental" in contrast to the "placental" eutherians is quite erroneous.

Chapter 8 Concluding Discussion

8.1 Summary of Results

In this thesis I have shown that in the fat-tailed dunnart, *Sminthopsis crassicaudata*, the mucoid coat is secreted by non-ciliated epithelial cells of the ampulla and isthmus which have heterogeneous and homogeneous electron-dense secretory vesicles, respectively. After embryos have passed through the oviduct secretory granules in both regions are largely depleted. A shell membrane is first seen surrounding embryos which have just entered the uterus. Immunocytochemistry employing polyclonal antibodies raised against the extracellular coats of the oocyte/embryo and histochemistry have revealed that the shell membrane is secreted by non-ciliated luminal epithelial cells of the utero-tubal junction and adjacent glands in the cranial pole of the uterus and by scattered glandular and luminal epithelia in the remaining endometrium. Secretory granules containing fine granular shell membrane precursors are comparatively electron-lucent and are particularly abundant in the glands of the utero-tubal junction.

When the shell membrane is first deposited it is thin and irregular but rapidly becomes very electron-dense, granular and compact. During unilaminar and bilaminar blastocyst stages the shell membrane loses its granular appearance, except for on the outer surface where it is still being deposited, and becomes fibrous. It is still being secreted at the bilaminar blastocyst stage but its deposition appears to have ceased around early primitive streak embryos. The shell membrane has been shed at the early somite stage (about day 10 of pregnancy) allowing implantation to proceed.

Embryos are apposed to the uterine luminal epithelium for several days during blastocyst differentiation. After the loss of the shell membrane, trophoblast adjacent to the embryo from the trilaminar region of the yolk sac (TYS) appears to fuse with uterine luminal epithelial cells. Therefore, implantation is centric but is not confined to any particular endometrial region with embryos being attached mesometrially, anti-mesometrially, or a combination of both.

On the last two days of pregnancy trophoblast in the TYS is non-invasive but in the bilaminar yolk sac (BYS), in an annular region adjacent to the sinus terminalis, multinucleate trophoblast penetrates the uterine luminal epithelium by intrusion, and possibly fusion. Trophoblast giant cells, which may be syncytial, erode the endometrium as far as the maternal endothelium, thus an endotheliochorial placenta develops. Trophoblast surrounds and phagocytoses uterine epithelial cells, occasional

glands and a variety of maternal stromal cells. These presumably comprise histolytic nutrition for the embryo. In response the uterine endothelium secretes a thickened basal lamina which may be contain factors which protect the endothelium from trophoblast invasion.

Invasive trophoblast has abundant smooth and rough endoplasmic reticulum, mitochondria with both tubular and lamellar cristae, widely dilated golgi complexes and predominantly euchromatic nuclei, each with a prominent nucleolus. These features indicate peptide, and possibly steroid, biosynthesis. Non-invasive trophoblast has a similar suite of organelles except that there is no smooth endoplasmic reticulum. Non-invasive trophoblast in the BYS is comparatively electron-dense with heterogeneous secretory granules which are alcian blue and PAS positive. Secretion with similar histochemical staining reactions is present at the feto-maternal interface and may be similar to placental fibrinoid of some eutherian species or, alternatively, may be a glycoproteinaceous hormone.

Late in pregnancy the TYS is non-invasive and follows the folded contours of the uterine luminal epithelium forming an epitheliochorial placenta. Trophoblast microvilli interdigitate with those of the maternal epithelium and in regions where exchange appears to take place the distance between fetal and maternal blood is reduced to 4μm with extreme attenuation of the intervening cell layers.

The allantois approaches but does not fuse with the chorion late in pregnancy.

Thus I have found that, despite the presence of the shell membrane around embryos for more than 2/3 of pregnancy, an invasive placenta develops in the dunnart which, although associated with the yolk sac, has feto-maternal cell interactions similar to those of the carnivore chorioallantoic placenta; this suggests nutrient uptake and possibly hormone synthesis and secretion.

8.2 Marsupial Mode of Reproduction

The path that the ureters and Müllerian / Wolffian ducts follow in therians differs to that of prototherians and sauropsids. Tyndale-Biscoe and Renfree (1987) argued that this is because of a change in excretory function rather than reproduction although it has obviously resulted in profound effects on the structure of the reproductive tract. The ureters in embryonic metatherians and eutherians migrate to enter the bladder ventrally, rather than dorsally in the wall of the urogenital sinus opposite the urethral openings of the bladder as occurs in monotremes and sauropsids. To reach the ventral wall of the bladder the ureters must follow one of two possible routes: in eutherians

they are lateral to the Müllerian / Wolffian ducts and in marsupials they develop medially. This results in the separation of the marsupial reproductive tract at the level of the vagina such that there are two lateral vaginae which terminate in the urogenital sinus (Sharman, 1965, 1970, 1976; Barbour, 1977). Despite this, there is some fusion of the female reproductive tract in the midline at the level of the cervices resulting in the anterior vaginal cul-de sac. In some species a median pseudo-vaginal canal opens at each parturition, while in others, *Didelphis*, *Tarsipes* and the macropods, the median pseudo-vagina remains open after the first parturition (Sharman, 1965, 1970, 1976; Barbour, 1977). By contrast, in eutherians there is always fusion of the Müllerian ducts at the level of the vagina, often at the cervix, and, in primates, a simplex uterus results from more extensive fusion. It is possible that the lack of a true vagina has effected the reproductive strategy which marsupials employ.

In marsupials the proportion of time spent *in utero* as opposed to suckling is relatively much less than that of eutherians. For up to 2/3 of intrauterine life the embryo is unattached and surrounded by the shell membrane. This is followed by a short period of embryonic attachment during which time a placenta rapidly develops. Neonates continue their development during a comparatively long period of lactation. This strategy is considered to be an alternative, rather than inferior, reproductive mode to that of eutherians (Renfree, 1983, 1993b).

It has been argued that the marsupial reproductive strategy is actually more efficient in terms of energy costs of reproduction than that of eutherians, particularly when the losses of embryos, neonates and juveniles are considered (Parker, 1977; Low, 1978; Hayssen *et al.*, 1985). Marsupials may abandon their young after birth with less cost to the mother than eutherians because of their short gestation period. Low (1978) argued strongly that it is less costly in terms of parental investment†, not merely energetics, for gestation to be as short as possible in uncertain environments. The actual energy cost is not all that should be examined here but the cost to future reproduction is also affected. The risk to the marsupial mother that abortion poses is less than that in an equivalent eutherian in late pregnancy because the marsupial birth weight is much lower in proportion to maternal weight. The marsupial litter never weighs more than 1% of maternal body weight as compared with rodents which can have litters that weigh over 50% of the maternal body weight (Lee and Cockburn, 1985).

---

†Parental investment includes energetics of reproduction as well as the cost and risks of mating, pregnancy and subsequent care and the risk to future reproductive potential with regard to an individual offspring (Low, 1978).

Also the cost of abortion to future reproduction is less in marsupials than in eutherians because the short gestation period is almost always within one oestrous cycle (there are two exceptions according to Low (1978), the swamp wallaby and the grey kangaroo). Pregnancy, therefore, does not interfere with subsequent ovulations and the animal can become pregnant again at the next oestrus. A marsupial is also able to abandon pouch young if necessary when being pursued by a predator or if food or water are in short supply while a female eutherian in late pregnancy cannot eliminate her fetuses quickly in order to save her own life.

8.2.1 Altriciality

Marsupials are distinguished by the fact that they produce altricial young after their short gestation period. Their neonates are significantly less developed than those of rodents, for example, which are considered altricial for the subclass. At birth marsupials appear to be no more advanced than early eutherian fetuses although they exhibit precocious development of structures that are necessary for extra-uterine survival (Kirsch, 1977; Tyndale-Biscoe and Renfree, 1987). The forelimbs have separate digits with deciduous claws and are required for the long journey to the teat; the lungs do not have alveoli but well vascularized bronchioles capable of gaseous exchange occur; the kidneys are mesonephric and functional; the alimentary system is complete with a particularly mature pancreas, gall bladder and the stomach is able to digest milk; the olfactory bulbs are well developed and the regions of the CNS necessary for neural transmission to these structures are precocious (Lee and Cockburn, 1985).

Tyndale-Biscoe and Renfree (1987) argued that altriciality in marsupial and monotreme neonates is symplesiomorphic and that the comparative precociality of eutherians at birth is apomorphic. Hayssen *et al.* (1985) suggested to the contrary that the immaturity of marsupials at birth is a highly derived character and cited the case of reptiles with precocious young that require little or no parental care after birth or hatching as evidence. They assume that the mammal-like reptiles, the therapsids, similarly required less maternal care than modern metatherians. So, for these authors, neonatal altriciality and the further development of lactation are apomorphic for marsupials. Lactation in the early therians must have been apomorphic compared to the non-lactating condition of reptiles.

In a study on the intrinsic rate of population increase in altricial and precocial eutherians, Hennemann (1984) argued that in small mammals it is advantageous to give birth to altricial young. First, small mammals are able to reproduce at an earlier

age if their offspring are small, often in their first year of life whereas small mammals that have large precocial neonates may not be able to reproduce until the second breeding season when they achieve full adult body size. Secondly, altricial species give birth to larger litters and therefore produce more offspring per year (Henneman, 1984). Extrapolating to marsupials a similar line of reasoning could be made for the carnivorous species but the large macropods have retained the altriciality even though it may not be particularly advantageous to animals of more than 500g (Henneman, 1984). In addition, macropods have evolved post-partum oestrus and delayed implantation which results in the capacity to increase population rapidly rather than by increasing litter size (Tyndale-Biscoe and Renfree, 1987).

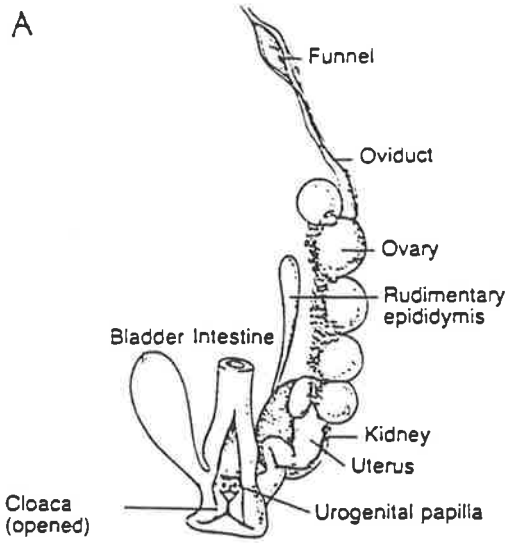
Lillegraven *et al.* (1987) argued that marsupial reproduction requires a greater time from conception to weaning than in comparable-sized eutherians and is therefore energetically more costly. Case (1978) examined the advantages of altriciality, parental care and endothermy and found that these three factors are intimately related. He contended that because altricial neonates are ectothermic they have low metabolic demands and can channel more of the nutrients from their mother's milk into growth than can endothermic precocial neonates. This also means that there is a lower energetic cost of early lactation in animals that give birth to altricial young. In the face of this argument Lillegraven's *et al.* (1987) assessment of the energetics of marsupial reproduction seems lacking.

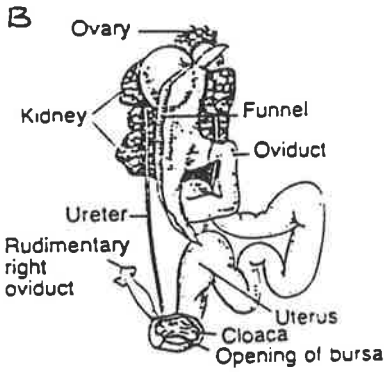
8.3 Shell Membrane Homology

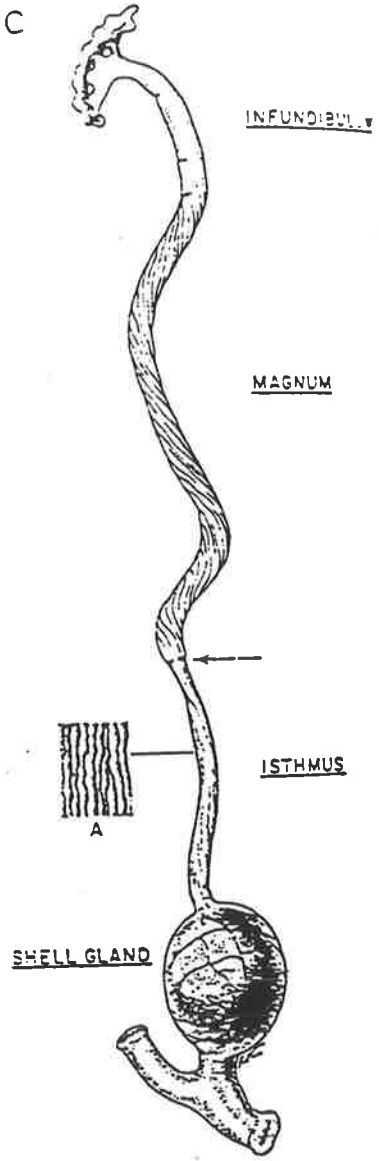
The gross morphology of the reproductive tracts of reptiles, birds, monotremes and marsupials is given in figure 8.1. The female reproductive tract proximal to the vagina in all vertebrates except cyclostomes and teleosts is derived from the Müllerian ducts (Wake, 1985). The regions of the reproductive tract in reptiles, birds and mammals have been given different names but, as they have the same embryonic derivation, they should be considered homologous.

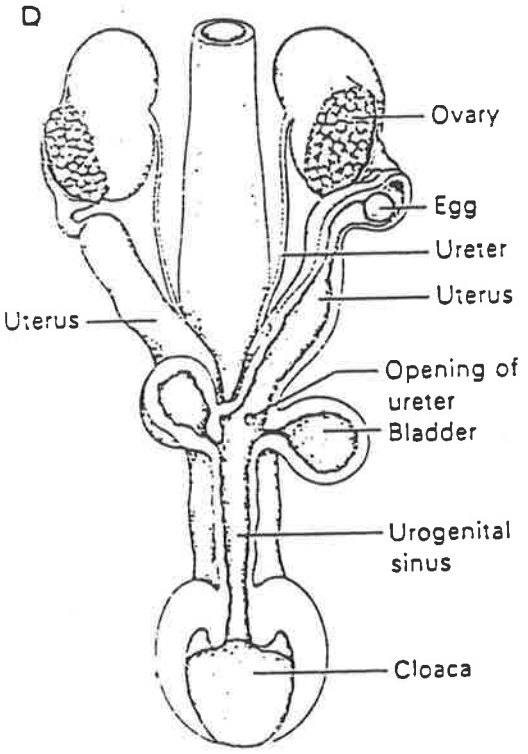
Figure 8.1 Female Reproductive Tracts.

