

**THE REGULATION OF HUMAN FETAL  
GONADAL DEVELOPMENT IN THE SECOND  
TRIMESTER**

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## TABLE OF CONTENTS

<b>Abstract</b>	7
<b>Declaration</b>	9
<b>Publications</b>	10
<b>Acknowledgments</b>	11
<b>Abbreviations</b>	13
<b>Chapter 1 Introduction</b>	17
1.1 Human gonadal proliferation and development	18
1.2 Mammalian gonadal development – indifferent stage	19
1.21 Maternal Gonadotrophins and the effect on fetal gonadal development	19
1.22 Human fetal testicular development	21
1.23 Human fetal ovarian development	24
1.3 C-kit	29
1.4 Matrix metalloproteinases and tissue inhibitors of metalloproteinases	33
1.5 Neurotrophins	39
1.6 Trk receptors	43
1.61 TrkA receptor	44
1.62 TrkB receptor	45
1.63 TrkC receptor	46
1.64 Trk receptor signal transduction	47
1.65 p75 receptor	48

1.7 K-252a	52
1.8 Fibroblastic growth factor	53
1.9 Summary	54
1.91 Hypothesis	54
1.92 Aims	54
<b>Chapter 2 Materials and methods</b>	<b>55</b>
2.1 Materials	56
2.2 Methods	56
2.21 Tissue collection	56
2.22 Development of tissue culture method	57
2.23 Tissue culture	54
2.24 Quantification and analysis of cultured tissue	59
2.3 Isolation of RNA and synthesis of cDNA	60
2.31 Polymerase chain reaction	61
2.32 Sequencing of DNA	64
2.33 Purification of products of sequencing	64
2.34 Method for sequencing DNA	65
2.35 Isolation of RNA by laser capture	66
2.36 In situ hybridization	70
2.37 Generation of riboprobes for in situ hybridization	70
2.38 In situ hybridization method	71
2.4 Immunohistochemistry	73
2.41 Haemotoxylin and eosin stain	75

2.5 Western Blot	76
2.51 Protein extraction from tissue	77
2.52 Protein separation	77
2.53 Immunoblotting method	78
2.6 Explant culture	79
2.7 Zymography	80
2.71 Reverse zymography	83
<b>Chapter 3 Germ cell specific expression of c-kit in the human fetal gonad</b>	85
3.1 Introduction	86
3.2 Materials and methods	89
3.3 Results	94
3.4 Discussion	101
<b>Chapter 4 Matrix metalloproteinases and tissue inhibitors of metalloproteinases in human fetal testis and ovary</b>	106
4.1 Introduction	107
4.2 Materials and methods	109
4.3 Results	114
4.3 Discussion	125

<b>Chapter 5</b>	<b>Expression of neurotrophins and their receptors in the human fetal ovary and their effect on germ cell survival</b>	129
	5.1 Introduction	130
	5.2 Materials and methods	132
	5.3 Results	142
	5.4 Discussion	157
<b>Chapter 6</b>	<b>Expression of neurotrophins and their receptors in the human fetal testis and their regulation of germ cell number</b>	165
	6.1 Introduction	166
	6.2 Materials and methods	168
	6.3 Results	173
	6.4 Discussion	184

<b>Chapter 7</b>	<b>General Conclusion</b>	190
	7.0 General conclusion	191
	7.1 C-kit receptor and ligand	191
	7.2 MMPs and TIMPs in human fetal testis and ovary	192
	7.3 The role of neurotrophins in human fetal ovarian and testicular development	193
	<b>Bibliography</b>	196
	<b>Appendix I</b> Sources of Materials	215
	<b>Appendix II</b> Recipes for Solutions	224
	<b>Appendix III</b> Presentations	240
	<b>Appendix IV</b> Publications	242

## ABSTRACT

Fetal gonadal development is a complex process dependent on maturation and differentiation of several cell types with different functions. Adequate development is important for normal sexual development and fertility potential. However, to date the factors that are involved in the regulation of gonadal growth and differentiation are not well understood. The aim of this thesis was to investigate the role of survival and proliferative factors, namely the c-kit proto-oncogene receptor and ligand and the family of neurotrophins in human gonadal development during mid-trimester. The involvement of metalloproteinases (MMPs) and their inhibitors (TIMPs) in tissue remodelling in both human fetal testes and ovaries was also considered.

C-kit and its ligand have been demonstrated to be essential to the processes of germ cell migration, proliferation and survival in the rodent and their expression was investigated in human fetal gonads. Expression of c-kit mRNA and the protein was demonstrated in both ovary and testis throughout mid-trimester. Testicular germ cell-specific expression of c-kit mRNA was confirmed using laser capture microscopy and c-kit protein was localised to the germ cells in both ovaries and testes. These data demonstrate that the expression of c-kit mRNA and protein is germ cell specific in human fetal gonads and are consistent with an important role for the c-kit/kit ligand signalling system in germ cell proliferation and survival in the developing human gonad.

Neurotrophins are survival and differentiation factors in the nervous system. The presence of neurotrophins and their receptors and their role in germ cell survival was investigated in the human fetal gonads. Expression and localisation of neurotrophins

and their receptors was detected throughout mid-trimester in both ovaries and testes. The effects of the tyrosine kinase receptor inhibitor K252a were studied in organ cultures. In the ovary, treatment with K252a resulted in a significant fall in germ cell number and proliferation. In the testis, cell-specific marked decrease in both gonocyte and peritubular cell number and proliferation was seen after treatment with K252a, with little effect on Sertoli cells. These findings therefore demonstrate the expression of neurotrophins and their receptors in human fetal gonads during the second trimester and indicate possible roles in the regulation of proliferation/survival of germ cells and peritubular cells.

MMPs and TIMPs are major regulators of tissue remodelling of the extracellular matrix (ECM) and may also be involved in the control of growth factor availability. Their production and localisation was investigated in the human fetal gonad. Tissue was collected and analysed for the presence of MMP-2 and -9 and for TIMP activities using zymographic techniques. These MMPs and in addition, MMP-1 and TIMPs were localised using immunohistochemistry. The secretion of MMP-2, -9 and all four TIMPs was demonstrated from both testis and ovary, the predominant gelatinase produced being MMP-2. In both gonads, MMP-1, -2, -9 and all TIMP family members were localised to specific cell types. This therefore indicates that MMPs and TIMPs are likely to play a role in ECM remodelling during fetal gonadal development and also in the cell and matrix interactions that control a range of cellular functions.



## **DECLARATION**

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Dr. Lynne Lochart Louise Robinson

## PUBLICATIONS

The following publications have arisen from work undertaken in this thesis:

Robinson LLL, Townsend J, Anderson RA. The human fetal testis is a site of expression of neurotrophins and their receptors: regulation of the germ cell and peritubular cell population. *Journal of Clinical Endocrinology and Metabolism* 88(8): 3943-3951

Spears N, Molinek MD, Robinson LLL, Fulton N, Cameron H, Shimoda K, Telfer EE, Anderson RA, Price DJ. The role of neurotrophin receptors in female germ cell survival in mouse and human *Development* 2003 ;130: 5481-5491

Anderson RA, Robinson LLL, Brooks J, Spears N. Neurotrophins and their receptors are expressed in the human fetal ovary. *Journal of Clinical Endocrinology and Metabolism* 87: 890-7, 2002

Robinson LLL, Gaskell T, Saunders PTK, Anderson RA. Germ cell specific expression of c-kit in the human fetal gonad. *Molecular Human Reproduction* 7: 845-852, 2001

Robinson LLL, Sznajder NA, Riley SC, Anderson RA. Identification and localisation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in human fetal gonads. *Molecular Human Reproduction* 7: 641-648, 2001

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## ABBREVIATIONS

ABC-HRP	<u>A</u> vidin- <u>B</u> iotin- <u>C</u> omplex, conjugated to <u>h</u> orse <u>r</u> adish peroxidase.
ACTH	<u>A</u> drenoc <u>c</u> orticotrophic <u>h</u> ormone
AMH	<u>A</u> nti <u>m</u> ullerian <u>h</u> ormone
AR	<u>A</u> ndrogen <u>r</u> eceptor
APS	<u>A</u> mmonium persulphate
BDNF	<u>B</u> rain <u>d</u> erived <u>n</u> eurotrophic <u>f</u> actor
$\beta$ ME	$\beta$ - <u>m</u> ercaptoethanol
BMP	<u>B</u> one <u>m</u> orphogenetic <u>p</u> roteins
bp	<u>B</u> ase pairs
BrdU	<u>B</u> romodeoxy <u>u</u> ridine
BSA	<u>B</u> ovine <u>s</u> erum <u>a</u> lbumin
CIS	<u>C</u> arcinoma <u>i</u> n <u>s</u> itu
DAB	3, 3' - <u>d</u> iaminobenzidine
DAX-1	<u>D</u> osage-sensitive sex reversal- <u>A</u> drenal hypoplasia congenita critical region on the <u>X</u> chromosome
DTT	<u>D</u> ithio <u>t</u> reit <u>o</u> l
DEPC	<u>D</u> iethylpyro <u>c</u> arbonate
DHPLC	<u>D</u> enaturing <u>h</u> igh pressure <u>l</u> iquid <u>c</u> hromatography
DHT	<u>D</u> ihydro <u>t</u> estosterone
DIG	<u>D</u> igoxigenin
DMSO	<u>D</u> imethylsulfo <u>x</u> ide
DNA	<u>D</u> eoxyribo <u>n</u> ucleic <u>a</u> cid

