Development of the Rete Ovarii in the Sheep Ovary

Peter Smith

A thesis submitted in partial fulfilment for the degree of Master of Science at the University of Otago, Dunedin, New Zealand

July 2011
Abstract

The rete ovarii is commonly described as a vestigial network of cells and tubules. While functions have been proposed for the rete during foetal ovarian development, its presence in the postnatal ovary is the subject of conflicting reports and no function has been attributed to the rete postnatally. The aim of this study was to assess the presence of the rete ovarii throughout foetal and adult life in the sheep ovary. An additional aim was to use structural information, cell proliferation data and gene expression data to review roles of the rete prenatally and propose roles postnatally.

Sheep ovaries were collected at days 55, 75 and 95 of gestation and at four weeks, seven months, two years and eight years of age postnatally. Serial sections were examined to determine structure, stereology performed to estimate rete volumes and BrdU immunohistochemistry performed to determine cell proliferations rates. In situ hybridisation was performed to determine expression of key genes involved in follicular development namely Follistatin (FST), Anti Mullerian Hormone (AMH), Aromatase (Cyp19A), Bone Morphogenetic Protein 4 (BMP4), Wilms Tumour (WT-1) as well as the Platelet Derived Growth Factors (PDGFA, PDGFB, PDGFC, PDGFD) and their receptors (PDGFRA, PDGFRB).

Results show that cells of the rete arise from the regressing glomeruli and collecting ducts of the mesonephros. Mesangial cells appear to be the predominant cell of origin. Migration of the rete into the ovary occurs as a large membrane bound structure from day 55 to day 95 of gestation, at this time non membrane bound mesonephric derived cell streams are also apparent in the foetal ovary.

Both CR and IR were present in all ovaries examined. While initially ER, CR and IR are connected, the IR becomes isolated from the CR and ER during mid to late gestation. Differences in morphology, PAS staining patterns and gene expression also become apparent during this time period. Stereology showed that both IR and CR reached peak volumes at four weeks postnatally and declined thereafter. Labelling of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) showed that peak proliferation rates of rete cells was achieved at day 95 of gestation. Significant levels of rete cell proliferation continued through until eight years of age. WT-1 gene was consistently expressed in rete cells and FST was expressed in the CR at most ages studied and at all ages in the IR. PDGFA and Cyp19A were expressed at foetal ages, while PDGFB and PDGFC were expressed in the IR at eight years of age.
The results show that elements of the rete system have the potential to play roles in ovarian function through until at least eight years of age in the sheep. Functions ascribed to the rete during foetal ovarian development, notably in the initiation of meiosis may not be attributed to the rete but rather may be a function of the mesonephric derived cell streams. Cells of the rete may play a role in the development of the vasculature during foetal development through an oestrogen/PDGFA mechanism. Differences between the ER, CR and IR point to functional differences between rete components. There is no evidence to suggest that the rete plays a role in the initiation of follicle growth, however throughout both prenatal and postnatal life, cells of the rete appear to play a role in populating the ovarian cortex.
Acknowledgements

I have been privileged to work on a subject that is both interesting and exciting and I acknowledge the support of AgResearch and, particularly Drs Jenny Juengel and Jimmy Suttie for allowing me the freedom to pursue this subject. This subject has intrigued me for many years but some, at times contentious debates, with Drs Ken McNatty and Heywood Sawyer in particular, developed this from some fleeting thoughts to the project contained here.

My mentors/supervisors Drs Peter Hurst and Jenny Juengel for their support, guidance, patience and knowledge, particularly their ability to keep me focused and on track (more or less) while not curbing my, at times, schoolboy enthusiasm.

I have also worked with a number of exceptional people, but special thanks to Dianne Sebelin whose histology expertise still astounds me, Laurel Quirke who managed to explain and teach the complex procedure of in situ hybridisation to an old dog, and my office buddy Dr Anne O’Connell who has had to endure my soap box speeches extolling the rete as the centre of the universe.

Finally a huge thank you to my wife. As mentioned, she not only managed to teach an old dog in situ hybridisations, but has also had to endure the soap box speeches, brainstorming sessions, and some long absences while I gazed down the microscope. Thanks Laurel.
# Contents

Abstract ........................................................................................................................ii

Acknowledgements .........................................................................................................iv

List of Figures ................................................................................................................viii

List of Tables .................................................................................................................. x

List of Abbreviations ..................................................................................................... xi

Chapter 1: Introduction .................................................................................................. 1

1.1: Ovarian Development and Function ................................................................. 1

1.2: The Rete Ovarii ..................................................................................................... 6

1.3: Hypothesis and Aims ............................................................................................. 13

1.4: Genes of interest .................................................................................................... 13

1.4.1: Anti Mullerian Hormone (**AMH**) .............................................................. 14

1.4.2: Follistatin (**FST**) ........................................................................................ 14

1.4.3: Platelet Derived Growth Factors and Receptors (**PDGF** and **PDGFR**)... 15

1.4.4: Bone Morphogenetic Protein 4 (**BMP4**) ................................................... 15

1.4.5: Wilms Tumour (**WT-I**) ............................................................................... 16

1.4.6: Aromatase (**Cyp19A**) .................................................................................. 16

1.5: Ages for Study ....................................................................................................... 17

Chapter 2: Materials and Methods ............................................................................. 18

2.1: Animals and Tissue Collection .......................................................................... 18

2.2: Sectioning ............................................................................................................... 19

2.3: Imaging .................................................................................................................... 19

2.4: Stereology ............................................................................................................... 20

2.5: 5-bromo-2'-deoxyuridine (**BrdU**) and Cell Proliferation ............................... 22

2.6: *In situ* Hybridisation ............................................................................................ 23

Chapter 3: Morphology of the Rete Ovarii ................................................................. 30

3.1: Overview ............................................................................................................... 30
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>Foetal Day 55</td>
<td>30</td>
</tr>
<tr>
<td>3.3</td>
<td>Foetal Day 75</td>
<td>33</td>
</tr>
<tr>
<td>3.4</td>
<td>Foetal Day 95</td>
<td>36</td>
</tr>
<tr>
<td>3.5</td>
<td>Postnatal Four Weeks</td>
<td>40</td>
</tr>
<tr>
<td>3.6</td>
<td>Puberty</td>
<td>45</td>
</tr>
<tr>
<td>3.7</td>
<td>Two Years</td>
<td>48</td>
</tr>
<tr>
<td>3.8</td>
<td>Eight Years</td>
<td>51</td>
</tr>
<tr>
<td>3.9</td>
<td>Discussion</td>
<td>53</td>
</tr>
<tr>
<td>4.1</td>
<td>Rete Volumes</td>
<td>56</td>
</tr>
<tr>
<td>4.2</td>
<td>Cell Proliferation</td>
<td>61</td>
</tr>
<tr>
<td>4.3</td>
<td>Discussion</td>
<td>66</td>
</tr>
<tr>
<td>5.1</td>
<td>Background and Interpretation</td>
<td>69</td>
</tr>
<tr>
<td>5.2</td>
<td>AMH</td>
<td>73</td>
</tr>
<tr>
<td>5.3</td>
<td>FST</td>
<td>75</td>
</tr>
<tr>
<td>5.4</td>
<td>CYP19A</td>
<td>79</td>
</tr>
<tr>
<td>5.5</td>
<td>WT-1</td>
<td>83</td>
</tr>
<tr>
<td>5.6</td>
<td>BMP4</td>
<td>86</td>
</tr>
<tr>
<td>5.7</td>
<td>PDGFA</td>
<td>88</td>
</tr>
<tr>
<td>5.8</td>
<td>PDGFB</td>
<td>91</td>
</tr>
<tr>
<td>5.9</td>
<td>PDGFC</td>
<td>93</td>
</tr>
<tr>
<td>5.10</td>
<td>PDGFD</td>
<td>95</td>
</tr>
<tr>
<td>5.11</td>
<td>PDGFRA</td>
<td>96</td>
</tr>
<tr>
<td>5.12</td>
<td>PDGFRB</td>
<td>98</td>
</tr>
<tr>
<td>5.13</td>
<td>Discussion</td>
<td>103</td>
</tr>
<tr>
<td>6.1</td>
<td>Conclusions</td>
<td>107</td>
</tr>
<tr>
<td>References</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------------------------------</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>Appendix - Protocols</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>Fixation and Processing</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>Fixation</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>Routine Processing Schedule</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Histological Stains</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>Haematoxylin and Eosin</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>Periodic-Acid-Schiff</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Heidenhains Aniline Blue</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>BrdU Immunohistochemistry Protocol</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>Protocols for In Situ Hybridisation</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Cell Culture</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>DNA Miniprep Purification</td>
<td>129</td>
<td></td>
</tr>
<tr>
<td>Restriction Enzyme Digests</td>
<td>129</td>
<td></td>
</tr>
<tr>
<td>Gel Extraction</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>In Situ Hybridisation</td>
<td>132</td>
<td></td>
</tr>
</tbody>
</table>
List of Figures

Figure 1: Foetal Ovarian Development in the Sheep ................................................................. 2
Figure 2: Stages of Follicular Growth ......................................................................................... 4
Figure 3: Cavaleiri Volume Estimations ..................................................................................... 21
Figure 4: Linearisations, Generation of DNA to Produce Sense and Antisense Probes ............ 27
Figure 5: Examples of Electrophoresis Gels During Probe Preparation ................................. 29
Figure 6: Rete Morphology at Day 55 of Gestation ................................................................. 32
Figure 7: Rete Morphology at Day 75 of Gestation .................................................................. 35
Figure 8: Day 95. Continuity Between Connecting Rete and Extraovarian Rete ................. 38
Figure 9. Rete Morphology at Day 95 of Gestation ................................................................. 39
Figure 10: Rete Morphology at Four Weeks of Age ................................................................. 42
Figure 11. Four Weeks of Age. Continuity Between Connecting Rete and Extraovarian Rete ................................................................. 43
Figure 12.Rete Morphology at four Weeks of Age ................................................................. 44
Figure 13: Rete Morphology at Puberty .................................................................................... 46
Figure 14. Blind Nature of Intraovarian Rete ........................................................................... 47
Figure 15: Rete Morphology at Two Years of Age ................................................................. 50
Figure 16: Rete Morphology at Eight Years of Age ................................................................. 52
Figure 17: Extraovarian Rete Volumes ..................................................................................... 58
Figure 18: Connecting Rete Volumes ....................................................................................... 58
Figure 19: Intraovarian Rete Volumes ..................................................................................... 59
Figure 20: Examples of BrdU Labelling ...................................................................................... 62
Figure 21: BrdU Labelling Index for Intraovarian Rete ............................................................ 64
Figure 22: BrdU Labelling Index for Connecting Rete ............................................................. 65
Figure 23: BrdU Labelling Index for Extraovarian Rete ........................................................... 65
Figure 24: Expression Intensity Levels. Example one, \( AMH \) in Follicular Granulosa Cells .......................................................................................................................... 70
Figure 25: Intensity Profile of Figure 24 .................................................................................... 70
Figure 26. Expression Intensity Levels. Example two, \( CYP19A \) in Intraovarian Rete ........... 71
Figure 27: Intensity Profile of Figure 16 .................................................................................... 71
Figure 28: Validation of \( AMH \) probe ..................................................................................... 73
Figure 29: \( AMH \) Expression ..................................................................................................... 74
Figure 30: Validation of \( FST \) Probe ........................................................................................ 76
Figure 31: Foetal Expression of \textit{FST} .......................................................... 77
Figure 32: Postnatal Expression of \textit{FST} ....................................................... 78
Figure 33: Validation of \textit{CYP19A} Probe ...................................................... 80
Figure 34: Expression of \textit{CYP19A} ............................................................... 81
Figure 35: Validation of \textit{WT-1} Probe ........................................................... 84
Figure 36: Expression of \textit{WT-1} ................................................................. 85
Figure 37: Validation of \textit{BMP4} Probe .......................................................... 86
Figure 38: Expression of \textit{BMP4} ................................................................. 87
Figure 39: Validation of \textit{PDGFA} Probe ......................................................... 89
Figure 40: Expression of \textit{PDGFA} ............................................................... 90
Figure 41: Validation of \textit{PDGFB} Probe ......................................................... 91
Figure 42: Expression of \textit{PDGFB} ............................................................... 92
Figure 43: Validation of \textit{PDGFC} Probe ......................................................... 93
Figure 44: Expression of \textit{PDGFC} ............................................................... 94
Figure 45: Validation of \textit{PDGFD} Probe ......................................................... 95
Figure 46: Validation of \textit{PDGFRA} Probe ...................................................... 96
Figure 47: Expression of \textit{PDGFRA} ............................................................. 97
Figure 48: Validation of \textit{PDGFRB} Probe ...................................................... 98
Figure 49: Expression of \textit{PDGFRB} ............................................................. 99
List of Tables

Table 1: Parameters Employed for Stereological Volume Estimations ............................................. 22
Table 2: \textit{In situ} Hybridisation Probe details ............................................................................. 24
Table 3: Restriction Enzymes ........................................................................................................ 25
Table 4: Homology of Sequenced Insert ....................................................................................... 26
Table 5: Volumes of Rete Components ......................................................................................... 27
Table 6: Percentage of Ovary Occupied by Rete Components .................................................... 57
Table 7: BrdU Labelling index for Rete Components .................................................................... 60
Table 8: Mean BrdU Labelling Index for Control Cells at Two Years of Age ............................... 64
Table 9: BrdU Labelling Index, Statistical Differences Between Groups ....................................... 66
Table 10: Definition of Gene Expression Levels ............................................................................ 69
Table 11: Gene Expression in the Extraovarian Rete ................................................................. 100
Table 12: Gene Expression in the Connecting Rete ...................................................................... 101
Table 13: Gene Expression in the Intraovarian Rete .................................................................... 102
List of Abbreviations

Note: Names of genes/proteins are formatted following the guidelines of the HUGO gene nomenclature committee [1]. Briefly gene references are italicized, proteins are in regular font. Large animals species (e.g. sheep) are capitalized, small animal species are lower case other than the first letter.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF</td>
<td>Antral Follicle</td>
</tr>
<tr>
<td>AMH</td>
<td>Anti Mullerian Hormone</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone Morphogenetic Protein 4</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pairs</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BV</td>
<td>Blood Vessel</td>
</tr>
<tr>
<td>CD</td>
<td>Collecting Ducts</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus Luteum</td>
</tr>
<tr>
<td>CO</td>
<td>Cortex</td>
</tr>
<tr>
<td>CR</td>
<td>Connecting Rete</td>
</tr>
<tr>
<td>CS</td>
<td>Cell Streams</td>
</tr>
<tr>
<td>CY</td>
<td>Cyst</td>
</tr>
<tr>
<td>CYP19A</td>
<td>Aromatase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EGVEGF</td>
<td>Endocrine Gland Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>EP</td>
<td>Epithelial Cell</td>
</tr>
<tr>
<td>ER</td>
<td>Extraovarian Rete</td>
</tr>
<tr>
<td>FOL</td>
<td>Follicle</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
</tr>
<tr>
<td>FST</td>
<td>Follistatin</td>
</tr>
<tr>
<td>GE</td>
<td>Glomeruli Epithelium</td>
</tr>
<tr>
<td>GL</td>
<td>Glomeruli</td>
</tr>
<tr>
<td>GR</td>
<td>Granulosa Cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>H and E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HAB</td>
<td>Heidenhains Aniline Blue</td>
</tr>
<tr>
<td>IR</td>
<td>Intraovarian Rete</td>
</tr>
<tr>
<td>MC</td>
<td>Mesangial Cells.</td>
</tr>
<tr>
<td>ME</td>
<td>Regressing Mesonephros</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MV</td>
<td>Microvasculature</td>
</tr>
<tr>
<td>OC</td>
<td>Ovigerous Cords</td>
</tr>
<tr>
<td>OO</td>
<td>Oocyte</td>
</tr>
<tr>
<td>OV</td>
<td>Ovary</td>
</tr>
<tr>
<td>OVM</td>
<td>Ovarian Medulla</td>
</tr>
<tr>
<td>PA</td>
<td>Preantral Follicle</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic Acid Schiff</td>
</tr>
<tr>
<td>PCOS</td>
<td>Polycystic Ovarian Syndrome</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet Derived Growth Factor Receptor</td>
</tr>
<tr>
<td>PI</td>
<td>Primary Follicle</td>
</tr>
<tr>
<td>PO</td>
<td>Primordial Follicle</td>
</tr>
<tr>
<td>POV</td>
<td>Periovarian Tissue</td>
</tr>
<tr>
<td>RE</td>
<td>Restriction Enzyme</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>SE</td>
<td>Surface epithelium</td>
</tr>
<tr>
<td>SRY</td>
<td>Sex determining region y</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TH</td>
<td>Theca Cells</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WT-1</td>
<td>Wilms Tumour</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1: Ovarian Development and Function.

In general mammalian ovaries develop and function in a similar manner and while differences between species are apparent in the timing of specific events, the overall chronology of developmental events is similar in all mammals.

In the sheep the developing gonad is first visible as a thickening of the coelomic epithelium on the medial aspect of the mesonephros around day 24 of gestation. The mesonephros is a temporary kidney in mammalian species and is composed of a longitudinal array of glomeruli and collecting ducts. Glomeruli occupy a ventral position and excretory tubules are located dorsal to these. The excretory tubules drain into a series of collecting tubules which in turn drain into the Wolffian duct that runs through the dorsolateral region of the organ [2]. The mesonephros is visible as early as day 18 in the sheep fetus, begins regression around day 30 of gestation and has completely regressed by day 75 [3]. However while the mesonephros may no longer be apparent as a discrete organ at day 75, throughout this study remnants of the glomeruli and collecting tubules were observed in the periovarian tissue until at least day 95 of gestation. From day 24 the gonad continues to grow and develop not only by cell proliferation but by recruiting cells from the coelomic epithelium, the mesenchyme of the mesonephros and the glomeruli and tubules of the mesonephros [4]. More recent genetic and organ culture studies by Capel et al. [5] in the mouse have demonstrated that around the time of gonadal sexual differentiation, migration of mesonephric cells into the ovary is dependent on expression of the y chromosome linked Sry gene. Should this contention hold true in the sheep, then contrary to earlier studies, mesonephric cell migration would not begin until around day 30 in the male and even later in the female. Capel et al. [5] showed that in the female mouse mesonephric cell migration into the ovary did not occur around the time of sexual differentiation. This does not preclude migration of these cells following sexual differentiation.
Figure 1: Foetal Ovarian Development in the Sheep

Figure 1: Foetal Ovarian Development in the Sheep. Schematic representation of foetal ovarian development between day 24 and day 100. Illustrating germ cell migration, ovigerous cord formation and follicle development. Also shown are different phases of mesonephric cell migration to the ovary. (From Juengel et al. [4])
Additionally, primordial germ cells migrate to the ovary from the endoderm of the yolk sac. Around day 30 to day 32 of gestation gonadal sexual differentiation takes place, and by day 35 the testes and the ovary can be morphologically distinguished from each other. In the ovary, germ cells proliferate through until around day 100, but beyond day 75 the rate of proliferation declines rapidly [6-8]. From day 40 ovigerous cords are visible and continue to develop radially in the outer (cortical) region of the ovary through until day 75. The ovigerous cords are bound by a basement membrane and contain germ cells and somatic cells (pregranulosa cells). Meiosis of germ cells is first apparent at day 55 and peaks around day 75. From day 75 the first follicles become apparent “budding off” from the innermost regions of the ovigerous cords [8]. Between day 75 and 90 approximately 70% of germ cells are lost through a massive wave of atresia. Around day 90 of gestation the first growing follicles are observed and by day 135 numerous antral follicles are present [9]. Gestation in the sheep ends at approximately 147 days.

In some species (e.g. human, sheep) ovarian development and formation of follicles occurs in utero, such that when animals are born they already contain their lifetime supply of follicles, while in other species (rodents, rabbits) development is completed early in postnatal life. This stock of quiescent follicles is often referred to as the primordial pool of follicles or the ovarian reserve. The concept of an ovary being endowed with its lifetime supply of follicles from around birth has been the subject of some controversy in recent years. Johnson [10] has proposed the concept of germ line stem cells which have the ability to replenish, to some extent, the stock of germ cells within an adult ovary [10]. While no attempt will be made here to debate the validity of the germline stem cell concept it should none the less be noted.

Throughout the reproductive life of an animal, follicles from the primordial pool are continually recruited into the growing pool. The mechanism and signalling by which follicles are recruited to grow is not known and elucidating this is the focus of much research in the reproductive field. Two morphological criteria are generally accepted as indicating the first stages of follicular growth, enlargement of the oocyte and a change in shape of the granulosa cells surrounding the oocyte from flattened to cuboidal. It is not known whether these events occur simultaneously or independently. Follicles continue to grow through proliferation of the granulosa cells [11].
Figure 2: Stages of Follicular Growth

Non Growing
Committed
Gonadotrophin Responsive
Gonadotrophin Dependent

Figure 2: Stages of Follicular Growth. Pictorial illustrations of the different stages of follicular growth from non-growing primordial follicles through to large antral/preovulatory follicles. Based on Scaramuzzi [12] and Lundy [11].
Sawyer et al. [8] have reported that during follicle formation in the sheep foetal ovary some follicles are formed with cuboidal granulosa cells. How long these cuboidal cells remain is unclear, but the potential for misclassification of follicles is clear. Additionally Lundy et al. [11] and McNatty et al. [13] have reported marked variations in the size of the oocytes and the number of granulosa cells surrounding these oocytes in primordial follicles of sheep ovaries. These studies highlight the morphological variation amongst non-growing and early growing follicles and the need for care in the application of these criteria when classifying early follicular growth.

Relatively early in growth a thecal layer becomes evident surrounding the follicle but separated from the granulosa cells by a basement membrane. The stage at which thecal cells are first present is also the topic of some debate, notably in the rodent, with some authors suggesting these cells are present from the earliest stages of growth [14] and others propose the presence of a population of thecal stem cells [15] while most indicate their presence from around the type three to the type four stage of development [16]. The thecal layer subsequently differentiates into two layers, the theca interna and the theca externa. At a stage generally accepted to be stage three (small preantral), follicles become responsive to gonadotrophin hormones, and then antral follicles from approximately 3mm in diameter become gonadotrophin dependent. (Figure 2)

Most follicles undergo atresia, such that as the stage of follicular development becomes more advanced fewer healthy follicles are present. In the sheep, for each oestrous cycle, between one and three follicles avoid atresia and go on to ovulate and form a corpus luteum. As follicles are continually recruited from the ovarian reserve this population becomes depleted and in some species such as humans the ovarian reserve becomes completely exhausted while in other species such as the sheep complete exhaustion of the ovarian reserve is not apparent.