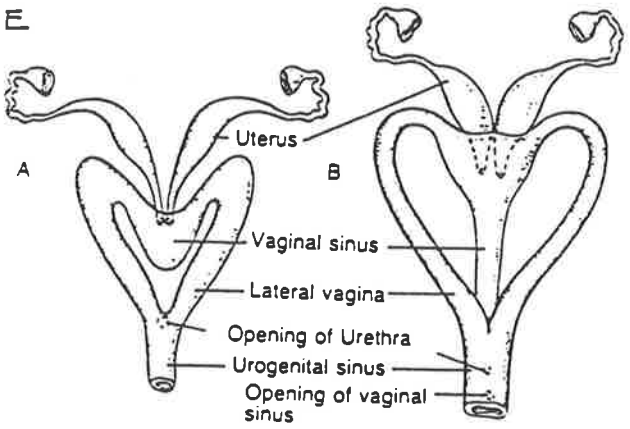
A. Lizard (from Romer and Parsons, 1986, p. 412.).
B. Pigeon (from Romer and Parsons, 1986, p. 413).
C. Japanese Quail (from Hoffer, 1971, p. 254).
D. Echidna (from Sharman, 1976, p. 39).
E. Kangaroo (from Romer and Parsons, 1986, p. 432).


A
Funnel
Oviduct
Ovary
Rudimentary epididymis
Bladder Intestine
Kidney
Uterus
Cloaca (opened)
Urogenital papilla


B
Ovary
Kidney
Funnel
Oviduct
Ureter
Rudimentary right oviduct
Uterus
Cloaca
Opening of bursa


C
INFUNDIBUL
MAGNUM
ISTHMUS
A
SHELL GLAND


D
Ovary
Egg
Ureter
Uterus
Uterus
Opening of ureter
Bladder
Urogenital sinus
Cloaca


E
Uterus
A
B
Vaginal sinus
Lateral vagina
Opening of Urethra
Urogenital sinus
Opening of vaginal sinus

A little digression here into what should be considered to be homologous is necessary. According to Ridley (1993) a homologous character is one that is shared by a set of species and was present in their common ancestor and according to Curtis and Barnes (1989) homology is when there is similarity in structure and/or position, assumed to result from a common ancestry, regardless of function, such as the wing of a bird and the forelimb of a mammal. Hence, the most parsimonious conclusion that can be drawn is that the morphology of amniote female reproductive tracts, as well as their secretions, are homologous, irrespective of their function due to their common embryological origin.

In each amniote group the proximal end of the oviduct is always dilated or funnel shaped and assists in egg pick-up after ovulation. The rest of the oviduct is usually divided into two regions which secrete either mucinous or proteinaceous secretions which either come to surround the newly fertilized egg in a visible layer (albumen in the sauropsids and mucin in monotremes, marsupials and a few eutherian species) or have a role at fertilization and in early embryonic development during the tubal life of the embryo in many eutherian species. The most distal region tends to be more glandular and is responsible for secretion of the calcareous or leathery shell in oviparous species. It is thought that the mammalian uterus has evolved from this glandular region of the oviduct of oviparous ancestors and the glandular secretions have been sequestered for nutrition of the embryo (Mossman, 1987).

Most eutherian species have a specialization of the yolk sac, chorioallantoic or para-placental regions which are able to absorb endometrial secretions. Even in humans the smooth chorion apposed to the parietal decidua is able to absorb the endometrial glandular secretions (Mossman, 1987). Although the chorion here is non-vascular it is thought that the digested products are transported from trophoblast through the mesoderm of the chorioallantois and adjacent fused amnion and into the amniotic fluid and are then ingested orally by the fetus (Mossman, 1987). Thus, the eutherian oviduct and uterus and its secretions are considered homologous to those of their oviparous ancestors but their functions have changed dramatically with the evolution of viviparity (Mossman, 1987).

8.4 Egg and Fetal Membranes in Oviparous Amniotes

In sauropsidan oviparous species the female reproductive tract is regionally specialized to secrete precursors of the albumen layer, which is thick in birds, turtles and crocodilians but thin in lizards and snakes, as well as the inner and outer shell membranes and shell (Mossman, 1987).

The fetal membranes of the domestic hen have been the most well-studied of all oviparous amniote species (Mossman, 1987). During the first three days early embryonic differentiation occurs and extra-embryonic epiblast migrates to surround the yolk and form the yolk sac (Luckett, 1977; Dumont and Brummett, 1985). Subsequent incubation can be divided into two phases when fetal membranes are important. During days 4-8 the yolk sac vasculosa is the principal site for gaseous exchange and uptake of yolk (Luckett, 1977; Reeves, 1984). On each of these days the oxygen requirement of the embryonic chick doubles. The allantois first makes contact with the chorion on day 6 and is completely attached by day 12. In the second phase of incubation (days 8-18) the chorioallantois becomes the most important fetal membrane for respiratory exchange (Luckett, 1977; Reeves, 1984). It is believed that the vitelline vessels cannot deliver enough oxygen to the embryo to sustain development by days 10-12, the allantoic vessels are more efficient but still the developing chick suffers progressive hypoxia which may be the stimulus to hatching (Reeves, 1984).

It is important to note that in the cleidoic egg the chorionic ectoderm associated with the yolk sac and allantois is principally an oxygen exchanger and it is the yolk sac endoderm which supplies yolk (nutrition) for the embryo throughout incubation (Morriss, 1975; Mossman, 1987). Also besides its role in respiration the allantois stores uric acid and then the whole structure is left behind at hatching (Morriss, 1975). In fact it seems that the allantois cannot expand and fuse with the chorion, and therefore fulfil its respiratory function, unless it is distended by the mesonephric excretions, as experimental obstruction of the mesonephric ducts prevents allantoic expansion and chorioallantoic membrane formation (Boyden, 1924). During the last day of incubation the yolk sac, with remaining yolk and fluid, is withdrawn into the abdominal cavity so that after hatching the chick has a small nutritional store upon which it can depend for the first few days after hatching (Mossman, 1987).

Electron microscopy of the fetal membranes of the chick has shown that the chorioallantoic membrane is adapted for maximizing gaseous exchange (Mossman, 1987). The allantoic capillaries indent the chorionic ectoderm such that there is only a thin layer of ectodermal cytoplasm intervening between the very thin capillary endothelium and the shell membrane (Mossman, 1987). This is reminiscent of the attenuation of intervening cell layers between the maternal and fetal blood in eutherian epitheliochorial and endotheliochorial placentae (Enders, 1982).

8.5 Fetal Membranes in Intermediate Forms

It is generally accepted that viviparity has evolved from oviparity (Hogarth, 1976; Luckett, 1977; Shine, 1984, 1985; Guillette, 1991; Mossman, 1987) and that during the course of its evolution there have been intermediate forms e.g. ovoviviparity (Shine, 1985). The fetal membranes in oviparous and ovoviviparous species are very similar so the changes that have taken place during evolution to allow retention of embryos have been essentially the physiological adaptations of the mother (Mossman, 1987) which are a consequence of the development of the corpus luteum and the prolongation of the cycle by progesterone forming the luteal phase (Amoroso, 1981). There are a large number of squamate species which retain eggs *in utero* and the embryo undergoes substantial development prior to oviposition, usually relying on the yolk for nourishment, and therefore these species are ovoviviparous (Yaron, 1985). In reptiles the longevity of the corpora lutea and the length of time that eggs are retained *in utero* are correlated (Yaron, 1985).

Monotremes are oviparous, although there is significant transfer of nutrients and gases between mother and embryo *in utero* prior to oviposition through the acellular coats which surround the developing embryo (Luckett, 1977). Therefore, monotremes can be considered to be an intermediate group in the development of viviparity. At ovulation the oocyte is about 4mm in diameter and grows to about 17mm during intrauterine 'gestation' (Luckett, 1977; Hughes, 1993). There is a prolonged period of intra-uterine development (about 18 days) during which time nutrients are taken up from uterine secretions by the yolk sac through the shell membrane. The yolk supply is too meagre to sustain the embryo for the whole period of development. The endoderm of the yolk sac must be involved in the uptake of the yolk and presumably trophoblast of the yolk sac is involved in gaseous exchange and nutrient uptake from maternal secretions. At oviposition the egg measures 16 by 18mm, the embryo has 19-20 somites, and the neural tube has begun to form. This is comparable to the stage at which the shell membrane is lost in many species of marsupials although a little later than in the dunnart. After oviposition eggs are incubated for about a further 10 days before hatching (Hughes, 1993). The allantois in monotremes does not develop and fuse with the chorion until the last 4-6 days of the 10 day incubation of the egg (Luckett, 1977; Hughes, 1993) and therefore trophoblast associated with it cannot be responsible for nutrient uptake but it presumably has only a respiratory function.

In addition the allantois occupies only half of the surface area of the conceptus in monotremes with the TYS encompassing the rest, whereas in all other oviparous

amniotes the chorioallantois spreads over the entire surface (Mossman, 1987). It may be that in monotremes the yolk sac has taken a more dominant role in nutrient and gaseous exchange with the mother than is found in other oviparous species.

8.6 Viviparity

Viviparity requires a reduction in the thickness and, in many cases, later loss of the shell membrane and hence a reduction in the number of shell glands within the endometrium. Viviparity also requires the development of a glandular endometrium capable of producing copious secretion under the influence of progesterone which is characteristic of all mammals and some squamates (Tyndale-Biscoe and Renfree, 1987).

The shell membrane in marsupials does not preclude the occurrence of viviparity and seems to play a supportive role in cleavage and pre-implantation development (Selwood, 1989). Once the shell membrane is shed implantation and placentation can begin. However, in the viviparous garter snake the shell membrane is present throughout gestation and does not prevent feto-maternal exchange through the placenta (Hoffman, 1970).

Viviparity has evolved independently at least 95 times in squamates (Shine, 1984; Guillette, 1991) but probably only once in therians and has been conserved from an early mammalian ancestor. In viviparous lizards and snakes there have been few changes to the gross structure of the fetal membranes when compared to oviparous species (Mossman, 1987). However, three types of histological structure of the fetal membranes have been recorded (1) in which both the uterine epithelial cells and the chorionic ectoderm are relatively tall columnar cells (epitheliochorial), (2) in which, at the light microscope level of resolution, the maternal capillaries appear to protrude through the uterine epithelium into direct contact with the chorionic ectoderm (possibly endotheliochorial), and (3) in which the maternal capillaries appear to be in contact with the fetal capillaries (possibly endotheliochorial) (Weekes, 1935; Yaron, 1985; Mossman, 1987). Reptilian placentae appear to be capable of amino acid uptake although in many species the yolk is the sole nutrient source (Yaron, 1985). The placentae of all viviparous squamates absorb water and some also take up maternal electrolytes while a few species, which also have a reduced yolk supply, take up maternal amino acids (Yaron, 1985).

In most viviparous reptiles the corpus luteum of pregnancy secretes progesterone at much higher circulating levels than prior to ovulation, and lutectomy during pregnancy

results in embryo resorptions or abortions, or prolongation of pregnancy, and significant reduction of neonatal survival (Yaron, 1985).

The occurrence of yolk sac placentation in marsupials does not simply constitute a change in fetal nutritional source from the yolk to the mother. In birds, the endoderm is responsible for absorbing nutrients from the yolk and transferring them to the embryo. By contrast, in marsupials it is trophoblast which is responsible for both gaseous and nutrient exchange between mother and fetus, their eggs being microlecithal. Interestingly in rodents, the yolk sac also plays a significant role in embryonic development but again it is the endoderm of the inverted yolk sac which is the important cell type in transfer of nutrients to the embryo. In viviparous reptiles trophoblast is also the absorptive cell type.

It is possible that the yolk sac is a limiting factor in marsupial intrauterine development and, hence, the marsupial strategy of a short pregnancy with a long lactation and extra-uterine development. However, the benefits that the marsupial reproductive strategy offers may make the assumed inefficiency of the yolk sac irrelevant. My results have shown that trophoblast associated with the yolk sac in a marsupial can be highly invasive and may be a further development of yolk sac placentation. Also, the bandicoots are one of the oldest marsupial groups and develop a chorioallantoic placenta, yet still give birth to altricial young. So, assuming that allantoic placentation is more efficient at feto-maternal exchange, the apparatus appears to be in place to extend gestation in the same way that eutherians have done. But, chorioallantoic placentation does not occur in most marsupials.

The occurrence of yolk sac placentation and a variety of other paraplacental exchange regions in eutherians as described in chapter 7 indicate that the eutherian chorioallantoic placenta is not the be-all-and-end-all of placental exchange. Also perhaps a long gestation is not necessarily the best possible reproductive strategy. Macropod reproduction is highly derived with embryonic diapause and highly developed lactation which enables a female to have a diapausing blastocyst in the uterus, a young joey in the pouch and an older but still suckling joey at foot which both drink milk of different composition to suit their different needs (Cowie, 1984).

Hill (1910) and Hartman (1916) thought that the three extant mammalian groups evolved one from the other i.e. eutherians evolved from marsupials which evolved from monotremes. This is a somewhat simplistic view which assumes little change in the living groups of monotremes and marsupials. There appears to be some evidence that the three mammalian groups constitute a morphocline (Luckett 1977) as in many

respects marsupial reproduction does represent an intermediate between egg-laying and the relatively long, intimate feto-maternal contact of eutherian mammals (Kirsch 1977). The expansion of the allantois, with the development of the mesonephros, can be considered to be a primitive character present in the cleidoic egg which in oviparous ancestors led to the formation of the chorioallantoic membrane that is the principal site of gaseous exchange in oviparous birds, reptiles and monotremes. The evidence leads one to speculate that the last common ancestor of therians had a very similar oviparous reproductive mode to the extant monotremes. Perhaps then the eutherian lineage further developed the role of the chorioallantoic placenta, necessary for an extended gestation and the marsupial lineage developed yolk sac placentation with a short pregnancy and extended lactation.

8.7 Future directions

My research has essentially been a morphological description of the shell membrane, implantation and placentation in the fat-tailed dunnart and has revealed new information as well as enabled some speculation to be made on the evolution of viviparity in mammals. However, the next step is to perform experiments which will demonstrate the physiology of feto-maternal interactions in marsupials, particularly in dasyurids as they clearly have an invasive placenta.