DRG	<u>D</u> orsal <u>r</u> oot <u>g</u> anglia
ECL	<u>E</u> nhanced <u>c</u> hemiluminescence visualisation system
ECM	<u>E</u> xtra <u>c</u> ellular <u>m</u> atrix
EDTA	<u>E</u> thylenediaminetetraacetic <u>a</u> cid
EGF	<u>E</u> pidermal <u>g</u> rowth <u>f</u> actor
bFGF	<u>B</u> asic <u>f</u> ibroblast <u>g</u> rowth <u>f</u> actor
FGF	<u>F</u> ibroblast <u>g</u> rowth <u>f</u> actor
FGFR	<u>F</u> ibroblast <u>g</u> rowth <u>f</u> actor <u>r</u> eceptor
FBS	<u>F</u> etal <u>b</u> ovine <u>s</u> erum
GAP	<u>G</u> TPase <u>a</u> ctivating protein
GAPDH	<u>G</u> lycer <u>a</u> ldehyde-3-phosphatase <u>d</u> ehydrogenase
GITC	<u>G</u> uanidine <u>i</u> sothi <u>c</u> yanate
hCG	<u>H</u> uman <u>c</u> horionic gonadotrophic hormone
IFN $\gamma$	<u>I</u> nterferon- $\gamma$
IGF	<u>I</u> nsulin <u>l</u> ike <u>g</u> rowth <u>f</u> actor
IGFBP	<u>I</u> nsulin <u>l</u> ike <u>g</u> rowth <u>f</u> actor <u>b</u> inding protein
IL-1	<u>I</u> nterleukin- <u>1</u>
IL-4	<u>I</u> nterleukin- <u>4</u>
IL-6	<u>I</u> nterleukin- <u>6</u>
IL-10	<u>I</u> nterleukin- <u>10</u>
ITS	<u>I</u> nsulin, <u>t</u> ransferrin, <u>s</u> odium selenite
JNK	<u>c</u> -jun <u>k</u> inase
kb	<u>K</u> ilo <u>b</u> ases
KL	<u>K</u> it <u>l</u> igand

LPS	<u>L</u> ipopolysaccharide
LRM	<u>L</u> eucine <u>r</u> ich <u>m</u> otifs
MGF	<u>M</u> ast cell growth <u>f</u> actor
$\alpha$ -MEM	$\alpha$ - <u>M</u> inimal <u>e</u> ssential <u>m</u> edium alpha
MIF	<u>M</u> ullerian <u>i</u> nhibitory <u>f</u> actor
MIS	<u>M</u> ullerian <u>i</u> nhibiting <u>s</u> ubstance
MMP	<u>M</u> atrix <u>m</u> etalloproteinase
MRC	<u>M</u> edical <u>R</u> esearch <u>C</u> ouncil
NGF	<u>N</u> erve growth <u>f</u> actor
NRS	<u>N</u> ormal <u>r</u> abbit <u>s</u> erum
NSS	<u>N</u> ormal <u>s</u> wine <u>s</u> erum
NT3	<u>N</u> eurotrophin <u>3</u>
NT4	<u>N</u> eurotrophin <u>4</u>
NT6	<u>N</u> eurotrophin <u>6</u>
PCR	<u>P</u> olymerase <u>c</u> hain <u>r</u> eaction
PDF	<u>P</u> olyvinylidene <u>f</u> luoride
PGC	<u>P</u> rimordial germ <u>c</u> ell
PGE <sub>2</sub>	<u>P</u> rostaglandin <u>E</u> <sub>2</sub>
PI-3K	<u>P</u> hosphatidylinositol <u>3</u> '- <u>k</u> inase
PLC $\gamma$	<u>P</u> hospholipase <u>C</u> - $\gamma$
RNA	<u>R</u> ibonucleic <u>a</u> cid
RT	<u>R</u> oom <u>t</u> emperature
SCF	<u>S</u> tem <u>c</u> ell <u>f</u> actor
SDS-PAGE	<u>S</u> odium <u>d</u> odecyl <u>s</u> ulfate <u>p</u> olyacrylamide-gel <u>e</u> lectrophoresis

SF-1	<u>S</u> teroidogenic factor-1
SLF	<u>S</u> teel factor
SLPI	<u>S</u> ecretory leucocyte protease inhibitor
SSC	<u>S</u> aline- <u>s</u> odium <u>c</u> itrate
STE	<u>S</u> odium <u>t</u> ris- <u>E</u> DTA buffer
TBS	<u>T</u> ris <u>b</u> uffered <u>s</u> aline
TBE	<u>T</u> ris, <u>b</u> oric acid, <u>E</u> DTA
TEA	<u>T</u> riethylamine
TEAA	<u>T</u> riethylammonium acetate
TEMED	N,N,N <sup>1</sup> - <u>T</u> etra- <u>m</u> ethylethylenediamine
TGFβ	<u>T</u> ransforming growth factor β
TIMP	<u>T</u> issue inhibitors of metalloproteinases
TNF-α	<u>T</u> umour <u>N</u> ecrosis <u>F</u> actor α
TNF-β	<u>T</u> umour <u>N</u> ecrosis <u>F</u> actor β
TRAF	<u>T</u> umour Necrosis Factor <u>R</u> eceptor <u>A</u> ssociated <u>F</u> actor
TrkA	<u>T</u> yrosine <u>k</u> inase <u>r</u> eceptor <u>A</u>
TrkB	<u>T</u> yrosine <u>k</u> inase <u>r</u> eceptor <u>B</u>
trTrkB	<u>T</u> runcated tyrosine <u>k</u> inase <u>r</u> eceptor <u>B</u>
TrkC	<u>T</u> yrosine <u>k</u> inase <u>r</u> eceptor <u>C</u>
TUNEL	<u>T</u> dT-mediated d <u>U</u> TP digoxigenin <u>n</u> ick <u>e</u> nd <u>l</u> abelling



## **CHAPTER 1**

### **INTRODUCTION**

## **1.1 HUMAN GONADAL PROLIFERATION AND DEVELOPMENT**

Although germ cells are not essential for the survival of the body they hold the key to the life cycle and the survival of mankind. The foundation for normal puberty and adult reproductive function is established during fetal life. The increasing identification of factors that regulate gonadal paracrine and autocrine function, as well as the emerging application of molecular biological techniques, have provided new insight into rodent and larger mammalian fetal growth and development. However, relatively little is known about the processes regulating fetal gonadal development in the human.

It is vitally important that the mechanisms involved in the development of the gonad during fetal life are better understood. Until this is achieved, therapeutic options available to treat pathological conditions such as premature ovarian failure and methods designed to promote germ cell development in vitro will remain sub-optimal.

The ensuing literature review will initially cover the development of the indifferent gonad and then specifically the ovary and the testis. The factors which are likely involved in the regulation of this development will then be considered.

## **1.2 MAMMALIAN GONADAL DEVELOPMENT – INDIFFERENT STAGE**

Gonadal life begins in the human embryo with the differentiation of primordial germ cells (PGC) in 4.5-day old blastocysts. At this time the embryo lacks any specific gonadal structures, but the ultimate development of a female or male gonad is predetermined by its chromosomal sex. The PGC start their amoeboid migration (Fujimoto et al 1977; Witschi E 1948) from the endoderm of the yolk sac through the gut endoderm and into the mesoderm of the mesentery to the coelomic epithelium of the urogenital ridge. By 5 to 6 weeks of gestation, the indifferent gonad is composed of PGC, supporting cells of the coelomic epithelium and the mesenchyme of the gonadal ridge. The future sexual development of the gonads is indistinguishable at this stage.

### **1.21 MATERNAL GONADOTROPHINS AND THE EFFECT ON FETAL GONADAL DEVELOPMENT**

Human chorionic gonadotrophic hormone (hCG) is secreted by the fertilized ovum from the blastocyst stage and is later secreted by the syncytiotrophoblast of the placenta. It stimulates oestrogen and progesterone production and peaks at 8-10 weeks of gestation, falling after this and plateauing at 18-20 weeks of gestation. hCG is responsible for sex differentiation in the male fetus by stimulating the Leydig cells to produce testosterone and initiating development of male secondary sexual characteristics. The fetal pituitary does not start to secrete LH until after the 12<sup>th</sup> week and it is responsible for the later development of male secondary sexual characteristics. In early pregnancy, oestrogen is mainly produced in the corpus

luteum and these levels reach a maximum by 10 weeks. From 6 weeks maternal and fetal androgens are converted into oestradiol and oestrone by the placenta. The fetal adrenals develop very early in pregnancy and undergo remarkable growth, becoming the main source of oestrogens in later pregnancy as maternal serum oestrogens rise progressively throughout the pregnancy. By 20 weeks, the fetal hypophyseal-portal system is fully developed and the fetal adrenals become progressively under control of the prolactin and adrenocorticotrophic hormone (ACTH) secreted by the fetal pituitary. Because of high maternal oestrogen and progesterone levels, follicle stimulating hormone (FSH) and luteinising hormone (LH) are suppressed. Progesterone levels progressively increase as pregnancy advances, first being produced by the corpus luteum until the 10<sup>th</sup> week and then by the placenta. It is regarded as the dominant pregnancy hormone as its levels are 10 times that of oestrogen (Edmonds DK 1995).

In sheep, a transient period of steroid synthesis occurs in the fetal ovary between days 35-55 of gestation and this broadly coincides with the onset of meiosis in the germ cells (Mauleon P 1977). The ontogeny of fetal hypothalamic function has been studied in sheep, demonstrating that LH secretion is essential for normal development of the testis and there is a critical window for this in late pregnancy (Brooks AN et al 1996; Brooks et al 1995). There is little data on the effect of serum gonadotrophins on ovarian development but these investigators have shown that GnRH positively regulates the synthesis and secretion of gonadotrophins in the fetus and that reduced fetal gonadotrophic support during the last half of gestation results in a reduction in testicular growth but has no effect on ovarian development (Thomas

et al 1994). Environmental oestrogens have also been suggested to be detrimental to fetal testis development, resulting in a reduction in testicular size and in the number of Sertoli cells (Sharpe et al 1998).