Following ovulation, production of progesterone by the corpus luteum is essential for the establishment of pregnancy. Recent work has shown that exposure of early sheep embryos in utero to elevated progesterone levels can enhance survival of these embryos [17] and further that uterine progesterone is most likely ovarian derived as the uterus itself does not have the ability to synthesise progesterone. Thus even following ovulation the ovary continues to play a key role in the production of offspring.
1.2: The Rete Ovarii

The classical definition of the rete ovarii refers to a vestigial network of cells and tubules within the ovary [18]. A number of studies have described the formation of the rete ovarii and postulated functions for this cellular network during foetal life. These studies which have been carried out in a variety of mammalian species vary in their description of the rete, its origin and also possible functions. In a review of the rete in 1985 Wenzel and Odend’hal [19] note that apart from its developmental function, the rete generally has been considered a functionless vestige in the adult ovary. Confusion surrounding the structure is compounded by interspecies variation and differences in terminology. While the classical definition of a vestigial network or cells and tubules is still widely held to, some authors have queried this. As early as 1923 Wilkerson [20] noted the rete ovarii was found in every ovary examined that had been sectioned longitudinally and spread as serial sections. Wilkerson concludes that the rete ovarii is a normal structure in the adult ovary and should be so recognised in describing the ovary.

Zamboni and Mauleon [3] provided a detailed description of mesonephric regression and migration of mesonephric cells into the developing sheep ovary. From as early as day 30 of gestation, small clusters of mostly mesangial cells along with a few epithelial cells from regressing glomeruli, congregated to form a semi continuum between the mesonephros and the developing gonad. The more recent studies in the mouse of Capel [21] would seem to contradict such an early migration of mesonephric cells, at least in the female. This mobilisation of mesangial and epithelial cells became more pronounced with increasing age and the cells began to form small delicate trabeculae. The trabeculae became surrounded by a basement membrane and merged with one another forming a conspicuous cell mass which extended uninterrupted from the mesonephros to the ovary. Upon entering the ovary, the cell mass became less compact and ended in a scattering of isolated cells. By day 40 of gestation this cell mass was composed almost entirely of epithelial cells, the intraovarian portion comprised a substantial portion of the ovarian medulla with the ventral aspect of this mass dispersing cells into the ovarian stroma. An increasing number of the dispersing cells became associated with germ cells located in the ovarian medulla. By day 58 of gestation the mesonephric derived cell mass displayed signs of atrophy and appeared smaller in size and surrounded by connective tissue containing collagen fibres and fibroblasts. This was exaggerated by only a small number of trabeculae departing exclusively from the few remaining tubules of the mesonephros. Aggregations of
mesonephric cells and germ cells peripheral to the mass continued to be present and appeared to take the form of fully fledged ovigerous cords. By day 70 of gestation emission of epithelial cells from the regressing mesonephros was no longer apparent, with the region dorsal to the ovary occupied exclusively by connective tissue. In the centre of the ovary a markedly atrophic, disorganised and hyalinised remnant of the previously prominent cell mass could be distinguished. The ovarian cortex was packed with radially orientated ovigerous cords demarcated by a continuous basal lamina with the innermost portions of the cords being more developmentally advanced than the peripheral portions. From this description Zamboni and Mauleon [3] conclude that the mesonephric cells colonising the sheep ovary from approximately day 30 of gestation are the source of pregranulosa cells.

Byskov and Lintern-Moore [22] describe the rete ovarii in the immature mouse ovary and proposed a role for the rete in follicle formation. They describe the rete as a continuous association of cell cords and tubules which extend from the ovary into the periovarian tissue. They describe 3 distinct regions the extraovarian rete (ER), tubes and cords in the periovarian tissue which extend to the region of the Wolffian duct. The intraovarian rete (IR) which is found only within the ovary and the connecting rete (CR) which joins the ER and IR. Highlighting the confusing differences in terminology, Wenzel and Odend’hal [19] note that the ER is sometimes (notably in human) referred to as transverse ductules of the eopophoron and that the IR can be variously termed primary sex cords, medullary cords or ovigerous cords, depending on the author, species or stage of development. At birth in the mouse the ER ends blindly with no connection to the Wolffian duct. As it enters the ovary it forms a compact cell cord (the CR) from which a number of small cords branch into the cortical regions of the ovary (the IR). These cords often appear to envelope oocytes. The epithelium of the rete changes as it enters the ovary from cuboidal to pseudocolumnar, cytoplasmic basophilia is reduced and the nuclear: cytoplasmic ratio is also reduced. Both the basophilic cytoplasm and the higher nuclear: cytoplasm ratio return as the IR forms. All rete components are bound by a basal lamina. During the first week of life cells from all rete components show evidence of mitotic activity and both the CR and IR increase in size. However by the second week of life the whole rete system appears smaller with mitotic figures rarely seen. Oocytes contained within rete tubules are less frequent and often are in various stages of atresia. Similar to Zamboni and Mauleon [3], Byskov and Lintern-Moore [22] conclude that in the mouse the rete system is a source of pregranulosa cells for developing follicles. Another important conclusion arising from this study is in terminology. Byskov and Lintern-Moore [22] refer to earlier studies which
describe both medullary cords and a central blastema, terms which are still used by some authors today. They conclude that the term medullary cords describe the IR, while the term central blastema describes the CR.

In a similar study in 1975 Byskov [23] concludes that in the cat, mink and ferret, a combination of rete and surface epithelial cells contributed to the pregranulosa cell layer of developing follicles. This study based solely on light microscope observations, proposes open connections between ovigerous cords and both the surface epithelium (at the periphery of the ovary) and the rete ovarii (in the medulla of the ovary). In contrast to both surface epithelial cells and pregranulosa cells, cells of the rete system displayed a positive Periodic Acid Schiff (PAS) reaction in their cytoplasm. Proposed connections between the IR and those germ cells in the early stages of meiosis prompted the conclusion that the rete ovarii also plays a role in the initiation of meiosis.

In a 1977 study Byskov et al. [24] transplanted whole and partial immature mouse ovaries into immunocompromised mice and monitored the degree of follicle formation. Transplanted cranial sections of ovary, rich in CR and ER generally resulted in follicles being formed while caudal sections of ovary containing only some IR did not. While the conclusion was that the rete was necessary for follicle formation, by most likely contributing granulosa cells, there remained questions. The role of the surface epithelium was inconclusive, and furthermore the numbers of follicles formed were markedly reduced in transplanted ovaries. Failure of a number of grafts to develop was also noted. While it was apparent that at least in the mouse the rete does in fact develop connections with at least some oocytes/follicles, and that the presence of the rete is most likely necessary for follicular development, it is still unclear whether this requirement is a cell contribution or a signalling mechanism.

In a 1976 study O and Baker [25] used tissue/organ culture techniques to study the role of the rete in ovarian development in the hamster. By trimming the cranial section of the ovary the ER could be removed from 12-16 day old ovaries. Ovaries subjected to this trimming at day 12 and 13 and subsequently cultured for eight days showed no germ cells entering meiosis. Similar ovaries not subjected to the trimming, and also those trimmed after day 13 showed germ cells in meiosis. If ovaries were cultured in close proximity to similar aged testes then meiosis could be induced in testicular germ cells. The data strongly supports the conclusion that the rete produces a factor which controls the initiation of meiosis. Follicle formation failed to occur normally in most cultured ovaries, either with or without rete. While this failure was ascribed to low gonadotrophin activity of the media, it
nonetheless prevented the authors from making worthwhile conclusions as to the role of the rete in follicle formation.

In a 2002 study in the sheep Sawyer et al. [8] arrived at a somewhat different conclusion, than the studies thus far presented. They note that the bulk of germ cell proliferation occurred while these cells were located in ovigerous cords, which were well delineated by a basal lamina with no evidence of open connections between cells within the cords and the ovarian stroma or rete, even during the formation of individual follicles. Thus while elements of the rete ovarii were often in close proximity to developing follicles it was concluded that the rete cells could not contribute directly to the follicle cell population. The ovigerous cords were, however, open to the surface epithelium. While somatic cells within the cords did not proliferate, cells of the surface epithelium did. Given the significant increase in both germ cell and associated pregranulosa cell numbers within these cords it was concluded that the majority of pregranulosa cells originated from the surface epithelium. The study did note the presence of a relatively small number of isolated medullary germ cells, which often were enclosed by the rete tubule network. Although no evidence was found that these rete enclosed germ cells developed into follicles their fate could not be determined at late gestational ages.

There seems to be consensus amongst these studies and their authors that the rete ovarii plays a critical role in the initiation of meiosis, although as O and Baker [25] conclude in the hamster, it is yet to be determined whether this is by intercellular contact or by a diffusible factor secreted by the rete. There are differing conclusions however as to the role of the rete in follicle formation and this may reflect true species differences. Given that in some species both the rete and surface epithelium are proposed as dual contributors to pregranulosa cells it may be possible that the source of granulosa cells is not important. It is conceivable that as long as the granulosa cells are derived from an epithelial cell type then the germ cell may have the ability to drive these cells to behave as a granulosa cell. There are two important aspects to all these studies which are critical to the final interpretation. The first is in the identification and the characterisation of basement membranes as to whether they are continuous or not. Indeed prior to the Sawyer study [8] these same authors had earlier noted that the proximity of the rete to developing follicles and germ cells in the sheep was consistent with the notion that these tubules contributed directly to the developing follicles [13]. It was only the use of specific basement membrane stains and electron microscopy that allowed a different conclusion to be reached. Most of the studies mentioned above refer to the establishment of contacts between the rete and
medullary germ cells. In the sheep study this population of germ cells is referred to as abnormal, in that they appear isolated and not part of ovigerous cords. Byskov and Lintern-Moore [22] note that many of these cells were undergoing atresia. The ultimate fate of this population of germ cells as to whether they can develop into normal follicles or degenerate is yet to be determined.

While the rete has been the subject of a number of studies during foetal and early postnatal development little is known about its presence and/or function during adult life. Postnatally rete components have been described in human [26], buffalo, camel [27], marmoset [28], cat, mink, ferret [23], European hedgehog [29], rabbit [30], hamster [25] and cow [31]. Many of these studies have been carried out on early postnatal subjects in species where ovarian development is incomplete at birth, and also on prepubertal subjects. The lack of in depth studies on the rete in postpubertal animals is a major impediment to our understanding of how this structure may influence ovarian function in the reproductively mature ovary.

Mossman and Duke [26] note that the rete ovarii probably exists in all mammals and should be considered a normal structure of the adult ovary. Sauramo [32] describes the adult human rete ovarii as variable in both its quantity, from little, in some ovaries to abundant during pregnancy where the rete also appears more active. With advancing age the rete becomes more atrophic. Shehata [27] describes the presence of medullary tubes in the adult camel ovary and the scanty appearance of medullary tubes in water buffalo. In the same study however the ovaries of cows, pigs, goats and sheep were devoid of medullary tubes. At odds with Shehata’s descriptions however Cassali et al. [33] reported the presence of rete ovarii in 55 of 64 adult sheep ovaries. The presence was noted in periovarian tissue, the ovarian hilus and both medullary and cortical regions of the ovary, the structure in each compartment being similar to that described in the mouse for ER, CR and IR. Also at odds with Shehata, Archibald et al. [31] in a preliminary study describe the rete ovarii in control and superovulated heifers at various stages following ovulation. Histologically the rete in the heifer was similar to that described in sheep and mouse and did not change significantly between four and 20 days following ovulation. However at days 4, 8 and 12 following ovulation an eosinophillic, PAS positive material was noted in the lumen of rete tubules, the histochemical features of this material suggestive of either muco-proteins or glycoproteins. This material was not present following regression of the corpus luteum at day 20. The presence of the material only during the dioestrous period is indicative of an endocrine influence on the rete cells.
Further evidence of changes in the structure and function of the rete have been reported in the cow with Miller [34] noting a more prominent epithelial structure and secretory component in the rete of pregnant cows. Wenzel et al. [35] also describes differences in the rete ovarii in the adult cow related to pregnancy and stage of the oestrus cycle. While the epithelium of the CR and IR varied considerably from cuboidal to columnar and simple to pseudostratified to fully stratified, there appeared to be no consistent pattern to the variations in epithelial structure. All rete components were surrounded by a continuous basement membrane composed of reticular fibres and an amorphous layer of glycosoaminoglycans. While elastic fibres did not surround the rete, the section of the CR immediately outside the ovary was surrounded by a collagenous connective tissue, whereas the section within the ovary was surrounded by smooth muscle. Additionally the lumen of the rete often contained cells and cellular debris, a feature also noted by Archibald et al. in the heifer [31], suggesting a form of holocrine secretion, where secretions are released by the rupture of cells. Interestingly Odendhal et al. describe in cattle and deer tubules connecting the most distal section of the CR with the infundibulum of the oviduct, referred to as a tubo-retial communication [36]. This connection theoretically provides a route for expulsion of secretions produced by the rete ovarii. The suggestion that secretions from the rete ovarii may influence the environment of the upper reproductive tract, especially the oviduct, opens a whole new range of possibilities regarding function of the rete in adult animals. Particularly given that the findings of Archibald et al. [31] concur with the study of Miller [34], in that PAS positive secretions in the lumen of the rete along with the cellular debris also present in the lumen were more prominent in the ovaries of cows during the early stages of pregnancy.

In the human Sauramo [32] notes a prominent rete during foetal development although childhood ovarian samples showed little rete. Post puberty however, the rete while prominent, varied greatly, being described variously as polycystic, metaplastic resembling Brenner Islands and long tubular structures. No attempt was made to correlate these differences to cycle stages, presumably because of insufficient information. At various stages of pregnancy the rete also varied, of interest it was noted that in some cases one ovary could contain significant rete while the contralateral ovary could contain hardly any. Again no attempt was made to explain the differences or to correlate them for instance to the presence of a corpus luteum on the ovary. In the ovaries from aged women the rete was reported as definitely not decreasing in size although it did become more atrophic with age. While Sauramo [32] concludes that the rete ovarii should be considered an ontogenetic
remnant with no necessary function, the paper does raise some interesting points. The quiescence of the rete from birth until puberty, followed by an increased presence post puberty is interesting. While this may merely be a response to the altered hormonal environment post puberty, it may also reflect a requirement for rete post puberty. The variation between patients and at times between ovaries from the same patient is curious and it is unfortunate that no descriptions were given to attempts to correlate these variations with stage of cycle and/or the presence of a Corpus Luteum.

In an immunohistochemical study in 1999 Khan et al. [37] measured proliferation rates and the presence of both oestrogen and progesterone receptors in human samples. The presence of rete ovarii in the periovarian tissue can cause complications in the diagnosis of endometriosis, thus the focus of this study was on comparing rete ovarii with foci of endometriosis. Nonetheless some interesting observations were made. Labelling of the cell proliferation marker, Ki-67, indicated that rete cells were proliferative (albeit significantly less than fallopian tube and foci of endometriosis) during the menstrual cycle and both steroid receptors were present. Although only two post menopausal samples were present both the proliferation rate and the presence of oestrogen receptor declined markedly in these two samples.

In reviewing the literature it would seem a comprehensive study of the rete is warranted. A study describing changes in the morphology of the rete at critical ages, stereological estimations of the amount of rete present at these ages combined with measures of proliferation and gene expression studies may help elucidate possible roles for the rete. The sheep is an ideal animal for such a study because development of the ovary during foetal life is well described as is the morphology of the ovary postnatally. Unpublished observations from our laboratory have noted the presence of tubules, thought to be rete in adult cycling animals. A number of gene expression studies aimed at understanding follicular development have established methodologies and validated reagents for use in the sheep. Finally previous studies have demonstrated that cell proliferation studies can be performed not only in adult but also in foetal samples.
1.3: Hypothesis and Aims

While there seems general agreement amongst authors that the rete plays a role during foetal ovarian development, opinion is still divided as to whether the rete plays a role in the ovary postnatally. Thus the hypothesis underlying this study is:

“That the rete is a dynamic system of cells and tubules which plays a role in ovarian development prenatally and plays a role in ovarian function postnatally.”

To provide evidence in support of this hypothesis, this project will use the sheep as a model to:

1. Provide a detailed morphological description of the rete ovarii at critical ages during foetal life, and also postnatally, including ovaries from animals past their reproductive prime.
2. Not only demonstrate that the rete ovarii is present during adult life, but also to use stereology to determine volumes of the rete ovarii present at these critical ages.
3. To use *in vivo* administration and subsequent immunohistochemical detection of 5-bromo-2'-deoxyuridine (BrdU) to determine the proliferative capacity of the rete ovarii both pre and postnatally.
4. To use *in situ* hybridisation to determine expression patterns within the rete ovarii of keys genes involved in the regulation of ovarian development and function.

1.4: Genes of interest

To provide some insights into possible roles of the rete ovarii *in situ* hybridisation will be performed on ovarian sections. The choice of genes for this study was underpinned by three observations or questions.

1. In a 2009 study Smith *et al.* [38] reported simultaneous changes in the developmental pattern of the rete ovarii and increased recruitment of non growing follicles into the growing follicle pool in the female offspring of prenatally androgenised ewes. While no direct link between these two events was drawn nonetheless genes involved in early follicular development are of interest to this study.
Observations on numerous foetal ovary sections suggests there may be an association between the rete ovarii and the vasculature, particularly during foetal ovarian development [8]. Thus genes involved in angiogenesis and cell differentiation are also of interest.

The precise cellular origins of the rete ovarii are still unclear, thus genes which may help determine to specific cell origins of the rete ovarii are also of interest.

1.4.1: Anti Mullerian Hormone (AMH)

AMH is a member of the TGBβ superfamily of growth factors and is expressed in the human ovary [39], the rat prenatal ovary [40] as well as the post natal sheep ovary[41] where expression is largely confined to granulosa cells. Amh is also expressed in the granulosa cells of newly recruited follicles in the mouse [42, 43] and has been implicated in the inhibition of Follicle Stimulating Hormone (FSH) stimulated preantral follicular growth [44] and the inhibition of selection of dominant follicles [45]. Of particular interest for this study AMH has been shown to inhibit the initiation of primordial follicular growth in the mouse [46]. If differences in the rete ovarii can trigger increased follicular recruitment this may be by direct stimulation of the follicle or reduction of a suppressive factor such as AMH.

1.4.2: Follistatin (FST)

FST is an autocrine glycoprotein that is expressed in nearly all tissues of higher animals. It was initially isolated from follicular fluid and was identified as a protein fraction that inhibited FSH secretion from the pituitary. Since then its primary function has been determined to be a binding and bio neutralization agent of members of the TGFβ superfamily including bone morphogenetic protein 15 (BMP15), bone morphogenetic protein 6 (BMP6) and particularly activin. Studies in humans with polycystic ovarian syndrome and also in transgenic mice have shown that FST plays a role in follicular growth from at least the primary stage of development [47, 48]. Expression of FST and the presence of protein are detected in sheep follicles shortly after the initiation of growth. Further FST protein is observed in granulosa cells of sheep follicles at an earlier stage of development than mRNA, suggesting perhaps that this protein is from an extra follicular source [49]. Expression and/or presence of FST has been shown in some cells of the rete in sheep ovaries [50]. Confirmation of this result, clarification as to which rete component expresses FST, and characterisation of this expression with respect age and stage of development may be important in trying to understand possible functions of the rete ovarii.
1.4.3: Platelet Derived Growth Factors and Receptors (*PDGF* and *PDGFR*)

PDGFs are dimeric glycoproteins, disulphide linked dimers of two polypeptide chains. Four PDGF chains are known PDGFA, PDGFB, PDGFC and PDGFD but only PDGFA and B can form heterodimers, thus 5 PDGF dimers have been identified. Two related receptors are known PDGFRα and PDGFRβ, these can also function as homo or heterodimers [51, 52] PDGF-AA, AB, BB and CC can bind to and activate the α receptor, while PDGF-BB and DD can bind and activate the β receptor. PDGF-AB, BB and CC can also activate the heterodimeric receptor PDGFR α/β [52]. PDGF’s have been shown to drive cellular responses including proliferation, migration, survival and deposition of extracellular matrix.

*PDGFA* and *PDGFRα* are expressed at a number of sites of epithelial - mesenchymal interaction, where *PDGFA* is expressed by the epithelia and *PDGFRα* by the mesenchyme. *PDGFB* and *PDGFRβ* are mainly expressed in the developing vasculature where *PDGFB* is expressed by endothelial cells and the receptor by pericytes and smooth muscle cells. *PDGFC* and *PDGFD* are usually expressed in different cell types from each other, but their expression has been recorded in a variety of cells and organs. PCR analysis has revealed the presence of mRNA for all PDGFs and their receptors in the rat ovary [53]. A combination of immunohistochemistry and *in situ* hybridisation has shown PDGFA and B as well as both receptors, α and β, to be present in human ovaries [54]. Expression in both human and rat ovary appeared to be quite widespread including the ovarian stroma although no specific mention of the rete ovarii was made. Treatment of four day old rat ovaries in culture with PDGF-AB resulted in a significant decrease in the percentage of primordial follicles present and a concomitant increase in the percentage of primary (early growing) follicles, suggesting that at least PDGF-AB may play a role in initiating follicular growth [55]. The diverse functional nature of PDGFs makes them a good candidate to determine whether the rete ovarii plays a role in ovarian and follicular development.

1.4.4: Bone Morphogenetic Protein 4 (*BMP4*)

*BMP4* is a member of the TGFβ superfamily and has been shown to promote the primordial to primary follicle transition in the rat, and also appears to be essential for oocyte survival [56]. The same study showed BMP4 to be present in theca cells of growing follicles. Given its apparent importance in follicular growth, the rete as a potential source of BMP4 for primordial or early growing follicles seems a concept worth investigating. In
both the rat and sheep \textit{BMP4} has also been shown to be expressed in islands of stromal cells and mesenchymal cells not directly associated with follicles [57, 58]. That the cells which give rise to this island pattern of expression in the ovarian stroma may be IR will be investigated.

1.4.5: Wilms Tumour (\textit{WT-1})

\textit{WT-1} is a transcriptional repressor protein essential for the development of gonads [59]. Expression of \textit{WT-1} gene is present in granulosa cells of primordial follicles from sheep ovaries [60]. Interestingly around day 75 of gestation it is reported in the same study that cells of the mesonephric glomeruli express \textit{WT-1}, but cells of the mesonephric tubules do not. Expression patterns of this gene may help in determining the origin of the ER and CR. Expression in the rete would suggest that these cells are of mesonephric glomeruli origin, notwithstanding the possibility that differentiation of cells during migration may occur.

1.4.6: Aromatase (\textit{Cyp19A})

\textit{Cyp19A} is the enzyme responsible for a key step in the biosynthesis of estrogens. Foetal ovaries secrete significant amounts of oestradiol and this secretion coincides with significant developmental effects in the cow [61]. This same study showed that this secretion was greatly reduced over the time period where follicle formation and initial growth were occurring. Activation of follicular growth could be inhibited in culture by the addition of oestradiol. Treatment of pregnant baboons with an aromatase inhibitor resulted in a 30-50% decrease in follicles in the foetal ovaries [62]. \textit{Cyp19A} is also thought to play a key role in the formation of ovigerous cords [63], germ cell meiosis and development of the vasculature [4, 64]. Additionally Garverick \textit{et al.} [65] reported \textit{CYP19A} expression in mesonephric derived cell streams and/or the rete in the cow. That the exact site of this expression which may include the rete needs to be identified, and the important and many roles proposed for oestradiol during ovarian and follicular development makes this gene an ideal choice to study.
1.5: Ages for Study

1. Eight year old animals were the oldest available ewes for this study and would give an indication as to how long the rete may persist, if at all, in the adult ovary.