8.7.1 Macromolecular Uptake by Pre- and Post-implantation Marsupial Embryos

In chapter 7 I argued that the yolk sac is involved in macromolecular uptake in a wide variety of eutherian species and that the ultrastructure of trophoblast in pre-implantation embryos and in the yolk sac placenta in the dunnart suggests that it is also actively involved in providing nutrients to the developing embryos.

A series of experiments on the tammar wallaby show that its yolk sac placenta is involved in macromolecular uptake and metabolism as yolk sac fluids contain amino acids, proteins and glucose of maternal, as well as fetal, origin and that there is a significant rise in their concentrations after implantation (Renfree, 1970, 1973). Also the composition of yolk sac fluids is very different to that of uterine luminal fluids (Renfree, 1973). However, there have been no investigations as to how this exchange takes place at the cellular level.

It would be appropriate now to assess macromolecular uptake by the trophoblast of both the bilaminar (avascular) and trilaminar (vascular) regions of the yolk sac placenta in the dunnart using tracer studies. Horseradish peroxidase should be injected intravenously into pregnant females which should then be examined at a

variety of times after injection using a method previously used for detection of endocytosis across the rat uterine epithelium and into the lumen (Parr, 1980) and TEM localization studies using the method of Schlafke and Enders (1973).

Pre-implantation uptake by trophoblast could be assessed by injection of ferritin into the uterine lumen of anaesthetized pregnant females. Using the method of Parr and Parr (1978) both LM and TEM studies could demonstrate the presence of iron, i.e. ferritin, at a series of times after the injection to demonstrate its uptake and fate.

Acid phosphatase activity in trophoblast and endoderm of the yolk sac could also be examined using the method of Barka (1960) to demonstrate the presence of lysosomes which would indicate that protein degradation occurs.

Autoradiography of sections of uteri with pre- and post-implantation embryos *in situ* could be performed on females which had been injected with either [^{3}H] uridine, [^{3}H] thymidine, [^{3}H] leucine, or [^{3}H] mannose to determine if RNA, DNA synthesis, or amino acid and carbohydrate uptake, respectively, occur.

In a further series of experiments embryos of pregnant females at different times 1-10 days *pc* should be incubated with either [^{3}H] uridine or [^{3}H] thymidine or [^{3}H] leucine and radioactivity counted after extraction of the TCA soluble fraction in a scintillation counter using the method of Shaw and Renfree (1986) to quantify RNA and DNA synthesis and amino acid uptake.

8.7.2 Endocrinology of Pregnancy

In eutherian mammals pre-implantation embryos are involved in biosynthesis of a variety of proteins and steroids which vary between species. Within 12 hours of the entry of rat embryos into the uterus they have increased both RNA and DNA synthesis as determined by radiolabelling studies suggesting an increase in metabolic activity (Heald *et al.*, 1975). Early pregnancy factor (EPF), a protein complex, secreted by the early embryo has been detected within the first few days after fertilization in women, mice and sheep and is thought to have immunosuppressive properties (Hodgen and Itskovitz, 1988). Pre-implantation eutherian embryos secrete a factor(s) which signal to the mother their presence and the response constitutes the maternal recognition of pregnancy. The identity of these factors varies between species and is not known for many. In sheep at least one of these factors is ovine trophoblastic protein (oTP-1), an acidic low-molecular weight polypeptide, which prolongs the life of the corpus luteum by inhibiting endometrial production of PGF_{2alpha} which is the luteolysin in sheep (Hodgen and Itskovitz, 1988). Early rabbit, rat, mouse, pig and hamster embryos

secrete oestradiol which may play a role in implantation (George and Wilson, 1978; Wu and Lin, 1982; Fischer *et al.*, 1985; Wu and Matsumoto, 1985). Oestradiol secretion by pre-implantation pig embryos stimulates secretion of protein from the uterine glandular epithelium which becomes part of the histotrophic nutrition for the embryo (Geisert *et al.*, 1982).

Most of the physiological work on the pre-implantation marsupial embryo has been performed on the blastocyst of the tammar wallaby during diapause and after reactivation at which time uptake of [^{3}H]leucine and [^{3}H]uridine increases. Labelled leucine is incorporated into secreted protein by day 4 after removal of pouch young and increased significantly by day 6 (Shaw and Renfree, 1986). However, there is no information of the control nor biosynthesis of pre-implantation embryonic development in dasyurid marsupials.

Radioimmunoassays have been developed for progesterone and oestradiol in wallaby serum (Fletcher and Renfree, 1988). Pre-implantation dunnart embryos could be cultured in the presence of pregnenolone or androstenedione and the supernatant (medium) assayed for progesterone or oestradiol 17ß respectively. The findings would indicate whether preimplantation embryos are synthesizing steroids. If steroids are found, immunolocalization of enzymes involved in steroid biosynthesis and the hormones using commercially available monoclonal antibodies should be performed at both the LM and TEM levels to identify the specific cell populations involved.

Pregnenolone is converted to progesterone by 3ß hydroxy steroid dehydrogenase in eutherian luteal tissue and the chorioallantoic placenta. Using a slight modification of an established enzyme histochemical method for this enzyme (Galil and Deane, 1966) fresh frozen sections could be incubated with pregnenolone to determine whether this steroid dehydrogenase is present in the yolk sac of the dunnart and, by implication, progesterone synthesis by the placenta. If present ultrathin plastic sections could be immunogold labelled with anti-3ß hydroxy steroid dehydrogenase and anti-progesterone monoclonal antibodies to determine whether progesterone is synthesized by the trophoblast or transported to the placenta from another source. The same technique using monoclonal antibodies to 17ß hydroxy steroid dehydrogenase to examine oestradiol synthesis could also be carried out.

8.7.3 Endometrial Cell Proliferation during Pregnancy

In the pregnant tammar wallaby there is a difference in endometrial weight independent of the corpus luteum (Renfree, 1972) as well as the glandular secretions in the gravid and non-gravid uteri which suggest that the embryo has a morphogenetic

effect on the endometrium of the uterus in which it resides (Renfree and Tyndale-Biscoe, 1973). In the brown antechinus (a dasyurid) there are histological differences in the thickness of the myometrium, endometrial stroma and epithelium, the gland density and lymphocyte numbers at the uterine epithelial basal lamina between pregnant and non-pregnant individuals (Cruz and Selwood, 1993). Although not quantified, I have found that the size of the uterus during pregnancy in the fat-tailed dunnart appears to be correlated with the number of embryos present and is not merely a function of the volume that the embryos occupy and also is evident before implantation occurs. I suggest that in the dunnart, too, the embryo has a morphogenetic effect on the endometrium and this could be quantified by examining cell proliferation in the uterus during pregnancy.

In rats radiolabelling has shown that the morphogenetic effect of preimplantation embryos on the uterus results in increased synthesis of the luminal epithelium, stroma and myometrium (Heald *et al.*, 1975). A new method employing immuno-cytochemistry and utilising antibodies to proliferating cell nuclear antigen (PCNA) detects proliferating cells for a longer period than that of DNA synthesis as PCNA is expressed in the late G1 phase of cycling cells as well as the S phase. This has been used to examine endothelial, glandular and luminal epithelial and stromal cell proliferation in the human menstrual cycle (Goodger and Rogers, 1993) and the pregnant rat (Goodger and Rogers, 1994). By using a double staining method, one for PCNA and the other to identify specific intermediate filaments, different cell populations could be targeted. Antibodies to either cytokeratin (for intermediate filaments in cells of epithelial origin) or vimentin (for intermediate filaments in cells of mesenchymal origin) to identify endothelial cells (Khong *et al.*, 1986) could be used.

8.7.4 Implantation

In the mouse, acidic glycoproteins have been found on the surface of both the blastocyst and the uterine epithelium between Days 4 and 7 *pc* (peri-implantation period) after binding with concavalin-A, ruthenium red and colloidal thorium (Enders and Schlafke, 1974). There is also evidence that at this time glycosyl transferases may catalyse the binding of sugars between oligosaccharides on the cell coats of trophoblast and uterine epithelial cells thus forming molecular bridges between these cells (Schlafke and Enders, 1975). Studies with colloidal iron and cationized ferritin have also shown that the extracellular coats of the trophoblast are negatively charged at adhesion in several eutherian species (Enders and Schlafke, 1979). This adhesive stage is thought to occur in all eutherian species that exhibit implantation whether or not the conceptus is subsequently invasive.

At implantation in the ewe there are also changes in the glycocalyx of trophoblast and investigations with ruthenium red, cationized ferritin, concavalin-A-peroxidase and phosphotungstic acid in HCl revealed that the cell coat of trophoblast is composed of an acidic glycoprotein high in carbohydrate with many negative charges (Guillomot *et al.*, 1982).

There is no information on the composition of the cell coats of the embryo or the uterus at adhesion in any marsupial and this feature of implantation should be investigated in the dunnart. As there are already two distinct regions (the bilaminar, or avascular, and the trilaminar, or vascular) of the yolk sac at implantation in the dunnart and their invasive properties are different the trophoblast cell coats in the two regions should be compared.

To determine if there are molecular changes to the glycocalyx of embryonic and maternal cells at the adhesive stage of implantation the cell coats on trophoblast populations from the bilaminar and trilaminar regions of the yolk sac and on the uterine luminal epithelium should be examined prior to the loss of the shell membrane (day 8), at implantation (day 10), and after (day 12). Embryos, endometrium and yolk sac could be studied at the ultrastructural level by incubating tissue during or after fixation in ruthenium red, cationized ferritin, concavalin-A-peroxidase, phosphotungstic acid, colloidal iron, tannic acid or colloidal thorium and then processed for TEM using the methods of Enders and Schlafke (1974, 1979), Enders *et al.* (1980) and Guillomot *et al.* (1982).

Remodelling of the endometrial stromal extracellular matrix (ECM) during pregnancy should also be examined by immunogold labelling of collagen type I and laminin at implantation (day 10) and during placentation (day 11 and 12) using the method of Clark *et al.*, (1993) who detected changes in the rat ECM at implantation.

In the chorioallantoic placenta of the bandicoots there is evidence that fetal and maternal syncytia fuse to produce cells with two different nuclear types (heterokaryons) (Padykula and Taylor, 1982). Feto-maternal fusion at implantation is a more common event than was previously thought (Wooding and Flint, 1994). Larsen (1970), from light microscopical studies, has suggested that both fusion and intrusion are occurring at implantation in humans and my own observations in the dunnart suggest that this is also the case in this marsupial species (Roberts and Breed, 1994b, this thesis). In the rabbit, too, the "trophoblast pegs" seen subsequent to fusion at implantation (Enders and Schlafke, 1971a) may in fact be the result of intrusion as

well. Therefore, the hypothesis that intrusion follows fusion at implantation in species in which trophoblast subsequently invades the endometrial stroma should be tested.

At fusion, expression of cell surface glycoproteins and cell adhesion molecules on both trophoblast and maternal epithelium are presumably different than prior to implantation. Uvomorulin has been found to be associated with the development of the junctional complex between many types of epithelial cells. When uvomorulin was blocked with specific monoclonal antibodies the formation of junctional complexes between epithelial cells of the Madin-Darby kidney cell line was inhibited (Gumbiner *et al.*, 1988).

Therefore, the hypothesis that at implantation, and as trophoblast migrates, the expression of uvomorulin changes should also be tested. Prior to implantation, when trophoblast cells are bound to each other by junctional complexes, uvomorulin expression should be maximally expressed. At adhesion of trophoblast to maternal epithelium this may decrease and once epithelial penetration is underway uvomorulin could be down-regulated. If feto-maternal cell fusion is taking place there may be no change in uvomorulin expression but if intrusion also occurs there may be a transient loss of its expression to allow breakage of the junctional complexes between trophoblast cells and also between uterine epithelial cells which are then reformed as hybrid junctions between these two cell types. Also migrating trophoblast may lose the ability to express cell adhesion molecules as it loses its epithelial characteristics.

8.7.5 Immunology of Placentation

The embryo possesses genetic information from both its mother and father such that half of its genes code for proteins that are antigenically foreign to the mother. However, there is no simple explanation as to why the fetal allograft is not rejected by its mother. Villous trophoblast itself is only weakly antigenic but non-villous trophoblast does express class 1 MHC antigens (Sargent and Redman, 1985) and the embryo, as early as the eutherian inner cell mass (ICM) stage, also has histocompatability antigens (Beer and Billingham, 1979). Hogarth (1982) suggested that several factors may prevent fetal rejection during eutherian pregnancies. Firstly, decidualization of the uterine endometrium confers some protection to the fetus because decidual cells are packed closely together and may not allow access of immune cells to the trophoblast (Bell, 1983a&b). However, this cannot be the sole means of protection of the fetus from the maternal immune system as it is possible, but rare, for ectopic pregnancies to go to term (Kirby and Wood, 1967). Also, not all species exhibit decidualization (Amoroso, 1952).

Secondly, it has been suggested that the maternal immune response during pregnancy is suppressed (Beer and Billingham, 1976). The evidence shows this is not the case in humans and mice although lymphocyte function is altered in pregnancy in an as yet unclear way (Hogarth, 1982; Rodger and Drake, 1987). Steroid and glycoprotein hormones secreted by the feto-placental unit are in high enough concentration in the immediate area of the fetal membranes to suppress the maternal immune response locally, leaving it relatively intact systemically (Amoroso and Perry, 1975; Szekeres-Bartho *et al.*, 1985; Feinberg *et al.*, 1992). Pregnancy-specific serum proteins secreted by the trophoblast may act in a similar way. Early pregnancy factor (EPF), a protein complex, secreted by the early embryo has been detected within the first few days after fertilization in women, mice and sheep and is thought to have immunosuppressive properties (Hodgen and Itskovitz, 1988).

During murine pregnancy maternal natural suppressor cells of T lymphocyte origin suppress natural killer cells and cytotoxic T lymphocytes by inhibiting interleukin-2 (IL-2) uptake and hence prevent their activation at the materno-fetal interface (Brooks-Kaiser *et al.*, 1992). Lymphocytes from lymph nodes draining the pregnant mouse uterus secrete a factor(s) which reduces protein kinase C activity thereby decreasing synthesis of both IL-2 and its receptor (Blumenthal *et al.*, 1993). In pregnant pigs there is an elevation of natural killer cell numbers in the endometrium between days 10 and 20 of pregnancy but by day 30 they have declined to undetectable levels (Yu *et al.*, 1993) possibly due to suppression. Megasuppressin, a glycoprotein found in uterine fluid of pregnant sheep, suppresses lymphocyte proliferation *in vitro* (Stephenson *et al.*, 1989). Presumably, a factor released by the embryo either directly or indirectly induces these inhibitory changes in the pregnant uterus.