## **1.22 HUMAN FETAL TESTICULAR DEVELOPMENT**

Gonadal differentiation into a testis is controlled by a multitude of genes beginning with Sry, located on the Y chromosome, which is believed to represent the testis determining factor (Berta et al 1990). Sry is active for a brief period in the genital ridge in order to initiate Sertoli cell differentiation and triggers a cascade of gene activity, acting on Sox9, steroidogenic factor-1 (SF-1) and the dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome (DAX-1) (Ramkissoon et al 1996; Wagner et al 1994). Sox9 is up-regulated by Sry and is a testis specific Sertoli cell transcription factor (Kent et al 1996; Morais et al 1996). SF-1, a member of the nuclear hormone receptor superfamily plays a critical role in gonad morphogenesis and transcriptional activation of several relevant genes, including Sox9, anti-Müllerian hormone (AMH) and DAX-1 (Lovell-Badge et al 2002). DAX-1 has an antagonistic effect on Sertoli cell differentiation and is down-regulated as testes develop whereas it continues throughout ovarian development (Swain et al 1996; Swain et al 1998).

Following migration of PGCs from the yolk sac to the nephrogonadoblastic ridge, the first sign of testicular differentiation is the appearance of testicular cords consisting of PGCs enclosed by Sertoli cells (Byskov 1986). In the human, this is initiated at 6-7 weeks gestation and continues for several weeks. The Sertoli cells have an

important paracrine (secretion of AMH) and endocrine (secretion of inhibin) function as well as inhibiting the onset of meiosis of the spermatogonia. The Leydig cells secrete testosterone, causing differentiation and growth of the Wolffian duct structures (Boehmer et al 1999). AMH is a glycoprotein produced by the fetal Sertoli cells. Its primary role in sex development is to cause a gradient of cranial to caudal regression of Müllerian ducts at 8-10 weeks of gestation in the human (Durlinger et al 2002).

Dihydrotestosterone, created by the metabolism of testosterone, causes growth of the prostate and phallus and fusion of the labiosacral folds (Thigpen et al 1993) (Wilson et al 1993).

Unlike their counterparts in the developing ovary, male germ cells do not start meiotic division until puberty, and the germ cells (termed gonocytes or prespermatogonia) continue mitotic division until approximately 22 weeks gestation (Hilscher 1991).

Steroid secreting Leydig cells appear in the interstitium at a gestational age of 8 weeks (Black V.H. 1969). They are intermingled with mesenchymal cells and small capillaries. Shortly after their appearance, androgen concentrations rise and reach a maximum at 15 – 18 weeks (George et al 1978; Skinner 1991). This corresponds with a peak in Leydig cell number which then declines and by term very few Leydig cells are seen (Niemi M 1967). The pattern of Leydig cell development parallels closely with fetal hCG levels during pregnancy and suggests a regulatory role for this

hormone (Pelliniem LJ 1969). Mesonephros-derived cells also contribute to the peritubular myoid cell population which form a layer of cells around the basement membrane of the cords (Buehr et al 1993). Around the time of Leydig cell proliferation, there is increased expression of steroidogenic enzymes (particularly  $3\beta$ -hydroxysteroid dehydrogenase and P450  $17\alpha$ -hydroxylase/C17-20-lyase) and expression of the androgen receptor (AR) in the peritubular myoid cells (Murray et al 2000). The migration of the testis from the lower pole of the kidney on the abdominal wall is a two-stage process of transabdominal migration and inguino-scrotal descent (Hutson J.M. 1992). The gubernaculum in the male contracts, thickens and develops a bulbar outgrowth which, with regression of the cranial suspensory ligaments, results in the testis located in the lower abdomen by the internal inguinal ring. Inguino-scrotal descent is androgen dependent and insulin-like factor 3 has also been implicated (Bernstein et al 1988; Lim et al 2001; Tomboc et al 2000).

### 1.23 HUMAN FETAL OVARIAN DEVELOPMENT

In the human the indifferent gonadal stage persists longer in the female than in the male. In the absence of the male sex determining gene Sry, Sertoli cell differentiation does not occur. Rather germ cells migrating into the primordial gonad differentiate as oogonia and cells derived from the mesonephros differentiate into stromal cells, some of these eventually becoming granulosa cells. In the absence of secretion of AMH by Sertoli cells and testosterone by Leydig cells, the Müllerian ducts differentiate and grow as female internal genitalia and the external genitalia are feminized. Between 7 and 9 weeks, primitive germ cells, most of which are oogonia, are scattered throughout the ovarian tissue and divide mitotically. Cytoplasmic cleavage may be incomplete and results in daughter cells sometimes remaining linked by cytoplasmic bridges allowing the transfer of macromolecules and cytoplasmic organelles (Pepling et al 1999). 'Nests' of syncytial germ cells tend to develop until associations break down at meiosis. These germ cell nests are interspersed with cell streams of somatic cells originating from the mesonephros (McNatty et al 2000). Meiosis begins in the human fetal ovary around 11-12 weeks gestation (Gondos et al 1971; Gondos et al 1986). The leptotene stage begins around 8 to 11 weeks (Baker 1963; Baker et al 1974; Gapienko 1975) and the maximum number of oocytes in this stage can be seen at 14 – 26 weeks (Kurilo 1981) amounting to approximately 2% of all germ cells (Baker 1963; Kurilo 1981). Oocytes enter the zygotene stage of meiosis between 10.5 and 12 weeks of gestation (Baker 1963) (Kurilo 1981) and cells at this stage subsequently become abundant. The maximum number of oocytes at zygotene occurs after 16 weeks of development and near term their amount does not exceed 5% (Kurilo 1981). Pachytene, the stage



during which pairing of chromosomal homologues takes place (Baker 1963; Kurilo 1981; Speed 1985), initially occurs between the 10.5–14 weeks of fetal life. There is some discrepancy over the timing of cells passing into the diplotene stage. Baker (Baker 1963) first found cells at this stage at 16 weeks, with cell numbers reaching a peak at 19 weeks of gestation. However a more recent study detected cells in diplotene 3–4 weeks earlier (Kurilo 1981). The oocyte is suspended in the diplotene stage of prophase until resumption of meiosis later in reproductive life.

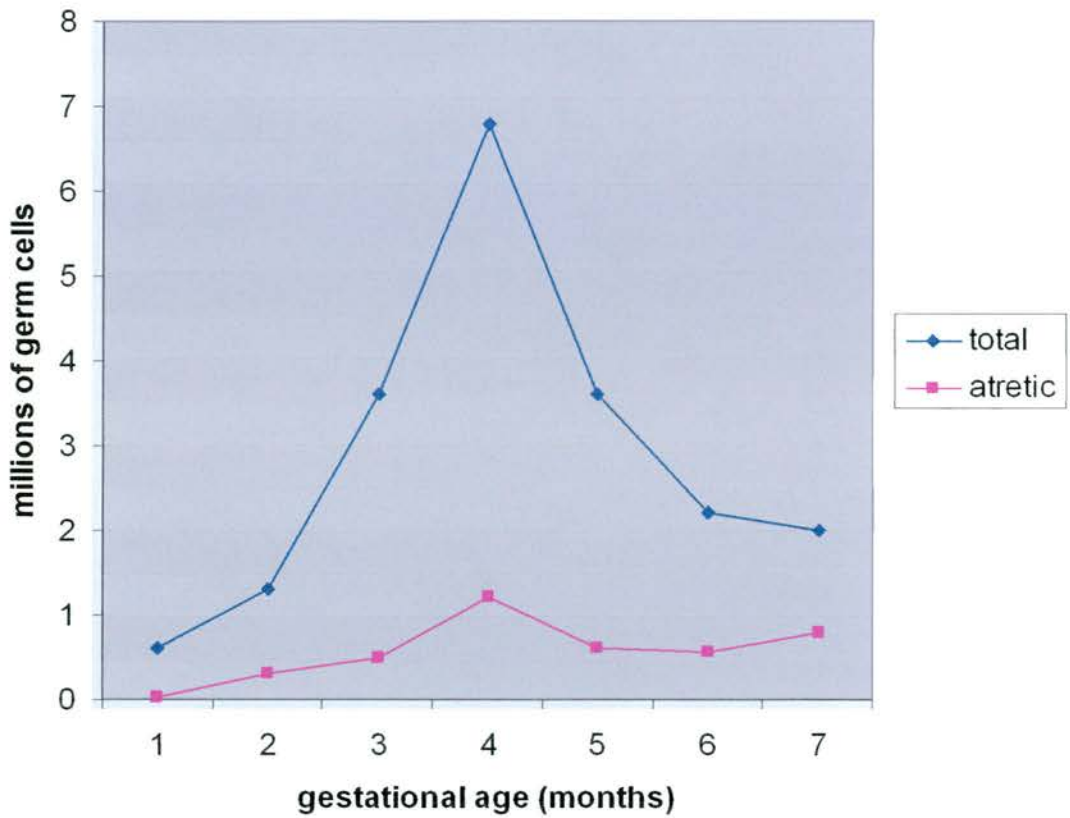
It is at the diplotene stage that genetic recombination occurs and in 1968, Henderson and Edwards (Henderson et al 1968) proposed a link between recombination and non-disjunction, suggesting that declining levels of recombination were the cause of the maternal age effect on trisomy. Their model, the so-called ‘production line’ hypothesis of maternal age non-dysjunction, suggested that meiotic chromosomes of older women were held together by fewer chiasmata, and that consequently the chromosomes were more likely to nondisjoin at meiosis I. It was not until the 1980s when DNA polymorphism analysis arrived that it became possible to test this hypothesis and it is now clear that this model cannot explain the maternal age effect on human trisomy. However, it seems that their major premise was correct, i.e. that alterations in recombination are an important determinant of human trisomy.

Three distinct waves of atresia occur in the human fetal ovary, affecting (1) oogonia undergoing mitosis (atretic divisions), (2) oocytes largely at the pachytene stage (‘Z’ cells), and (3) oocytes at the diplotene stage. The number of germ cells rises steadily from 600 000 at 8 weeks of gestation and peaks at approximately 7 million at 16–20

weeks (Baker 1963) with a subsequent reduction to about 2 million cells at term (Figure 1.1).