2. Two year old animals are at their reproductive prime and as such an ideal age to study factors which may influence ovarian function and fertility.

3. Animals at puberty experience numerous metabolic, hormonal and behavioural changes. The status of the rete at this time would be of interest.

4. At four weeks of age the sheep ovary goes through a short period of rapid growth and contains a large number of growing follicles [66]. Should the rete play some role in follicular growth, the status of the rete at this age may provide evidence to support such a contention.

Foetal ages were chosen to be able to draw on existing knowledge from previous studies of ovarian development and to coincide with key developmental events.

5. At day 95 of gestation the first growing follicles are observed within the sheep ovary.

6. At day 75 of gestation germ cell atresia and follicle formation are proceeding.

7. At day 55 of gestation germ cell meiosis begins, this is also the first age at which a discrete rete like structure has been observed extending into the ovary.
Chapter 2: Materials and Methods

The experimental procedures carried out in this study were performed in accordance with 1999 Animal Protection (Codes of Ethical Conduct) Regulations. Approval for all work was granted by the Animal Ethics Committee of the Invermay Agricultural Centre.

2.1: Animals and Tissue Collection

All animals were selected from the Invermay breeding flock and were of mixed Romney x Texel breed. Samples from animals at puberty, two years of age and eight years of age were collected during the breeding season. Two year old and eight year old ewes were run with a vasectomised ram fitted with a marking harness and crayon. Daily checks were carried out to determine the onset of oestrus with day one being that day on which a mating mark was first observed, ovaries were subsequently collected on day 12 of the cycle. For animals at puberty, immature animals approximately seven months of age were run with a vasectomised ram as above, from the beginning of the breeding season. Ovaries were collected 12 days following the first mating mark. This was taken to be the first reproductive cycle that the animal had experienced. Ovaries were also collected from selected lambs at four weeks of age.

Foetal ovaries were collected from animals at known gestational ages (days 55, 75 and 95) following mating with an intact Texel ram fitted with a marking crayon. Day one of gestation was considered to be the day on which a mating mark was first observed.

Prior to euthanasia all animals were given two intravenous injections of 250mg of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) (Roche Diagnostics, Auckland, New Zealand). This procedure was based on that used by Sawyer et al. [8], however to allow for an expected lower proliferation rate in rete cells the number of injections was increased from one to two. Briefly 250mg of BrdU was dissolved in 10 ml of warmed sterile saline, two drops of 2M Sodium Hydroxide was added to aid solubility. Each animal (pregnant ewes in the case of foetal animals) received injections at two hours and one hour prior to euthanasia.

All ewes were euthanised with a 20ml intravenous injection of a solution containing 500mg/ml of Sodium Pentobarbitone (Pentobarb 500, South Island Chemicals,
Christchurch, New Zealand). In the case of foetal collections this dose was administered slowly to the pregnant ewes to facilitate euthanasia of the foetus. Ovaries were removed and fixed in 4% paraformaldehyde at 4°C overnight. Following an overnight wash in 70% ethanol ovaries, were processed for histology following a standard procedure (see appendix).

2.2: Sectioning

All sections were cut at five µm on a Leica Rotary microtome. At least one ovary from each animal was serially sectioned. Every 10th section was stained with haematoxylin and eosin (H&E) and remaining sections stored at 4°C until required. Sections were examined to identify that region of the ovary which contained rete components. Sections within this region were then randomly assigned for further use.

H&E staining (see appendix) was performed on some sections to provide series of serially stained sections. PAS was performed to highlight membranes, stain glycogen and glycoproteins. On some sections prior to PAS staining, a salivary amylase digestion was performed to remove glycogen to allow further identification of stained material (see appendix). Heidenhains Aniline Blue (HAB) was performed to differentiate connective tissues and muscle cells. (see appendix). Sections were also assigned for BrdU immunohistochemistry and in situ hybridisation.

2.3: Imaging

Sections were examined on an Olympus BX50 microscope fitted with a Nikon camera. Darkfield illumination was used to view in situ hybridisation sections.

Nikon Elements Research software was used for counting of cells, assessment of in situ hybridisation labelling and volume estimations of rete components.

For low magnification work, ovarian volume estimations were performed on an Olympus BH2 microscope fitted with a drawing arm.
2.4: Stereology

All volume estimations were performed using the Cavaleiri method [67]. Essential to the Cavaleiri volume estimation are serial sections and the application of an independent uniform random sampling strategy. For example, if every 10\textsuperscript{th} section was to be used, a random number between one and ten is chosen. If this number were for example seven, then the sections to be examined would be 7, 17, 27, 37 etc until the sections contain no more ovary. The Cavaleiri estimation applies a grid of known dimension over the sections; the area of the item of interest on each section is estimated by point counting. The sum of these areas is multiplied by the distance between the sections examined to obtain the volume. For example if every 10\textsuperscript{th} 5\textmu m section were used, the area is multiplied by 50\textmu m (Figure 3).

Important factors to ensure accuracy in the application of the Cavaleiri method are the number of sections to examine and the size of the grid (number of points falling on the object of interest). In a review of stereological methods, Gundersen states that providing appropriate sampling strategies are used then a maximum of 200 points on the object of interest is sufficient to obtain a reliable volume estimate [67]. For both ovarian volume and rete volume estimations, the number of points falling on the object of interest either approached or exceeded 200 for each ovary or rete component. Parameters used in volume estimations are presented in Table 1. To further assess the accuracy in some cases the distance between sections was doubled and in other cases the grid size was increased by 25%. Values calculated using these parameters were compared to those already obtained using the stated parameters and did not differ by more than 10\%.
In estimating volumes of rete components, high magnifications were required. On each section studied the whole section was systematically scanned. Where a field contained rete components, the grid points over the rete components were then counted.
Table 1: Parameters Employed for Stereological Volume Estimations

<table>
<thead>
<tr>
<th>Object of interest</th>
<th>Grid size</th>
<th>Magnification</th>
<th>Distance between sections</th>
<th>Average number of points per ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraovarian rete (adult)</td>
<td>20µm</td>
<td>40x</td>
<td>100µm</td>
<td>357</td>
</tr>
<tr>
<td>Connecting rete (adult)</td>
<td>40µm</td>
<td>20x</td>
<td>100µm</td>
<td>226</td>
</tr>
<tr>
<td>Intraovarian rete (foetal)</td>
<td>20µm</td>
<td>40x</td>
<td>50µm</td>
<td>294</td>
</tr>
<tr>
<td>Connecting rete (foetal)</td>
<td>40µm</td>
<td>20x</td>
<td>50µm</td>
<td>186</td>
</tr>
<tr>
<td>Extraovarian rete (foetal)</td>
<td>40µm</td>
<td>20x</td>
<td>50µm</td>
<td>195</td>
</tr>
<tr>
<td>Adult ovary</td>
<td>1250µm</td>
<td>12x</td>
<td>250µm</td>
<td>623</td>
</tr>
<tr>
<td>Foetal ovary</td>
<td>420µm</td>
<td>24x</td>
<td>50µm</td>
<td>316</td>
</tr>
</tbody>
</table>

2.5: 5-bromo-2'-deoxyuridine (BrdU) and Cell Proliferation

BrdU is a thymidine analogue which is incorporated into cells in the S phase of mitosis and can subsequently be detected using immunohistochemistry. It is widely used in many species to study cell proliferation. Importantly for this study, Sawyer et al. [8] used intravenous administration of BrdU in pregnant sheep and immunohistochemistry to detect BrdU incorporation in proliferating cells of the foetus.

As detailed previously, two 250mg injections of BrdU were administered intravenous to each animal, two hours and one hour prior to euthanasia.

Immunohistochemistry was performed using a BrdU kit (Invitrogen Corporation, Camarillo, California, USA). The kit utilizes a biotinylated monoclonal anti BrdU antibody and a streptavidin-biotin detection system. See appendix for detailed protocol.
Within the region of each ovary identified as containing rete components, 3 random sections were used for BrdU immunohistochemistry. Fields used to count BrdU labelling index were selected in an unbiased fashion. Fields were selected at a low magnification and slightly out of focus, such that rete components were identifiable but individually labelled cells were not. The magnification was then increased and counting performed.

2.6: In situ Hybridisation

In situ hybridisation utilizes the fact that DNA and RNA will undergo hydrogen bonding to complimentary sequences of DNA or RNA. By labelling sequences of DNA or RNA of sufficient length, selective probes can be made to detect particular sequences of DNA or RNA. The application of these probes to tissue sections allows DNA or RNA to be localized within tissue regions and cell types.

For this study frozen bacterial glycerol stocks were available which contained vectors with sequences of DNA coding for the genes of interest. Thus prior to this work, primers had been designed to allow amplification of DNA within the genes of interest. This DNA had then been ligated into a vector (PGMTeasy) and then transformed into DH5α Escherichia coli cells. Primer information and length of the DNA of interest is presented in Table 2. Published in situ hybridisation results are available for WT-1 [60], FST [68] and BMP4 [58].

Cell stocks were plated onto a Luria Broth –Agar plate containing 50µg/ml ampicillin and incubated overnight at 37°C. A single colony was then selected to inoculate 10ml Luria Broth and incubated with shaking at 150rpm overnight at 37°C. One ml aliquots were then used to inoculate 10ml Luria Broth and again incubated overnight with shaking at 150rpm at 37°C. Plasmid DNA was purified from the bacterial culture using a Miniprep purification kit (Qiagen, Hilden, Germany) as per the manufacturer’s instructions (see appendix). A 1:100 dilution of the sample was used to measure DNA concentration in a Pharmacia Ultraspec 3 Spectrophotometer. Absorbance was measured at 260nm and 280nm with the concentration calculated from the absorbance at 260nm and the purity from the 260:280 absorbance ratio.
Table 2: *In situ* Hybridisation Probe details

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>For: GGC GCC GGC CTG GGT TAG CCC TTA CCC TG&lt;br&gt;Rev: CCC GCG GCC CAC AGA GGG GAC GAC TTA CCC TG&lt;br&gt;Rev: CCC GCG GCC CAC AGA GGG GAC TTG GGA CC</td>
<td>478</td>
</tr>
<tr>
<td>FST</td>
<td>Prepared from a sub clone of 0FS2 [68]</td>
<td>801</td>
</tr>
<tr>
<td>Cyp19A</td>
<td>For: AAT TAC TTC CCC TGA GAT CAA G&lt;br&gt;Rev: GCA ATG ACT TGG GCT ATG TG</td>
<td>586</td>
</tr>
<tr>
<td>WT-1</td>
<td>For: GAC TAA TTC GTC TGA CCG CGC&lt;br&gt;Rev: GCG GCG CAG TTC CCC AAC CA</td>
<td>795</td>
</tr>
<tr>
<td>BMP4</td>
<td>For: CAT CAC ACG ACT ACT GGA C&lt;br&gt;Rev: CCT CTA CTA CGA TCT CCT G</td>
<td>607</td>
</tr>
<tr>
<td>PDGFA</td>
<td>For: GTC AGA TCC ACA GCA TCC G&lt;br&gt;Rev: GCT ACA ATA CTT GCT TTG ATG TCA C</td>
<td>670</td>
</tr>
<tr>
<td>PDGFB</td>
<td>For: CGC CAA CTT CCT GGT GTG&lt;br&gt;Rev: CTC CGA GGC TCT CCT TCA G</td>
<td>403</td>
</tr>
<tr>
<td>PDGFC</td>
<td>For: CTT ATT CGG TAT CTT CAA CCA G&lt;br&gt;Rev: AAC AGG CAC AGT TCC CAC CAC</td>
<td>268</td>
</tr>
<tr>
<td>PDGFD</td>
<td>For: TCC CAG GAG AAA ACA MGG ATA CA&lt;br&gt;Rev: CTG TAA CGC TTG RCA TCA TCA TTA</td>
<td>559</td>
</tr>
<tr>
<td>PDGFRA</td>
<td>For: AAA CCC AGG TAT GAA ATC CG&lt;br&gt;Rev: CTT CTC TGG ATG GTG GCT C</td>
<td>447</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>For: TCA ACC TGC TGG GGG C&lt;br&gt;Rev: AGG CTG TTG AAG ATG CTC TC</td>
<td>642</td>
</tr>
</tbody>
</table>
To confirm that the insert was present (DNA fragment coding for the gene of interest previously ligated into the vector), a 5µl aliquot was subjected to a restriction enzyme digest (see Table 3: Restriction Enzymes for specific enzymes) for two hours at 37°C. Agarose gel electrophoresis was then used to separate fractionated DNA, which was visualized using Sybr green on a UV transilluminator. Samples were compared to known standards to check the size of the DNA bands (Figure 5A).

Table 3: Restriction Enzymes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Insert Release</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>EcoR1</td>
<td>Sal1</td>
<td>Nco1</td>
</tr>
<tr>
<td>FST</td>
<td>EcoR1 and Xba1</td>
<td>Xba1</td>
<td>EcoR1</td>
</tr>
<tr>
<td>Cyp19A</td>
<td>Not1</td>
<td>Sal1</td>
<td>Nco1</td>
</tr>
<tr>
<td>WT-1</td>
<td>Sal1 and Apa1</td>
<td>Apa1</td>
<td>Sal1</td>
</tr>
<tr>
<td>BMP4</td>
<td>EcoR1</td>
<td>Nco1</td>
<td>Spe1</td>
</tr>
<tr>
<td>PDGFA</td>
<td>EcoR1</td>
<td>Sal1</td>
<td>Nco1</td>
</tr>
<tr>
<td>PDGFB</td>
<td>EcoR1</td>
<td>Sal1</td>
<td>Nco1</td>
</tr>
<tr>
<td>PDGFC</td>
<td>EcoR1</td>
<td>Sal1</td>
<td>Nco1</td>
</tr>
<tr>
<td>PDGFD</td>
<td>EcoR1</td>
<td>Sal1</td>
<td>Nco1</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>EcoR1</td>
<td>Sal1</td>
<td>Nco1</td>
</tr>
</tbody>
</table>

A Sample of the insert was sequenced and this sequence was compared to known gene sequences using the Blast Local alignment search tool of the National Centre for Biotechnology Information. These results are presented in table 4.
**Table 4. Homology of Sequenced Insert**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Homology/Species</th>
<th>Reference</th>
<th>Base Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>93% Bos Taurus</td>
<td>MN173890.1</td>
<td>672-1176</td>
</tr>
<tr>
<td>FST</td>
<td>100% Ovis Aries</td>
<td>M63123.1</td>
<td></td>
</tr>
<tr>
<td>Cyp19A</td>
<td>99% Ovis Aries</td>
<td>Emb/AJ012153.1</td>
<td>2-587</td>
</tr>
<tr>
<td>WT-1</td>
<td>94% Homo Sapiens</td>
<td>NM024426.4</td>
<td>896-1690</td>
</tr>
<tr>
<td>BMP4</td>
<td>99% Ovis Aries</td>
<td>NM001110277.1</td>
<td>675-1247</td>
</tr>
<tr>
<td>PDGFA</td>
<td>96% Bos Taurus</td>
<td>NM001075231.1</td>
<td>192-655, 734-932*</td>
</tr>
<tr>
<td>PDGFB</td>
<td>98% Ovis Aries</td>
<td>NM001009471.1</td>
<td>363-721**</td>
</tr>
<tr>
<td>PDGFC</td>
<td>98% Bos Taurus</td>
<td>NM002694444.1</td>
<td>598-854</td>
</tr>
<tr>
<td>PDGFD</td>
<td>98% Bos Taurus</td>
<td>NM001083706.1</td>
<td>377-935</td>
</tr>
<tr>
<td>PDGFRA</td>
<td>98% Bos Taurus</td>
<td>NM001192345.1</td>
<td>1859-2265</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>98% Bos Taurus</td>
<td>XM.002689291.1</td>
<td>2402-2713</td>
</tr>
</tbody>
</table>

*Stop codon between 655 and 734
** primers from different species removed for product

Linearisations were then performed on the DNA samples (see appendix). Linearisation requires two separate restriction enzyme digests to produce two DNA samples, one for generation of a sense probe and one for an antisense probe (see for specific enzymes). Restriction enzymes were chosen on the basis of the location of their restriction site on the plasmid in order to produce linearised plasmids which were subsequently used to generate sense and antisense RNA plasmids.
Figure 4. Digesting with the green restriction enzyme allows T7 RNA polymerase to later transcribe RNA from the DNA which includes the DNA corresponding to the gene of interest, while digesting with the orange restriction enzyme allows SP6 RNA polymerase to transcribe the DNA with the gene of interest. As both SP6 and T7 only transcribe one DNA strand, it is apparent that the RNA sequences produced are opposite. The sequence of the antisense RNA produced is complimentary to the cellular RNA and will thus bind to RNA in the cell which has the complimentary sequence from the gene of interest. The RNA generated from the sense DNA has the same sequence as the cellular RNA and thus should not bind. Thus when observing completed *in situ* hybridisation those slides incubated with an antisense probe should show specific hybridisation while those incubated with the sense probe should show background or non specific hybridisation.

Following restriction enzyme digestion, agarose gel electrophoresis was used to separate fractionated DNA using a 1.5% agarose gel, at 67 volts for 40 minutes (see appendix). Fractionated DNA bands were excised from the gel under UV illumination, and DNA extracted from the excised gel using gel extraction kit (Qiagen. Hilden, Germany) and following the manufacturer’s instructions. Agarose gel electrophoresis is used to check the purity of the samples obtained (Figure 5B).
The *in situ* hybridisation protocol is based on the method of Tisdall *et al.* [69] and has been published previously [70]. “Briefly, for all *in situ* hybridisations, 5µm sections were incubated overnight at 55 °C in hybridisation solution containing 45000cpm/µl of 33P labelled antisense or sense RNA that had been generated with T7 or SP6 RNA polymerase using the Riboprobe Gemini system (Promega. Madison, Wisconsin, USA). Nonspecific hybridisation of RNA was removed by RNase A digestion followed by stringent washes (double strength SSC, 50% formamide, 65°C, and 0.2 x strength SSC at 37°C; single strength SSC is 0.15 M sodium chloride and 0.015 M sodium citrate). After washing, sections were dehydrated, air dried, and coated with autoradiographic emulsion (LM-1 emulsion; Amersham Pharmacia Biotech New Zealand, Auckland, New Zealand). The emulsion coated slides were exposed at 4°C for two to three weeks. Slides were then developed and fixed. The sections were stained with haematoxylin and viewed and photographed using both light and darkfield illumination. Nonspecific hybridisation was monitored by hybridising at least one tissue section from each genotype as well as tumour tissue with approximately equal concentrations of the sense mRNA for each gene. No specific hybridisation was observed for any section hybridised with the sense mRNA for any gene” (for detailed protocol see appendix).
Figure 5: Examples of Electrophoresis Gels During Probe Preparation

Figure 5: [A] Cyp19a insert release gel. DNA fragment released in digested sample corresponds to expected size of insert 586bp. [B] Assessment of purity for BMP4 showing single DNA fragment for both sense and antisense DNA.
Chapter 3: Morphology of the Rete Ovarii

3.1: Overview

Ovaries for morphological (and stereology) studies were serial sectioned. Initially every third section was stained with H&E. Having used these sections to establish the region(s) where rete components were present then sections within this region were stained with PAS to determine the presence of glycogen and secretory proteins. Where a positive reaction was observed an amylase digestion step was used to distinguish between glycogen and secretory proteins. Heidenhians Aniline Blue (HAB) was used to highlight and differentiate connective tissue components. Remaining sections were then stained with H&E. Examination of serial sections was critical to determine features such as the continuity between rete components.

3.2: Foetal Day 55

At Day 55 the ER was located in the periovian tissue and was linked anatomically to the CR (Figure 6A). The ER appeared to arise from a coalescing of mesonephric collecting tubules and remnants of regressing glomeruli. The ER had an organized structure, in that large aggregations of epitheloid cells were noted in close contact with each other and with regions of basement membrane, usually forming tubes (Figure 6B). The tubes often displayed a lumen with some epithelial cells showing numerous fine projections on their luminal aspect. While these projections were often visible they usually appear as a matted amorphous layer. The depth of this layer, approximately 10µm would suggest that these projections are cilia and not microvilli. The epithelium appeared to be pseudostratified in nature and exhibits a positive PAS reaction. Large accumulations of an amorphous material were present, close examination would suggest that this is composed of thin fibres and connective tissue staining would suggest that these are predominantly collagen fibres. The structure was largely surrounded by a thin layer of collagenous material and a loose connective tissue.

The CR was present in the medulla of the ovary, encapsulated by a nondescript connective tissue containing fibroblastic cells and collagen fibres (Figure 6C). The boundary of the CR was discrete; the structure was encased by both a basement membrane.
and a thin layer of material which had staining characteristics suggestive of collagen (Figure 6F). This material was also scattered throughout the CR usually associated with short segments of basement membrane. The majority of the structure was occupied by a highly cellular connective tissue. However aggregations of cuboidal epitheloid cells were present attached to segments of basement membrane scattered throughout the structure. While tubules appeared to have developed, there was little or no lumen apparent. The epitheloid cells exhibited a PAS positive reaction (Figure 6E). The periphery was generally populated by a concentration of long thin fibroblastic cells. Occasional projections from the main body were apparent and these sometimes appeared to envelope isolated clumps of germ cells located in the medulla of the ovary (Figure 6C). Occasional signs consistent with apoptosis were seen; acute nuclear condensation, blebbing of plasma membranes and the appearance of cell fragments. Rarely were mitotic figures seen at this age.

There was little evidence of IR at this age. However numerous streams of densely packed mesenchymal cells appeared, largely in the medulla region (Figure 6D). These cell streams appeared to be of mesonephric origin and their function is unclear. While these streams often appeared to be closely associated with the innermost ends of some ovigerous cords previous studies have indicated that the cords are completely enclosed in a basement membrane and the cell streams are unable to contribute cells. In some cases however the cell streams made contact with either isolated or small aggregations of germ cells (Figure 6D), it appeared likely that cells from the cell streams can become attached to this population of germ cells. The fate of this population of germ cells and follicle like structures is uncertain.
Figure 6: Rete Morphology at Day 55 of Gestation

Figure 6: [A] Continuity between extraovarian rete (ER) and connecting rete (CR). (ME) regressing mesonephros, (OV) ovary. [B] Extraovarian rete showing branches (arrows). [C] Connecting rete, note early signs of branching (arrow). [D] Connecting rete (CR) showing putative germ cells, based on size and shape (OO) and cell streams (CS) associated with germ cells. [E] PAS stained connecting rete showing positive PAS reaction (arrows). [F] HAB stained connecting rete illustrating collagenous material within the body of the connecting rete (arrows).
3.3: Foetal Day 75

At this age the ER was still present in the periovarian tissue (Figure 7A), its structure was unchanged from day 55 and it remained continuous with the CR. Branches from the ER extended deep into the periovarian tissue (Figure 7A). From the sections examined it is unclear whether these branches were blind.