Kiso *et al.* (1990) speculated that in *Suncus murinus* maternal endothelial hypertrophy protects the fetus once the syncytiotrophoblast layer becomes discontinuous on Day 24 of gestation. However, this also does not occur in all eutherian species. It has also been suggested that in the black mastiff bat placenta endothelial hypertrophy of maternal capillaries of the endometrial tufts plays an important role in trophoblast growth and differentiation (Rasweiler, 1991, 1993).

Research in the last 15 years has shown that the female eutherian mammal during pregnancy is immunocompetent (Beer and Billingham, 1979; Sargent and Redman, 1985; Rodger and Drake, 1987) and that trophoblast is undoubtedly antigenic (Jenkinson and Billington, 1974; Billingham and Head, 1981).

There is a little evidence to suggest that in marsupial pregnancy, too, there is an as yet unknown mechanism(s) to prevent immunological rejection of the fetus. Pre-immunization of female tammar wallabies with skin grafts from their prospective male mates did not effect the success of pregnancy (Walker and Tyndale-Biscoe, 1978; Rodger *et al.*, 1985). My own observations of pregnant dunnarts found that there did not seem to be an inordinate number of maternal leukocytes in the endometrium during pregnancy and that trophoblast does not appear to be subject to immunological rejection by the mother. In female brown antechinus during pregnancy the density of maternal lymphocytes in the endometrium is similar to that in non-pregnant females except on days 1 and 15 when there are significantly more in pregnant animals (Cruz and Selwood, 1993). In this species implantation occurs on days 23 and pregnancy lasts 27 days so there would be more than enough time for rejection if there was immunological activation on day 15. A mixed lymphocyte reaction should be performed with splenic cells from a female after parturition with those from her male mate to see if there has been any immunization effect of pregnancy in the dunnart.

8.8 Conclusion

Although this is the end of my PhD research it is really only the beginning of work in an interesting and understudied area. I predict that in the future there will be an increased interest in yolk sac placentation, especially when one considers that there is substantial pre-implantation loss in many domestic species, particularly the ungulates, at the time that the yolk sac is functional as a region of exchange.

References

Abbie A.A. (1941) Marsupials and the evolution of mammals. Aust. J. Sci. **4**, 77-92.

Abe H. (1994) Regional variations in the ultrastructural features of secretory cells in the rat oviductal epithelium. Anat. Rec. **240**, 77-85.

Abe H., Numazawa C., Abe M., Onodera M. and Katsumi A. (1993) Immunocytochemical localization of oviduct-specific glycoproteins in the oviductal epithelium from cows at follicular and luteal phases. Cell Tiss. Res. **274**, 41-47.

Abe H. and Oikawa T. (1991) Regional differences in the ultrastructural features of secretory cells in the golden hamster (*Mesocricetus auratus*) oviductal epthelium. J. Anat. **175**, 147-158.

Alberts B., Bray D., Lewis J., Raff M., Roberts K. and Watson J.D. (1994) Molecular Biology of the Cell 3rd. ed. Garland Publishing Inc., New York.

Allen W.R. (1975) Endocrine functions of the placenta. In: Steven D.H. (ed.) Comparative Placentation Academic Press, London, pp. 214-267.

Allen W.R., Hamilton D.W. and Moor R.M. (1973) The origin of equine endometrial cups. II. Invasion of the endometrium by trophoblast. Anat. Rec. **177**, 485-502.

Amoroso E.C. (1952) Placentation In: Parkes A.S. (ed.) Marshall's Physiology of Reproduction Vol. 2, 3rd. ed. Longmans, London, pp.127-311.

Amoroso E.C. (1981) Viviparity. In: Glassock S.R. and Bullock D.W. (eds.) Cellular and Molecular Aspects of Implantation Plenum Press, New York, pp. 3-25.

Amoroso E.C. and Perry J.S. (1975) The existence during gestation of an immunological buffer zone at the interface between maternal and fetal tissues. Phil. Trans. R. Soc. Lond. B. **271**, 343-361.

Amoroso E.C. and Perry J.S. (1977) Ovarian activity during gestation. In: Zuckerman S. and Weir B.J. (eds.) The Ovary Vol. 2, 2nd. ed., Academic Press, New York. pp. 316-398.

Anderson D.J., Stoyan N.C. and Ricklefs R.E. (1987) Why are there no viviparous birds? A comment. Am. Nat. **130**, 941-947.

Anderson L.L. (1978) Growth, protein content and distribution of early pig embryos. Anat. Rec. **190**, 143-154.

Baggott L.M., Davis-Butler S. and Moore H.D.M. (1987) Characterization of oestrus and timed collection of oocytes in the grey short-tailed opossum, *Monodelphis domestica* J. Reprod. Fert. **79**, 105-114.

Baggot L.M. and Moore H.D.M. (1990) Early embryonic development of the grey short-tailed opossum, *Monodelphis domestica in vivo* and *in vitro*. J. Zool. Lond. **222**, 623-639.

Bancroft B.J. (1973) Embryology of *Schoinobates volans* (Kerr) (Marsupialia : Petauridae) Aust. J. Zool. **21**, 33-52.

Barbour R.A. (1977) Anatomy of marsupials. In: Stonehouse B. and Gilmore D. (eds.) The Biology of Marsupials Macmillan, London, pp. 237-272.

Barka T. (1960) A simple azo-dye method for histochemical demonstration of acid phosphatase. Nature **187**, 248.

Beckman D.A., Brent R.L. and Lloyd J.B. (1994) Pinocytosis in the rat visceral yolk sac: potential role in amino acid nutrition during the fetal period. Placenta **15**, 171-176.

Bedford J.M. (1991) The coevolution of mammalian gametes. In: Dunbar B.S. and O'Rand M.G. (eds.) A Comparative Overview of Mammalian Fertilization. Plenum Press, New York, pp. 3-35.

Bedford J.M. and Breed W.G. (1994) Regulated storage and subsequent transformation of spermatozoa in the fallopian tubes of an Australian marsupial, *Sminthopsis crassicaudata*. Biol. Reprod. **50**, 845-854.

Beer A.E. and Billingham R.E. (1976) The Immunobiology of Mammalian Reproduction Prentice-Hall, Englewood Cliffs.

Beer A.E. and Billingham R.E. (1979) Maternal immunological recognition mechanisms during pregnancy. In: Maternal Recognition of Pregnancy. Ciba Foundation Symposium **64** (new series) Excerpta Medica, Amsterdam, pp. 293-322.

Behrman S.J. (1971) Implantation as an immunologic phenomenon. In: Blandau R.J. (ed.) Biology of the Blastocyst University of Chicago Press, Chicago, pp. 479-494.

Bell S.C. (1979) Immunochemical identity of 'decidualization-associated protein' and alpha$_2$ acute-phase macroglobulin in the pregnant rat. J. Reprod. Immunol. **1**, 193-206.

Bell S.C. (1983a) Decidualization and associated cell types: implications for the role of the placental bed in the materno-fetal immunological relationship. J. Reprod. Immunol. **5**, 185-194.

Bell S.C. (1983b) Decidualization: regional differentiation and associated function Oxford Rev. Reprod. Biol. **5**, 220-271.

Benirschke K. and Kaufmann P. (1990) Pathology of the Human Placenta 2nd. ed. Springer-Verlag, New York.

Bennett J.H., Breed W.G., Hayman D.L. and Hope R.M. (1990) Reproductive and genetical studies with a laboratory colony of the dasyurid marsupial *Sminthopsis crassicaudata*. Aust. J. Zool. **37**, 207-222.

Billingham R.E. and Head J.R. (1981) Current trends in reproductive immunology: an overview. J. Reprod. Immnnol. **3**, 253-265.

Billington W.D. (1971) Biology of the trophoblast. In: Bishop M.W.H. (ed.) Advances in Reproductive Physiology Logos Press, London, pp. 27-66.

Blackburn D.G. (1993) Chorioallantoic placentation in squamate reptiles: structure, function, development, and evolution. J. Exp. Zool. **266**, 414-430.

Blackburn D.G. and Evans H.E. (1986) Why are there no viviparous birds? Am. Nat. **128**, 165-190.

Blackburn D.G., Taylor J.M. and Padykula H.A. (1988) Trophoblast concept as applied to therian mammals. J. Morphol. **196**, 127-136.

Blackburn D.G., Vitt L.J. and Beuchat C.A. (1984) Eutherian-like reproductive specializations in a viviparous reptile. Proc. Natl. Acad. Sci. U.S.A. **81**, 4860-4863.

Blandau R.J. (1961) Biology of eggs and implantation. In: Young W.C. (ed.) Sex and Internal Secretions Vol. 2. 3rd. ed. Williams and Wilkins Co., Baltimore, pp. 797-882.

Blankenship T.N. and Given R.L. (1992) Penetration of the uterine epithelial basement membrane during blastocyst implantation in the mouse. Anat. Rec. **233**, 196-204.

Blumenthal E.J., Copestake C., Hite G. and Hoversland R.C. (1993) Pregnancy-associated, lymphocyte-derived suppressor factor inhibits protein kinase C activity. J. Reprod. Immunol. **24**, 97-109.

Boshier D.P. (1969) A histological and histochemical examination of implantation and early placentome formation in sheep. J. Reprod. Fert. **19**, 51-61.

Boshier D.P. (1976) Effects of the rat blastocyst on neutral lipids and non-specific esterases in the uterine luminal epithelium at the implantation area. J. Reprod. Fert. **46**, 245-247.

Boyd J.D. and Hamilton W.J. (1952) Cleavage, early development and implantation of the egg. In: Parkes A.S. (ed.) Marshall's Physiology of Reproduction. Vol. 2, 3rd. ed. Longmans, London, pp.1-126.

Boyd J.D. and Hamilton W.J. (1970) The Human Placenta W. Heffer and Sons, Cambridge.

Boyden E.A. (1924) An experimental study of the development of the avian cloaca, with special reference to a mechanical factor in the growth of the allantois. J. Exp. Zool. **40**, 437-472.

Bradbury S., Billington W.D. and Kirby D.R.S. (1965) A histochemical and electron microscopical study of the fibrinoid of the mouse placenta. J. Roy. Microsc. Soc. **84**, 199-211.

Bradshaw S.D., McDonald I.R., Hähnel R. and Heller H. (1975) Synthesis of progesterone by the placenta of a marsupial. J. Endocr. **65**, 451-452.

Brambell F.W.R. (1958) The passive immunity of the young mammal. Biol. Rev. **33**, 488-531.

Brambell F.W.R. and Perry J.S. (1945) The development of the embryonic membrane of the shrews, *Sorex araneus* Linn. and *Sorex minutus* Linn. Proc. Zool. Soc. Lond. **115**, 251-278.

Breed W.G. (1994) How does sperm meet egg? In a marsupial. Reprod. Fertil. Dev. **6**, 485-506.

Breed W.G. and Leigh C.M. (1988) Morphological observations on sperm-egg interactions during *in vivo* fertilization in the dasyurid marsupial *Sminthopsis crassicaudata*. Gamete Res. **19**, 131-149.

Breed W.G. and Leigh C.M. (1990) Morphological changes in the oocyte and its surrounding vestments during *in vivo* fertilization in the dasyurid marsupial, *Sminthopsis crassicaudata*. J. Morph. **204**, 177-196.

Breed W.G. and Leigh C.M. (1992) Marsupial fertilization: some further ultrastructural observations on the dasyurid *Sminthopsis crassicaudata*. Mol. Reprod. Devel. **32**, 277-292.

Breed W.G., Leigh C.M. and Bennett J.H. (1989) Sperm morphology and storage in the female reproductive tract of the fat-tailed dunnart, *Sminthopsis crassicaudata* (Marsupialia: Dasyuridae). Gamete Res. **23**, 61-75.

Brooks-Kaiser J.C., Murgita R.A. and Hoskin D.W. (1992) Pregnancy associated suppressor cells in mice: functional characteristics of $CD3^{+}4^{-}8^{-}45R^{+}T$ cells with natural suppressor activity. J. Reprod. Immunol. **21**, 103-125.

Brower L.K. and Anderson E. (1969) Cytological events associated with the secretory process in the rabbit oviduct. Biol. Reprod. **1**, 130-148.

Bulmer D. and Dickson A.D. (1960) Observations on carbohydrate materials in the rat placenta. J. Anat. **94**, 46-58.

Caldwell H. (1884) On the arrangement of the embryonic membranes in marsupial animals. Q. J. Microsc. Sci. **24**, 655-658.

Candlish J.K. (1972) The role of the shell membranes in the functional integrity of the egg. In: Freeman B.M. and Lake P.E. (eds.) Egg Formation and Production British Poultry Science, Edinburgh, pp. 87-105.

Carr K.E. and Toner P.G. (1982) Cell Structure 3rd. ed. Churchill Livingstone, Edinburgh.

Case T.J. (1978) Endothermy and parental care in the terrestrial vertebrates. Am. Nat. **112**, 861-874.

Challis J.R.G. and Olson D.M. (1988) Parturition. In: Knobil E. and Neill J. (eds.) The Physiology of Reproduction Vol. 2 Raven Press, New York, pp. 2177-2216.

Christensen A.K. (1975) Leydig Cells. In: Handbook of Physiology Section 7 Endocrinology Vol. 5 American Physiological Society, Washington, pp. 57-94.

Christensen A.K. and Gillim S.W. (1969) The correlation of fine structure and function in steroid-secreting cells, with emphasis on those of the gonads. In: McKerns K.W. (ed.) The Gonads Appleton-Century-Crofts, New York, pp. 415-488.

Clark D.E., Hurst P.R., McLennan I.S. and Myers D.B. (1993) Immunolocalization of collagen type I and laminin in the uterus on days 5-8 of embryo implantation in the rat. Anat. Rec. **237**, 8-20.

Clemens W.A. (1977) Phylogeny of the marsupials. In: Stonehouse B. and Gilmore D. (eds.) The Biology of Marsupials. Macmillan, London, pp. 401-406.