The primary oocytes at diplotene stage and some argue also the pachytene stage (Ohno 1962; Ohno 1961) become associated with stromal cells, the precursors of granulosa cells and form primordial follicles. The first of these are seen at around 16-21 weeks gestation (Baker et al 1974; Konishi et al 1986), although some report follicles as early as 11.5-12 weeks (Kurilo 1981). Preantral follicles develop around 24 weeks gestation and antral follicles can be found in ovaries near term or in the neonate (Peters et al 1978). However, such follicles are rarely observed and constitute an insignificant portion (0.1-0.5%) of the total number of oocytes in the follicles. Some of the disparity in the gestations at which these stages of oocyte development are observed may be due to researchers variously using the date of the last menstrual period or the date post coitum.

## Total germ cells in human fetal ovaries



**Figure 1.1** Graph showing numbers of germ cells in human ovaries throughout fetal life (based on figure from (Baker 1963)).

The interstitial cells of the ovary appear to reach their maximal numbers at around 18 weeks and thereafter decline in number. Since ovarian interstitial cells are not associated with the developing follicles, they appear to be distinct from thecal cells. It is only in the latter stages of pregnancy that theca cells with steroidogenic potential are found surrounding the developing follicles (Gondos et al 1971; Guraya 1977). In the human ovary, folliculogenesis appears to be independent of gonadotrophins, as little or no steroid synthesis can be detected prior to this process (Payne et al 1971). The aromatizing system appears to function as early as the 8<sup>th</sup> week but the ovary is incapable of synthesizing steroids *de novo* (Bloch 1974; Noumora 1966).

It is clear that many factors are involved in the normal progression of the germ cell lineage during gonadogenesis. From the initial step of migration of the PGCs to the urogenital ridge there is activation of various pathways, such as bone morphogenetic proteins (BMPs), the c-kit receptor and its ligand and Bcl-x (a cell survival factor) and Bax (a cell death factor) (Kierszenbaum et al 2001). Once the PGCs have colonized the developing gonad, an immense amount of tissue remodelling occurs, along with PGC proliferation, differentiation and apoptosis. An array of proliferative and apoptotic factors are implicated in these processes in the mammalian gonad. The ensuing paragraphs will discuss in detail: (1) the structure and function of the c-kit receptor and ligand and its role in cell migration, proliferation and survival; (2) extracellular matrix (ECM) cleavage and remodelling under the influence of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs); (3) the structure of neurotrophins and their receptors and their participation in cell differentiation, migration, proliferation, and survival in neuronal and non neuronal tissue; (4) K252a,

a compound known to inhibit the actions of neurotrophic growth factor (NGF) and other neurotrophins; (5) fibroblastic growth factors (FGFs), a family of mitogenic factors which act via tyrosine kinase receptors and appear not to be inhibited by K252a.

### 1.3 C-KIT

The tyrosine kinase receptor C-kit and its ligand have previously been demonstrated in rodents to affect the proliferation, migration and survival of germ cells, melanocytes and haematopoietic progenitor cells during embryogenesis and adult life. Both receptor and ligand are encoded at the White spotting (*W*) and Steel (*Sl*) loci respectively (Chabot et al 1988; Copeland et al 1990; Geissler et al 1988; Nocka et al 1990; Zsebo et al 1990). In homozygous mice most mutations are lethal, and those surviving are generally sterile (Russell 1979). In *Sl/Sl* homozygotes, it is evident PGCs have not migrated from the hindgut to the gonadal ridge. In *W/W* homozygous mice, some migration does occur (Loveland et al 1997).

C-kit is predominantly expressed by germ cells in the rodent testis although it has also been suggested to be expressed by Leydig cells (Manova et al 1990), whereas kit ligand (KL; also known as stem cell factor (SCF), mast cell growth factor (MGF) and steel factor (SLF)) is expressed by a wider range of cell types. The presence of a functional c-kit receptor has been implicated in spermatogonial proliferation, survival and adhesion to Sertoli cells (Loveland et al 1997). Monoclonal antibodies have been used to block the effect of c-kit and KL in the postnatal testis of genetically normal mice. This resulted in disruption of the proliferation of Type A

spermatogonia, hence indicating that c-kit is involved in mitotic activity of germ cells (Tajima et al 1994; Yoshinaga et al 1991). The expression of C-kit by pachytene spermatocytes has been demonstrated and it has been proposed that the C-kit/Kl interaction is also essential for meiosis (Vincent et al 1998). Consistent with the suggestion that signalling via C-KIT is important in normal male germ cell development and function, alterations in C-KIT/KL expression have also been demonstrated in some patients with defective spermatogenesis (Mauduit et al 1999), with reduced expression associated with increased germ cell apoptosis.

Messenger RNA encoding *C-kit* and *Kl* has been detected in fetal mouse ovaries between embryonic days 8 and 14.5 consistent with a role in germ cell migration and proliferation (reviewed by (Driancourt et al 2000)). Functional effects of Kl/C-kit in the ovary may persist into adult life, for example in the regulation of persistence of meiotic arrest (Horie et al 1991) (Ismail et al 1997) and activation of primordial follicle growth (Parrott et al 1999; Yoshida et al 1997). In mouse mutants, some *Sl* mutant phenotypes display reduced follicle numbers and fail to initiate follicular growth from the primordial follicle stage (Driancourt et al 2000). Several steps of folliculogenesis also appear to have graded requirements for C-kit and Kl. A limited reduction in amounts of Kl seen in heterozygous mice results in alterations in oocyte growth, granulosa cell proliferation in preantral follicles and in antrum formation (Reynaud et al 2001).

C-kit is a receptor glycoprotein in the platelet – derived growth factor receptor family with tyrosine kinase activity encoded in a split kinase domain. In humans, the *C-KIT*

gene on the proximal arm of chromosome 4 covers 20 kb and is comprised of 21 exons (Giebel et al 1992; Vandenbark et al 1992). Mutations at the *W* locus have been described and include large deletions, point mutations and rearrangements. These can alter the amount of C-kit protein expressed and the level of kinase activity (Dubreuil et al 1990; Nocka et al 1989). The ligand appears to interact with the C-kit receptor as a dimer and the subsequent dimerisation of C-kit results in autophosphorylation of the C-kit subunits to affect signal transduction (Blume-Jensen et al 1991; Lev et al 1992; Reith et al 1991). Autophosphorylation of C-kit activates intracellular signaling pathways that can affect cell proliferation, survival and adhesion.

The ligand for C-kit is an integral membrane glycoprotein encoded at the *Sl* locus (Anderson et al 1990; Copeland et al 1990; Flanagan et al 1990; Martin et al 1990; Nocka et al 1990; Zsebo et al 1990). KL is encoded by nine exons in the human (Martin et al 1990). Two different forms of *Kl* mRNA exist, one including and one excluding exon 6, and these show tissue specific patterns of expression (Huang et al 1992). The cleavage of the extracellular portion of the growth factor can produce a soluble form of the ligand, incorporating exon 6, while the form lacking this exon can be cleaved at other sites at a slower rate (Huang et al 1992). The soluble (sKl) and membrane-bound (mKl) forms have different effects on c-kit in target cells. After ligand binding, signal transduction cascades are initiated by the stimulation of receptor autophosphorylation (Ullrich et al 1990). In the case of sKl, tyrosine phosphorylation of C-kit is rapid, followed by a decline in phosphorylation. This decline in phosphorylation coincides with receptor internalization and endocytosis,

(Gommerman et al 1997) leading ultimately to receptor degradation. In contrast, phosphorylation of c-kit by mKl persists over much longer periods (Miyazawa et al 1995). This persistence in tyrosine phosphorylation is attributed to the enhanced stability of the C-kit receptor on the cell surface after mKl stimulation, likely to be due to prevention of receptor internalization by this form of ligand. In the mouse testis at birth, mKl is predominant in early development, but sKl is prevalent in the adult (Manova et al 1990). Both forms produced by Sertoli cells could interact with the C-kit receptor on spermatogonia, while the soluble form could potentially reach distal targets such as c-kit on Leydig cells.



#### **1.4 MATRIX METALLOPROTEINASES AND TISSUE INHIBITORS OF METALLOPROTEINASES**

Within gonadal tissue, the extra cellular matrix (ECM) provides the scaffolding which cells attach to and also by binding to specific cell surface receptors modulate their activity. Remodelling of the ECM may play an integral role in fetal gonadal development, including cell migration and organisation. Matrix metalloproteinases (MMPs), so called because of their dependence on metal ions for catalytic activity, are a family of enzymes that are primarily responsible for the cleavage of components of ECM, both interstitial matrix and basement membrane. They can be categorized, in relation to their specificity for the ECM and location, into subgroups such as collagenases, gelatinases, stromelysins, membrane-type MMPs and a miscellaneous group. In total, seventeen MMPs have been characterized (see Table 1.1).

MMPs have been identified in the rat ovary and are known to be produced by various cell types (Bagavandoss 1998). Specific inhibitors of MMPs (TIMPs) have also been found in gonadal tissue (Curry, Jr. et al 1990) and these not only inhibit MMPs but also promote proliferation of various cell types (Edwards et al 1996a).

MMPs are zinc dependent metallopeptidases, secreted as latent soluble or membrane bound forms. These inactive proMMPs or zymogens, are activated by proteolytic cleavage to give the active enzyme (Hulboy et al 1997). At the amino terminus is the 'pre' domain, which signals for cellular export. Cleavage and removal of the 'pro' domain which is adjacent to the 'pre' domain converts the proMMPs to the active

enzyme. A variety of activators have been described, such as other MMPs including membrane bound MMPs (Foresta et al 1997), serine proteases (plasminogen activator and kallikreins) (Espey L 1992) and mast cell proteases (Suzuki et al 1995) and these convert proMMPs to the active enzyme. The disengagement of a cysteine residue (cysteine switch mechanism) in the pro domain sequence PRCGVPDV is the key mechanism in pro domain cleavage. This reveals a zinc ion which is held in position by binding to 3 histidines in the active site (amino acid sequence HEXGHXXGXXHS) in the adjacent catalytic domain. It is the binding of water to the zinc ion in the active site that completes the MMP activation. MMP-2 is refractory to activation by serine proteinases and is instead activated at the cell surface through a unique multistep pathway involving MT-MMPs and TIMP-2 (Strongin et al 1995).