The CR had developed further, its appearance resembling that of the ER at day 55. The structure contained mostly epitheloid cells arranged loosely in tubules, in a simple columnar or pseudostratified arrangement (Figure 7C). The cells had a high nuclear to cytoplasm ratio. Occasionally, the tubules contained a small lumen, but there was no evidence of holocrine secretion (i.e. cell debris within the lumen) and cilia were absent from the luminal aspect of the cells. However the luminal aspect of the epithelial cells exhibited a positive PAS reaction (Figure 7C). The basement membrane surrounding the CR did not appear to be continuous. Some large cells with a low nuclear to cytoplasm ratio, and a clear staining cytoplasm were present, these cells contained a distinctive nucleus and were morphologically similar to macrophages. Aggregations of collagen like material were still present throughout the structure and appeared to form an almost continuous layer surrounding its periphery (Figure 7D), and similar to day 55 connective tissue with a high density of fibroblastic cells surrounded the structure. At numerous locations branches from the CR were seen (Figure 7D), these either remained connected to the CR or were isolated, at this stage they were referred to as IR.

The IR was dispersed throughout the ovarian medulla and comprised numerous tubule structures isolated from the CR (Figure 7F). The epithelia were varied and could be a simple columnar or a pseudostratified arrangement, although as the tubules became more remote from the CR the epithelium became cuboidal. The integrity of the IR also varied, with some tubules completely enclosed in a membrane, and with a well organized epithelium, while other tubules, usually located closer to the ovarian cortex, appeared to have lost large sections of basement membrane and in these structures the epithelium appeared disorganized and contained few if any columnar cells. Well defined IR displayed the presence of a small lumen (Figure 7F). Medullary germ cells with no pregranulosa cells attached appeared in close proximity to some tubules of the IR. There was no evidence of direct cell to cell contact between IR and the medullary germ cells, however contact between these germ cells and the cell streams was still apparent (Figure 7E). Cells of the IR
did not exhibit a positive PAS reaction (Figure 7F). Apoptotic cells were present at a similar frequency to day 55 and occasionally mitotic figures were seen.
Figure 7: Rete Morphology at Day 75 of Gestation

[A] Extraovarian rete (ER) extending from the periovarian tissue towards the ovary (OV). Note branches extending into the periovarian tissue (arrows). [B] Connecting rete (CR) showing separation from ovigerous cords (OC) with cell streams (CS) in an intermediate position. [C] PAS stained connecting rete showing positive reaction. [D] HAB stained connecting rete showing collagenous material still within body of the connecting rete, note branch with minimal collagenous staining within its structure (arrow). [E] Connecting rete (CR) and cell streams (CS), showing proximity of medullary germ cells
(OO) and the association of these germ cells with cell streams (CS). [F] PAS stained intraovarian rete, note lack of reaction compared to connecting rete.

**3.4: Foetal Day 95**

At day 95 of gestation the 3 components of the rete remained present with the CR still continuous with the ER (Figure 8). The ER remained a compact structure (Figure 9C) with a comparatively well organized tubular structure and simple or pseudostratified epithelium, generally although not exclusively columnar. The cells of the ER contained numerous cytoplasmic vesicles not seen to any extent previously, many exhibited a positive PAS reaction. Where a lumen was present there was little evidence of cilia being present. The ER was enclosed by a basement membrane and a layer of collagenous material which in turn was surrounded by a loose connective tissue. The CR appeared to be a less compact and discrete structure when compared to both earlier ages (Figure 9A), and also to the ER at this age. Invaginations of the surrounding connective tissue had the effect of isolating individual tubules within the CR. The CR epithelium was again varied being either pseudostratified columnar or simple cuboidal, this variation could occur within the same tubule. Epithelial cells were less vesiculated than those of the ER. Intercellular spaces and ducts were often present, these ducts often contained accumulations of PAS positive material (Figure 9E) and the cells adjacent or lining these ducts appeared ciliated. Large macrophage like cells were present and appeared to lie within a ductal lumen. While in the ER there is abundant strong staining of collagenous material throughout the structure, this staining is reduced in extent and intensity in the CR (Figure 9D). Both CR and ER appeared to be completely enveloped in the collagenous material.

Examination of sequential sections indicated that the IR was generally isolated from the CR (Figure 9D) and its epithelium was similar to the CR, either simple cuboidal or pseudostratified columnar. The collagenous material seen throughout the ER and CR was generally confined to the periphery of the IR tubules (Figure 9D). The smaller of the IR structures were completely enclosed by this material and a basement membrane (Figure 9F), whereas in the larger of these structures both this staining and the basement membrane appeared discontinuous. Much of the IR displayed a striking resemblance to small follicles, however by applying a number of criteria a distinction could be made between the two structures. Each criterion was not necessarily exclusive but when applied collectively a
clear distinction could be drawn. Examination of sequential sections showed the absence of an oocyte in the IR. The IR was generally located in the medulla of the ovary while small follicles were generally located in the cortex. Cells of the IR were predominantly columnar and arranged in a radial pattern while those cells of small follicles were flattened and arranged concentrically around the oocyte. A clear distinguishing feature of the IR was the absence of a PAS reaction in cells of the IR (Figure 9F), this was in stark contrast to cells of the CR (Figure 9E), follicular granulosa cells and also cells associated with the developing vasculature.
Figure 8: Day 95. Continuity Between Connecting Rete and Extraovarian Rete

Figure 8: Day 95 images from every 3rd (5µm) section showing continuity between extraovarian rete (ER) and connecting rete (CR).
Figure 9. Rete Morphology at Day 95 of Gestation.

Figure 9: Day 95. [A] Connecting rete illustrating less discrete nature of the structure and isolation of tubules (arrows). [B] Connecting rete showing columnar epithelium and appearance of lumen (arrows). [C] HAB stain showing extraovarian rete (ER), connecting rete (CR) and intraovarian rete (IR). Note collagenous material surrounding CR and ER. [D] HAB stained connecting rete (CR) and intraovarian rete (IR). [E] PAS stained connecting rete showing positive reaction (arrows). [F] PAS stained intraovarian rete (IR) showing no reaction within the tubules.
3.5: Postnatal Four Weeks

At this stage all rete components were prominent. As a percentage of the ovary occupied, this age was where the rete was most prominent. The ER consisted of a largely simple columnar epithelium arranged in a number of tubules (Figure 10B). The tubules often contained a lumen which frequently was filled with cell debris and an amorphous material which exhibited a positive PAS reaction (Figure 10B). The structure and individual tubules were surrounded by a continuous basement membrane. A collagenous material containing fibroblastic cells filled the spaces between tubules. The surrounding connective tissue gave the appearance of invading the structure and isolating tubules. A number of tubules connected to the ER ran into the periovarian tissue (Figure 10E). The ER remained continuous with the CR (Figure 11).

The CR was similar in appearance to the ER, epithelial cells arranged around a series of tubules (Figure 10D), interspersed with collagenous material containing fibroblasts surrounded by a basement membrane (Figure 10F). While the ER was surrounded by a collagenous rich connective tissue, the CR was surrounded by layers of smooth muscle cells (Figure 10F). The lumen of the tubules contained the amorphous material and cell debris (Figure 10D) seen in the ER, although not to the same extent. Intermediate sized pieces of CR were seen throughout the medulla generally although not exclusively continuous with the main body of the CR. These were distinguished from the IR by the presence of significant amounts of collagenous material within the structure.

The IR rete comprised a large number of tubules (Figure 12A&B), many were interconnected with each other and also with the CR, a number however could not be shown to be connected to the CR. The membrane enclosing the IR tubules was surrounded by a layer of thin fibroblastic cells. The epithelium was predominantly columnar, and both simple and stratified. The IR showed a graded, most likely sequential variation in structure, from a discrete membrane bound structure with a well organized epithelium, to structures with no or little basement membrane and a disorganized epithelial structure. This graded structure seemed to represent a breakdown of the IR tubules and a disbursement of the cells into the surrounding stroma as there seemed to be no increase in the amount of degenerating cells associated with this change in integrity of the tubules (Figure 12A&B). The tubules were largely located in the ovarian medulla (Figure 10A&C), some however extended towards the ovarian cortex (Figure 10C). IR occasionally showed a lumen (Figure 12A) and the epithelial cells contained large numbers of vesicles. While a positive PAS reaction was
observed in cells of the CR (Figure 12E) and follicular granulosa cells (Figure 12F) cells of the IR displayed no PAS reaction (Figure 12D).
Figure 10: Four weeks. [A] Showing connecting rete (CR) extending into periovarian tissue and intraovarian rete (IR). [B] Extraovarian rete showing columnar epithelium and lumen containing cell debris (arrows). [C] Connecting rete (CR) and intraovarian rete (IR), note variation in size of IR. [D] Connecting rete showing lumen containing cell debris (arrows) and pockets of mesenchymal cells embedded in collagenous material. [E] HAB stained extraovarian rete showing numerous tubules and predominance of collagen (blue) surrounding the tubules. [F] HAB stained connecting rete, note thin (red) layer of muscle cells surrounding the structure (arrow).
Figure 11. Four Weeks of Age. Continuity Between Connecting Rete and Extraovarian Rete

Figure 11: Four weeks. Images from every 10th (5µm) section showing continuity between extraovarian rete (ER) and connecting rete (CR).
Figure 12: Rete Morphology at four Weeks of Age.

Figure 12: Four weeks. [A and B] HAB stained intraovarian rete showing the variation in integrity from well defined tubules to isolated cells. [C] HAB stained intraovarian rete (IR) extending to near the ovarian cortex. [D] PAS stained intraovarian rete showing no positive reaction in the cells of either well defined tubes or disintegrating tubes. [E] PAS stained connecting rete showing positive reaction (arrows). [F] PAS stained follicle around antrum formation showing granulosa cells displaying a positive PAS reaction (arrow).
3.6: Puberty

In animals which had recently reached puberty the appearance of the ER had changed from that seen at four weeks of age. In addition to the discrete structure of multiple tubules observed at earlier ages, individual tubules were also now prominent, and these individual tubules were largely interconnected. The epithelium was a simple columnar arrangement, displayed a positive PAS reaction (Figure 13E) and cilia on their luminal aspect.

The CR was continuous with the ER and appeared as a membrane bound series of interconnecting tubules (Figure 13A) with a predominantly simple cuboidal epithelium interspersed with a collagen rich connective tissue (Figure 13C&D), although in many sections the connective tissue within the membrane bound structure had all but disappeared. Variation in the structure of the CR was observed within the same ovary, similar to that seen in the ER. In some sections a discrete structure of multiple tubules was observed while in other sections interconnected individual tubules were apparent (Figure 13A&C). This variation appeared to arise from invasion of the structure by connective tissue of the ovarian stroma through breaks in the surrounding basement membrane. The lumen of the tubules was more pronounced than at four weeks and contained PAS positive material as well as cells and cellular debris (Figure 13B&D). A PAS positive reaction was also apparent in the epithelial cells (Figure 13F). These cells also displayed cilia on their luminal aspect.

Aggregations of IR tubules were often seen, usually in close proximity to the CR, these differed from the CR in that they lacked the connective tissue and collagenous material between tubules, additionally the cells did not display a significant PAS reaction, thus these structures were classified as IR. IR was distributed throughout the ovarian medulla, at times up to the cortex-medulla interface. While predominantly they appeared to be isolated from the CR (Error! Reference source not found.), occasionally they could be followed in serial sections to the CR. In contrast to the CR and ER (and also granulosa cells of follicles), cells of the IR did not display a PAS reaction (Figure 13F), this feature was useful in distinguishing between follicles and IR tubules. As at four weeks of age the IR showed a graded structure, from well defined tubules enclosed in a continuous basement membrane to a structure with little or no basement membrane and disorganized cell content. Where the IR and its basement membrane appeared intact a layer of thin flattened cells often surrounded the tubules.

45
Figure 13: Rete Morphology at Puberty

Figure 13: Puberty. [A] Connecting rete showing a series of interconnecting tubules. [B] Connecting rete with considerable cell debris within the lumen (arrows). [C and D] HAB stained connecting rete illustrating variation in the structure when compared to [A]. Note pronounced lumen when compared with earlier ages and presence of cell debris within the lumen (arrow). [E] PAS stained extraovarian rete showing positive reaction and debris contained within the lumen (arrow). [F] PAS stained connecting rete (CR) showing positive
reaction while intraovarian rete (IR) shows no reaction. Note also positive reaction in pericytes surrounding blood vessel (BV)

Figure 14. Blind Nature of Intraovarian Rete
Figure 14: Puberty. Images from serial sections showing blind nature of intraovarian rete tubules. IR tubule, is not present in [A], appears in [B to I] (arrow) and not present in [J].

3.7: Two Years

At two years of age the appearance of all rete components was very similar to that observed at puberty (Figure 15). The ER displayed both individual tubules and the multiple tubular structure. While it appeared that many of the ER tubules ended blindly, this could not be determined with any certainty from the sections available. The epithelium was predominantly of a simple columnar type and exhibited a PAS positive reaction, cilia were noted on the luminal aspect of the epithelial cells. On occasions tubules resembling Brenner tumours were apparent in the periovarian tissue (Figure 15B) (Brenner tumours are benign ovarian tumours thought to be of follicular origin, they are characterized by islands of stratified epithelial cells).

In some animals as well as the multiple tubular structure seen at previous ages (Figure 15C) the CR could be seen as a series of interconnecting tubules (Figure 15D) and on occasions comprised a smaller structure of one to two tubules with some connective tissue all enclosed in an layer of thin fibroblastic cells, this structure while small in diameter extended some distance through the medulla of the ovary. Within the main body of the CR the collagenous material seen previously often formed its own tubular like structure (Figure 15C) filled with cells and cell debris, these structures also resembled the Brenner tumour like structures seen in the ER. Cells of the CR displayed a strong PAS positive reaction (Figure 15E). The CR showed breaks in the integrity of the basement membrane surrounding the structure and invasion of the structure by the surrounding ovarian stroma through these breaks. Cells with little or no cytoplasmic staining were often observed within the epithelial layer (Figure 15E). These cells were morphologically similar to goblet cells of the intestinal tract. Whether these cells are goblet cells or are degenerating is unclear, nonetheless their presence reinforces the holocrine or possible apocrine secretory nature of the CR.

IR had not changed in appearance compared to those animals at puberty, only occasionally was a lumen present (Figure 15F) and most if not all of the IR was not connected to the CR. Cells of the IR did not display a PAS positive reaction. IR continued to exhibit variation in appearance from well defined tubules with an organized epithelial
structure to tubules with poor boundary definition, compromised basement membrane and a disorganized epithelial structure.
Figure 15: Two years. [A] Extraovarian rete (ER) in periovarian tissue. [B] Extraovarian tissue showing structures resembling Brenner tumours. [C] HAB stained connecting rete. Collagenous material is beginning to delineated individual tubular like structures (arrows). [D] Connecting rete seen as a series of interconnecting tubules. [E] PAS stained connecting rete, note cells with little cytoplasm (arrows) whose structure suggests an apocrine secretion. [F] Intraovarian rete (arrows) showing lack of a prominent lumen.
3.8: Eight Years

At eight years of age most rete components had undergone a marked change in morphology from that seen at earlier ages. The ER was now comprised largely of individual tubules with a large lumen and, on occasions, large cystic like structures were apparent (Figure 16A). The epithelium of the cyst like structures was a pseudostratified cuboidal arrangement, while for the tubules a simple cuboidal arrangement was apparent. The lumen of the cysts contained an amorphous material which was PAS positive while the lumen of the tubules was generally clear with occasional cells/debris present. Continuity between the ER and the CR was apparent.

The CR was greatly diminished in size (Figure 16C), and often comprised a single tube spread over many sections (Figure 16D&F). This tubule appeared to provide the continuity between the CR and ER. The tube displayed a pseudostratified cuboidal epithelium with a prominent lumen. The tubule originated from the remnants of the classical CR described up to this stage and was similar to the structure seen in the ER at two years. As at previous ages the CR cells displayed a positive PAS reaction while those cells of the IR did not display such a reaction (Figure 16F). The lumen of CR showed signs of holocrine secretion with cells and cell debris evident in the lumen.

Significant amounts of IR were present in all ovaries at this age. Epithelium in the IR while largely columnar varied from simple to stratified (Figure 16E). As at other postnatal ages the IR varied in its degree of cellular organisation, size and integrity of the surrounding basement membrane.
Figure 16: Rete Morphology at Eight Years of Age

[A] Extra ovarian rete (ER) and cyst (CY) in the periovarian tissue. [B] Connecting rete (CR) with similar classical structure as seen at earlier ages. [C] Connecting rete (CR) more commonly seen at this age, smaller in size and resembling the structures seen in the extraovarian rete at two years of age. [D] HAB stained connecting rete showing collagenous material surrounding a tubule like structure (arrow). [E] Intraovarian rete showing variation in size. [F] PAS stained connecting rete (CR) displaying a positive reaction while intraovarian rete (IR) does not display any reaction.
3.9: Discussion

There seems little doubt that the rete ovarii is of mesonephric origin. The descriptive evidence presented here suggests that the rete forms from the coalescing of remnants of both the regressing glomeruli and the collecting tubules. Three factors would appear to support this contention; firstly both the ER and CR at day 55 are already discrete membrane bound structures. Secondly collecting tubules and glomeruli are in close proximity to, and at times continuous with the ER. Thirdly the mixed cellular composition of the rete is consistent with having derived from the collecting tubules and glomeruli, with epithelial cells originating from the epithelia of these structures and the highly cellular connective tissue from the remnants of mesangial cells of the glomeruli.

In contrast to the study of Zamboni and Mauleon [3], the CR reported here at day 55 appears to be in a discrete membrane bound structure, this would also appear to be at odds with the findings of Byskov [71] who reported connections between the rete and germ cells and proposed a role for the rete in the initiation of meiosis which begins around this age in the sheep. This apparent discrepancy may to some extent be attributed to terminology and definitions applied to the rete, a factor which has been contributing to confusion over the years. It should also be noted that the interpretation of static histological sections can differ from author to author. For the purposes of this study for any structure to be considered a component of the rete it must be either wholly or substantially bound by a basement membrane. McNatty et al. [72] reported a continuum of cells migrating from the mesonephros to the ovary prior to day 55, these cells gave rise to “cell streams” within the ovary. These cell streams were observed in close proximity with the medullary regions of ovigerous cords thus making these streams a more likely candidate to play a role in the initiation of meiosis. The CR makes contact and envelopes a number of germ cells. These germ cells however are not part of ovigerous cords, but, rather isolated cells located in the ovarian medulla, remote from the ovigerous cords. They comprise a minority of ovarian germ cells and their fate is unclear, many seem to undergo degeneration while a few form structures closely resembling follicles. Given the reported interspecies variation in the origin of granulosa cells it is not inconceivable that these structures could continue to develop as normal follicles with the oocyte having the ability to differentiate cells from differing origins into granulosa cells. This concept could merely be considered an extension of the oocytes ability to drive granulosa cell function during follicular development as has been the topic of numerous studies over the last decade.
The presence of a positive PAS reaction in the epithelial cells of both ER and CR at all ages, pre and post natal, suggests that these cells are active throughout the life of the rete system. This PAS reaction has been previously reported, at least postnatally, and was suggestive of production and secretion of mucoproteins or glycoproteins [35]. Evidence of holocrine secretion, particularly in the CR and ER was not seen until four weeks post birth. Odend’hal et al. noted in both the cow and deer that the CR and ER were continuous with the uterine tube [36] and suggested that the tubules of the rete could serve to transport secretions to the infundiblim and hence uterus. In the sheep the uterus develops during gestation but up until four weeks of age is a small structure. Thereafter the uterus expands rapidly, a time which coincides with the first evidence of holocrine secretion. Given also the findings of Miller [34], that the rete exhibits a more prominent secretory component during pregnancy the idea that secretions of the rete play a role in the rapid expansion of the uterus, either during development or pregnancy seems a concept worthy of further study.

That the IR is a development of the CR is supported by the progressive appearance of projections from the CR and the pinching off of these projections to form tubules isolated from the CR. The application of the term tubules to the IR is perhaps misleading as while initially tubules are apparent, the IR increasingly develops into a number of structures which morphologically resemble prenatal follicles. The application of a number of criteria, notably the lack of an oocyte, and the absence of PAS staining however allows a distinction to be drawn between the two structures. The difference in PAS staining between follicles and IR has not to my knowledge been published previously. Cells of the IR also appeared less vesiculated than those of both the CR and the extra ovarian rete. Combined with the loss of the PAS reaction this would suggest that once isolated from the CR the IR either loses much of its previous activity or its activity changes. Should the components of the rete system indeed have a function then it is likely that the IR has a function different from both CR and ER. The identification of IR is further complicated in that even from its initial appearance at day 75 of gestation a number of the tubules lose the integrity of the enclosing basement membrane and spill their cell contents into the ovarian stroma. This process occurs throughout the adult life of the sheep and without any marked degeneration of these cells. The continual addition of rete derived epithelial cells to the ovarian stromal cell population is an intriguing phenomenon and raises some interesting questions, namely do these cells play a specific role in ovarian function? The appearance of these IR structures from day 75 coincides with some important developmental events in the ovary. Firstly the appearance and expansion of the cortical stromal tissue, secondly follicle formation begins
around this period and shortly thereafter the first signs of follicular growth are apparent. One possibility is that these cells populate the cortex of the ovary and are predestined to be recruited as follicular thecal cells. Little is known about the developmental origins of thecal cells, while not morphologically distinguishable until follicular growth is well underway (type three to type four) some authors have shown that thecal cells are present from the initiation of follicular growth [14]. As follicles grow and either ovulate or undergo atresia their thecal cells are effectively lost to the ovary, the ongoing breakdown of IR tubules may represent a mechanism by which a putative population of prethecal cells is replenished. Another possibility is that these cells may play a role in the initiation of follicular growth. Developmental differences in the rete in prenatally androgenised animals which display a polycystic ovarian phenotype have been reported [38]. These animals also exhibit as part of their PCOS phenotype, increased recruitment of follicles from the primordial pool into the growing pool of follicles. The factors which control the initiation of follicular growth are not understood and widely regarded as one of the “holy grails” of reproductive biology research. While these two observations may indeed be unrelated, the possibility that the rete plays a role in initiation of growth is an exciting possibility.
Chapter 4: Stereology and Cell Proliferation

While some authors have reported the presence of the rete ovarii in ovarian sections, other authors have reported its complete absence. On occasions these contradictory reports apply to the same species [27, 33]. These contradictions may arise through true species differences, application of different or inadequate sampling strategies, differing definitions of rete or a failure to recognise rete or distinguish it from similar structures. The aim of this chapter is to apply rigorous stereological sampling strategies combined with the knowledge of rete morphology (Chapter 3) to accurately determine the presence or absence of the rete in sheep, the volume of rete components, and the proportion of the ovary occupied by these components. In addition cell incorporation of the thymidine analogue BrdU will be used to assess the proliferative capacity of rete cells. The protocol of two injections of 250mg BrdU, two hours and one hour prior to tissue collection was based on previous studies which give high labelling levels in the granulosa and thecal cells of growing follicles and has been shown to be incorporated into foetal tissue [8]. Two alternative immunohistochemical methods were considered to measure cell proliferation, antibodies against Proliferating Cell Nuclear Antigen (PCNA) and Ki-67. PCNA is present in varying amounts in all cell cycle stages, the proportion of cells staining varies dependent on fixation [73] and tissue [74]. Both PCNA and Ki-67 while useful for comparative purposes produce an overestimate of cell proliferation rates [74].