Clemens W.A., Richardson B.J. and Baverstock P.R. (1989) Biogeography and phylogeny of the Metatheria. In: Walton D.W. and Richardson B.J. (eds.) Fauna of Australia: Mammalia Australian Government Publishing Service, Canberra, pp. 527-548.

Cohen J. (1977) Reproduction Butterworths, London.

Cowie A.T. (1984) Lactation. In: Austin C.R. and Short R.V. (eds.) Reproduction in Mammals Vol. 3, 2nd. ed. Cambridge University Press, Cambridge, pp. 195-231.

Cruz Y.P. and Selwood L. (1993) Uterine histology of the dasyurid marsupial, *Antechinus stuartii*: relationship with differentiation of the embryo. J. Reprod. Fert. **99**, 237-242.

Curtis H. and Barnes N.S. (1989) Biology 5th ed. Worth Publishers, New York.

Dantzer V., Björkman N. and Hasselager E. (1981) An electron microscopic study of histiotrophe in the interareolar part of the porcine placenta. Placenta **2**, 19-28.

Dantzer V. and Leiser R. (1994) Initial vascularization in the pig placenta: 1. Demonstration of non-glandular areas by histology and corrosion casts. Anat. Rec. **238**, 177-190.

Dearden L., Ockleford C.D. and Gupta M. (1983) Structure of human trophoblast: correlation with function. In: Loke Y.W. and Whyte A. (eds.) Biology of Trophoblast Elsevier Publishers, Amsterdam, pp. 69-110.

de Kretser D.M. and Kerr J.B. (1988) The cytology of the testis. In: Knobil E. and Neill J. (eds.) The Physiology of Reproduction Vol. 1 Raven Press, New York, pp. 837-932.

Denker H-W. (1983) Cell lineage determination and differentiation in earliest developmental stages in mammals. Biblthca. anat. **24**, 22-58.

Denker H-W. (1993) Implantation: a cell biological paradox. J. Exp. Zool. 266: 541-558.

Denker H-W. and Tyndale-Biscoe C.H. (1986) Embryo implantation and proteinase activities in a marsupial (*Macropus eugenii*). Cell Tiss. Res. **246**, 279-291.

Deren J.J., Padykula H.A. and Wilson T.H. (1966) Development of structure and function in the mammalian yolk sac 2. Developmental morphology and Vitamin B_{12} uptake of the rat yolk sac. Dev. Biol. **13**, 349-369.

Draper M.H., Davidson M.F., Wyburn G.M. and Johnston H.S. (1972) The fine structure of the fibrous membrane forming region of the isthmus of the oviduct of *Gallus domesticus*. Q. J. Exp. Physiol. **57**, 297-309.

Dumont J.N. and Brummett A.R. (1985) Egg envelopes in vertebrates. In: L.W. Browder (ed.) Developmental Biology Vol. 1 Oogenesis, Plenum Press, New York, pp. 235-288.

Drury R.A.B. and Wallington E.A. (1980) Carleton's Histological Technique 5th ed Oxford University Press, Oxford.

Eisenberg J.F. (1981) The Mammalian Radiations University of Chicago Press, Chicago.

Ellington J.E., Farrell P.B., Simkin M.E., Foote R.H., Goldman E.E. and McGrath A.B. (1990) Development and survival after transfer of cow embryos cultured

from 1-2-cells to morulae or blastocysts in rabbit oviducts or in a simple medium with bovine oviduct epithelial cells. J. Reprod. Fert. **89**, 293-299.

Enders A.C. (1971) The fine structure of the blastocyst. In: Blandau R.J. (ed.) Biology of the Blastocyst University of Chicago Press, Chicago, pp. 71-94.

Enders A.C. (1982) Whither studies of comparative placental morphology? J. Reprod. Fert. Suppl. **31**, 9-15.

Enders A.C. and Enders R.K. (1969) The placenta of the four-eyed opossum (*Philander opossum*). Anat. Rec. **165**, 431-450.

Enders A.C. and Hendrickx A.G. (1980) Morphological basis of implantation in the rhesus monkey. Prog. Reprod. Biol. **7**, 270-283.

Enders A.C., Hendrickx A.G. and Schlafke S. (1983) Implantation in the rhesus monkey: initial penetration of endometrium. Am. J. Anat. **167**, 275-298.

Enders A.C. and Liu I.K.M. (1991a) Lodgement of the equine blastocyst in the uterus from fixation through endometrial cup formation. J. Reprod. Fert. Suppl. **44**, 427-438.

Enders A.C. and Liu I.K.M. (1991b) Trophoblast-uterine interactions during equine chorionic girdle cell maturation, migration, and transformation. Am. J. Anat. **192**, 366-381.

Enders A.C. and Schlafke S. (1969) Cytological aspects of trophoblast-uterine interaction in early implantation. Am. J. Anat. **125**, 1-30.

Enders A.C. and Schlafke S. (1971a) Penetration of the uterine epithelium during implantation in the rabbit. Am. J. Anat. **132**, 219-240.

Enders A.C. and Schlafke S. (1971b) Implantation in the ferret: epithelial penetration. Am. J. Anat. **133**, 291-316.

Enders A.C. and Schlafke S. (1974) Surface coats of the mouse blastocyst and uterus during the preimplantation period. Anat. Rec. **180**, 31-46.

Enders A.C. and Schlafke S. (1979) Comparative aspects of blastocyst-endometrial interactions at implantation. In: Maternal Recognition of Pregnancy. Ciba Foundation Symposium **64**. (new series) Excerpta Medica, Amsterdam, pp.3-32.

Enders A.C., Schlafke S. and Welsh A.O. (1980) Trophoblastic and uterine luminal epithelial surfaces at the time of blastocyst adhesion in the rat. Am. J. Anat. **159**, 59-72.

Ertzeid G. and Storeng R. (1992) Adverse effects of gonadotrophin treatment on pre- and postimplantation development in mice. J. Reprod. Fert. **96**, 649-655.

Ewing L.L. and Zirkin B. (1983) Leydig cell structure and steroidogenic function. Rec. Prog. Horm. Res. **39**, 599-635.

Fawcett D.W. (1966) The Cell W.B. Saunders Co., Philadelphia.

Feinberg B.B., Tan N.S., Walsh S.W., Brath P.C. and Gonik B. (1992) Progesterone and estradiol suppress human mononuclear cell cytotoxicity. J. Reprod. Immunol. **21**, 139-148.

Finn C.A. (1989) Species variation, location and attachment of blastocysts. In: Yoshinaga K. (ed.) Blastocyst Implantation. Serono Symposia, Boston, pp. 47-54.

Fischer H.E., Bazer F.W. and Fields M.J. (1985) Steroid metabolism by endometrial and conceptus tissues during early pregnancy and pseudopregnancy in gilts. J. Reprod. Fert. **75**, 69-78.

Fleming M.W. and Harder J.D. (1981) Uterine histology and reproductive cycles in pregnant and non-pregnant opossums, *Didelphis virginiana*. J. Reprod. Fert. **63**, 21-24.

Fletcher T.P. and Renfree M.B. (1988) Effects of corpus luteum removal on progesterone, oestradiol-17ß and LH in early pregnancy of the tammar wallaby, *Macropus eugenii*. J. Reprod. Fert. **83**, 185-191.

Flood P.F., Betteridge K.J. and Diocee M.S. (1982) Transmission electron microscopy of horse embryos 3-16 days after ovulation. J. Reprod. Fert. Suppl. **32**, 319-327.

Flynn T.T. (1923) The yolk sac and allantoic placenta of *Perameles*. Q. J. Microsc. Sci. **67**, 123-182.

Flynn T.T. (1930) The uterine cycle of pregnancy and pseudo-pregnancy as it is in the diprotodont marsupial *Bettongia cuniculus*. Proc. Linn. Soc. NSW **55**, 506-531.

Flynn T.T. and Hill J.P. (1939) The development of the Monotremata: Part 4. Growth of the ovarian ovum, fertilization and early cleavage. Trans. Zool. Soc. Lond. **24**, 445-622.

Fredricsson B. (1969) Histochemistry of the oviduct. In: Hafez E.S.E. and Blandau R.J. (eds.) The Mammalian Oviduct University of Chicago Press, Chicago, pp. 311-332.

Freeman S.J., Beck F. and Lloyd J.B. (1981) The role of the visceral yolk sac in mediating protein utilization by rat embryos cultured *in vitro*. J. Embryol. exp. Morph. **66**, 223-234.

Freeman S.J. and Lloyd J.B. (1983) Evidence that protein ingested by the rat visceral yolk sac yields amino acids for the synthesis of embryonic protein. J. Embrol. exp. Morph. **73**, 307-315.

Fujimoto T., Miyayama Y. and Fuyuta M. (1977) The origin, migration and fine morphology of human primordial germ cells. Anat. Rec. **188**, 315-330.

Galil A.K.A. and Deane H.W. (1966) Δ^5-3ß-Hydroxysteroid dehydrogenase activity in the steroid hormone producing organs of the ferret (*Mustela putorius furo*). J. Reprod. Fert. **11**, 333-338.

Gandolfi F., Passoni L., Modina S., Brevini T.A.L., Varga Z. and Lauria A. (1993) Similarity of an oviduct-specific glycoprotein between different species. Reprod. Fertil. Dev. **5**, 433-443.

Geisert R.D., Renegar R.H., Thatcher W.W., Roberts R.M. & Bazer F.W. (1982) Establishment of pregnancy in the pig. I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. Biol. Reprod. 27, 925-939.

Gemmel R.T. (1981) The role of the corpus luteum of lactation in the bandicoot *Isoodon macrourus* (Marsupialia: Peramelidae). Gen. Comp. Endocrin. **44**, 13-19.

George F.W. and Wilson J.D. (1978) Oestrogen formation in the early rabbit embryo. Science **199**, 200-201.

Goodger A.M. and Rogers P.A.W. (1993) Uterine epithelial proliferation before and after embryo implantation in rats. J. Reprod. Fert. **99**, 451-457.

Goodger A.M. and Rogers P.A.W. (1994) Endometrial endothelial cell proliferation during the menstrual cycle. Hum. Reprod. **9**, 399-405.

Gould S.J. (1977) Sticking up for marsupials. Nat. Hist. **86**, 22-30.

Greenwald G.S. (1962) The role of the mucin layer in development of the rabbit blastocyst. Anat. Rec. **142**, 407-415.

Greenwald G.S. (1969) Endocrinology of oviductal secretions. In: Hafez E.S.E. and Blandau R.J. (eds.) The Mammalian Oviduct University of Chicago Press, Chicago, pp. 183-201.

Grubb J.D., Koszalk T.R., Drabick J.J. and Metrione R.M. (1991) The activities of thiol proteases in the rat visceral yolk sac increase during late gestation. Placenta **12**, 143-151.

Guillette L.J. Jr. (1991) The evolution of viviparity in amniote vertebrates: new insights, new questions. J. Zool., Lond. **223**, 521-526.

Guillomot M., Fléchon J-E. and Wintenberger-Torres S. (1982) Cytochemical studies of uterine and trophoblastic surface coats during blastocyst attachment in the ewe. J. Reprod. Fert. **65**, 1-8.

Gumbiner B., Stevenson B. and Grimaldi A. (1988) The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. J. Cell Biol. **107**, 1575-1587.

Gupta M., Gulamhusein A.P. and Beck F. (1982) Morphometric analysis of the visceral yolk sac endoderm in the rat *in vivo* and *in vitro*. J. Reprod. Fert. **65**, 239-245.

Hamilton W.J. and Day F.T. (1945) Cleavage stages of the ova of the horse, with notes on ovulation. J. Anat. **79**, 127-130.

Hamilton W.J. and Mossman H.W. (1972) Human Embryology. 4th. ed. W Heffer and Sons, Cambridge.

Hamlett W.C., Eulitt A.M., Jarrell R.L. and Kelly M.A. (1993) Uterogestation and placentation in elasmobranchs. J. Exp. Zool. **266**, 347-367.

Harder J.D. and Fleming M.W. (1981) Oestradiol and progesterone profiles indicate a lack of endocrine recognition of pregnancy in the opossum. Science **212**, 1400-1402.

Harder J.D., Hinds L.A., Horn C.A. and Tyndale-Biscoe C.H. (1984) Oestradiol in follicular fluid and in utero-ovarian venous and peripheral plasma during parturition and post-partum oestrus in the tammar, *Macropus eugenii*. J. Reprod. Fert. **72**, 551-558.

Harder J.D., Stonerook M.J. and Pondy J. (1993) Gestation and placentation in two New World opossums: *Didelphis virginiana* and *Monodelphis domestica*. J. Exp. Zool. **266**, 463-479.

Hartfield P.J., Williams K.E., Geddes R. and Lloyd J.B. (1989) Glycogen metabolism in the rat visceral yolk sac. I. Glycogen content and gestational age. Placenta **10**, 45-54.

Hartman C.G. (1916) Studies in the development of the opossum *Didelphis virginiana* L. J. Morph. **27**, 1-84.

Hartman C.G. (1925a) On some characters of taxonomic value appertaining to the egg and the ovary of rabbits. J. Mamm. **6**, 114-121.

Hartman C.G. (1925b) The interruption of pregnancy by ovariectomy in the aplacental opossum: a study in the physiology of implantation. Am. J. Physiol. **71**, 436-454.

Hayssen V., Lacy R.C. and Parker P.J. (1985) Metatherian reproduction: transitional or transcending. Am. Nat. **126**, 617-632.

Heald P.J., O'Grady J.E., O'Hare A. and Vass M. (1975) Nucleic acid metabolism of cells of the luminal epithelium and stroma of the rat uterus during early pregnancy. J. Reprod. Fert. **45**, 129-138.

Heap R.B., Perry J.S. and Challis J.R.G. (1973) Hormonal maintenance of pregnancy. In: Greep R.O. and Astwood E.B. (eds.) Handbook of Physiology Section 7 Endocrinology Vol. II Female Reproductive System Part 2 American Physiological Society, Washington, pp. 217-260.

Heap R.B., Renfree M.B. and Burton R.D. (1980) Steroid metabolism in the yolk sac placenta and endometrium of the tammar wallaby, *Macropus eugenii*. J. Endocr. **87**, 339-349.

Heap R.B. and Flint A.P.F. (1984) Pregnancy. In: Austin C.R. and Short R.V. (eds.) Reproduction in Mammals. Vol. 4, 2nd. ed. Cambridge University Press, Cambridge, pp. 153-194.