All MMPs except MMP-7 possess other regions that bestow diversity when they associate with the ECM or substrate. Fibronectin domains (MMP-2 and MMP-9) facilitate gelatin binding, haemopexin-like domains (all MMPs) allow association to the ECM and MMP inhibitors (Baragi et al 1994), Mt-MMPs contain a transmembrane domain that localizes the enzyme to the plasma membrane and Mt-MMPs and MMP-11 possess a region which binds furins, which are protein-processing enzymes.

Expression of pro-MMP genes is subject to control during transcription, translation, by changes in mRNA stability, at the activation of the pro-enzymes by proteolytic cleavage and by the association and dissociation of MMPs with their specific

inhibitors. MMP release can be stimulated by cytokines (interleukin-1 (IL-1), tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6), ECM components, MMPs, lipopolysaccharides (LPS), nitric oxide and PGE<sub>2</sub> and inhibited by secretory leucocyte protease inhibitor (SLPI), cytokines (interleukin-4 (IL-4), interleukin-10 (IL-10), tumour necrosis factor  $\beta$  (TNF- $\beta$ ) and interferon  $\gamma$  (IFN- $\gamma$ ), oestrogen and progesterone (Hulboy et al 1997).

The principal inhibitors of MMP function are the liver derived, serum borne  $\alpha_2$ -macroglobulin and the natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (Hulboy et al 1997). TIMPs consist of two domains linked by disulphide bonds. The TIMPs bind to each MMP using non-covalent 1:1 stoichiometric binding to their active site. To date, four TIMPs have been identified (Table 1.2) and they do not appear to be selective among the MMPs they act upon.

Although MMPs can cleave virtually all structural ECM molecules, they can also potentially influence cell behaviour by cleaving cell-cell adhesion proteins, by cleaving cell surface molecules that transduce signals, or by releasing bioactive cell surface molecules (Sternlicht et al 2001). For example MMP-3 and MMP-7 cleave the adherens junction protein E-cadherin and the soluble fragment which is released disrupts cell aggregation and promotes cell invasion via a paracrine mechanism separate from cleavage itself (Lochter et al 1997; Noe et al 2001). The disruption of the matrix can induce apoptosis in cells anchored in the ECM. Therefore, in the case of normal physiological cell death such as involution of the mammary gland, MMPs can play a role (Alexander et al 1996). Another potential function of MMPs is in the

regulation of paracrine signaling and in breaking down non-matrix proteins (Sternlicht et al 2001). MMP-2, -7, -9, and -12 are capable of cleaving plasminogen to generate angiogenesis inhibitor angiostatin, thereby generating a breakdown product with an entirely new biological function (O'Reilly et al 1999; Patterson et al 1997). The expression of MMPs can be induced by many factors including growth factors, cytokines, chemical agents, physical stress and oncogenic cellular transformation. This enhanced MMP expression may in turn be down-regulated by suppressive factors such as transforming growth factor  $\beta$  (TGF  $\beta$ ), retinoic acids and glucocorticoids (Nagase et al 1999). Certain signaling pathways can lead to expression of a particular MMP gene. Ultraviolet B irradiation can up-regulate MMP-1, MMP-3 and MMP-9 expression in human dermal fibroblasts by stimulating ERK, JNK and p38 MAP kinase pathways (Brenneisen et al 1998; Fisher et al 1996). TIMPs are also multifunctional proteins, inhibiting cell invasion *in vitro*, tumour cell growth, metastasis *in vivo* and angiogenesis (Gomez et al 1997). However, they exhibit additional biological functions. TIMP-1 and TIMP-2 have mitogenic activities on several cell types, but an over expression of these inhibitors reduces tumour cell growth (Gomez et al 1997). TIMP-3 has been shown to induce apoptosis of human colon carcinoma cells (Smith et al 1997) and TIMP-2 inhibits basic fibroblast growth factor-induced human endothelial cell growth (Murphy et al 1993). These varied biological activities of TIMPs are independent of MMP-inhibitory activities (Chesler et al 1995; Hayakawa et al 1994).

<b>Group</b>	<b>Name</b>	<b>MMP numbers</b>	<b>MW latent</b>	<b>Substrate specificity</b>
<b>Collagenases</b>	Interstitial collagenase	MMP-1	52000 (42000) 56000 (46000)	Collagens I, II, III, VII, VIII Gelatin, IGFBP-3, TNF- $\alpha$
	Neutrophil collagenase	MMP-8	75000 (65000)	Collagens I, II, III
	Collagenase 3	MMP-13	53700	Collagen II
<b>Gelatinases</b>	72kDa Gelatinase	MMP-2	72000 (62000)	Collagen II Collagens I, IV, V, VII, X, elastin, gelatin, fibronectin,
	92kDa Gelatinase	MMP-9	92000 (84000)	TNF- $\alpha$ Collagens IV, V, elastin, gelatin, TNF- $\alpha$
<b>Stromelysins</b>	Stromelysin	MMP-3	56000 (45000) 59000 (28000)	Proteoglycan, fibronectin, laminin, elastin, collagens III, IV, V, VII, X, gelatins, IGFBP-3, TNF- $\alpha$ , MMP-1, -8, -9
	Matrilysin	MMP-7	28000 (19000)	Fibronectin, laminin, elastin, collagen IV, gelatin, IGFBP-3, TNF- $\alpha$ , MMP-1 and -9, proteoglycan
	Stromelysin 2	MMP-10	60000 (55000)	Proteoglycan, fibronectin, laminin, elastin, collagens III, IV, V, gelatins, MMP-8
	Stromelysin 3	MMP-11		Fibronectin, laminin
<b>Membrane-type MMPs</b>	Mt-MMP-1	MMP-14	63000	MMP-2
	Mt-MMP-2	MMP-15	?	?
	Mt-MMP-3	MMP-16	?	MMP-2
	Mt-MMP-4	MMP-17	?	?
<b>Other</b>	Metalloelastase	MMP-12	?	Elastin, fibronectin, collagen IV

**Table 1.1** Matrix metalloproteinases – nomenclature and substrate specificity

<b>Inhibitor</b>	<b>Relative Molecular Mass</b>	<b>Glycosylation</b>	<b>Extracellular Location</b>
<b>TIMP-1</b>	28 000	Glycosylated	Soluble in ECM and body fluids
<b>TIMP-2</b>	21 000	Not glycosylated	Soluble in ECM and body fluids
<b>TIMP-3</b>	24 000	Not glycosylated	Bound to ECM
<b>TIMP-4</b>	22 000	?	?

**Table 1.2** Tissue inhibitors of matrix metalloproteinases – nomenclature and substrate specificity

## 1.5 NEUROTROPHINS

The neurotrophins are a family of related neurotrophic factors which are critical in mediating the differentiation, migration, proliferation, and survival of neurons in the developing brain and peripheral nervous system (Davies 1994; Henderson et al 1993; Snider 1994). In addition, there is accumulating evidence for non-neuronal roles of the neurotrophins, particularly in mesenchymal-epithelial interactions (Brill et al 1995; Dissen et al 1995; Mitsiadis et al 1995; Ojeda et al 1992; Sariola et al 1991; Wheeler et al 1992; Yaar et al 1991).

The neurotrophin hypothesis states that the survival of developing neurons depends on the supply of a neurotrophic factor that is synthesised in limiting amounts in their target fields (Levi-Montalcini et al 1968; Thoenen et al 1980). Neurotrophin synthesis commences in the peripheral target fields of sensory and sympathetic neurons with the arrival of the earliest axons (Davies 1987; Korsching et al 1988). When neuronal death occurs in the sensory ganglia, the level of neurotrophin present is proportional to the final innervation density; high levels in future densely innervated areas and low levels in future sparsely innervated areas (Harper et al 1990). After uptake by sensory and sympathetic fibres in their target fields, neurotrophins are conveyed by fast axonal transport to the cell bodies of the innervating neurons where they exert their survival promoting effects (Hendry et al 1974; Korsching et al 1983). The best known and most extensively studied among these factors are the nerve growth factor (NGF) family of neurotrophins. NGF is the founder member (Levi-Montalcini 1966) and several other neurotrophins have been

identified, including brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3), neurotrophin 4 (NT4, also known as NT5) and more recently neurotrophin 6 (NT6) (Barde et al 1982; Ernfors et al 1990a; Gotz et al 1994; Hallbook et al 1991). All the neurotrophins are initially produced as 30–35 kDa precursor proteins and these unprocessed proneurotrophins often represent abundant forms of secreted neurotrophin (Lee et al 2001). The proneurotrophins contain a signal peptide, sites for glycosylation, and pairs of basic amino acids that are recognized by processing enzymes. These enzymes, including the calcium-dependent serine protease furin cleave each of the neurotrophins, releasing the biologically active 12–14 kDa C-terminal product (Seidah et al 1996). The neurotrophin monomer has an elongated shape with the central part of the molecule formed by two pairs of twisted, antiparallel  $\beta$ -strands. There are three hairpin loops on one end, and the other end carries a cysteine-knot motif, a step-like arrangement of three disulfide bridges. In the biologically active form, two monomers are arranged in a parallel manner to form a close-packed homodimer (Figure 1.2). All neurotrophins share a pair-wise sequence identity of approximately 50%.