4.1: Rete Volumes

Volumes of individual rete components are presented in Table 5, and in graphical form for ER (Figure 17), CR (Figure 18) and for IR (Figure 19). At sample collection the amount of extraovarian tissue collected postnatally varied and did not include all tissue extending to the oviduct. Given the reports that the ER extends to the oviduct, at least in the cow and deer [36], it is unclear what proportion of the ER is contained within the samples. Accordingly volumes for ER were not calculated at postnatal ages.

All rete components experience a four to five fold increase in volume during the foetal period day 55 to day 95 of gestation. From Day 95 of gestation to four weeks postnatal both the CR and IR more than double in volume. Significant amounts of CR and IR
are present at puberty and two years of age. While at eight years of age, the volumes of both CR and IR have fallen markedly, they were nonetheless present in all ovaries at this age.

Table 5: Volumes of Rete Components

<table>
<thead>
<tr>
<th>Age</th>
<th>Extraovarian rete mm$^3$</th>
<th>Connecting rete mm$^3$</th>
<th>Intraovarian rete mm$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 55 n=6</td>
<td>0.0100 (0.0026)</td>
<td>0.0084 (0.00200)</td>
<td>0.0027 (0.0009)</td>
</tr>
<tr>
<td>Day 75 n=6</td>
<td>0.0290 (0.0048)</td>
<td>0.0228 (0.0061)</td>
<td>0.0228 (0.0041)</td>
</tr>
<tr>
<td>Day 95 n=5</td>
<td>0.0479 (0.0104)</td>
<td>0.0346 (0.0064)</td>
<td>0.0492 (0.0092)</td>
</tr>
<tr>
<td>Four weeks n=5</td>
<td>.0607 (0.0180)</td>
<td>.1209 (0.0429)</td>
<td></td>
</tr>
<tr>
<td>Puberty n=5</td>
<td>0.0247 (0.0055)</td>
<td>0.0281 (0.0017)</td>
<td></td>
</tr>
<tr>
<td>Two years n=5</td>
<td>0.0449 (0.0148)</td>
<td>0.0507 (0.0139)</td>
<td></td>
</tr>
<tr>
<td>Eight years n=5</td>
<td>0.0089 (0.0020)</td>
<td>0.0198 (0.0059)</td>
<td></td>
</tr>
</tbody>
</table>

Values presented are means (and standard errors).
n = number of animals.
Figure 17: Extraovarian Rete Volumes

Values presented are means and standard errors

Figure 18: Connecting Rete Volumes

Values presented are means and standard errors
Figure 19: Intraovarian Rete Volumes

Values presented are means and standard errors

Should the rete play some role in ovarian development and/or function, its relatively small size and lack of vascularity suggests such an effect is likely to be mediated via a paracrine effect. Notwithstanding the proximity of the rete to any target tissue the proportion of the ovary occupied by the rete components may give some indication of its ability to influence events within the ovary. This data is presented in Table 6. These values are considerably less than those reported in mice [71], however with the CR being a discrete structure and the IR being more concentrated near the CR, sampling across the entire ovary is required to produce an accurate result. Values presented here are based on stereological volume estimations of both the rete components and the ovary and thus give an accurate reflection, while figures for the mouse appear to be based on observations of single sections.
Table 6: Percentage of Ovary Occupied by Rete Components

<table>
<thead>
<tr>
<th>Age</th>
<th>Connecting rete (% of ovarian stroma)</th>
<th>Intraovarian rete (% of ovarian stroma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 55 n=6</td>
<td>0.16</td>
<td>0.41</td>
</tr>
<tr>
<td>Day 75 n=6</td>
<td>0.47</td>
<td>0.47</td>
</tr>
<tr>
<td>Day 95 n=5</td>
<td>0.63</td>
<td>0.47</td>
</tr>
<tr>
<td>Four weeks n=5</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>Puberty n=5</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Two years n=5</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Eight years n=5</td>
<td>0.006</td>
<td>0.002</td>
</tr>
</tbody>
</table>

The proportion of the ovary occupied by rete components drops markedly, from its peak at day 95 of gestation, this drop is particularly evident from four weeks of age. The low percentage of rete in cycling ovaries (puberty, two years and eight years of age) suggests that should a paracrine mediated effect be present then the proximity of rete components to any target cells will be an important consideration.
4.2: Cell Proliferation

Incorporation of the thymidine analogue BrdU is commonly used to measure cell proliferation. Figure 20 shows examples of BrdU incorporation obtained in this study.

BrdU labelling index values are presented in Table 7 and in graphical form in Figure 21 for IR, Figure 22 for CR and Figure 23 for ER. For each rete component, at each age, an average of 4256 cells was counted (min 2684, max 8640). For each animal three sections were chosen randomly for BrdU immunohistochemistry from within that region of the ovary where the rete had been noted.

For comparative purposes the BrdU labelling index was calculated from three animals at two years of age for granulosa cells from small antral follicles where growth is rapid, and from cells of the corpus luteum, which at this stage of the cycle, although the CL size is static, small luteal cells are still proliferating [75]. Stromal cells are included although their labelling index and thus proliferation rate is negligible at this age. From a total of 5236 stromal cells counted, no labelled cells were observed. This data is presented in Table 8.

The labelling index for all rete components is highest during the period day 55 to day 95 of gestation, the levels approaching those seen in granulosa cells of small antral follicles where growth is rapid. This indicates that both cell proliferation as well as migration play a role in the four to five fold increase in volume for both CR and IR during this period. At each age the labelling index of the separate rete components is also remarkably similar, this similarity between the ER (external to the ovary) and both the CR and IR (within the ovary) suggests that proliferation is globally rather than locally driven.

At four weeks postnatal labelling indices have dropped from their peak at day 95 but remain relatively high, in general values being between those seen for the CL and follicular granulosa cells.

While values declined from puberty a noteworthy feature is that even in animals at eight years of age proliferating cells are observed in all rete components, with values for all components being higher than that seen in stromal cells, and for the IR at eight years of age the values were similar to those seen in the corpus luteum.
Figure 20: Examples of BrdU Labelling

Table 7: BrdU Labelling index for Rete Components

<table>
<thead>
<tr>
<th>Age</th>
<th>Intraovarian Rete</th>
<th>Connecting Rete</th>
<th>Extraovarian Rete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 55</td>
<td>n/a</td>
<td>2.98 (0.37)</td>
<td>3.84 (0.58)</td>
</tr>
<tr>
<td>(n=5-6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 75</td>
<td>4.83 (0.62)</td>
<td>3.95 (0.56)</td>
<td>3.70 (0.78)</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 95</td>
<td>6.36 (0.92)</td>
<td>5.75 (0.67)</td>
<td>6.20 (0.52)</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Four weeks</td>
<td>3.29 (0.86)</td>
<td>3.42 ((1.08)</td>
<td>3.47 (0.53)</td>
</tr>
<tr>
<td>(n=4-5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Puberty</td>
<td>1.10 (0.22)</td>
<td>1.59 (0.43)</td>
<td>1.64 (1.42)</td>
</tr>
<tr>
<td>(n=4-5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two years</td>
<td>2.20 (1.51)</td>
<td>0.08 (0.05)</td>
<td>1.48 (0.83)</td>
</tr>
<tr>
<td>(n=3-5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eight years</td>
<td>1.61 (1.61)</td>
<td>0.22 (0.22)</td>
<td>0.32 (0.32)</td>
</tr>
<tr>
<td>(n=3-5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values presented are means and standard errors in brackets.
n/a indicates intraovarian rete not present at this age. As sections were selected randomly for some animals ER was not present.
Table 8: Mean BrdU Labelling Index for Control Cells at Two Years of Age

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>BrdU labelling Index (labelled cells per 100 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulosa Cells (small antral follicles)</td>
<td>7.42</td>
</tr>
<tr>
<td>Corpus Luteum</td>
<td>1.86</td>
</tr>
<tr>
<td>Stroma *</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*5236 stromal cells were counted with no labelled cells observed

Figure 21: BrdU Labelling Index for Intraovarian Rete

![Graph showing BrdU labelling index](image)

Fig 21: Intraovarian Rete: BrdU Labelling Index. (Labelled cells per 100 cells). Values presented are means and standard errors
Figure 22: BrdU Labelling Index for Connecting Rete

![Graph showing BrdU Labelling Index for Connecting Rete.](image)

Fig 22: Connecting Rete: BrdU Labelling Index. (Labelled cells per 100 cells). Values presented are means and standard errors

Figure 23: BrdU Labelling Index for Extraovarian Rete

![Graph showing BrdU Labelling Index for Extraovarian Rete.](image)

Fig 23: Extraovarian Rete: BrdU Labelling Index. (Labelled cells per 100 cells). Values presented are means and standard errors
While the purpose of the BrdU labelling experiment was to answer the question “are cells of the rete ovarii proliferating?” nonetheless some rudimentary statistics were able to be performed. Statistical analysis was restricted by low n values at each age and a number of 0 values at post pubertal ages. Therefore data was combined into 3 groups, based on their differing stages of life, representing foetal ages (day 55, 75, and 95), prepuberty (four weeks) and post puberty (puberty, two years and eight years of age). To meet a key requirement for analysis of variance, data was log transformed to equalize variances between groups and the analysis of variance was then performed using the Minitab statistical software package. Individual differences between groups were tested for using Tukeys multiple range test. Results are summarised in Table 9.

Table 9: BrdU Labelling Index, Statistical Differences Between Groups.

<table>
<thead>
<tr>
<th>Age group</th>
<th>IR</th>
<th>CR</th>
<th>ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetal vs Prepuberty</td>
<td>Not sig</td>
<td>Not sig</td>
<td>Not sig</td>
</tr>
<tr>
<td>Foetal vs Postpuberty</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Prepuberty vs Postpuberty</td>
<td>Not sig</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

A regression analysis was performed to test for any relationship between volume of individual rete components and their corresponding BrdU labelling index. At individual ages no correlations existed although n values are particularly low for this type of analysis. Combining data into foetal, prepuberty and postpuberty groups also showed no correlations.

### 4.3: Discussion

While previous reports have used observations on individual sections to estimate the relative levels of rete components in the ovary [23, 71] this is the first report to use stereology to calculate rete volumes, both during foetal development and in the post natal ovary.

The first and arguably most important finding is that both CR and IR are present in the sheep ovary until at least eight years of age. Further, BrdU labelling indicates that cells of these components are proliferating throughout post natal life until at least eight years of age. While these results are not definitive proof, they are nonetheless consistent with the hypothesis that the rete plays a role in post natal ovarian function.
The two to three fold increase in both CR and IR volumes from day 55 to day 95 of gestation, appears to arise from a combination of migration of cells into the ovary and also proliferation of these cells. The proportion of the ovary occupied by rete components is also at its highest level during this foetal period. This relatively high concentration of rete during this important developmental period may serve to maximize any paracrine effects of factors produced by the rete.

Peak volumes and high BrdU labelling of CR and IR are reached at four weeks post natal. At this time the sheep ovary experiences a temporary phase of rapid growth with growing follicle numbers being significantly higher than seen at any adult age [66] and the ovary also increasing significantly in size. The BrdU labelling results show high labelling not just in cells of growing follicles and rete cells, but also stromal cells of the ovarian medulla. The only cells not showing significant labelling at this age were cells of the ovarian cortex, and yet the ovarian cortex shows a seven fold increase from day 140 of gestation until 10 months of age [38]. One interpretation of this result is that the ovarian cortex is being populated by cells from rete tubules whose integrity has become compromised and whose cells are disbursed into the ovary, a similar concept being previously proposed by Wartenburg [76].

The decline in both volume and BrdU labelling from four weeks of age to puberty and two years followed by a further decline out to eight years may indicate a declining importance of the rete. However this decline may also reflect a relative decline in follicular activity, especially in early growing follicles in the ovary over this time period [77, 78]. As already mentioned at four weeks of age a temporary phase of high follicular growth is evident in the ovary. At puberty and also two years of age the amount of follicular growth, as reflected by numbers of growing follicles, is less than that seen at four weeks. By eight years of age, while animals still manage to ovulate, the ovulation rate is less than that seen in younger animals. The numbers of growing follicles present in the ovary appears to be less than seen at younger ages. As the decline in the rete follows the trend in follicle numbers while it may reflect a lesser importance for this structure it is not inconsistent with the rete playing some role in ovarian function. Should such a role exist this late in adult life it is likely that this role would be performed by the IR, which appears to largely retain its proliferation rate and shows a lesser decline in volume when compared to the CR. From puberty through to eight years of age the labelling index for the CR dropped by 87% and for the ER an 80% drop was noted over this period. The IR however appears to retain its
labelling index. Additionally while the volume of the CR falls by 64% over this period the drop in volume for the IR is 30%.
Chapter 5: *In Situ* Hybridisation

5.1: Background and Interpretation

Radioactively labelled *in situ* hybridisation slides are observed using darkfield microscopy where silver grains are visible as white dots. In general high concentrations of white dots compared to background on antisense slides indicate expression. Sense slides should give either no grains or a uniform intensity of grains across the section. Most authors rely on a subjective interpretation of results while some authors have quantified *in situ* hybridisation results, usually by counting and comparing the number of white dots (silver grains), however results are variable. The major problem with quantification of *in situ* hybridisation results lies in variability of results, the technique is a complex and involved procedure and results can vary markedly between *in situ* runs, the factors underlying this variability are many and not well understood. In the course of this project 32 *in situ* hybridisation procedures were performed and the intensity of silvers grains varied between individual procedures even for the same probe. To help counter this, each *in situ* hybridisation run comprised usually one (sometimes two) genes of interest and multiple sections of each age were used in each *in situ* hybridisation procedure.

For the purpose of this study gene expression is described as none, light, moderate or strong. These descriptions are based an intensity profile graph generated using Nikon Elements software. The classification is based on the ratio of the average intensity over the area of interest compared to the intensity over tissue not expressing above background levels.

Table 10: Definition of Gene Expression Levels

<table>
<thead>
<tr>
<th>Level of Expression</th>
<th>Intensity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area of interest: normal tissue</td>
</tr>
<tr>
<td>None</td>
<td>&lt;1 – 1.1</td>
</tr>
<tr>
<td>Light</td>
<td>1.1-1.25</td>
</tr>
<tr>
<td>Moderate</td>
<td>1.25-1.5</td>
</tr>
<tr>
<td>Strong</td>
<td>&gt;1.5</td>
</tr>
</tbody>
</table>
Examples: *AMH* in follicular granulosa cells at two years of age (Figure 24, Figure 25) and *CYP19A* in IR at day 95 of gestation (Figure 26, Figure 27)

An intensity profile graph is generated along the horizontal line and the values for each point on the y scale are generated. The average intensity is then calculated for the points along the line which correspond to the region of interest (ROI) and compared to the average values generated over the remainder of the line.

Figure 24: Expression Intensity Levels. Example one, *AMH* in Follicular Granulosa Cells

![Figure 24: Expression Intensity Levels. Example one, AMH in Follicular Granulosa Cells](image)

Figure 25: Intensity Profile of Figure 24

![Figure 25: Intensity Profile of Figure 24](image)
In the example above the average intensity profile in the area of interest (granulosa cells of a small antral follicle, in between white bands) is 117, while for the remaining tissue on the horizontal line the value is 71. The ratio of 1.6 indicates strong expression.

Figure 26. Expression Intensity Levels. Example two, CYP19A in Intraovarian Rete

Figure 27: Intensity Profile of Figure 16
In this example the average intensity value for the region of interest between the white bands (CR) is 93, while over the remaining tissue the average intensity value is 41. The ratio of 2.3 indicates strong expression.

While the focus of this study is on the rete ovarii, gene expression has been noted and recorded in other ovarian cell types. The reasons for this are threefold;

1. To understand the significance of expression in the rete ovarii, where noted, it is important to know how widespread in the ovary expression of the gene is and also which other cell types express the gene.

2. Where no expression in the rete ovarii is noted it is important to verify that the in situ hybridisation procedure has worked thus validating the negative result.

3. In the case of the PDGFs and their receptors there are few publications describing their cell specific expression in the ovary and so the descriptions for the expression pattern of these genes is broader than for the other genes studied.

Examples of images obtained using a sense probe and an antisense probe for each gene are presented throughout the results section. These images were not necessarily obtained from adjacent sections but the sections were chosen from the same ovary so that the same structure (e.g. follicle, CR) was present in both sections.
5.2: AMH

At the three foetal ages studies (days 55, 75 and 95) there was no expression of AMH in any rete components. It appeared that there was no specific expression of AMH in any cell type within the ovary at these stages of development. This result is in agreement with previously published reports [39].

At four weeks of age there was again no expression of AMH in any rete components. There was moderate to strong expression in the granulosa cells of some healthy follicles from the large preantral stage of development. The same pattern was evident in animals at puberty, two years and eight years of age.

Figure 28: Validation of AMH probe

Figure 28: Validation of AMH Probe. Four weeks of age, sections through the same small antral follicle. [A] Brightfield image. [B] Sense probe showing sparse and consistent pattern of silver grains. [C] Antisense section showing specific binding of probe to granulosa cells.
Figure 29: AMH Expression

5.3: *FST*

At day 55 there was some expression of *FST* in the ER. Although this expression was light, it was consistently present in all animals. The CR displayed strong expression which was consistently present in all animals.

At day 75 expression of *FST* was either not present or greatly reduced in both the ER and CR when compared to day 55, being classified as moderate (Figure 31A&B). There was some punctate expression in the ovarian medulla. This expression was predominantly not localised to any rete component including the IR, rather the expression appeared to be associated with some, but not all, cells of the cell streams. Occasionally some IR tubules showed moderate *FST* expression, this expression was confined to cells within the tubule and was absent from cells being disbursed where the basement membrane was not intact.

At day 95 *FST* expression as absent from the ER and CR, other than in one ovary where very light expression was noted in the CR. Strong punctate expression was noted in the ovary corresponding to IR (Figure 31C&D). This expression did not extend to those cells being disbursed from tubule undergoing breakdown. Where IR tubules were seen in close proximity to developing follicles the expression remained in the IR cells but expression was absent from the follicular granulosa cells (Figure 31E&F). All follicles up to the type two stage of development did not exhibit *FST* expression. In all samples some distinctive tubes were present which did not exhibit *FST* expression. These tubes were generally small, always rounded, had a relatively large lumen for their size. These were considered to be part of the lymphatic system and not IR.

At four weeks of age the pattern of *FST* expression mimicked that of day 95. There was no expression in either the ER or the CR. Strong expression was however exhibited in cells of the IR, regardless of their location in the ovary (Figure 32A&B). The expression did not extend to cells being disbursed from IR tubules.

At puberty the ER did not exhibit *FST* expression. In contrast to day 75, day 95 and four weeks the CR did show some, albeit faint expression. The IR displayed a complete range of expression, from some tubules showed strong expression of *FST* right through to some tubules showing no expression at all. This variation occurred within the same ovary.

At two years of age the expression pattern was the same as at puberty. The ER showed no expression of *FST* while both the CR and IR showed a range of expression from strong through to no expression (Figure 32C&D). The intensity of expression did not
appear to be related to its location within the ovary or its proximity to other structures such as the CR as in some cases adjacent tubules displayed this variation.

At eight years of age there was again no FST expression in the ER. The CR also at this age did not show any appreciable expression of FST. It is difficult to determine whether expression has been completely lost or whether this is merely a reflection of the CR being a much smaller structure at this age. The IR still showed the same pattern as at puberty and two years with expression varying from strong through to no expression at all.

Figure 30: Validation of FST Probe

Figure 30: Validation of FST Probe, two years of age, sections through connecting rete from the same ovary. [A] Brightfield image. [B] Sense probe showing consistent pattern of silver grains. [C] Antisense section showing specific binding of probe to connecting rete.
Figure 31: Foetal Expression of *FST*

[A] Day 75 showing connecting rete (CR) and intraovarian rete (IR). [B] Darkfield image of [A] showing light expression of *FST* in the connecting rete and no expression in the intraovarian rete. [C] Day 95 showing several intraovarian rete tubules, some indicated by arrows. [D] Darkfield image of [C] showing strong expression of *FST* in the intraovarian rete. [E] Day 95 showing a primordial follicle (PO) a primary follicle (PI) and intraovarian rete (IR). [F] Darkfield image of [E] showing strong expression of *FST* in the three intraovarian rete tubules present. Close examination shows that this expression does not extend to the granulosa cells of either follicle.
Figure 32: Postnatal Expression of FST

[A] Four weeks of age showing connecting rete (CR) and intraovarian rete (IR). [B] Darkfield of [A] showing no expression of FST in the connecting rete and strong expression in the intraovarian rete. [C] Two years of age showing connecting rete (CR). [D] Darkfield image of [C] with connecting rete now showing expression of FST in contrast to four weeks. [E] Four weeks age showing intraovarian rete (IR) extending towards the ovarian cortex where growing follicles (FOL) and numerous primordial follicles (PI) are present. [F] Darkfield image of [E] showing strong expression in the intraovarian rete. Note also expression in the granulosa cells of growing follicles but not in the primordial follicles.
### 5.4: CYP19A

At day 55 there was no expression of CYP19A in any rete component (ER, CR or IR). Expression was confined to the mesonephric derived cell streams comprising long slender fibroblastic like cells, and was light.

At day 75 there was no expression in either the ER or the CR. There was a strong punctate expression pattern throughout the medulla of the ovary. This expression did not correlate to the mesonephric derived cell streams or to the well defined IR. The expression was restricted to IR and CR where the basement membrane is compromised, cell organisation was poor and the outer boundary was less well defined and few columnar cells are present (Figure 34A&B).

At day 95 there was still no expression of CYP19A in either the ER or the CR. The IR however now exhibited strong CYP19A expression (Figure 34C&D), this expression was restricted to those tubules where the basement membrane appeared compromised, as was observed at day 75.

At four weeks of age there was no expression of CYP19A in either the ER or the CR. Some ovaries exhibited CYP19A expression in the IR, following the same pattern as at day 75 and 95, while other ovaries showed no expression in the IR (Figure 34 E&F). This pattern of expression appeared to be related to the stage of development of the ovary.

Around four weeks of age sheep ovaries exhibit a burst of growth and follicular activity [66]. The CYP19A expression in the IR appears related to this burst of growth, smaller ovaries with little/normal follicular activity showed CYP19A expression in the IR, while the larger ovaries with numerous growing follicles did not show CYP19A expression in the IR. In these ovaries the IR was predominantly enclosed in a well defined basement membrane.