Hennemann W.W. III (1984) Intrinsic rates of natural increase of altricial and precocial eutherian mammals: the potential price of precociality. Oikos **43**, 363-368.

Hill C.J. (1941) The development of the Monotremata: Part 5. Further observations on the histology and the secretory activities of the oviduct prior to and during gestation. Trans. Zool. Soc. Lond. **25**, 2-31.

Hill C.J. and Hill J.P. (1933) The development of the Monotremata Part 1. The histology of the oviduct during gestation. Part 2. The structure of the eggshell. Trans. Zool. Soc. Lond. **21**, 413-455.

Hill J.P. (1898) Contributions to the embryology of the Marsupialia. 1. The placentation of *Perameles*. Q. J. Microsc. Sci. **40**, 385-446.

Hill J.P. (1900) On the fetal membranes, placentation and parturition of the native cat *Dasyurus viverrinus* Anat. Anzeig. **18**, 364-373.

Hill J.P. (1910) The early development of the marsupialia, with special reference to the native cat, (*Dasyurus viverrinus*) Q. J. Microsc. Sci. **56**, 1-134.

Hill J.P. (1918) Some observations on the early development of *Didelphis aurita*. Contributions to the embryology of the Marsupialia, 5. Q. J. Microsc. Sci. **63**, 91-139.

Hinds L.A. and Selwood L. (1990) Plasma progesterone concentrations during pregnancy in the dasyurid marsupial, *Antechinus stuartii*: relationship with differentiation of the embryo. Reprod. Fertil. Dev. **2**, 61-70.

Hinds L.A. and Tyndale-Biscoe C.H. (1982) Plasma progesterone levels in the pregnant and non-pregnant tammar, *Macropus eugenii*. J. Endocr. **93**, 99-107.

Hobson B.M. (1983) An appraisal of the mouse uterine weight assay for the bioassay of chorionic gonadotrophin in the macaque term placenta. J. Reprod. Fert. **68**, 457-463.

Hodgen G.D. and Itskovitz J. (1988) Recognition and maintenance of pregnancy. In: Knobil E. & Neill J. (eds.) Physiology of Reproduction Raven Press, New York., pp. 1995-2021.

Hoffer A.P. (1971) The ultrastructure and cytochemistry of the shell membrane-secreting region of the Japanese quail oviduct. Am. J. Anat. **131**, 253-288.

Hoffman L.H. (1970) Placentation in the garter snake, *Thamnophis sirtalis*. J. Morphol. **131**, 57-88.

Hoffman L.H. and Wooding F.B.P. (1993) Giant and binucleate trophoblast cells of mammals. J. Exp. Zool. **266**, 559-577.

Hogarth P.J. (1976) Viviparity Edward Arnold Publishers, London.

Hogarth P.J. (1982) Immunological Aspects of Mammalian Reproduction Blackie, London.

Hollenberg F. and Wourms J.P. (1994) Ultrastructure and protein uptake of the embryonic trophotaeniae of four species of Goodeid fishes (Teleostei: Atheriniformes). J. Morphol. **219**, 105-129.

Hollis D.E. and Lyne A.G. (1977) Endoderm formation in blastocysts of the marsupials *Isoodon macrourus* and *Perameles nasuta*. Aust. J. Zool. **25**, 207-223.

Hughes R.L. (1969) A light and electron microscopical study of the tertiary egg membranes of three marsupial species: *Trichosurus vulpecula, Macropus robustus* and *Magaleia rufa* J. Anat. **104**, 407.

Hughes R.L. (1974a) The tertiary egg membranes of the marsupial *Trichosurus vulpecula*. Ph.D. Thesis, The University of New South Wales.

Hughes R.L. (1974b) Morphological studies on implantation in marsupials. J. Reprod. Fert. **39**, 173-186.

Hughes R.L. (1977) Egg membranes and ovarian function during pregnancy in monotremes and marsupials. In: Calaby J.H. and Tyndale-Biscoe C.H. (eds.) Reproduction and Evolution Australian Academy of Science, Canberra, pp. 281-291.

Hughes R.L. (1984) Structural adaptations of the eggs and the fetal membranes of monotremes and marsupials for respiration and exchange. In: Seymour R.S. (ed.) Respiration and Metabolism of Embryonic Vertebrates pp. 389-421.

Hughes R.L. (1993) Monotreme development with particular reference to the extraembryonic membranes. J. Exp. Zool. **266**, 480-494.

Hughes R.L., Carrick E.N. and Shorey C.D. (1975) Reproduction in the platypus, *Ornithorynchus anatinus*, with particular reference to the evolution of viviparity. J. Reprod. Fert. **43**, 374-375.

Hughes R.L. and Green R.H. (1994) Placentation and adaptations for survival at birth in the common wombat *Vombatus ursinus*. Proceedings Wombats in Australia Adelaide Sep. 1994, Royal Zoological Society S.A.

Hughes R.L., Hall L.S., Aplin K.P. and Archer M. (1987) Organogenesis and fetal membranes in the New Guinea pen-tailed possum, *Distoechurus pennatus (Acrobatidae : Marsupialia)*. In: Archer M. (ed.) Possums and Opossums: Studies in Evolution Vol. 2 Surrey Beatty and Sons, Sydney, pp. 715-724 .

Hughes R.L., Hall L.S., Archer M. and Aplin K.P. (1990) Observations on placentation and development in *Echymipera kaluba*. In: Seebeck J.H., Brown P.R. Wallis R.L. and Kemper C.M. (eds.) Bandicoots and Bilbies Surrey Beatty and Sons, Sydney, pp 259-270.

Hughes R.L. and Shorey C.D. (1973) Observations on the permeability properties of the egg membranes of the marsupial, *Trichosurus vulpecula*. J. Reprod. Fert. **32**, 25-32.

Hughes R.L., Thomson J.A. and Owen W.H. (1965) Reproduction in natural populations of the Australian ringtail possum, *Pseudocheirus peregrinus* (Marsupialia : Phalangeridae), in Victoria. Aust. J. Zool. **13**, 383-406.

Jenkinson E.J. and Billington W.D. (1974) Studies on the immunobiology of mouse fetal membranes: the effect of cell-mediated immunity on yolk sac cells *in vitro*. J. Reprod. Fert. **41**, 403-412.

Jansen R.P.S. and Bajpai V.K. (1982) Oviduct acid mucus glycoproteins in the estrous rabbit: ultrastructure and histochemistry. Biol. Reprod. **26**, 155-168.

Johnson M.H. (1971) The molecular and cellular basis of preimplantation mouse development. Biol. Rev. **56**, 463-498.

Johnson M.H., Pratt H.P.M. and Handyside A.H. (1981) The generation and recognition of positional information in the pre-implantation mouse embryo. In: Glasser S.R. and Bullock D.W. (eds.) Cellular and Molecular Aspects of Implantation Plenum Press, New York, pp. 55-74.

Johnson M.H. and Rossant J. (1981) Molecular studies on cells of the trophectodermal lineage of the post-implantation mouse embryo. J. Embryol. exp. Morph. **61**, 103-116.

Johnson M.H. and Ziomek C.A. (1981) The foundation of two distinct cell lineages within the mouse morula. Cell **24**, 71-80.

Jones B.M. and Kemp R.B. (1969) Self-isolation of the fetal trophoblast. Nature **221**, 829-831.

Kane M.T. (1975) Inhibition of zona shedding of rabbit blastocysts in culture by the presence of a mucoid coat. J. Reprod. Fert. **44**, 539-542.

Kane M.T. (1987) *In vitro* growth of preimplantation rabbit embryos. In: Bavister B.D. (ed.) The Mammalian Preimplantation Embryo Plenum Press, New York, pp.193-217.

Kapur R.P. and Johnson L.V. (1988) Ultrastructural evidence that specialized regions of the murine oviduct contribute a glycoprotein to the extracellular matrix of mouse oocytes. Anat. Rec. **221**, 720-729.

Kaufman M.H. (1983) The origin, properties and fate of trophoblast in the mouse. In: Loke Y.W. and Whyte A. (eds.) Biology of Trophoblast Elsevier Publishers, Amsterdam, pp. 23-68.

Kaye M.D. (1979) The immunobiology of placentation. Aust. N. Z. J. Obstet. Gynaec. **19**, 34-39.

Kemp T.S. (1983) The relationships of mammals. Zool. J. Linn. Soc. **77**, 353-384.

Khong T.Y., Lane E.B. and Robertson W.B. (1986) An immunocytochemical study of fetal cells at the maternal-placental interface using monoclonal antibodies to keratins, vimentin and desmin. Cell Tiss. Res. **246**, 189-195.

King B.F. (1993) Development and structure of the placenta and fetal membranes of non-human primates. J. Exp. Zool. **266**, 528-540.

King B.F. and Enders A.C. (1970) Protein absorption and transport by the guinea-pig visceral yolk sac placenta. Am. J. Anat. **129**, 261-288.

King G.J., Atkinson B.A. and Robertson H.A. (1982) Implantation and early placentation in domestic ungulates. J. Reprod. Fert. Suppl. **31**, 17-30.

Kirby D.R.S., Billington W.D., Bradbury S. and Goldstein D.J. (1964) Antigen barrier of the mouse placenta. Nature **204**, 548-549.

Kirby D.R.S. and Wood C. (1967) Embryos and Antigens. Science J. Dec. 1967. pp. 56-60.

Kirsch J.A.W. (1977) The six-percent solution: second thoughts on the adaptedness of the Marsupialia. Amer. Sci. **65**, 276-288.

Kiso Y., Yasufuku K., Matsuda H. and Yamauchi S. (1990) Existence of an endothelio-endothelial placenta in the insectivore *Suncus murinus*. Cell Tiss. Res. **262**, 195-197.

Knoth, M. and Larsen J.F. (1972) Ultrastructure of a human implantation site. Acta. Obstet. Gynec. Scand. **51**, 385-393.

Krause W.J. and Cutts J.H. (1983) Ultrastructural observations on the shell membrane of the North American opossum (*Didelphis virginiana*). Anat. Rec. **207**, 335-338.

Krause W.J. and Cutts J.H. (1985a) The allantois of the North American opossum (*Didelphis virginiana*) with preliminary observations on the yolk sac endoderm and trophectoderm. Anat. Rec. **211**, 166-173.

Krause W.J. and Cutts J.H. (1985b) Placentation in the opossum, *Didelphis virginiana*. Acta anat. **123**, 156-171.

Larsen J.F. (1970) Electron microscopy of nidation in the rabbit and observations on the human trophoblastic invasion. In: Hubinot P.O., Leroy F., Robyn C. and Leleux P.(eds.) Ovo-implantation, Human Gonadotrophins and Prolactin S. Karger, Basel, pp. 38-51.

Lawn A.M. and Chiquoine A.D. (1965) The ultrastructure of the placental labbyrinth of the ferret (*Mustela putorius furo*). J. Anat. Lond. **99**, 47-69.

Lee A.K. and Cockburn A. (1985) Evolutionary Ecology of Marsupials Cambridge University Press, Cambridge.

Leiser R. (1982) Development of the trophoblast in the early carnivore placenta of the cat. Biblthca. anat. **22**, 93-107.

Leiser R. and Enders A.C. (1980a) Light- and electron microscopic study of the near-term paraplacenta of the domestic cat I. Polar zone and paraplacental junctional areas. Acta anat. **106**, 293-311.

Leiser R. and Enders A.C. (1980b) Light- and electron microscopic study of the near-term paraplacenta of the domestic cat II. Paraplacental haematoma. Acta anat. **106**, 312-326.

Leiser R. and Koob B. (1993) Development and characteristics of placentation in a carnivore, the domestic cat. J. Exp. Zool. **266**, 642-656.

Lelkes P.I. and Pollard H.B. (1991) Cytoplasmic determinants of exocytotic membrane fusion. In: Wilschut J. and Hoekstra D. (eds.) Membrane Fusion Marcel Dekker Inc., New York, pp. 511-551.

Lillegraven J.A. (1985) Use of the term trophoblast for tissues in therian mammals. J. Morph. **183**, 293-299.

Lillegraven J.A., Thompson S.D., McNab B.K. and Patton J.L. (1987) The origin of eutherian mammals. Biol. J. Linn. Soc. **32**, 281-336.

Low B.S. (1978) Environmental uncertainty and the parental strategies of marsupials and placentals. Am. Nat. **112**, 197-213.

Luckett W.P. (1971) The origin of extra-embryonic mesoderm in the early human and rhesus monkey embryos. Anat. Rec. **169**, 369-370.

Luckett W.P. (1977) Ontogeny of amniote fetal membranes and their application to phylogeny. In: Hecht M.K., Goody P.C. and Hecht B.M. (eds.) Major Patterns in Vertebrate Evolution. Plenum Press, New York, pp. 439-516.

Luckett W.P. (1980) The use of reproductive and developmental features in assessing tupaiid affinities. In: Luckett W.P. (ed.) Comparative Biology and Evolutionary Relationships of Tree Shrews Plenum Publishers, New York, pp. 245-266.

Luckett W.P. (1985) Superordinal and intraordinal affinities of rodents: developmental evidence from the dentition and placentation. In: Luckett W.P. and Hartenberger J-L. (eds.) Evolutionary Relationships Among Rodents Plenum Press, New York, pp. 227-276.

Luckett W.P. (1993) Uses and limitations of mammalian fetal membranes and placenta for phylogenetic reconstruction. J. Exp. Zool. **266**, 514-527.

Lyne A.G. and Hollis D.E. (1976) Early embryology of the marsupials *Isoodon macrourus* and *Perameles nasuta*. Aust. J. Zool. **24**, 361-382.

Lyne A.G. and Hollis D.E. (1977) The early development of marsupials with special reference to bandicoots. In: Calaby J.H. and Tyndale-Biscoe C.H. (eds.) Reproduction and Evolution Australian Academy of Science, Canberra, pp. 293-302.

Marsh M. and Quinn P. (1991) Membrane cycling through the endocytotic and exocytotic pathways. In: Wilschut J. and Hoekstra D. (eds.) Membrane Fusion Marcel Dekker Inc., New York, pp. 421-448.

Marshall L.G. (1979) Evolution of metatherian and eutherian (mammalian) characters: a review based on cladistic methodology. Zool. J. Linn. Soc. **66**, 369-410.

Martin L. (1979) Early cellular changes and circular muscle contraction associated with the induction of decidualization by intrauterine oil in mice. J. Reprod. Fert. **55**, 135-139.