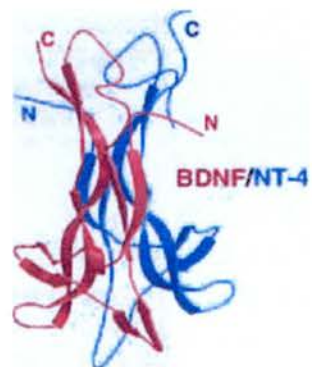
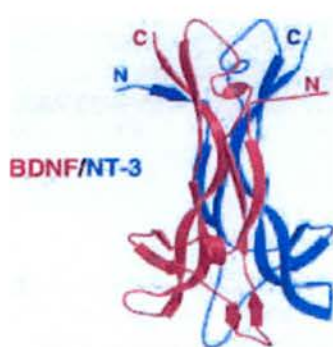
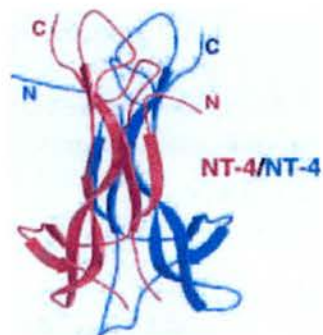
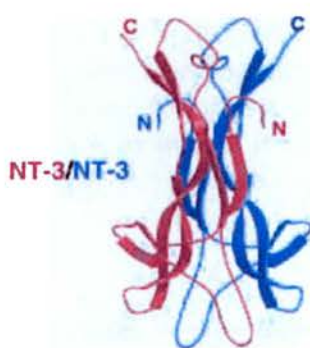
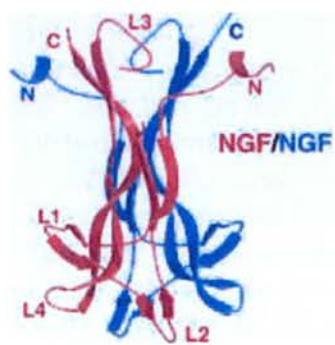
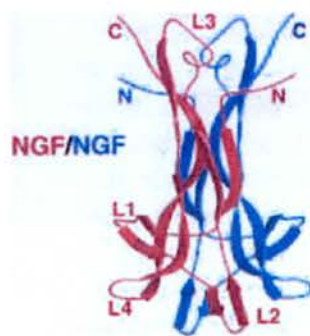
The main function of NGF in neuronal tissue is to support the survival of sympathetic ganglion neurons and subpopulations of sensory neurons in dorsal root ganglia (DRG) and trigeminal ganglia. BDNF and NT3 also support DRG and trigeminal ganglia but in addition support nodose and vestibular ganglion cells (Korsching 1993). However, neurotrophin expression is not restricted to neuronal cells, and as previously mentioned, they have been found in many non-neuronal tissues. Neurotrophins were first identified in the ovary in *Xenopus laevis* (Hallbook



et al 1991) and have since been described in the mouse and rat ovary and in the adult human ovary (Berkemeier et al 1991; Dissen et al 1995; Dissen et al 2001; Ernfors et al 1990a; Klein et al 1989; Lamballe et al 1991; Lara et al 1990; Seifer et al 2002). The homozygote *Ngf*-null mice have markedly reduced numbers of primary and secondary ovarian follicles and reduced somatic cell proliferation (Dissen et al 2001).

Several members of the neurotrophin family and their receptors have also been identified in the developing rodent testis (Cupp et al 2000; Djakiew et al 1994; Levine et al 2000b; Russo et al 1999). NT3, NGF and the neurotrophin receptor tyrosine kinase C (TrkC) appear to be critical for cord formation in the rodent testis and they may be important in the differentiation and maturation of Leydig cells and germ cells as suggested by neurotrophin tyrosine kinase receptor knockout mice (Cupp et al 2000; Cupp et al 2002; Levine et al 2000b).

**Figure 1.2** Ribbon diagram demonstrating the structure of neurotrophin dimers. N and C termini are labelled. (Wiesmann et al 2001)



## 1.6 Trk RECEPTORS

The biological actions of the neurotrophins are mediated by tyrosine kinase receptors which were first described in 1986, as the product of the *trk* oncogene, a chimeric oncoprotein found in a human colon carcinoma (Martin-Zanca et al 1986; Martin-Zanca et al 1989). In 1991 it was discovered that the physiological role of the *trk* tyrosine protein kinase (TrkA) was to serve as a signalling receptor for NGF (Hempstead et al 1991; Kaplan et al 1991). Subsequent studies have revealed that TrkA belongs to a small family of closely related receptors that include TrkB and TrkC (Barbacid 1994). These receptors have strong affinities for particular neurotrophins, TrkA binding preferentially to NGF (Klein et al 1991), TrkB to BDNF and NT4 (Berkemeier et al 1991; Ip et al 1992; Klein et al 1992), and TrkC is the primary receptor for NT3 (Lamballe et al 1991). NT3 is also capable of binding to and signalling through TrkA and TrkB at high concentrations. Both TrkB and TrkC have a truncated form of receptor lacking the tyrosine kinase domain, but the function of the truncated form is still unclear (Barbacid et al 1991). In addition, all neurotrophins are recognised by a more widely-expressed low affinity receptor known as p75, which is a member of the tumour necrosis receptor super family (Chao 1994) (discussed in Section 1.65).

The generation of strains of transgenic mice deficient for each of the Trk receptors has helped define the role neurotrophin signalling plays in mammalian development. *TrkA*<sup>-/-</sup> mice are small and display a wide array of sensory defects and have extensive neuronal cell loss in trigeminal, dorsal root and sympathetic ganglia (Smeyne et al 1994). *TrkB* targeted mice develop to birth but most die within the

first postnatal week. They do not respond to stimuli, are unable to feed and their main neurological deficiencies are in the trigeminal and nodose/petrosal ganglia (Klein et al 1993). Unlike the targeted *TrkA*<sup>-/-</sup> and *TrkB*<sup>-/-</sup> mice, *TrkC*<sup>-/-</sup> develop to birth and appear normal, respond to painful stimuli and take nourishment. However, after birth they display abnormal movements, suggesting a defect in proprioception. They have a limited lifespan (most die by P21) and most of their neuronal defects are in the dorsal root ganglia and in muscle afferents (Klein et al 1994). With regard to gonadal development, testes of *TrkA*<sup>-/-</sup> and *TrkC*<sup>-/-</sup> mice contain reduced numbers of germ cells and impaired seminiferous tubule development compared to wild type (Cupp et al 2002) and *TrkB*<sup>-/-</sup> lack primordial follicle formation (Molinek M 2000). Mice carrying a null mutation of the *p75* gene appear to have normal numbers of ovarian follicles (unpublished data cited in (Dissen et al 1995)).

### **1.61 TrkA Receptor**

The Trk proto-oncogene encodes two tyrosine protein kinase isoforms of 790 and 796 amino acid residues, designated as TrkA (also known as Trk, or gp140<sup>trk</sup>) (Barker et al 1993; Horigome et al 1993; Martin-Zanca et al 1989; Meakin et al 1992). Both isoforms have a structure which includes a 32-amino-acid-long putative signal peptide, followed by an ectodomain which includes cysteine clusters, leucine rich motifs (LRM) and immunoglobulin-like motifs responsible for the interaction with NGF. They also have a transmembrane region and a cytoplasmic region that encompasses the tyrosine kinase catalytic domain. (Fig1.3A). It is the 790 amino acid long isoform that has been found mainly in cells of non-neuronal origin (Barker et al 1993; Horigome et al 1993), although it appears that both isoforms have similar

biological properties (Meakin et al 1992). During murine development TrkA is expressed mainly in the PNS and has also been detected in both mammalian ovaries and testes (Barbacid 1994; Dissen et al 1995; Levine et al 2000b)

### **1.62 TrkB Receptor**

The TrkB receptor exists in two forms; the full length TrkB receptor (TrkB<sup>TK+</sup>) and the truncated TrkB receptor (TrkB<sup>TK-</sup>). The full length form is a heavily glycosylated molecule of 821 amino acid residues and it contains all the canonical motifs of the tyrosine kinase receptors. Most of the homology between the receptors is in the catalytic domain and like TrkA; this receptor has a 15 amino acid cytoplasmic tail.

The truncated form of the TrkB receptor has the same extracellular and transmembrane domain as the full length TrkB. However it has a very short cytoplasmic tail and lacks the entire kinase catalytic region. The size of the full length and truncated receptors are 145,000-daltons and 95,000-daltons respectively.

In general, in mice TrkB<sup>TK+</sup> is located preferentially in neuronal tissue and TrkB<sup>TK-</sup> in non-neuronal cells. TrkB is expressed widely in the mouse CNS and PNS and has been detected in the ovary, lung, muscle and adult rat testis (Klein et al 1989; Schultz et al 2001).

### 1.63 TrkC Receptor

TrkC locus encodes two classes of cell surface receptors. To date, four TrkC tyrosine kinase isoforms have been described (Lamballe et al 1991; Lamballe et al 1993; Tsoulfas et al 1993; Valenzuela et al 1993). TrkC K1 was first identified and is a 145 kd glycoprotein. The other TrkC tyrosine kinases differ from TrkC K1 in the presence of 14 (TrkC 14), 25 (TrkC 25), or 39 (TrkC39) additional amino residues and these sequences are encoded by alternatively spliced exons. All TrkC isoforms engage in downstream signalling and induce resting cells to initiate DNA synthesis upon addition of NT3 (Lamballe et al 1993).

The second class of TrkC receptors are similar in structure to the TrkB<sup>TK-</sup> receptors and are termed TrkC<sup>TK-</sup>. They have the same extracellular and transmembrane domains as the TrkC tyrosine kinases but lack the kinase domain. To date, four different TrkC<sup>TK-</sup> receptor isoforms have been described, termed as TrkC<sup>TK-</sup> 158, TrkC<sup>TK-</sup> 143, TrkC<sup>TK-</sup>113 and TrkC<sup>TK-</sup> 108, based on the number of cytoplasmic residues (Tsoulfas et al 1993; Valenzuela et al 1993). These isoforms are the result of alternatively spliced exons. The precise physiological role of TrkB<sup>TK-</sup> and TrkC<sup>TK-</sup> non-catalytic isoforms is not clear but they may act as dominant inhibitory modulators of Trk signalling, concentrate or prevent diffusion of neurotrophins or mediate signalling events (Baxter et al 1997; Eide et al 1996; Kryl et al 2000).