At puberty ER, CR and IR did not exhibit any CYP19A expression in all ovaries examined. This same pattern of expression was also evident in ovaries at two years and eight years of age.
Figure 33: Validation of CYP19A Probe. Two years of age sections through the same small antral follicle. [A] Brightfield image. [B] Sense probe. [C] Antisense probe showing specific binding of probe to granulosa cells.
Figure 34: Expression of $CYP19A$

Figure 34: $CYP19A$ Expression. [A] Day 75 showing connecting rete (CR). Note poorly defined boundary indicative of a compromised basement membrane, lack of cellular organization and few columnar cells. White chevron indicates an intact well defined intraovarian rete tubule and CS indicates cell stream. [B] Darkfield of [A] showing $CYP19A$ expression in the compromised intraovarian rete while the intact tubule and cell stream show no $CYP19A$ expression. [C] Day 95 showing numerous intraovarian rete (IR) whose boundaries are poorly defined. [D] Darkfield of [C] showing strong expression in the intraovarian rete (IR). [E] Puberty showing connecting rete (CR) well defined intraovarian...
rete (IR) and poorly defined intraovarian rete (white chevron). [F] Darkfield of [E] showing no CYP19A expression in either the connecting rete or the well defined rete, while strong CYP19A expression is evident in the poorly defined intraovarian rete.
5.5: WT-1

At day 55 WT-1 expression was widespread in the ovary. Strong expression was exhibited in both the ER and CR. From the regressing mesonephros WT-1 expression was noted in the mesangial cells of the regressing glomeruli, but not the epithelial cells of either the glomeruli or the collecting ducts (Figure 36A&B).

At day 75 the expression pattern of WT-1 was the same as at day 55, with the expression now extending to the IR which is now present (Figure 36C&D). Expression in the periovarian tissue was somewhat reduced at day 75, representing that the few structures left did not contain mesangial cells.

At day 95 expression appeared more specific than at day 75 and 55. There was strong expression throughout the cortex (with the exception of oocytes) and expression in the medulla was strong in CR, IR and cell streams (Figure 36E&F). The appearance of more specific expression is reflective of a more developed ovary, particularly in the medulla where the vasculature is rapidly developing and does not express WT-1.

At four weeks of age WT-1 expression was exhibited in the ER, the CR and the IR. While there appeared to be significant expression in ovarian stromal cells the high level of expression in the IR did not extend to cells being disbursed from tubules. The lower expression levels of WT-1 by cells surrounding both the CR and IR likely reflects an inherent level of expression by stromal cells rather than these cells being derived from the IR.

Expression at puberty was similar, with strong expression in all rete components as well as some adjacent cells. Close examination of the CR suggests that it is the epithelial cells in this structure which are expressing WT-1. In one instance a cyst was present in the periovarian tissue. In contrast to the ER, cells of the cyst did not express WT-1. While cysts of the rete ovarii are not uncommon [79] without further investigation it would be premature to classify this cyst as of rete origin.

At two years of age the pattern of WT-1 expression mimicked that at puberty. Expression was displayed in the epithelial cells of the ER, the CR and the IR. Once cells were disbursed from the rete then expression ceased.

At eight years of age the expression pattern remained unchanged.
Figure 35: Validation of WT-1 Probe

Figure 35: Validation of WT-1 Probe. Sections through the connecting rete from a four week ovary. [A] Brightfield image. [B] Sense probe showing no binding. [C] Antisense probe showing specific binding of probe to cells of the connecting rete.
Figure 36: Foetal expression of WT-1. [A] Day 55 periovarian tissue showing collecting ducts (CD), glomerular epithelium (GE) and glomerular mesangial cells (MC). [B] Darkfield of [A] showing strong expression of WT-1 in glomerular mesangial cells. [C] Day 75 showing connecting rete (CR) and cell streams (CS). [D] Darkfield of [C] showing expression in connecting rete and cell streams. Note also strong expression in ovarian cortex and surface epithelium. [E] Day 95 showing connecting rete (CR) and intraovarian rete (IR). Note poorly defined boundary of connecting rete common at this age. [F] Darkfield of [E] showing WT-1 expression in both connecting rete and intraovarian rete.
5.6: **BMP4**

At day 55 BMP4 moderate expression was present in the ER (Figure 38A&B) but not in the CR. There was no other significant expression in any other cells type, either ovarian or mesonephric.

At day 75 and day 95 the expression seen at day 55 in the ER was no longer present in seven out of eight samples, with only one animal at day 75 still exhibiting BMP4 expression. There was also no expression in the CR or IR C&D).

At four weeks of age there was also no expression of BMP4 in any rete components. At all adult ages studied, puberty, two years and eight years there was no expression of BMP4 in any rete component, either ER, CR or IR (Figure 38E&F). Light but consistent expression was apparent at all ages either in the granulosa cells of some but not all follicles or in a concentric ring surrounding follicles just beyond the theca layer. Expression was also noted in cells surrounding the blood vessels.

Figure 37: Validation of **BMP4** Probe

![Figure 37: Validation of BMP4 Probe](image)

Figure 37: Validation of BMP4 probe. Four weeks of age, sections through the hilus of the same ovary. [A] Brightfield image. [B] Sense probe showing sparse and consistent pattern of silver grains. [C] Antisense probe showing specific binding of probe to endothelial cells surrounding blood vessels.
Figure 38: Expression of BMP4

5.7: PDGFA

From day 75 of gestation expression of PDGFA was evident in most ovarian cell types (Figure 40A&B). Using the intensity profile ratio method as described in Table 10 may have the effect of describing the expression as lower than it appears. This occurs as expression in the rete is compared to other cell types which appear to have an inherent level of expression of PDGFA. However in considering a specific role for rete components it is the expression level above the inherent expression level that is important. Unless specified, the description of expression levels for PDGFA from day 75 refers to the level above the inherent expression level.

At day 55 there was no significant expression of PDGFA in either the ER or CR. Expression of PDGFA was evident in both the epithelial and mesangial cells of regressing glomeruli. Ovarian expression was evident in the cortex particularly in the surface epithelium.

At day 75 expression of PDGFA was strong in most ovarian cell types (Figure 40A&B). In ER, CR and IR expression was moderate when compared to other cell types.

At day 95 expression of PDGFA was still widespread throughout the ovary. Expression was however particularly strong in all rete components as well as cells surrounding the developing vasculature and oocytes of formed follicles (Figure 40C&D).

By four weeks of age expression in both ER and CR had decreased to such an extent that it was not discernible above background. The IR on some occasions appeared to show moderate expression of PDGFA while on other occasions there was no expression. The presence of expression appeared to be related to the position of the tubule within the ovary. In general those tubules in the central medulla, especially those adjacent to the CR did not show elevated expression of PDGFA. Those tubules with a more cortical location were more likely to show elevated PDGFA expression. Expression of PDGFA was also noted in smooth muscle cells surrounding the vasculature and in the oocytes and granulosa cells of follicles up to at least the small antral stage of development.

By puberty significant expression of PDGFA was no longer apparent in any rete component (Figure 40E&F). No evidence of expression was observed in ER, CR and IR was also observed at two years and eight years of age.

At puberty, two years and eight years expression was apparent in the granulosa cells and theca of healthy follicles and the granulosa cells of atretic follicles, expression was also noted in cells surrounding the vasculature.
Figure 39: Validation of \textit{PDGFA} Probe

Figure 39: Validation of \textit{PDGFA} probe. Day 75, sections through the same region of periovarian tissue. [A] Brightfield image. [B] Sense probe showing sparse and consistent pattern of silver grains. [C] Antisense section showing specific binding of probe to endothelial cells surrounding developing microvasculature.
Figure 40: Expression of PDGFA

[A] Day 75 showing glomeruli remnant (GL) extraovarian rete (ER) and ovarian cortex (OVC). [B] Darkfield of [A] showing expression in the glomeruli, ER and ovarian cortex. [C] Day 95 showing intraovarian rete (IR) and microvasculature (MV). [D] Darkfield of [C] showing expression above other cell types in the intraovarian rete and cells surrounding the microvasculature. [E] Puberty showing connecting rete (CR) and intraovarian rete (IR). [F] Darkfield of [E] showing no expression of PDGFA in the connecting rete and intraovarian rete above other cell types.
5.8: \textit{PDGFB}

At day 55 there was no expression of \textit{PDGFB} in any rete component. While expression in most ovarian cell types was low, results suggested that there was some expression above background in the cortical region of the ovary and also around the tubules of the regressing mesonephros.

At day 75 there was also no expression of \textit{PDGFB} in any rete component. Expression was concentrated in the ovarian outer cortex (Figure 42A&B) and appeared to be expressed in oocytes in this region. Expression was also noted surrounding some ducts in the periovarian tissue.

At day 95 the expression pattern was similar to day 75. There was no expression in any rete component. The expression in the ovary was confined to a very narrow band at the outer edge of the ovarian cortex.

At postnatal ages four weeks, puberty, and two years there was no expression of \textit{PDGFB} in any rete component (Figure 42C&D). Expression at these ages was evident in follicular granulosa cells and thecal cells of some follicles. At puberty, two years and eight years of age \textit{PDGFB} was strongly expressed in cells of the corpus luteum. At eight years of age moderate \textit{PDGFB} expression was evident in both IR and CR (Figure 42E&F).

Determination of expression in the ER at eight years was not possible owing to a lack of this structure in most sections.

Figure 41: Validation of \textit{PDGFB} Probe

![Image A](image1.png) ![Image B](image2.png) ![Image C](image3.png)

Figure 41: Validation of \textit{PDGFB} probe. Two years of age, sections through the same region at the interface of stromal tissue and corpus luteum. [A] Brightfield image. [B] Sense probe showing sparse and consistent pattern of silver grains. [C] Antisense probe showing specific binding of probe to cells of the corpus luteum and cells surrounding the vasculature.
Figure 42: Expression of PDGFB.

[A] Day 75 low power showing cortex (OVC) and medulla (OVM) of ovary. [B] Darkfield of [A] showing light expression of PDGFB in the outer cortex. [C] Four weeks of age showing connecting rete (CR) and intraovarian rete (IR). [D] Darkfield of [C] showing no expression of PDGFB in both connecting rete and intraovarian rete. Note region of low connective tissue cell density (stars) with corresponding low density of silver grains, suggesting that there is low expression of PDGFB in connective tissue cells. [E] Eight years of age showing connecting rete (CR). [F] Darkfield of [E] showing expression in and around the connecting rete.

92
5.9: *PDGFC*

At all foetal ages examined day 55, day 75 and day 95 there was no expression of *PDGFC* in the ER, the CR or the IR (Figure 44A&B). Expression was limited to low expression in the ovarian surface epithelium and the endothelium of the developing vasculature. Expression was not evident in any other cell type either within the ovary or in the periovarian tissue.

At four weeks of age there was again no expression of *PDGFC* in ER, CR or IR. Expression was evident in the granulosa and theca cells of follicles around the time of antrum formation (Figure 44C&D), especially those follicles which displayed some evidence of atresia. Expression, although low, was also evident in the corpus luteum, and in regions of the outer ovarian cortex.

The pattern of expression for *PDGFC* at puberty and at two years of age was the same as for four weeks. At eight years of age the pattern of *PDGFC* was similar to that seen at other postnatal ages. However on some occasions (seen in two ovaries) some elements of the IR showed moderate expression of *PDGFC* (Figure 44E&F). This expression is highly variable as within the same section some elements of IR may show expression while other elements would not.

Figure 43: Validation of *PDGFC* Probe

Figure 43: Validation of *PDGFC* probe. Day 95, sections through the same region of an ovary. [A] Brightfield image. [B] Sense probe showing little or no labelling. [C] Antisense probe showing specific binding cells of the surface epithelium.
Figure 44: Expression of PDGFC

[A] Day 75 showing extraovarian rete (ER) and ovary with surface epithelium highlighted (SE). [B] Darkfield of [A] showing no expression of PDGFC in the ER, but expression present in the ovarian surface epithelium. [C] Two years of age showing a small antral follicle with granulosa cells (GC) and theca cells (TH) highlighted. [D] Darkfield of [C] showing expression of PDGFC in both granulosa cells and theca cells from the large preantral follicle. [E] Eight years of age showing intraovarian rete (IR). [F] Darkfield of [E] showing expression of PDGFC in the intraovarian rete.
5.10: *PDGFD*

There was no expression of *PDGFD* at all ages studied for both foetal and adult, either in the ER, CR or IR. Further the only cell type which exhibited expression of *PDGFD* was cells of the corpus luteum where expression was light.

Figure 45: Validation of *PDGFD* Probe

Figure 45: Validation of *PDGFD* probe. Day 75, sections through the same region of an ovary at the interface between the corpus luteum and connective tissue. [A] Brightfield image. [B] Sense probe showing consistent pattern of silver grains. [C] Antisense section showing specific binding of probe to cells of corpus luteum.
5.11: *PDGFRA*

At all foetal ages studied (day 55, 75 and 95) there was no expression of *PDGFRA* in ER, CR or IR (Figure 47A&B). Further there appeared to be little if any expression in any specific ovarian cell type. Expression was evident in cells surrounding the developing uterus.

At four weeks of age there was again no expression of *PDGFRA* in ER, CR or IR. Specific expression patterns were however evident in the ovary. The granulosa cells of atretic follicles expressed *PDGFRA*, while the theca cells often, but not always, showed expression of *PDGFRA* (Figure 47C&D). Thecal expression increased in atretic follicles. Pockets of expression were often noted in the outer cortex (Figure 47E&F), this expression was often associated with the microvasculature or in close proximity to early growing follicles.

At puberty, two years and eight years of age there was no expression of *PDGFRA* in any rete component. The expression pattern mimicked that displayed at four weeks, with the addition of expression in the corpus luteum, where expression was largely localised to cells of the vasculature.

Figure 46: Validation of *PDGFRA* Probe

![Figure 46: Validation of *PDGFRA* Probe](image)

Figure 46: Validation of *PDGFRA* probe. Puberty, sections through the same region of an ovary. [A] Brightfield image. [B] Sense probe showing consistent pattern of silver grains. [C] Antisense probe showing specific binding of probe to cells of the ovarian cortex.
Figure 47: Expression of *PDGFRA*

[A] Day 75 showing connecting rete (CR).

[B] Darkfield of [A] showing no expression of *PDGFRA* in connecting rete.

[C] Four weeks showing theca cells (TH), Granulosa cells (GR) and oocyte (OO) from a preantral follicle.

[D] Darkfield of [C] showing *PDGFRA* expression in thecal cells and some surrounding cortical cells.

[E] Two years showing a primordial follicle (PI) in the ovarian cortex.

[F] Darkfield of [E] showing expression of *PDGFRA* on cortical stromal cells adjacent to the primordial follicle. Note autofluorescence in blood vessel (star).
5.12: PDGFRB

At all foetal ages studied days 55, 75 and 95 there was no specific expression of PDGFRB in either the ER or the CR (Figure 49A&B). Pockets of strong expression were noted in the ovary and these corresponded to the developing vasculature.

At four weeks of age there was again no expression of PDGFRB in either ER, CR or IR. Similar to that expression pattern for PDGFRA this lack of expression was so marked that often rete components gave the appearance of “black holes” in the section (Figure 49C&D). This phenomenon also extended to granulosa cells of healthy follicles (Figure 49E&F). Expression was noted similar to PDGFRA in the theca of a large number of healthy and atretic follicles. For PDGFRB however this expression in the theca seemed to be more restricted to the theca interna in the larger follicles. Unlike PDGFRA regionalised clusters of expression in the outer cortex were not apparent for PDGFRB.

At puberty, two years and eight years of age the pattern of expression for PDGFRB was similar to that observed at four weeks. Where a corpus luteum was present, punctate expression of PDGFRB was evident.

Figure 48: Validation of PDGFRB Probe

![Figure 48: Validation of PDGFRB Probe](image)

Figure 48: Validation of PDGFRB Probe. Two years of age. Sections through the same region of an ovary. [A] Brightfield image. [B] Sense probe showing consistent pattern of silver grains. [C] Antisense probe showing specific binding of probe to cells of the ovarian cortex and absence of binding to follicular granulosa cells.
Figure 49: Expression of PDGFRB. [A] Day 95 showing connecting rete. [B] Darkfield of [A] showing no expression of PDGFRB in connecting rete. [C] Four weeks of age showing connecting rete (CR) and intraovarian rete (IR). [D] Darkfield of [C] showing no expression of PDGFRB in both connecting rete and intraovarian rete. [E] Four weeks of age showing numerous preantral/small antral follicles (FOL) and numerous early growing, primary follicles (PI). [F] Darkfield of [E] showing no expression of PDGFRB in granulosa cells of follicles, note complete lack of specific binding leading to the typical appearance of “black holes”.

99
Table 11: Gene Expression in the Extraovarian Rete

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day 55</th>
<th>Day 75</th>
<th>Day 95</th>
<th>Four weeks</th>
<th>Puberty Two years</th>
<th>Eight years</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FST</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP19A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WT-1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>BMP4</td>
<td>++</td>
<td>++/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFA</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>n/p</td>
<td>-</td>
</tr>
<tr>
<td>PDGFC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFRA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 11: Gene Expression in the Extraovarian Rete. +++ indicates strong expression, ++ indicates moderate expression and + indicates light expression. – indicates no expression while +/- indicates variable expression at the indicated level. n/p indicates no extraovarian rete found in the sections for this gene.
### Table 12: Gene Expression in the Connecting Rete

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day 55</th>
<th>Day 75</th>
<th>Day 95</th>
<th>Four weeks</th>
<th>Puberty</th>
<th>Two years</th>
<th>Eight years</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FST</td>
<td>+++</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++/-</td>
<td>+/-</td>
</tr>
<tr>
<td>CYP19A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WT-1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>BMP4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFA</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PDGFC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 12: Gene Expression in the Connecting Rete. +++ indicates strong expression, ++ indicates moderate expression and + indicates light expression. – indicates no expression while +/- indicates variable expression at the indicated level. n/p indicates no extraovarian rete found in the sections for this gene.
Table 13: Gene Expression in the Intraovarian Rete

<table>
<thead>
<tr>
<th>Gene</th>
<th>AMH</th>
<th>FST</th>
<th>CYP19A</th>
<th>WT-1</th>
<th>BMP4</th>
<th>PDGFA</th>
<th>PDGFB</th>
<th>PDGFC</th>
<th>PDGFD</th>
<th>PDGFRB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 55</td>
<td>Day 75</td>
<td>Day 95</td>
<td>Four weeks</td>
<td>Puberty</td>
<td>Two years</td>
<td>Eight years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMH</td>
<td>N/P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FST</td>
<td>N/P</td>
<td>++/-</td>
<td>+++</td>
<td>+++</td>
<td>++/-</td>
<td>++/-</td>
<td>++/-</td>
<td>++/-</td>
<td>++/-</td>
<td>++/-</td>
</tr>
<tr>
<td>CYP19A</td>
<td>N/P</td>
<td>+++/-</td>
<td>+++/-</td>
<td>++/-</td>
<td>+++/-</td>
<td>+++/-</td>
<td>+++/-</td>
<td>+++/-</td>
<td>+++/-</td>
<td>+++/-</td>
</tr>
<tr>
<td>WT-1</td>
<td>N/P</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>BMP4</td>
<td>N/P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFA</td>
<td>N/P</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFB</td>
<td>N/P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>PDGFC</td>
<td>N/P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++/-</td>
<td>+</td>
</tr>
<tr>
<td>PDGFD</td>
<td>N/P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>N/P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 13: Gene Expression in the Intraovarian Rete. +++ indicates strong expression, ++ indicates moderate expression and + indicates light expression. – indicates no expression while +/- indicates variable expression at the indicated level. n/p indicates no extraovarian rete found in the sections for this gene.
5.13: Discussion

In selecting genes for study three questions were raised.

Firstly could gene expression patterns indentify the specific origins of rete cells? From the earliest age studied (day 55 of gestation) WT-1 was strongly expressed in all rete components. In the regressing mesonephros WT-1 was strongly expressed in the mesangial cells but not epithelial cells of regressing glomeruli. While this suggests that the rete is predominantly of mesangial cell origin further studies need to be undertaken to confirm this. Selection of a gene without the inherent widespread expression throughout the foetal ovary typical of WT-1 would help to confirm this finding. WT-1 is thought to be a differentiation inhibitor and has been shown to suppress transcription of several genes involved in differentiation [80, 81]. The widespread levels of expression in the developing ovary, particularly in the rete components and stromal cells of mature ovaries may seem counterproductive, but may serve to prevent wholesale differentiation of these cells.

Secondly does the rete play a role in the development of the vasculature? Of the PDGFs, PDGFB is most commonly associated with angiogenesis [51]. While there was no expression of PDGFB in any rete component (other than at eight years), the observed pattern of expression in the foetal ovary would suggest that PDGFB may not play a major role in the establishment of the vasculature. During foetal development expression was confined to avascular regions of the ovary, the outer cortex and oocytes within ovigerous cords. As described earlier oestrogen has also been implicated as being important for development of the vasculature [64, 82]. Expression of CYP19A in IR tubules with a compromised basement membrane at day 75 and 95 of gestation is an important result. This expression also coincides with expression of PDGFA in the IR, given the often observed co-localisation of IR and vasculature it seems likely that rete produced oestrogen and PDGFA play a role in developing the vasculature of the ovary. The development of the ovarian vasculature may also be controlled by other angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) or Endocrine Gland Vascular Endothelial Growth Factor (EGVEGF). While VEGF is a promising candidate for this role, effective in situ hybridisation studies could not be undertaken. VEGF has both an angiogenic form and an inhibitory form [83, 84], the subtle difference between these forms means that production of a probe specifically for the angiogenic form was not feasible for this study.

The final question was could gene expression patterns support a possible link between the rete and follicular growth, particularly recruitment of primordial follicles. To
support such a link expression would need to be consistent, particularly at postnatal ages. Only two genes displayed this consistent expression, WT-1 and FST. There are no published reports implicating WT-1 in early follicular growth. While in all animals expression of FST was noted in the IR, it was also noted that as the IR loses its integrity and contributes its cells to the ovarian stroma then FST appears to be switched off. Thus the possibility of rete derived FST having an effect on primordial follicles in a paracrine manner seems unlikely. The IR expression of FST in some sections but not others is an interesting finding, suggesting some paracrine regulation of this gene, although no pattern to this variation was evident in this study. Sex steroids have been implicated in the regulation of FST in the pituitary [85] but further study is required to see if this is true in the ovary.

While the results do not support a role in follicular growth they are nonetheless not inconsistent with a role for the rete. Certainly during foetal development the expression of CYP19A in IR tubules whose integrity has been compromised and PDGFA is an exciting result. This suggests that along with a possible role in vascular development, other roles ascribed to the rete during this time period, notably in follicle formation may well be mediated by rete derived oestrogen and/or PDGFA, certainly oestrogen is known to play a role in follicle formation[86, 87].

The expression patterns do not provide any evidence for a specific role for the rete postnatally. However the reacquiring of FST expression in the CR postpuberty combined with, at eight years of age, the expression of PDGFB (CR and IR) and PDGFC (IR) illustrate that the cells of the rete are not static and supports the overall hypothesis that the rete is a dynamic system.