Martin L. (1984) On the source of uterine 'luminal fluid' proteins in the mouse. J. Reprod. Fert. **71**, 73-80.

Martin P.G. (1977) Marsupial biogeography and plate tectonics. In: Stonehouse B. and Gilmore D. (eds.) The Biology of Marsupials Macmillan, London, pp. 97-115.

Massa H. and Martin L. (1994) Porcine relaxin differentially inhibits uterine longitudinal and circular muscles *in vivo* in oestrous and early pregnant rats. Proceedings Australian Society for Reproductive Biology **26**, 39.

Mastroianni L. Jr. (1969) Epilogue. In: Hafez E.S.E. and Blandau R.J. (eds.) The Mammalian Oviduct University of Chicago Press, Chicago, pp. 507-511.

McCrady E. (1938) The embryology of the opossum. Amer. Anat. Memoirs **16**, 1-233.

Metcalfe J. and Stock M.K. (1993) Current topic: oxygen exchange in the chorioallantoic membrane, avian homologue of the mammalian placenta. Placenta **14**, 605-613.

Moran T. and Hale H.P. (1936) Physics of the hen's egg 1. Membranes in the egg J. Exp. Biol. **13**, 35-40.

Morriss G. (1975) Placental evolution and embryo nutrition. In: Steven D.H. (ed.) Comparative Placentation Academic Press, London, pp. 87-107.

Mossman H.W. (1937) Comparative morphogenesis of the fetal membranes and accessory uterine structures. Carn. Inst. Contr. Embryol. **26**, 129-246.

Mossman H.W. (1952) The genital system and the fetal membranes as criteria for mammalian phylogeny and taxonomy. J. Mammal. **34**, 289-298.

Mossman H.W. (1987) Vertebrate Fetal Membranes Rutgers Press, New Brunswick.

Mossman H.W. and Owers N. (1963) The shrew placenta: evidence that it is endothelio-endothelial in type. Am. J. Anat. **113**, 245-271.

Murphy C.R. (1992) Structure of the plasma membrane of uterine epithelial cells in blastocyst attachment: a review. Reprod. Fertil. Dev. **4**, 633-43.

New D.A.T. (1973) Studies on mammalian fetuses *in vitro* during the period of organogenesis. In: Austin C.R. (ed.) The Mammalian Fetus *in vitro* Chapman and Hall, London, pp. 15-65.

Nilsson O. and Reinius S. (1969) Light and electron microscopic structure of the oviduct. In: Hafez E.S.E. and Blandau R.J. (eds.) The Mammalian Oviduct University of Chicago Press, Chicago, pp. 57-83.

Oriol J.G., Sharan F.J. and Betteridge K.J. (1993) Developmentally regulated changes in the glycoproteins of the equine embryonic capsule. J. Reprod. Fert. **99**, 653-664.

Packard M.J. and Packard G.C. (1984) Comparative aspects of calcium metabolism in embryonic reptiles and birds. In: Seymour R.S. (ed.) Respiration and Metabolism of Embryonic Vertebrates Junk, Dordrecht, pp. 155-179.

Padykula H.A., Deren J.J. and Wilson T.H. (1966) Development of structure and function in the mammalian yolk sac 1. Developmental morphology and vitamin B uptake of the rat yolk sac. Dev. Biol. **13**, 311-348.

Padykula H.A. and Taylor J.M. (1971) Ultrastructural differentiation of the endometrium of the opossum (*Didelphis marsupialis virginiana*) during pregnancy Anat. Rec. **169**, 394-395.

Padykula H.A. and Taylor J.M. (1976) Ultrastuctural evidence for the loss of the trophoblastic layer in the chorioallantoic placenta of Australian bandicoots (Marsupialia: Peramelidae). Anat. Rec. **186**, 357-386.

Padykula H.A. and Taylor J.M. (1977) Uniqueness of the bandicoot chorioallantoic placenta (Marsupialia: Peramelidae) cytological and evolutionary implications. In: Calaby J.H. and Tyndale-Biscoe C.H. (eds.) Reproduction and Evolution Australian Academy of Science, Canberra, pp. 303-323.

Padykula H.A. and Taylor J.M. (1982) Marsupial placentation and its evolutionary significance. J. Reprod. Fert. Suppl. **31**, 95-104.

Palmer B.D., DeMarco V.G. and Guillette L.J. Jr. (1993) Oviductal morphology and eggshell formation in the lizard, *Sceloporus woodi*. J. Morphol. **217**, 205-217.

Palmer B.D. and Guillette L.J. Jr. (1988) Histology and functional morphology of the female reproductive tract of the tortoise *Gopherus polyphemus*. Am. J. Anat. **183**, 200-211.

Parker P. (1977) An ecological comparison of marsupial and placental patterns of reproduction. In: Stonehouse B. and Gilmore D. (eds.) The Biology of Marsupials. Macmillan, London, pp. 273-286.

Parr E.L. and Parr M.B. (1989a) The barrier role of the primary decidual zone. In: Yoshinaga K. (ed.) Blastocyst Implantation Serono Symposia, Boston, pp. 163-169.

Parr E.L. and Parr M.B. (1989b) Epithelial cell death during rodent embryo implantation. In: Yoshinaga K. (ed.) Blastocyst Implantation Serono Symposia, Boston, pp. 105-115.

Parr E.L., Tung H.N. and Parr M.B. (1987) Apoptosis as the mode of uterine cell death during embryo implantation in mice and rats. Biol. Reprod. **36**, 211-225.

Parr M.B. (1980) Endocytosis at the basal and lateral membranes of rat uterine epithelial cells during early pregnancy. J. Reprod. Fert. **60**, 95-99.

Parr M.B. and Parr E.L. (1978) Uptake and fate of ferritin in the uterine epithelium of the rat during early pregnancy. J. Reprod. Fert. **52**, 183-188.

Perry J.S. (1981) The mammalian fetal membranes. J. Reprod. Fert. **62**, 321-335.

Phillips D.M. and Fadem B.H. (1987) The oocyte of a New World marsupial, *Monodelphis domestica:* structure, formation, and function of the enveloping mucoid layer. J. Exp. Zool. **242**, 363-371.

Pijnenborg R., Robertson W.B., Brosens I. and Dixon G. (1981) Trophobalast invasion and the establishment of haemochorial placentation in man and laboratory animals. Placenta **2**, 71-92.

Plattner H. (1991) Ultrastructural aspects of exocytosis. In: Wilschut J. and Hoekstra D. (eds.) Membrane Fusion Marcel Dekker Inc., New York, pp. 571-598.

Poelmann R.E. and Mentink M.M.T. (1982) Parietal yolk sac in early gestation mouse embryos: structure and function. Biblthca. anat. **22**, 123-127.

Porter D.G., Heap, R.B. and Flint A.P.F. (1982) Endocrinology of the placenta and the evolution of viviparity. J. Reprod. Fert. Suppl. **31**, 113-138.

Potts D.M. (1968) The ultrastructure of implantion in the mouse. J. Anat. **103**, 77-90.

Ramsey E.M., Houston M.L. and Harris J.W.S. (1976) Interactions of the trophoblast and maternal tissues in three closely related primate species. Am. J. Obstet. Gynec. **124**, 647-652.

Rasweiler J.J. (1990) Implantation, development of the fetal membranes and placentation in the captive black mastiff bat, *Molossus ater*. Am. J. Anat. **187**, 109-136.

Rasweiler J.J. (1991) Development of the discoidal haemochorial placenta in the black mastiff bat *Molussus ater*: evidence for a role of maternal endothelial cells in the control of trophoblastic growth. Am. J. Anat. **191**, 185-207.

Rasweiler, J.J. iv (1993) Pregnancy in Chiroptera. J. Exp. Zool. **266**, 495-513.

Reeves R.B. (1984) Blood oxygen affinity in relation to yolk-sac and chorioallantoic gas exchange in the developing chick embryo. In: Seymour R.S. (ed.) Respiration and Metabolism of Embryonic Vertebrates Junk, Dordrecht, pp. 231-244.

Renfree M.B. (1970) Protein, amino acids and glucose in the yolk sac fluids and maternal blood sera of the tammar wallaby, *Macropus eugenii* (Desmarest). J. Reprod. Fert. **22**, 483-492.

Renfree M.B. (1972) Influence of the embryo on the marsupial uterus. Nature **240**, 475-477.

Renfree M.B. (1973) The composition of fetal fluids of the marsupial *Macropus eugenii* Dev. Biol. **33**, 62-79.

Renfree M.B. (1977) Feto-placental influences in marsupial gestation. In: Calaby J.H. and Tyndale-Biscoe C.H. (eds.) Reproduction and Evolution Australian Academy of Science, Canberra, pp. 325-331.

Renfree M.B. (1980) Endocrine activity in marsupial placentation. In: Cumming I.A., Funder J.W. and Mendelsohn F.A.O. (eds.) Proceedings of 6th. International Congress on Endocrinology Australian Academy of Science, Canberra, pp. 83-86.

Renfree M.B. (1982) Implantation and placentation. In: Austin C.R. and Short R.V. (eds.) Reproduction in Mammals Vol. 2, 2nd. ed. Cambridge University Press, Cambridge, pp. 26-69.

Renfree M.B. (1983) Marsupial reproduction: the choice between placentation and lactation. In: Finn C.A. (ed.) Oxford Rev. Reprod. Biol. Vol. 5 Clarendon Press, Oxford, pp. 1-27.

Renfree M.B. (1993a) Diapause, pregnancy, and parturition in Australian marsupials. J. Exp. Zool. **266**, 450-462.

Renfree M.B. (1993b) Ontogeny, genetic control, and phylogeny of female reproduction in monotreme and therian mammals. In: Szalay F.S., Novacek M.J. and McKenna M.C. Mammal Phylogeny Springer-Verlag, New York, pp. 4-20.

Renfree M.B. (1994) Endocrinology of pregnancy, parturition and lactation in marsupials. In: Lamming G.E. (ed.) Marshall's Physiology of Reproduction Vol. 3 Pregnancy and Lactation Part 2 Fetal Physiology, Parturition and Lactation 4th. ed. Chapman and Hall, London, pp. 677-766.

Renfree M.B. and Tyndale-Biscoe C.H. (1973) Transferrin variation between mother and fetus in the marsupial, *Macropus eugenii*. J. Reprod. Fert. **32**, 113-115.

Renfree M.B. and Young I.R. (1979) Steroids in pregnancy and parturition in the marsupial, *Macropus eugenii*. J. Steroid Biochem. **11**, 515-522.

Renton J.P., Boyd J.S., Eckersall P.D., Ferguson J.M., Harvey M.J.A., Mullaney J. and Perry B. (1991) Ovulation, fertilization and early embryonic development in the bitch (*Canis familiaris*). J. Reprod Fert. **93**, 221-231.

Reynolds E.S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**, 208-212.

Ridley M. (1993) Evolution Blackwell Scientific Publications, Oxford.

Roberts C.T. and Breed W.G. (1994a) Embryonic-maternal cell interactions at implantation in the fat-tailed dunnart, a dasyurid marsupial. Anat. Rec. **204**, 59-76.

Roberts C.T. and Breed W.G. (1994b) Placentation in the dasyurid marsupial, *Sminthopsis crassicaudata*, the fat-tailed dunnart, and notes on placentation of *Monodelphis domestica*. J. Reprod. Fert. **100**, 105-113.

Roberts C.T., Breed W.G. and Mayrhofer G. (1994) The origin of the oocyte shell membrane of a dasyurid marsupial, an immunohistochemical study. J. Exp. Zool. **270**, 321-331.

Rodger J.C. and Bedford J.M. (1982) Induction of oestrus, recovery of gametes and the timing of fertilization events in the opossum, *Didelphis virginiana*. J. Reprod. Fert. **64**, 159-169.

Rodger J.C., Breed W.G. and Bennett J.H. (1992) Gonadotrophin-induced oestrus and ovulation in the polyovulatory marsupial *Sminthopsis crassicaudata*. Reprod. Fertil. Dev. **4**, 145-152.

Rodger J.C. and Drake B.L. (1987) The enigma of the fetal graft. Amer. Scientist **75**, 51-57.

Rodger J.C., Fletcher T.P. and Tyndale-Biscoe C.H. (1985) Active anti-paternal immunisation does not affect the success of marsupial pregnancy. J. Reprod. Immunol. **8**, 249-256.

Rogers P.A.W., Murphy C.R., Squires K.R. and MacLennan A.H. (1983) Effects of relaxin on the intrauterine distribution and anti-mesometrial positioning and orientation of rat blastocysts before implantation. J. Reprod. Fert. **68**, 431-435.

Romanoff A.L. and Romanoff A.J. (1949) The Avian Egg John Wiley and Sons, New York.

Romer A.S. and Parsons T.S. (1986) The Vertebrate Body Saunders College Publishing, Philadelphia.

Rose R.W. (1992) Maternal recognition of pregnancy in the Tasmanian bettong, *Bettongia gaimardi* (Marsupilia : Macropodoidea). Reprod. Fertil. Dev. **4**, 35-41.

Rossant J. and Frels W.I. (1981) The origin of trophoblast and its role in implantation. In: Glasser S.R. and Bullock D.W. (eds.) Cellular and Molecular Aspects of Implantation Plenum Press, New York, pp. 43-54.

Sadleir R.M.F.S. (1973) The Reproduction of Vertebrates Academic Press, New York.

Sargent I.L. and Redman C.W.G. (1985) Maternal cell-mediated immunity to the fetus in human pregnancy. J. Reprod. Immunol. **7**, 95-104.

Schindler J.F. and Hamlett W.C. (1993) Maternal-embryonic relations in viviparous teleosts. J. Exp. Zool. **266**, 378-393.

Schlafke S. and Enders A.C. (1975) Cellular basis of interaction between trophoblast and uterus at implantation. Biol. Reprod. **12**, 41-65.

Schlafke S., Welsh A.O. and Enders A.C. (1985) Penetration of the basal lamina of the uterine epithelium during implantation in the rat. Anat. Rec. **212**, 47-56.

Schröder H., Schoch C., Elwers W. and Leichtweiß H.-P. (1991) The artificially perfused guinea-pig yolk sac placenta: transfer and uptake of water, glucose and amino acids. Placenta **12**, 495-509.

Selwood L. (1980) A timetable of embryonic development of the dasyurid marsupial *Antechinus stuartii* (Macleay). Aust. J. Zool. **28**, 649-668.