TrkC is expressed throughout the CNS, in certain PNS structures in the mouse and uniquely in the ganglia of the enteric nervous system (Lamballe et al 1994; Tessarollo et al 1993). Outside of neural tissue it has been detected in both the ovary and testis (Lamballe et al 1991; Tessarollo et al 1993). Interestingly, *TrkC* null

mutations have reduced germ cell numbers and seminiferous cord area compared with the wild type mice (Cupp et al 2002).

#### **1.64 Trk Receptor signal transduction**

The binding of neurotrophins to Trk receptors produces biological responses through a two step process of activation. This involves the ligand mediated oligomerization of receptor molecules at the cell surface followed by autophosphorylation of their tyrosine residues. The phosphorylated tyrosine residues in the Trk cytoplasmic domain serve as anchors for binding downstream signalling elements (Schlessinger et al 1992). The membrane proximal domain of the Trk receptors has been identified as the ligand binding site (Ultsch et al 1999) and upon binding, enzymes become phosphorylated on tyrosine residues, which is required for their activation. The adaptor molecules facilitate the interaction between other signalling molecules by bringing them together at the cell membrane. Several proteins have been characterized as substrates for the Trk family of receptors. They include phospholipase C- $\gamma$  (PLC $\gamma$ ) (Ohmichi et al 1991), the regulatory subunit (p85) of phosphatidylinositol 3'-kinase (PI-3K) (Soltoff et al 1992), MAP kinase (Schanen-King et al 1991), Shc (a SH2-containing adaptor protein) (Obermeier et al 1993) and possibly the Ras GTPase activating protein (GAP) (Obermeier et al 1993).



## 1.65 p75 Receptor

After cleavage of its 28-amino acid signal peptide, human P75 is a 399-amino acid Type I transmembrane protein. It has an extracellular domain that contains four cysteine-rich domains and multiple O- and N-linked glycosylation sites. The intracellular domain contains a palmitoylation site at cysteine 279, two potential tumour necrosis factor receptor associated factor (TRAF) binding sites, a Type II death domain, a potential G protein activating domain and a PDZ (an amino acid binding motif) domain binding motif (Fig 1.3B). The p75 receptor is highly conserved among species and there are no similarities between p75 and the Trk receptors in terms of sequences in the ligand-binding or cytoplasmic domains. This receptor will bind to each mature neurotrophin with similar affinity and can be co-expressed with any of the Trk proteins. Truncated p75 isoforms do exist and are produced by alternative splicing and proteolysis.

In the rodent p75 is expressed widely in neural tissue, mainly in Schwann cells, motor neurons and meningeal cells. Outside of the CNS, it has been localised to the peritubular cells of the testis and to the stromal cellular compartment of the ovary (Campagnolo et al 2001; Dissen et al 1995; Levine et al 2000b; Russo et al 1999).

Although p75 and Trk receptors are co-expressed in many cells, independent expression of p75 and individual members of the trk family is also observed. The co-expression of p75 and trk receptors contributes to the formation of high affinity binding. p75 enhances the affinity of TrkA for NGF, while decreasing affinity for NT-3. Similarly, p75 differentially modulates the interactions of TrkB for BDNF, NT-3, and NT-4/5 (Bibel et al 1999). It can increase the binding affinity of NGF,

either by passing the ligand to the Trk receptor, or altering the conformation of TrkA receptors through allosteric interactions (Esposito et al 2001).

A role mediating apoptosis has been widely postulated for p75 (Barrett et al 1994; Rabizadeh et al 1993). The receptor has a recognised death domain which shares significant homology with the Fas and the TNF receptor family. The pathway responsible for p75 signalling transduction has not been completely identified but there is evidence for a number of second messenger systems, including ceramide, c-jun kinase (JNK), protein kinase B (PKB or Akt) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Barrett 2000). It also interacts with several adaptor proteins and of these neurotrophin receptor interacting MAGE homolog (NRAGE) (86 kDa member of the melanoma associated antigen (MAGE) family of proteins), p75 NTR associated cell death executor (NADE) and neurotrophin receptor interacting factors (NRIF1 and NRIF2) (closely related 94 kDa zinc finger proteins) have been associated with the induction of apoptosis and fas associated phosphatase-1 (FAP-1), receptor interacting protein-2 (RIP-2) and TRAF6 appear to promote survival (Roux et al 2002).

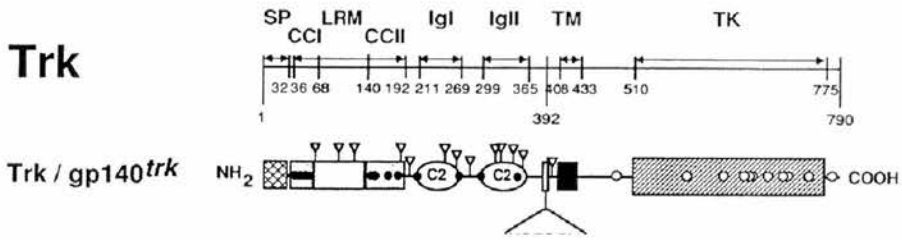
Differential effects of p75 during development have also been described, being required for NGF-mediated survival in neurons at the stage of target innervation but mediating an apoptotic signal at a later stage of cell development (Barrett et al 1994). Different responses can be elicited depending on whether or not p75 and Trk receptors are co-expressed: p75 alone can induce cell death, whereas in the presence of Trk receptors p75 can promote cell survival (Casaccia-Bonnet et al 1999). However, the dual receptor system is complex and the form of neurotrophin which

binds is crucial. The immature form of NGF, termed proNGF has a greater affinity for p75 than the mature form of NGF (Lee et al 2001). However, proNGF bound TrkA receptors much less strongly than mature NGF. The discovery that NGF may be secreted as proNGF, which preferentially activates p75, or as mature NGF, which preferentially activates TrkA, suggests that the balance between cell death and cell survival may be in part determined by the ratio of proNGF and mature NGF secreted by cells.

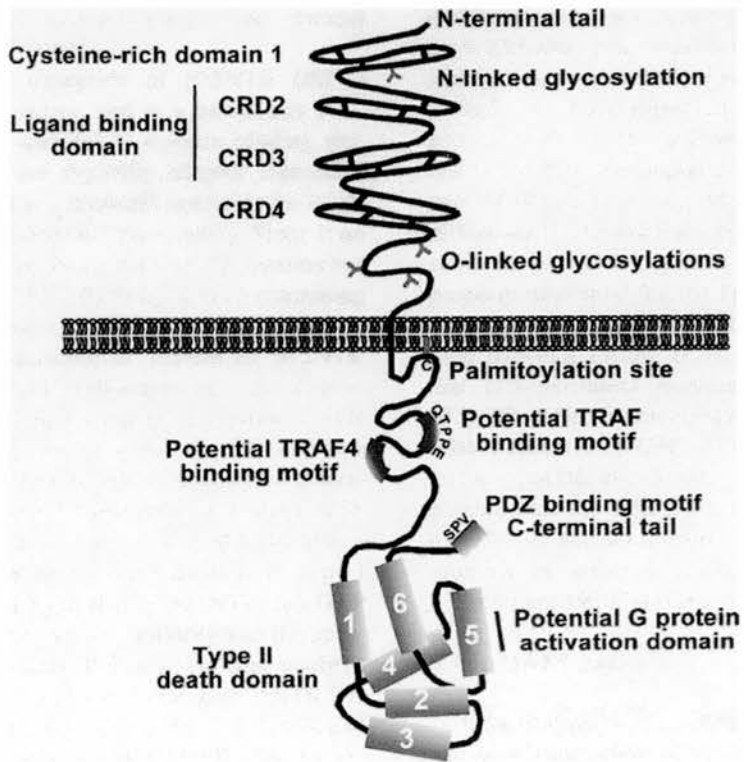
**Figures 1.3A and B. A shows Trk receptor structure (Barbacid 1994); B shows p75 receptor structure (Roux et al 2002)**



A



B



## 1.7 K-252a

K252a, a microbial alkaloid isolated from the culture broth of *Norcardiopsis* species, possesses a polyaromatic aglycon structure was originally characterised as a potent inhibitor of C kinase and cyclic nucleotide-dependent protein kinases, acting by interfering at or near the ATP binding site. However, chemical modifications in their sugar moiety can result in high specificity of the inhibitory action and, furthermore, can induce other stimulatory and inhibitory effects on nerve cells. K252a is of particular interest because it has been shown to inhibit the actions of NGF and other neurotrophins without diminishing comparable actions of other growth factors which act via tyrosine kinase receptors. This has been noted using PC12 and NIH3T3 cells, where the actions of neurotrophins were blocked by nanomolar concentrations of K252a but epidermal growth factor (EGF) and FGF pathways remained intact (Berg et al 1992; Hashimoto 1988; Nye et al 1992). Although it is known as a protein kinase C blocker, the action of K252a does not affect the protein kinase C pathway at these concentrations and rather the compound is thought to act directly on the tyrosine kinase catalytic domain (Berg et al 1992; Hashimoto 1988). The mechanism is thought to involve preventing ligand induced tyrosine kinase autophosphorylation by competitively blocking the kinase nucleotide binding site. K252a appears to act early in the NGF signalling pathway as almost all events induced by NGF in PC12 cells are affected by the compound (Berg et al 1992). These include increases in ornithine decarboxylase activity, stimulation of phosphatidylinositol breakdown, activation of MAP2 kinase (Miyasaka et al 1990; Tsao et al 1990), increases in intracellular calcium and the increase in *c-fos* transcription (Lazarovici et al 1989).

K252a is therefore a specific Trk receptor antagonist but does not distinguish between TrkA, TrkB and TrkC. Several more specific reagents have been previously used in order to distinguish between the actions of each receptor. Chimeric fusion proteins containing a Trk extracellular domain fused to human immunoglobulin (IgG) have been developed that inhibit receptor dimerisation and bind to their corresponding ligand to block biological activity (Shelton et al 1995).