Differences are also apparent between ER and CR/IR, notably in FST expression, raising the possibility that these components may have differing roles. If as suggested by Odend’hal et al. [36] that the CR and ER are continuous with the fallopian tube then the temporal expression of CYP19A in the CR at four weeks of age raises an intriguing possibility. The uterus at four weeks of age is a small structure but undergoes rapid growth from this age. The ovary is essential for this growth as evidenced in sheep homozygous for the Inverdale gene, these animals develop streak, non functional ovaries and their uterus does not develop beyond the level achieved at four weeks of age [88]. Oestrogens are essential for this growth as demonstrated in aromatase knockout mice [89]. It may be that the rete-fallopian tube connection is a direct conduit for rete derived oestrogen to stimulate the growth of the uterus.
The expression of *BMP4* in the ER at days 55 and 75 is an interesting finding. While BMP4 has been shown to promote primordial to primary follicle transition and also act as an oocyte survival factor in rodents its expression in the sheep ovary and the rete ovarii in particular, appears to be minimal. This result appears to be in line with the study of Juengel *et al.* [58]. BMP4 plays some critical roles in early embryo development and is essential for the development of primordial germ cells, at least in the mouse embryo [90]. The expression at days 55 and 75, in the ER appears to be highly specific to the ER and is not seen in elements of the regressing mesonephros. As the ER appears to develop from mesonephric cell remnants this shows that the *BMP4* gene is switched on as this structure develops, even if only for a short time frame, suggesting BMP4 plays a role beyond early embryo development although not necessarily at the level of the developing ovary.

In foetal ovaries expression of *PDGFA* and *PDGFB* was widespread while *PDGFC* was considerably less and *PDGFD* was not detectable. It is difficult to determine from the *in situ* hybridisation results the nature of *PDGFRA* expression. Whether there is widespread expression with no specific cell type showing elevated expression levels or whether the observed intensity level is merely background is unclear. The density of silver grains within these sections tends to reflect the cell density which suggests a widespread expression at similar levels in most cell types within the ovary. These conclusions are supported by PCR work which shows the presence of receptor expression in foetal ovaries [54]. Within post natal ovaries *PDGFA* was noted in granulosa cells of follicles. Most expression of *PDGFs* was detected in cells or regions where angiogenesis is likely to be occurring, notably the theca of developing follicles, granulosa of atretic follicles, the corpus luteum and directly associated with cells of the vasculature. This same general pattern was true for both PDGF receptors. This suggests a major role of PDGFs in the ovary is in angiogenesis.

With regard to expression in the rete ovarii elevated expression levels of *PDGFA* were noted in the ER, CR and IR at days 75 and 95 of gestation with expression in the IR maintained until at least four weeks of age. PDGFs can drive numerous cell responses including migration and survival [91]. With the rete being a migrating system of cells during foetal life it seems a logical conclusion that *PDGFA* expression may help facilitate this mesonephric-ovary migration. It was also noted that *PDGFA* was expressed in cells of regressing glomeruli, at least at day 55. This expression may confer some protective attributes to these cells, allowing them to survive regression of the mesonephros and migration to the ovary.
At four weeks of age the pattern of $PDGFA$ and $PDGFRA$ provides an interesting possibility. Expression of $PDGFA$ in the outer or most cortical pieces of IR coincides with pockets of expression of $PDGFRA$ within the ovarian cortex. While immediately adjacent expression was not detected the reasonable proximity of expression of ligand and receptor suggests that the IR may have some effect on development or differentiation of cortical cells.

The appearance of expression of $PDGFB$ and $PDGFC$ in the IR at eight years of age is surprising. At this age the IR is considerably smaller than seen previously, both in tubule number and size of tubules present. Additionally at this age the ovary appears markedly different from earlier adult ages. Fewer growing follicles tend to be present and numerous areas of scar tissue are present (most likely being remnants from either atretic follicles and/or regressing corpora lutea). $PDGFB$ and C could play roles in the remodelling of the ovarian stroma/medulla at later ages.
Chapter 6: Conclusions

Combined, these results add substantially to our knowledge and understanding of the rete ovarii. While the results do not provide definitive proof of a role for the rete, particularly postnatally, they do suggest that such a role is feasible, at least in the sheep. The continued presence of the rete through until at least eight years of age, the changing pattern of gene expression and its continued ability to proliferate support the hypothesis "That the rete is a dynamic system of cells and tubules which plays a role in both ovarian development prenatally and in ovarian function postnatally." Of the specific roles proposed in this study the strongest support is for a role in vascular development during foetal life.

It seems clear that mesonephric derived cells play multiple roles in ovarian development during foetal life (initiation of meiosis, follicle formation and growth). This study highlights the need for caution in ascribing these functions to the rete. The presence of mesonephric derived cell streams with a different morphology and gene expression profile from the rete argues that these functions could be fulfilled by either cell type/structure.

At the foetal ages studied strong expression of WT-1 gene in both the mesangial cells of regressing glomeruli and in components of the rete would suggest that the cells of the rete ovarii are largely comprised of mesangial cells. However WT-1 appears to be widely expressed within the ovary and this conclusion should be treated with some caution, particularly given that the morphological evidences suggests a mixed origin. The association between rete and the developing vasculature, combined with expression of CYP19A and PDGFA suggests that the rete may play a role in vascular development during foetal life. This is the first time that evidence for such a role has been presented.

Roles for the rete postnatally are less clear. The evidence does not appear to support roles in follicular growth or angiogenesis, two functions initially selected as likely roles for the rete. The lack of consistent expression of genes associated with early follicular growth, combined with the lack of a close physical association between the rete and non-growing or early growing follicles does not support the hypothesis that the rete has a role in regulating early follicular growth. However the list of genes involved in early follicular growth is extensive and such a role cannot be ruled out.

Of the PDGFs, PDGFB is known to have the strongest role in angiogenesis. A lack of expression of PDGFB by the rete, other than at eight years of age, would suggest that the
rete does not play a role in angiogenesis. Other angiogenic factors, such as VEGF and EGVEGF would need to be studied to confirm this contention however.

While a specific role for the rete postnatally appears elusive, perhaps the key to this role lies in the morphological development of this structure. The breakdown of rete tubules and disbursement of cells into the ovarian stroma at all ages studied appears to be a normal facet of rete development, given that these cells do not undergo any form of degeneration. The fate of these cells may well hold the key to understanding the role of the rete. In a recent study Honda et al. described the isolation and characterisation of a putative thecal stem cell population [15]. In culture these cells formed round colonies with intercellular spaces filled with basement membranes and collagen. This in vitro description bears a remarkable resemblance to the description of the CR in vivo. Thus it seems plausible that the rete may be a source of pretheical or thecal stem cells. Further anecdotal support for this contention comes from the study by Smith et al. [38] where early alterations in the development of the rete were noted in prenatally androgenised ewes, a model commonly used to mimic the symptoms of Polycystic Ovarian Syndrome (PCOS). In both PCOS and the prenatal androgenisation model hyperandrogenism caused by thecal hyperplasia and/or increased androgen production by thecal cells are common symptoms. While a link between the rete and the theca is perhaps tenuous it would seem an idea worthy of further study, especially given the prevalence of PCOS in the human population.

While a definitive role for the rete postnatally remains unclear, the results of this study, namely the continued presence of the rete, its continued proliferative ability and its changing pattern of gene expression support the hypothesis “that rather than a vestigial structure the rete is a dynamic system of cells and tubules which plays a role in ovarian development prenatally and plays a role in ovarian function postnatally.”
References


27. Shehata R. Medullary tubes in the ovary of the camel and other mammals. The Veterinary Record. 1964.; 76: 750-753.


34. Miller RI. Anatomical and pathological studies of the upper genital tract of the cow. James Cook University of N Queensland, australia; 1977.


63. Shemesh M. Estradiol-17 beta biosynthesis by the early bovine fetal ovary during the active and refractory phases. Biol Reprod 1980; 23: 577-582.


80. Werner H, Rauscher FJ, Sukhatme VP, Drummond IA, Roberts CT, LeRoith D. Transcriptional repression of the insulin-like growth factor I receptor (IGF-I-R) gene by the tumor suppressor WT1 involves binding to sequences both upstream and downstream of the IGF-I-R gene transcription start site. Journal of Biological Chemistry 1994; 269: 12577-12582.


Appendix - Protocols

Fixation and Processing

Fixation

On day of use mix 8% paraformaldehyde and 0.2M Phosphate buffer 1:1. pH to 7.4.
Fix tissue in 4% paraformaldehyde overnight at 4°C.
Replace fixative with 70% Ethanol and leave overnight.
Process to paraffin as per schedule.

Solutions

8% Paraformaldehyde:
Add 16g paraformaldehyde to approximately 180mls water.
Heat to 60°C while stirring (if temperature exceeds 70°C, throw away and start again)
While at 60°C add 5M NaOH drop wise until solution clears. This should take about 5-10 drops for 200mls and take 2-3 minutes, although if you are unsure or there are lumps leave stirring for 30 minutes.
Make up to 200mls with water.

Double Strength Phosphate Buffered Saline (0.2M):
For 500mls
NaCl..........8.0g
KCl............0.2g
Na₂HPO₄.....1.15g
KH₂PO₄......0.2g

Check pH and adjust as necessary to between 7.2-7.4
### Routine Processing Schedule

1. 70% Alcohol 2 Hours
2. 80% Alcohol 1.50 Hours
3. 95% Alcohol 1 Hour
4. 95% Alcohol 1 Hour
5. Absolute Alcohol 1.50 Hours
6. Absolute Alcohol 1.15 Hours
7. Absolute Alcohol 1.15 Hours
8. Abs Alcohol/Xylene 1.50 Hours
9. Xylene 1 Hour
10. Xylene 1 Hour
11. Wax 1.50 Hours
12. Wax(with Vacuum) 2 Hours
Histological Stains

Haematoxylin and Eosin
(For 5µm Paraffin Sections)

Procedure
1. Deparaffinise and hydrate sections to running tap water. Bouin’s fixed sections need to be washed in running tap water for 10-20 minutes to remove the yellow pigment.

2. Stain Nuclei in Gill’s Haematoxylin 4 minutes
3. Wash well in running tap water.

4. Blue in Scott’s tap water. 1 minute
5. Wash in running tap water. 5 minutes
6. Counter stain in Alcoholic Eosin 3 minutes
7. Wash well in running tap water.

8. Dehydrate, briskly in two changes of 95% Alcohol then two changes of 100% Alcohol, leaving in last 100% Alcohol 2 minutes.

9. Clear in three changes of Xylene,

10. Leave in last Xylene 5 minutes.
11. Mount in DPX.

Results

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other elements</td>
<td>Varying shades of pink</td>
</tr>
</tbody>
</table>

Solutions

Gill’s Haematoxylin No. 3

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematoxylin</td>
<td>6 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>690 ml</td>
</tr>
<tr>
<td>Ethylene Glycol</td>
<td>250 ml</td>
</tr>
<tr>
<td>Sodium Iodate</td>
<td>0.6 g</td>
</tr>
<tr>
<td>Aluminium Sulphate</td>
<td>52.8 g</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>60 ml</td>
</tr>
</tbody>
</table>
Add acid after all solids have dissolved. Mix in order given. Can be used at once. Requires no differentiation. Maintain acid content by adding 1 drop of Acetic acid per 100 ml of stain, do this weekly. Change solution monthly.

**Alcoholic Eosin**

- 1% aqueous Eosin (C. I. 45380)  
  - 100 ml
- 1% aqueous Phloxine  
  - 10 ml
- 95% ETOH  
  - 880 ml
- Acetic acid  
  - 5 ml

**Scotts Tap Water**

- Sodium Bicarbonate  
  - 7 g
- Magnesium Sulphate  
  - 40 g
- Distilled Water  
  - 2 litres

Add a crystal of Thymol to stop bacterial growth.
Periodic-Acid-Schiff

Procedure
1. Deparaffinise and hydrate sections to running tap water.
2. 1% Periodic acid. 5-7 minutes
3. Rinse well in running water.
4. Stain with Schiff’s reagent. 10-15 minutes
5. Wash in running water. 5-10 minutes
6. Stain with Gill’s Haematoxylin. 30 seconds
7. Rinse in water.
8. Scotts tap water. 1 minute
9. Rinse in water.
8. Dehydrate, clear and mount in DPX.

Results

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.A.S. positive material</td>
<td>Bright red</td>
</tr>
<tr>
<td>Nuclei</td>
<td>Blue</td>
</tr>
<tr>
<td>Background</td>
<td>Pink</td>
</tr>
</tbody>
</table>

Solutions

Schiff’s Reagent
Basic Fuchsin (C.I. 42500) 1 g
Distilled water. 300 ml
Place on stirrer. Then add;
Sodium metabisulphite (Na2S205) 3 g
Hydrochloric Acid (conc.) 3 ml
Stopper the flask with a good fitting bung
and leave on stirrer for 2 hours.
Add: Activated charcoal 1 g
Stir for further 1 minute. Filter into 100mls bottles x3

Keep in the refrigerator. Use one bottle at a time as working solution. Can be used repeatedly and keeps for months. Discard if solution becomes pink.
**Heidenhains Aniline Blue**

**Procedure**

1. Deparaffinise and hydrate to water.
2. If formalin fixed, mordant in Zenker’s overnight, then remove mercuric chloride crystals with iodine and clear with Sodium Thiosulfate.
3. Wash in running tap water.
4. Rinse in distilled water.
5. Place in preheated Azocarmine B solution at 56°C for 15 minutes.
6. Rinse in distilled water.
7. Differentiate in Aniline-Alcohol solution until cytoplasm and connective tissue are pale Pink and nuclei stand out sharply. (Control differentiation by rinsing in 1% Acetic Alcohol).
8. Mordant in Phosphotungstic Acid solution for 15 minutes.
9. Rinse in distilled water.
10. Aniline Blue solution for 15 minutes.
11. Rinse in distilled water.
12. Dehydrate in 95% Alcohol, absolute alcohol, and clear in xylene, two changes, 2 minutes each.
13. Mount in DPX.

**Solutions**

**1% Azocarmine**

| Azocarmine B | 4 g |
| Distilled water | 400 ml |

Bring to a boil, filter at 56°C, cool and add 4ml Glacial Acetic acid. Must be kept in refrigerator and filtered before use.

**1% Aniline Alcohol**

| Aniline | 4 ml |
| 95% Alcohol | 400 ml |
1% Acetic Acid
Glacial Acetic Alcohol 4 ml
95% Alcohol 400 ml

5% Phoshotungstic Acid
Phosphotungstic acid 20 g
Distilled water 400 ml

Aniline Blue
Aniline Blue, water soluble 1 g
Orange G 4 g
Distilled Water 600 ml
Glacial Acetic Acid 16 ml

Results
Chromatin, osteocytes, neuroglia Red
Collagen, reticulum Blue
Muscle Red to yellow
Osteoid material in decalcified sections Red
BrdU Immunohistochemistry Protocol

1. 2 x Xylene 10 min washes.
2. 2 x 100% ethanol, 5 min each.
3. 90% ethanol, 5 min.
4. 70% ethanol, 5 min.
5. 50% ethanol, 5 min.
6. PBS, 2 x 5 min.
7. 10 min quench in 3% H₂O₂-methanol (23 ml H₂O₂ + 200 ml methanol).
8. PBS 10 min wash.
9. Carefully blot each slide on tissue, then wipe each slide with cloth avoiding sections. Draw wax circle surrounding sections, quickly put slide back into PBS.

10. Set up large plastic container with metal slide rack inside. Lay slides in rack, pipette PBS onto sections to stop them drying out.
11. Trypsin (1:3 1A:1B) – preheated to 37°C. Add trypsin to sections with pipette, making sure all sections are covered. Have to do 1 slide at a time – have 1 slide draining while putting trypsin on first slide. Work in 37°C room – give each slide 10 min in trypsin.
12. After each slides incubation, put into rack in ddH₂O black box.
13. Wash slides in ddH₂O 3 x 2 min.
14. Denaturing solution (reagent 2) – cover sections, incubate 30 min (cover box with lid during every incubation.)
15. Wash slides in PBS 3 x 2 min.
16. Blocking solution (reagent 3) - cover sections, incubate 10 min. Drain slides and blot around sections with cloth.
17. Primary Ab – biotinylated mouse anti-BrdU (R4) - cover sections, incubate 30 min. Negative controls – cover sections with PBS.
18. Wash slides in PBS 3 x 2 min.
19. Streptavidin-HRP (R5) - cover sections, incubated 10 min.
20. Washed slides in PBS 3 x 2 min.
21. DAB (chromagen) – make 15 min before use, wrapped tube in tinfoil (1 drop of each 3 reagents to 1 ml ddH₂O). Cover sections, incubate 5 min.
22. Plunge slides into ddH$_2$O.
23. Transfer slides immediately to rack and put into ddH$_2$O
24. Dip slides in filtered haemotoxylin for 3 seconds
25. Put under running tap water until it runs clear
26. Put in PBS for 30 sec
27. Put in ddH$_2$O
28. 50% ethanol for 5 min
29. 70% ethanol for 5 min
30. 90% ethanol for 5 min
31. 100% ethanol for 5 min
32. Xylene – 2 x 10 min
33. Mount in DePeX – put coverslips on.
Protocols for *In Situ* Hybridisation

**Cell Culture**

*Luria broth (LB) media – 1 litre*

Yeast extract 5 g  
Bacto-tryptone 10 g  
NaCl 10 g  

Weigh contents into beaker. Make up with MQ water and stir until dissolved – make to 1 L. Autoclave

**Luria Broth Plates**

Weigh out 15 g bacto-agar and put into a 1 L Schott bottle, add 1L LB. Autoclave.  
While molten add 1µl 50 mg/ml Ampicillin per ml LB agar (1L LB Agar = 1 ml Amp).  
Mix well, then pour plates – approximately 45 plates per 1 L agar.  
Leave at room temp to set.  
Can leave at room temperature overnight to dry, then store in plastic bag at 4°C.

1. Take appropriate cell stock out of -70°C freezer (on dry ice), take loopful frozen stock and streak onto LB-Amp plate.  
2. Grow overnight at 37°C.

**Colony Selection**

1. Add 10µl of ampicillin stock (50 mg/ml) to one vial containing 10 ml LB.  
2. Using a sterile plastic loop or flamed metal loop, carefully select a white colony and put into the LB, stirring vigorously.  
3. Put cultures at 37°C overnight, shaking at 225 rpm.  
4. Add 1ml aliquots to each of 10 vials containing 10ml LB and 10µl ampicillin  
5. Put cultures at 37°C overnight, shaking at 225 rpm.
**DNA Miniprep Purification**

**Use QIAprep spin miniprep kit.**

1. Spin down glass vials at 3000 rpm for 10 min in lab centrifuge.
2. Resuspend pelleted bacterial cells in 250µl Buffer P1 by pipette or vortex (lid on!) and transfer to eppendorf.
3. Add 250µl Buffer P2 and gently invert tube 6 times. Incubate at RT for 5 min.
4. Add 350µl Buffer N3 and invert tube 6 times.
5. Centrifuge at 13,000 rpm for 10 min.
6. Apply supernatants to QIAprep columns and centrifuge for 1 min, discard flowthrough.
7. Wash column in 0.75 ml Buffer PE and centrifuge 1 min.
8. Centrifuge an extra min to remove residual wash buffer.
9. Place 3 columns in clean eppendorfs and add 55µl elution buffer to centre of column. Stand 1 min, centrifuge 1 min.
10. Use the elute from each of the 3 columns as the solution to elute the remaining 3 columns – stand 1 min, centrifuge 1 min – increases concentration.
11. Store at −20°C.

**Restriction Enzyme Digests**

**Insert Release**

1. Digestions set up as per table

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>5µl</td>
<td></td>
</tr>
<tr>
<td>10 X RE buffer</td>
<td>1µl</td>
<td></td>
</tr>
<tr>
<td>RE</td>
<td>1.5µl</td>
<td></td>
</tr>
<tr>
<td>TAE buffer</td>
<td>2.5µl</td>
<td></td>
</tr>
</tbody>
</table>

2. Digest at 37°C for at least 3 hours.
3. Stop reaction at 65°C for 10 min
4. Samples prepared for electrophoresis as indicated and left for 15 minutes at room temperature
DNA  | DNA  | Loading Buffer | Sybr Green | TAE  
---|---|---|---|---
Undigested Sample  | 1μl | 3μl | 1μl | 5μl 
Digested Sample    | 1μl | 3μl | 1μl | 5μl 
Marker              | 2μl | 3μl | 1μl | 4μl 

5. Electrophoresis: 1.5% agarose gel, in TAE buffer, 67v, 40 minutes  
6. Compare digested band to marker to ensure DNA is appropriate size for insert.

**Linearisations**

1. Work out amount of DNA required for 10μg.  
2. Set up digest: - require 10μg DNA for linearisations

<table>
<thead>
<tr>
<th>Plasmid DNA</th>
<th>10μg/ 24μl total</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X RE buffer</td>
<td>3μl</td>
</tr>
<tr>
<td>RE</td>
<td>3μl</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>To total of 30μl</td>
</tr>
</tbody>
</table>

3. Digest at 37°C for at least 3 hours. If using Eco RI, preferably digest overnight.  
4. Stop reaction at 65°C for 10 min.  
5. Samples prepared for electrophoresis as indicated and left for 15 minutes at room temperature.