Selwood L. (1982) A review of maturation and fertilization in marsupials with special reference to the dasyurid: *Antechinus stuartii*. In: Archer M. (ed.) Carnivorous Marsupials Royal Zool. Soc. NSW, Sydney, pp.65-76.

Selwood L. (1986) The marsupial blastocyst - a study of the blastocysts in the Hill Collection. Aust. J. Zool. **34**, 177-187.

Selwood L. (1987) Embryonic development in culture of two dasyurid marsupials, *Sminthopsis crassicaudata* (Gould) and *Sminthopsis macroura* (Spencer), during cleavage and blastocyst formation. Gamete Res. **16**, 355-370.

Selwood L. (1989) Development *in vitro* of investment-free marsupial embryos during cleavage and early blastocyst formation. Gamete Res. **23**, 399-413.

Selwood L. (1992) Mechanisms underlying the development of pattern in marsupial embryos. Current Topics Dev. Biol. **27**, 175-233.

Selwood L. and Sathananthan A.H. (1988) Ultrastructure of early cleavage and yolk extrusion in the marsupial, *Antechinus stuartii*. J. Morphol. **195**, 327-344.

Selwood L. and Smith D. (1990) Time-lapse analysis and normal stages of development of cleavage and blastocyst formation in the marsupials the brown antechinus and the stripe-faced dunnart. Mol. Reprod. Devel. **26**, 53-62.

Selwood L. and Woolley P.A. (1991) A timetable of embryonic development and ovarian and uterine changes during pregnancy in the stripe-faced dunnart, *Sminthopsis macroura* (Marsupialia : Dasyuridae). J. Reprod. Fert. **91**, 213-227.

Selwood L. and Young G.J. (1983) Cleavage *in vivo* and in culture in the dasyurid marsupial *Antechinus stuartii* (Macleay). J. Morphol. **176**, 43-60.

Sharman G.B. (1961) The embryonic membranes and placentation in five genera of diprotodont marsupials. Proc. Zool. Soc. Lond. **137**, 197-220.

Sharman G. B. (1965) Marsupials and the evolution of viviparity. Views Biol. **4**, 1-27.

Sharman G. B. (1970) Reproductive physiology of marsupials. Science **167**, 1221-1228.

Sharman G.B. (1976) Evolution of viviparity in mammals. In: Austin C.R. and Short R.V. Reproduction in Mammals Book 6. Cambridge University Press, Cambridge, pp.32-70.

Shaw G. and Renfree M.B. (1986) Uterine and embryonic metabolism after diapause in the tammar wallaby, *Macropus eugenii*. J. Reprod. Fert. **76**, 339-347.

Shaw G. and Rose R.W. (1979) Delayed gestation in the potoroo *Potorous tridactylus* (Kerr). Aust. J. Zool. **27**, 901-912.

Shine R. (1984) Physiological and ecological questions on the evolution of reptilian viviparity. In: Seymour R.S. (ed.) Respiration and Metabolism of Embryonic Vertebrates Junk, Dordrecht, pp. 147-154.

Shine R. (1985) The evolution of viviparity in reptiles: an ecological analysis. In: Gans C. and Billett F. (eds.) Biology of the Reptilia Vol. 15. John Wiley and Sons, New York, pp. 605-694.

Smith C.A., Moore H.D.M. and Hearn J.P. (1987) The ultrastructure of early implantation in the marmoset monkey (*Callithrix jacchus*). Anat. Embryol. **175**, 399-410.

Smith M.J., Bennett J.H. and Chesson C.M. (1978) Photoperiod and some other factors affecting reproduction in female *Sminthopsis crassicaudata* (Gould) (Marsupialia: Dasyuridae) in captivity. Aust. J. Zool. **26**, 449-463.

Spurgeon C.H. and Brooks R.J. (1915) The implantation and early segmentation of the ovum of *Didelphis virginiana*. Anat. Rec. **10**, 385-395.

Stephenson D.C., Hansen P.J., Newton G.R., Bazer F.W. and Low B.G. (1989) Inhibition of lymphocyte proliferation by uterine fluid from the pregnant ewe. Biol. Reprod. **41**, 1063-1075.

Steven D.H. (ed.) (1975) Comparative Placentation Academic Press, London.

Steven D.H. (1982) Placantation in the mare. J. Reprod. Fert. Suppl. **31**, 41-55.

Stevens A. and Lowe J. (1992) Histology Gower Medical Publishing, London.

Stewart J.R. (1993) Yolk sac placentation in reptiles: structural innovation in a fundamental vertebrate fetal nutrition system. J. Exp. Zool. **266**, 431-449.

Stewart J.R. and Blackburn D.G. (1988) Reptilian placentation: structural diversity and terminology. Copeia 1988 (4), 839-852.

Stewart J.R. and Thompson M.B. (1994) Placental structure of the Australian lizard, *Niveoscincus metallicus* (Squamata: Scincidae). J. Morphol. **220**, 223-236.

St-Jacques S., Malette B., Chevalier S., Roberts K.D. and Bleau G. (1992) The zona pellucida binds to the mature form of an oviductal glycoprotein (Oviductin). J. Exp. Zool. **262**, 97-104.

Stroband H.W.J. and Van der Lende T. (1990) Embryonic and uterine development during early pregnancy in pigs. J. Reprod. Fert. Suppl. **40**, 261-277.

Szekeres-Bartho J., Hadnagy J. and Pacsa A.S. (1985) The suppressive effect of progesterone on lymphocyte cytotoxicity: unique progesterone sensitivity of pregnancy lymphocytes. J. Reprod. Immunol. **7**, 121-128.

Tachi S., Tachi C. and Lindner H.R. (1970) Ultrastructural features of blastocyst attachment and trophoblastic inavsion in the rat. J. Reprod. Fert. **21**, 37-56.

Takashina T. (1987) Haemopoiesis in the human yolk sac. J. Anat. **151**, 125-135.

Thomas T., Southwell B.R., Schreiber G. and Jawarowski A. (1990) Plasma protein synthesis and secretion in the visceral yolk sac of the fetal rat: gene expression, protein synthesis and secretion. Placenta **11**, 413-430.

Thompson J. (1977) Embryo-maternal relationships in a viviparous skink *Sphenomorphous quoyi* (Lacertilia: Scincidae). In: Calaby J.H. and Tyndale-Biscoe C.H. (eds.) Reproduction and Evolution Australian Academy of Science, Canberra, pp. 279-280.

Tiedemann K. (1976) On the yolk sac of the cat: endoderm and mesothelium. Cell Tiss. Res. **173**, 109-127.

Tiedemann K. (1977) On the yolk sac of the cat: erythropoietic phases, ultrastructure of aging primitive erythroblasts, and blood vessels. Cell Tiss. Res. **183**, 71-89.

Tiedemann K. (1979) The amniotic, allantoic and yolk sac epithelia of the cat: SEM and TEM studies. Anat. Embryol. **158**, 75-94.

Tyndale-Biscoe C.H. (1973) Life of Marsupials Arnold, London.

Tyndale-Biscoe C.H. and Hinds L.A. (1989) The hormonal milieu during early development in marsupials. In: Development of Pre-implantation Embryos and their Environment Alan R. Liss Inc., New York, pp.237-246.

Tyndale-Biscoe C.H., Hinds L.A., Horn C.A. and Jenkin G. (1983) Hormonal changes at oestrus, parturition and post-partum oestrus in the tammar wallaby (*Macropus eugenii*). J. Endocrinol. **96**, 155-161.

Tyndale-Biscoe C.H. and Janssens P.A. (eds.) (1988) The Developing Marsupial Springer-Verlag, Berlin.

Tyndale-Biscoe C.H. and Renfree M.B. (1987) Reproductive Physiology of Marsupials Cambridge University Press, Cambridge.

Vaughan T.A. (1986) Mammalogy 3rd. ed. Saunders College Publishing, Philadelphia.

Wake M.H. (1985) Oviduct structure and function in non-mammalian vertebrates. In: Duncker H.R. and Fleischer G. (eds.) Functional Morphology in Vertebrates Gustav Fischer Verlag, Stuttgart, pp. 427-434.

Wake M.H. (1993) Evolution of oviductal gestation in amphibians. J. Exp. Zool. **266**, 394-413.

Walker K.Z. and Tyndale-Biscoe C.H. (1978) Immunological aspects of gestation in the tammar wallaby, *Macropus eugenii*. Aust. J. Biol. Sci. **31**, 173-182.

Walker M.T. and Hughes R.L. (1981) Ultrastructural changes after diapause in the uterine glands, corpus luteum and blastocyst of the red-necked wallaby, *Macropus rufogriseus banksianus*. J. Reprod. Fert. Suppl. **29**, 151-158.

Walker M.T. and Rose R. (1981) Prenatal development after diapause in the marsupial *Macropus rufogriseus*. Aust. J. Zool. **29**, 167-187.

Ward S. and Renfree M. (1988) Reproduction in females of the feathertail glider *Acrobates pygmaeus* (Marsupialia). J. Zool. Lond. **216**, 225-239.

Wathes D.C. and Wooding F.B.P. (1980) An electron microscopic study of implantation in the cow. Am. J. Anat. **159**, 285-306.

Weekes H.C. (1927) Placentation and other phenomena in the scincid lizard *Lygosoma (Hinulia) quoyi*. Proc. Linn. Soc. N.S.W. **52**, 499-554.

Weekes H.C. (1930) On placentation in reptiles II. Proc. Linn. Soc. N.S.W. **55**, 550-576.

Weekes H.C. (1935) A review of placentation among reptiles, with particular regard to the function and evolution of the placenta. Proc. Zool. Soc. London **2**, 625-645.

Weitlauf H.M. (1988) Biology of implantation. In: Knobil E. and Neill J. (eds.) The Physiology of Reproduction Vol. 1 Raven Press, New York, pp. 231-262.

Welsh A.O. and Enders A.C. (1991) Chorioallantoic placenta formation in the rat 1. Luminal epithelial cell death and extracellular matrix modifications in the mesometrial region of the implantation chambers. Amer. J. Anat. **192**, 347-365.

Welsh A.O. and Enders A.C. (1993) Chorioallantoic placenta formation in the rat. III. Granulated cells invade the uterine luminal epithelium at the time of epithelial cell death. Biol. Reprod. **49**, 38-57.

Williams K.E., Kidston E.M., Beck F. and Lloyd J.B. (1975a) Quantitative studies of pinocytosis. I. Kinetics of uptake of [^{125}I]polyvinylpyrrolidone by rat yolk sac cutured *in vitro*. J. Cell Biol. **64**, 113-122.

Williams K.E., Kidston E.M., Beck F. and Lloyd J.B. (1975b) Quantitative studies of pinocytosis. II. Kinetics of protein uptake and digestion by rat yolk sac cultured *in vitro*. J. Cell Biol. **64**, 123-134.

Wimsatt W.A. (1975) Some comparative aspects of implantation. Biol. Reprod. **12**, 1-40.

Wimsatt W.A. and Enders A.C. (1980) Structure and morphogenesis of the uterus, placenta and paraplacental organs of the neotropical disc-winged bat *Thyroptera tricolor spix* (Microchiroptera: Thyropteridae). Am. J. Anat. **159**, 209-243.

Wimsatt W.A., Enders A.C. and Mossman H.W. (1973) A re-examination of the chorioallantoic placental membrane of a shrew, *Blarina brevicauda*:: resolution of a controversy. Am. J. Anat. **138**, 207-234.

Wong M., Hendrix M.J.C., von der Mark K., Little C. and Stern R. (1984) Collagen in the egg shell membranes of the hen. Dev. Biol. **104**, 28-36.

Wooding F.B.P. (1992) Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. Placenta **13**, 101-113.

Wooding F.B.P. and Flint A.P.F. (1994) Placentation. In: Lamming G.E. (ed.) Marshall's Physiology of Reproduction Vol. 3 Pregnancy and Lactation. Part 1 Ovulation and Early Pregnancy. 4th. ed. Churchill Livingstone, Edinburgh, pp.233-460.

Wu J-T. and Lin G-M. (1982) The presence of 17ß-hydroxysteroid dehydrogenase activity in preimplantation rat and mouse blastocysts. J. Exp. Zool. **220**, 121-124.

Wu J-T. and Matsumoto P.S. (1985) Possible function of 17ß-hydroxysteroid dehydrogenase in preimplantation hamster embryos. J. Exp. Zool. **223**, 243-246.

Wynn R.M. (1971) Immunological implications of comparative placental ultrastructure. In: Blandau R.J. (ed.) Biology of the Blastocyst University of Chicago Press, Chicago, pp. 495-514.

Xu K.P., Yadav B.R., Roeie R.W., Plante L., Betteridge K.J. and King W.A. (1992) Development and viability of bovine embryos derived from oocytes matured and fertilized *in vitro* and co-cultured with bovine oviducal epithelial cells. J. Reprod. Fert. **94**, 33-43.

Yaron Z. (1977) Embryo-maternal interrelations in the lizard *Xantusia vigilis*. In: Calaby J.H. and Tyndale-Biscoe C.H. (eds.) Reproduction and Evolution. Australian Academy of Science, Canberra, pp. 271-277.

Yaron Z. (1985) Reptilian placentation and gestation: structure, function, and endocrine control. In: Gans C. and Billett F. (eds.) Biology of the Reptilia Vol. 15. John Wiley and Sons, New York, pp. 527-603.

Young I.R. and Renfree M.B. (1979) The effects of corpus luteum removal during gestation on parturition in the tammar wallaby (*Macropus eugenii*). J. Reprod. Fert. **56**, 249-254.

Yu Z., Croy B.A., Chapeau C. and King G.J. (1993) Elevated endometrial natural killer cell activity during early porcine pregnancy is conceptus mediated. J. Reprod. Immunol. **24**, 153-164.

Roberts, C.T., Breed, W.G., and Mayrhofer, G., (1994) Origin of the oocyte shell membrane of a dasyurid marsupial: an immunohistochemical study.
Journal of Experimental Zoology, v. 270 (3), pp. 321-331.

Roberts, C.T., and Breed, W.G., (1994) Embryonic-maternal cell interactions at implantation in the fat-tailed dunnart, a dasyurid marsupial.
The Anatomical Record, v. 240 (1), pp. 59-76.

Roberts, C.T., and Breed, W.G., (1994) Placentation in the dasyurid marsupial, *Sminthopsis crassicaudata*, the fat-tailed dunnart, and notes on placentation of the didelphid, *Monodelphis domestica.*
Journal of Reproduction and Fertility, v. 100 (1), pp. 105-113.