<table>
<thead>
<tr>
<th>DNA</th>
<th>DNA Vol</th>
<th>Loading Buffer</th>
<th>Sybr Green</th>
<th>TAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undigested</td>
<td>1μl</td>
<td>6μl</td>
<td>1μl</td>
<td>5μl</td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Digested   | 30μl    | 6μl            | 1μl        | To 30μl |
| sample     |         |                |            |        |

| Digested   | 30μl    | 6μl            |            | To 30μl |
| sample     |         |                |            |        |

| Marker     | 2μl     | 3μl            | 1μl        | 4μl   |

| Mass Ladder | 4μl     | 2μl            | 1μl        | 3μl   |

6. Electrophoresis: 1.5% agarose gel, in TAE buffer, 67v, 40 minutes.  
7. Cut out the 2 linearised plasmids from the gel, and extract the DNA.
**Gel Extraction**

1. Run gel at 65 volts for small gel, 100 V for large gel.
2. Cut band out under UV light using small sharp sterile scalpel, put into eppendorf.
3. Use Qiagen Gel extraction kit to extract DNA from agarose.
4. Weigh gel slice, add 3 volumes of QG buffer, incubate for 10 min at 50°C.
5. Add 1 volume isopropanol, mix, add to gel extraction column, spin 1 min.
6. Wash column with 500 µl QG buffer, spin 1 min.
7. Add 750 µl PE buffer, incubate 4 min, spin 1 min.
8. Discard supernatant, spin column 1 min.
9. Elution: put column into eppendorf tube, add 30 µl DEPC water, incubate 1 min.
10. Spin 1 min to elute DNA, store at −20°C.
11. Electrophoresis: 1.5% agarose gel, in TAE buffer, 67v, 40 minutes

<table>
<thead>
<tr>
<th>DNA</th>
<th>DNA</th>
<th>Loading Buffer</th>
<th>Sybr Green</th>
<th>TAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undigested Sample</td>
<td>1µl</td>
<td>3µl</td>
<td>1µl</td>
<td>5µl</td>
</tr>
<tr>
<td>Digested sample</td>
<td>2µl</td>
<td>3µl</td>
<td>1µl</td>
<td>4µl</td>
</tr>
<tr>
<td>Mass ladder</td>
<td>2µl</td>
<td>3µl</td>
<td>1µl</td>
<td>4µl</td>
</tr>
</tbody>
</table>

12. If band not pure, rerun all of extract on another gel and re-extract. Recheck purity.
13. Use the linearised plasmid DNA as riboprobe in *in situ* hybridisations.
**Solutions;**

**TAE Buffer (50x concentrate)**
- Tris: 242g
- Glacial Acetic Acid: 57.1ml
- EDTA (0.5M, pH8): 100ml
- H$_2$O: to 1L

**Loading Buffer**
- Bromophenol Blue: 0.025g
- Sucrose: 4.0g
- H$_2$O: to 10ml

**Markers**
- Roche DNA Molecular Weight Marker VIII: Cat number 11336045001
- Roche DNA Molecular Weight Marker III: Cat number 10528552001

**Mass Ladder**
- Invitrogen low DNA mass ladder: Cat number 10068-013

**In Situ Hybridisation**

**Pre Insitu**

Make sure that you have prepared linearised DNA (total 3.3µl for 1 reaction each of sense and antisense).

It is important that the restriction digestion be performed to completion. A small amount of undigested plasmid DNA can give rise to very long transcripts, which may incorporate a substantial fraction of the radiolabeled rNTP.

Check there is enough solutions and reagents
Probe Preparation

This procedure may be completed in one day if both precipitation steps are performed in dry ice or over two days if an overnight precipitation step is included. Check for any 3’ overhangs in linearised DNA. Extraneous transcripts have been reported to appear in addition to the expected transcript when templates contain 3’ overhangs. The extraneous transcripts can contain sequences complementary to the expected transcript as well as sequences corresponding to vector DNA. Therefore, it is recommended that plasmids should not be linearised with any enzyme that leaves a 3’ overhang. (Commonly used restriction enzymes that leave a 3’ overhang: Aat II, Apa I, Ban II, Bgl I, Bsp1286 I, BstX I, Cfo I, Hae II, HgiA I, Hha I, Kpn I, Pst I, Pvu I, Sac I, Sac II, Sfi I, Sph I). If there is no alternative restriction site, the 3´ overhang should be converted to a blunt end using the 3´→5´ exonuclease activity of DNA Polymerase I Large (Klenow) Fragment.

1. Preheat water-bath in Low Level Isotope Room to 37 °C
2. Pre-cool Centrifuge to 4 °C
3. Use screw cap tubes
4. If not already done, then dilute rUTP (10mM stock) 1 in 100 in nuclease-free H₂O. rUTP is found in the Riboprobe kit.
5. If using a large number of slides, then make a double reaction of antisense and sense probe. Combine the following reagents into 2 tubes (1 for sense, 1 for antisense)
6. The mixture should be kept at room temperature while each successive component is added, since DNA can precipitate in the presence of spermidine if kept at 4 °C.
7. Using Promega Riboprobe *invitro* Transcription Systems kit. cat #P1460)
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Single Reaction</th>
<th>Double Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Transcription Buffer</td>
<td>4µl</td>
<td>8µl</td>
</tr>
<tr>
<td>0.1M DTT 100mM</td>
<td>2µl</td>
<td>4µl</td>
</tr>
<tr>
<td>RNasin</td>
<td>1µl</td>
<td>2µl</td>
</tr>
<tr>
<td>template linearised DNA</td>
<td>3.3µl</td>
<td>6.6µl</td>
</tr>
</tbody>
</table>

*If template DNA has a 3’ overhang add Klenow fragment and incubate for 15 mins at 22°C*

| rATP 10mM                           | 1µl             | 2µl             |
| rCTP 10mM                           | 1µl             | 2µl             |
| rGTP 10mM                           | 1µl             | 2µl             |
| rUTP (1:100 dil)                    | 0.8µl           | 1.6µl           |
| [33P]UTP                            | 5µl             | 10µl            |
| Appropriate RNA polymerase (either SP6, T7 or T3) | 1µl             | 2µl             |

Incubate 37°C for 1 hour

| RQ1 DNase                           | 1µl             | 2µl             |

Incubate 37°C for 15 minutes.

| 10mg/ml tRNA                        | 4µl             | 8µl             |
| 7.5 M Ammonium Acetate pH 7.0       | 12µl            | 24µl            |
| Ice Cold 100% Ethanol               | 80µl            | 160µl           |

Place in Dry Ice 1-2 hours

Centrifuge probes for 20 minutes, 13,000rpm @ 4°C

Incubate reaction at 37°C for 1 hour

From now on add the same amount of reagents to both the sense and antisense probes:
8. Aspirate EtOH, resuspend pellet in 30µl DEPC H₂O
9. Add 15µl 7.5M Ammonium Acetate-MIX
10. Add 90µl 100% EtOH, put on dry ice for 1-2 hours or at -20 °C vernight
11. Centrifuge 13,000rpm 20 minutes @ 4°C
12. Aspirate EtOH, let pellet dry briefly.
15. Put 400µl of the optiphase into the trilux scintillation vials and add 1µl of probe.
16. Count in scintillation counter

**Slide Washing**

17. Preheat 37 °C waterbath in Low Level Isotope Room

Prepare the following wash solutions. The volume is amount needed to wash one 100mL glass container of slides. If using 2 containers - make up double the volume.

<table>
<thead>
<tr>
<th>Wash Solution</th>
<th>Volume</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% Ethanol / DEPC</td>
<td>200ml</td>
<td>190mls Ethanol 10mls autoclaved DEPC Water</td>
</tr>
<tr>
<td>70% Ethanol / DEPC</td>
<td>200ml</td>
<td>140mls Ethanol 60mls autoclaved DEPC Water</td>
</tr>
<tr>
<td>0.2M HCl</td>
<td>100ml</td>
<td>1.72mL cHCl, made up to 100mL with autoclaved DEPC-m’Q H₂O</td>
</tr>
<tr>
<td>Proteinase K Digestion Buffer (TE Buffer)</td>
<td>100ml</td>
<td>0.2M Tris HCl (pH 7.2) 20mL of 1M stock 50mM EDTA (pH 8.0) autoclaved DEPC H₂O Prewarm the solution to 37°C</td>
</tr>
<tr>
<td>2 x SSC / DEPC</td>
<td>200ml</td>
<td>20mls 20xSSC 180mls autoclaved DEPC Water</td>
</tr>
<tr>
<td>2M Triethanolamine</td>
<td>200ml</td>
<td>To 10ml of 2M of triethanolamine, pH 8.0 add 190ml autoclaved DEPC H₂O</td>
</tr>
</tbody>
</table>
Deparafinisation

18. Wearing gloves and using tweezers, transfer slides and place back-to-back into a sterile (ie. baked) glass bottomed dish. Place on platform in fume hood with gentle shaking.

<table>
<thead>
<tr>
<th>Step Description</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene 3X at RT 10min each</td>
<td>Discard into solvent waste</td>
</tr>
<tr>
<td>Rinse container with 100% EthOH</td>
<td>Discard into solvent waste.</td>
</tr>
<tr>
<td>100% EthOH 2X 5min each</td>
<td>Discard down sink</td>
</tr>
<tr>
<td>95% EthOH / DEPC 5min</td>
<td>Discard down sink</td>
</tr>
<tr>
<td>70% EthOH / DEPC 5min</td>
<td>Discard down sink</td>
</tr>
<tr>
<td>DEPC H₂O 5min</td>
<td>Discard down sink</td>
</tr>
</tbody>
</table>

Pretreatment This step removes basic proteins (ie positively charged), that may bind to the probe. Proteinase K opens up the tissue allowing the probe to stick. NB. Proteinase K self digests, therefore should not be thawed too long.

19. Wash slides in 0.2M HCl, 20min at RT.
20. Wipe across the surface of the solution with clean piece of Whatman filter paper to remove any lead residue from pencil.

21. Wash in 2xSSC, 30min at RT.

22. Wipe across the surface of the solution with clean piece of Whatman filter paper to remove any lead residue from pencil.

23. Thaw Proteinase K (10mg/ml) on ice. Just before use add 20µl Pro K to the preheated TE buffer. Incubate slides at 37°C for 5min (time varies) with Proteinase K digestion buffer solution.

**Acetylation of slides.** This step is to reduce non-specific binding of probe, ie the acetyl groups from the acetic anhydride will react with any protein amino groups thus eliminating their positive charge. This step also serves to eliminate any residual Proteinase K activity.

24. Pipette 250µl of acetic anhydride between slides (but not on sections), followed immediately by 100mL of triethanolamine. Incubate at RT 5min. **REPEAT.**

25. Wash slides in 2 X SSC-DEPC 5min RT

**Dehydration**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Discard down sink</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% EthOH</td>
<td>5min</td>
<td></td>
</tr>
<tr>
<td>95% EthOH / DEPC</td>
<td>5min</td>
<td></td>
</tr>
<tr>
<td>100% EthOH / DEPC</td>
<td>5min x2</td>
<td></td>
</tr>
</tbody>
</table>

At this stage the slides can be stored overnight at 4°C in 100% ethanol with a lid and wrapped in tinfoil, or dried and the protocol continued.
Hybridising

26. Air dry slides flat for 2 hours at room temperature.

27. Label slides SP6 or T7 as appropriate with pencil

Calculate amount of diluted probe required to cover sections. (See below for example). Aim for 45,000 counts /ul. For the calculation you will need counts per minute and volume of hybe mix needed to cover sections. This is calculated on that small sections will need approximately 40µl to cover them, while large will need approximately 70µl. Add a little extra to cover viscosity.

Eg:

<table>
<thead>
<tr>
<th>Probe Type</th>
<th>CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 Sense</td>
<td>3155979.50</td>
</tr>
<tr>
<td>SP6 Antisense</td>
<td>4923966.50</td>
</tr>
</tbody>
</table>

\[
\text{T7 } 45000 \times \frac{1210}{3155979} = 17.3 \text{ ul riboprobe}
\]

\[
\text{SP6 } 45000 \times \frac{770}{4923966} = 7.04 \text{ ul riboprobe}
\]
**Hybe Mix**

28. Make up the following in eppendorf tubes. 1ml per tube.

<table>
<thead>
<tr>
<th></th>
<th>Stock conc.</th>
<th>Final</th>
<th>Vol per mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5M</td>
<td>0.3M</td>
<td>60µL</td>
</tr>
<tr>
<td>Tris HCl pH 6.8</td>
<td>1M</td>
<td>10mM</td>
<td>10µL</td>
</tr>
<tr>
<td>NaP pH 6.8</td>
<td>0.5M</td>
<td>10mM</td>
<td>20µL</td>
</tr>
<tr>
<td>EDTA pH 8.0</td>
<td>0.5M</td>
<td>5mM</td>
<td>10µL</td>
</tr>
<tr>
<td>Denhardts</td>
<td>50X</td>
<td>1X</td>
<td>20µL</td>
</tr>
<tr>
<td>DTT</td>
<td>1.0M</td>
<td>50mM</td>
<td>50µL</td>
</tr>
<tr>
<td>tRNA</td>
<td>10mg/ml</td>
<td>1mg/ml</td>
<td>100µL</td>
</tr>
<tr>
<td>DEPC m’Q</td>
<td></td>
<td></td>
<td>30µL</td>
</tr>
</tbody>
</table>

29. Heat Dextran Sulphate at 65°C to melt it prior to use. Slowly add 200µL of 50% Dextran Sulphate, vortex thoroughly.

30. Add 500µL of deionised Formamide, vortex.

31. Incubate on ice 10min.

32. Spin at 13000X g 10min.
33. Transfer calculated amount hybe buffer to screwcap tubes and add calculated amount of labelled probe to buffer.
34. Boil probe for 5min (don’t forget tube lock if you didn’t use screw cap tubes!)
35. Vortex probes, leave to cool 2-3 min.
36. Add Xµl of appropriate probe to slides.
37. Cover with glass coverslips and hybridise overnight @ 55°C with equilibration solution in click-clack box. (50°C for non-homologous probes or for receptor probes. If signal is very faint, cpm/ul can be increased to 60,000).
38. Equilibration solution should be renewed monthly.

The Next Day
39. Preheat waterbath in to 37°C
40. Preheat waterbath to 65°C
41. Preheat waterbath to 50°C. Prepare the following w’sh solutions. The volume is amount needed to wash one 100mL glass container of slides. If using 2 containers - make up double the volume.
<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (mls)</th>
<th>Waterbath Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x SSC in DEPC</td>
<td>200</td>
<td>50°C</td>
</tr>
<tr>
<td>2 x SSC / 50% formamide /DEPC</td>
<td>100</td>
<td>65°C</td>
</tr>
<tr>
<td>2 x SSC/DEPC</td>
<td>400</td>
<td>37°C</td>
</tr>
<tr>
<td>Wash Solution</td>
<td>100</td>
<td>37°C</td>
</tr>
<tr>
<td>2 x SSC / 50% formamide m’Q</td>
<td>100</td>
<td>65°C</td>
</tr>
<tr>
<td>2 x SSC m’Q H₂O</td>
<td>100</td>
<td>37°C</td>
</tr>
<tr>
<td>0.2x SSC MQ</td>
<td>N/A</td>
<td>37°C</td>
</tr>
</tbody>
</table>

- **Wash Solution**: In `RNase flask`, add 10mL of Wash solution with 90mL Sterile MQ H₂O
- **0.2x SSC MQ**: make a 2L volume no matter how many dishes
42. Take slides out of clickclack boxes, remove coverslips and place slides back-to-back in glass dishes. Take care not to let slides dry out so place 1st wash solution into glass dishes. Also take care as slides are radioactive.
43. Wash slides twice with 5 x SSC/DEPC at 50°C, 15 min each wash. Pour solution into liquid R/A waste.
44. Wash slides twice with 5 x SSC/DEPC at 50°C, 15 min each wash. Pour solution into liquid R/A waste.
45. Wash slides in 2 x SSC/DEPC (4 times) 5 mins each at 37°C.

**RNase Treatment**
46. Add 100µl RNaseA (preboiled) to warmed Wash Solution
47. Incubate slides at 37°C for 30 min in Wash Solution.
48. Wash slides in 2 x SSC / 50% formamide /DEPC for 30 mins at 65°C
49. Wash slides in 2 x SSC / DEPC for 30 mins at 65°C
50. Transfer slides to metal slide basket submerged in 0.2x SSC
51. Wash slides in 0.2 x SSC MQ H2O for 15 min at 37°C
Dehydration (re-usable solutions)

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 % Ethanol/0.3M Ammonium Acetate</td>
<td>2min</td>
</tr>
<tr>
<td>60 % Ethanol/0.3M Ammonium Acetate</td>
<td>2min</td>
</tr>
<tr>
<td>80 % Ethanol/0.3M Ammonium Acetate</td>
<td>2min</td>
</tr>
<tr>
<td>95 % Ethanol/0.3M Ammonium Acetate</td>
<td>2min</td>
</tr>
<tr>
<td>100 % Ethanol</td>
<td>5min</td>
</tr>
<tr>
<td>100 % Ethanol</td>
<td>5min</td>
</tr>
</tbody>
</table>

52. Dry slides for 1-2 hours at RT. At this stage you can continue with Slide dipping or put the slides under film and leave o/n - 2 days.

Dipping Slides

53. Prewarm dark-room water bath to 42°C
54. Prepare dipping jar by placing it in a 250mL beaker of water and taping the top to prevent the jar from moving about. Place in dark-room water bath.
55. Preheat small 100mL beaker of water in water bath.
56. Bring emulsion (Amersham LM1 hypercoat emulsion) to room temperature in black bag in dark room.
57. Prepare black slide boxes by adding desiccant wrapped in tissue paper

NB: Do not expose emulsion to any light! Always handle in the dark.

58. After approx. 40 minutes transfer sealed jar of emulsion to 100ml beaker of water in water bath.
59. After approx. 40 minutes pour emulsion via glass funnel into dipping jar.
60. Dip slides – 5 sec in emulsion then draw out slowly, scraping slide back on dipping jar lip to remove excess emulsion.

61. Wipe back of slide with a kimwipe tissue and put into slot in drying box (slides dry upright). Leave slides to dry for 3+ hours in the dark. The alternative to this is to place straight into the black boxes, however if large sections are used, be careful of uneven coating of emulsion as slides will be drying on their sides.

62. When emulsion is dry, package slides into black boxes, wrap in aluminium foil (3 layers), label and place at 4°C for 3-4 weeks.

2-4 weeks later - Develop Emulsion

63. Move slide boxes from 4°C to darkroom at least 1 hour before developing the slides

64. Equilibrate Developer, Fix and Stop Solutions to between 15-18°C. Use black staining containers.

65. In the dark transfer slides from black boxes to metal trays.

66. Develop for 3 min 30 seconds

67. Stop bath for 1 minute.

68. Fix for 10 minutes in the dark

69. Running tap water for at least 1 hour

Staining the Slides -

70. Use black staining boxes

71. Filter Gills Haemotoxylin through filter paper (recycle after use)

72. 3-5 seconds Haemotoxylin

73. Running tap water until free of colour

74. Scotts Tap Water for 1 minute

75. Running Tap water for 2 minutes

76. 70% Ethanol 2 minutes

77. 95% Ethanol 2 minutes

78. 95% Ethanol 2 minutes

79. 100% Ethanol 5 minutes

80. 100% Ethanol 5 minutes

81. Xylene 10 minutes

82. Xylene 10 minutes
83. Mount with DePeX
84. Put on Coverslip
85. Allow to dry overnight before viewing.
In Situ Hybridisation Solutions

DEPC Water
1 Litre of milliQ water
Add 1ml of DEPC (Diethylpyrocarbonate)
Stir overnight with lid loose
Autoclave

7.5M Ammonium Acetate pH 7.0
Ammonium Acetate 57.81g
Make up to 100mls with unautoclaved DEPC water
pH to 7.0
Autoclave

1M Tris
Tris 60.55g
Autoclaved DEPC water 400ml
pH to 6.8, 7.2 or 7.5
Autoclaved DEPC water to 500mls
Autoclave

500mM EDTA pH 8.0
EDTA 73.06g
Make up to 400mls with unautoclaved DEPC water
pH to 8.0.
Note that EDTA will not dissolve until it has reached a certain pH so do the pHing first.
Make volume up to 500mls.
Autoclave.
20xSSC
3M NaCl 175.3g
0.3M Na Citrate.2H$_2$O 88.2g
Make up to 1 Litre with unautoclaved DEPC water (use MQ water and add DEPC while stirring overnight)
pH to 7.0 and autoclave.

Wash Solution (10xWS) 1L stock
NaCl 233.9g
1M Tris pH 7.5 100mL
0.5M EDTA (MW 372.24) 18.61g
MQ H$_2$O to final volume 1L.

NB Autoclave.

Proteinase K 10mg/ml
Make up to required concentration with 50mM TrisHCl pH 8.0 (made in DEPC).
Filter sterilise through 0.22µm filter.
Store in aliquots at –20°C.

2M Triethanolamine pH8.0
Triethanolamine 53ml
pH to 8.0 with cHCl
Make up to 200ml with unautoclaved DEPC treated water
Wrap bottle in tin foil and autoclave
Store at room temp.

5M NaCl
NaCl 146.1g
Add unautoclaved DEPC water up to 500mls
Autoclave
**0.5M NaPO₄ pH 6.8**

Na₂HPO₄ 7.09g
Make up to 100mls with unautoclaved DEPC water
NaH₂PO₄ 7.8g
Make up to 100mls with unautoclaved DEPC water – autoclave

pH 0.5M Na₂HPO₄ with 0.5M NaH₂PO₄ to pH 6.8.
0.5M Na₂HPO₄ alone cannot be autoclaved because it precipitates out.
Heat to 68°C to drive off the DEPC and then filter sterilise the solution.
Note that the solution should not be autoclaved as precipitation occurs.

**Denhardt's 50x**

PVP (Polyvinylpyrrolidone) 0.2g
BSA 0.2g
Ficoll 400 0.2g
Add autoclaved DEPC water to 20ml.
Filter sterilise through 0.22µm filter.
Store in aliquots at –20°C.

**1.0M DTT**

DTT (Dithiothreitol) 1.54g
Make up to 10mls with Autoclaved DEPC water
Filter sterilise through 0.22µm filter
Store in aliquots at –20°C

**tRNA 10mg/ml**

Make up the whole vial to the required concentration with autoclaved DEPC water.
Filter sterilise through 0.22µm filter.
Store in aliquots at –20°C
**Dextran Sulphate 50%**
Use only Pharmacia brand

Dextran Sulphate 10g

Make up to 20mls with autoclaved DEPC water.

Heat to help dissolve – very viscous solution!

Filter through 0.22µm filter

Store in aliquots at −20°C

**Rnase A 20mg/ml**

To the little vial of unopened RnaseA (100mg), add 5mls of sterile DEPC milliQ.

Vortex, filter sterilise through 0.22µm filter and pipette aliquots of 110ul.

Cap and boil for 2 minutes in boiling water bath.

Cool to room temperature and freeze at −20°C

**0.3 Ammonium Acetate / Ethanol solutions**

For 500mls per solution;

30% Ethanol = 20mls of 7.5M Ammonium Acetate + 150mls Ethanol, and make up to 500mls with Autoclaved DEPC water

60% Ethanol = 20mls of 7.5M Ammonium Acetate + 300mls Ethanol, and make up to 500mls with Autoclaved DEPC water

80% Ethanol = 20mls of 7.5M Ammonium Acetate + 400mls Ethanol, and make up to 500mls with Autoclaved DEPC water

95% Ethanol = 20mls of 7.5M Ammonium Acetate + 475mls Ethanol, and make up to 500mls with Autoclaved DEPC water

**1% Acetic Acid**

1 Litre of MilliQ water + 10mls of glacial acetic acid

**Scotts Tap Water**

Potassium Bicarbonate (Potassium hydrogen carbonate) 2.0g

Magnesium Sulphate (7H₂O) 20.0g

Formalin as a preservative 1.0ml

Make up to 1000 mls with distilled water
**Gills Haematoxylin**

Haematoxylin 6.0g  
Distilled H20 690ml  
Ethanediol 250ml  
Sodium Iodate 0.6g  
Aluminium Sulphate 52.8g  
Dissolve all solids  
Add 60ml Glacial Acetic Acid

**Equilibration Buffer**

For 200mls;  
NaCl 5M 12ml  
1M Tris pH 6.8 2ml  
0.5M NaP pH 6.8 4ml  
0.5M EDTA pH 8.0 2ml  
Formamide 100ml  
Sterile water 80ml