## **1.8 FIBROBLAST GROWTH FACTOR**

Fibroblast growth factors (FGFs) are members of a family of polypeptides synthesized by a variety of cell types during the processes of embryonic development and in adult tissues. There are at least 23 members of the FGF family and, of these, 10 are expressed in the developing CNS along with four FGF receptors (FGFR). FGFs have mitogenic and angiogenic activity with a crucial role in cell differentiation and development. They use a dual receptor system based on tyrosine kinases and heparin sulphate proteoglycans and they are modulated by transforming growth factor  $\beta$  (TGF $\beta$ ). Basic FGF (bFGF) is recognized as a multifunctional growth factor which promotes the survival and neurite growth of brain neurons in vitro and in vivo (Botta et al 2000; Lynch et al 2000). Outside the nervous system, it has been located in the testis and ovary and acts as a survival and mitogenic factor for germ cells (Dissel-Emiliani et al 1996; Han et al 1993; Lynch et al 2000) As mentioned previously, although FGF acts via tyrosine kinase receptors, their actions do not appear to be inhibited by K252a.

## 1.9 SUMMARY

To summarise, the literature review has considered some of the factors including c-kit receptor and ligand, neurotrophins and their receptors and MMPs and TIMPs likely involved in the control and regulation of the development of the human fetal gonad. Current knowledge as to their roles in cell migration, survival and differentiation and in mammalian gonadal development was then considered.

### 1.91 HYPOTHESIS

It was then **hypothesised** that these factors play a vital role in the development of the human fetal testis and ovary, in particular in germ cell differentiation and survival.

### 1.92 AIMS

The specific **aims** of this study were therefore to:

1. To investigate the expression and possible role of c-kit and its ligand in germ cell development in the human fetal gonads during mid-trimester
2. To establish the secretion and localisation of MMPs and TIMPs to determine their possible role in the development of the fetal testis and ovary.
3. To investigate the expression and localisation of neurotrophins and their receptors in human fetal gonads and their effect on germ cell survival.



**CHAPTER 2**  
**MATERIALS AND METHODS**

## **2.1 MATERIALS**

The sources of all the reagents used for the experiments described in all the following chapters are detailed in Appendix I.

## **2.2-2.7 METHODS**

### **TISSUE COLLECTION AND CULTURE METHODS**

#### **2.21 TISSUE COLLECTION**

Human fetal ovaries and testes were obtained following medical termination of pregnancy. Women gave consent according to national guidelines (Polkinghorne, 1989) and the study was approved by the Lothian Paediatrics / Reproductive Medicine Research Ethics Sub-Committee. Termination of pregnancy was induced by treatment with mifepristone (200mg orally) followed by prostaglandin E1 analogue (Gemeprost, Beacon Pharmaceuticals, Tunbridge Wells, UK) 1mg 3-hourly per vaginum. None of the terminations were for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Gestational age was determined by ultrasound examination prior to termination and confirmed by subsequent direct measurement of foot length.

Gonadal tissue was dissected free, and either fixed or snap frozen and stored at –70°C. Fixation was carried out in Bouins for 5 hours, followed by transfer to 70% ethanol prior to processing in paraffin using standard methods.

## **2.22 DEVELOPMENT OF TISSUE CULTURE METHOD**

Fetal ovaries and testes were placed in a sterile petri dish in a small volume of culture media. They were dissected free of adherent tissues using sterile technique, bisected longitudinally and then cut into slices ~0.5mm thick using a dissecting microscope with a measuring tape placed under the petri dish to ensure the slices were approximately equal sizes. Samples of fresh tissue were fixed for histological analysis (time 0 samples).

Initially, a culture method similar to that described by Dr. J. Tilly (Morita et al 1999) was used. Several pieces of tissue were placed on sterile preformed 2% agarose gel discs which were placed in each well of a 6-well organ culture dish. Enough culture medium was then added to the well to form a meniscus with the gel but not submerge the tissue. In order to create the optimum culture conditions  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) was used, adding either 10% fetal bovine serum (FBS) or bovine serum albumin (BSA) and insulin/transferrin/selenium (ITS).  $\alpha$ -MEM has been shown (Wright et al 1999) to be the most effective medium at supporting human ovarian tissue in culture, possibly because it contains amino acids, vitamins, ribonucleosides and deoxyribonucleosides that simple salt solutions do not have. Serum albumin and ITS have also been shown to be effective replacements for serum in culture (Wright et al 1999), and having cultured tissue in both serum and serum-free media an appreciable difference in the tissues at the end of culture was not observed. Hence, the serum-free conditions were used thereafter as it would allow testing of growth factor effects without the influence of serum in the media. The initial culture method using agarose gel was time consuming to set up and when

compared with the method described below using Millicell CM filters the latter method was more effective at supporting tissue viability. After preliminary experiments varying the culture time periods over 48 hours, 5 days and 7 days, significant differences between treated and control groups after 48 hours was observed and hence this time period used for all subsequent culture experiments.

### **2.23 TISSUE CULTURE**

Tissue fragments were cultured on 0.4µm pore Millicell CM filters in a 24 well plate. To each well 0.4ml of medium was added, enough to just cover the tissue fragments. Any remaining wells were partially filled with medium to maintain humidity in the culture vessel.

The medium comprised MEM $\alpha$  containing 3mg/ml BSA, antibiotics (100IU/ml penicillin, 100 µg/ml streptomycin sulphate and 0.125 µg/ml amphotericin), ITS (5 µg/ml bovine insulin, 5 µg/ml transferrin and 5 µg/ml sodium selenite), 2 mmol/l glutamine and 2 mmol/l pyruvate. Factors added were the Trk inhibitor K252a at a concentration of 100 nM and 0.05ng/µl basic fibroblast growth factor (bFGF). As K252a was dissolved in dimethylsulfoxide (DMSO), an equivalent amount of DMSO was added to control wells. The cultures were maintained at 37°C in a humidified incubator under 5% CO<sub>2</sub> in air for 48 hrs. Every 24hrs the media was removed and replaced with fresh media. Twenty-four hours prior to the end of culture, 9 µg/ml of bromodeoxyuridine (BrdU) was added to the medium in order to label proliferating cells. At the end of the culture period, tissue was fixed for 1 hr in Bouins fluid and then transferred to 70% ethanol before embedding in paraffin wax for histological

analysis. The tissue was sectioned at 5  $\mu\text{m}$  thickness and using immunohistochemistry, stained for BrdU protein using the described protocol (Section 2.4 and Section 5.2).

## **2.24 QUANTIFICATION ANALYSIS OF CULTURED TISSUE**

Sections of uncultured control and cultured tissue were analysed to investigate the effects of culture and of K252a on the number and proliferation of the various cell types present. Analysis was carried out blind using the Area Fraction Probe in the Stereologer software programme (Systems Planning and Analysis Inc, Alexandria, VA, USA). This was a randomised stage microscope which chose grids to count within the tissue, therefore minimising observer bias. Confirmation of accurate identification of cell types was confirmed by a second observer. Numbers of germ cells were counted for both ovary and testis, and numbers of Sertoli cells and peritubular cells for the testis were recorded. The numbers of each of these cell types that were proliferating (i.e. immunostained for BrdU) were recorded. Tissue was serially sectioned at 5 $\mu\text{m}$  thickness and 4 sections of tissue were mounted onto each slide to minimise the number of slides required to be stained. Several sections greater than 20  $\mu\text{m}$  apart were counted, ensuring the same cells were not counted more than once. The counting was done using a 121 point grid in the eyepiece of the microscope and only the cells whose nuclei lay beneath the intersections on the grid were counted. The numbers of points lying out-with any tissue were also recorded and the total cell numbers counted were corrected for this (using the formula: cells counted  $\times$  121/ points on tissue). The primary outcome was total numbers of germ cells. In order to decide the sample size required to be analysed to give standard error

of 5%, the percentage of tissue that the germ cells occupied in both ovaries and testes was estimated and the numbers of frames that would be required to be counted were calculated in preliminary experiments. This was carried out using the following formulae (Bancroft 1996). To calculate standard error:

$$\text{Standard error} = \text{square root } [P_i(100-P_i)/P_T]$$

Where,

$P_i$  = Percentage of volume occupied by the object (i) counted ie: the number of points intercepting with,  $i$ , expressed as a percentage of the total number of points counted

$P_T$  = Total number of points counted

To calculate percentage standard error:

$$\text{Percentage standard error} = (\text{standard error}/P_i) \times 100$$

Using these formulae I estimated that I needed to count approximately 600 points for each sample of ovarian tissue and 3025 points for testicular tissue. The average number of each cell type present per grid was calculated for each experimental condition, and data were analysed using SPSS statistics package. The parametric statistical test used to analyse data was the Students paired t test.

## **MOLECULAR BIOLOGICAL TECHNIQUES**

### **2.3 ISOLATION OF RNA AND SYNTHESIS OF cDNA**

To identify and study gene expression within tissues the very small amounts of RNA present need first of all to be amplified. Firstly, RNA is converted to complementary DNA using the enzyme reverse transcriptase. The polymerase chain reaction (PCR)

is then performed on the cDNA using specific primers to manufacture enough DNA copies to detect the required sequence.

Total RNA was extracted from snap frozen samples of fetal gonads (12-21 weeks) using the RNeasy mini kit. Tissue was homogenised and lysed in a volume of buffer, proportional to the amount of tissue, containing  $\beta$ -mercaptoethanol ( $\beta$ ME) and guanidine isothiocyanate (GITC). This was centrifuged and 70% ethanol added to provide appropriate binding conditions. This was mixed, added to a silica gel based spin column (binding capacity approximately 100  $\mu$ g RNA) and centrifuged at 12,000G at room temperature (RT). This allows adsorption of the RNA onto the silica gel based membrane. A washing buffer was then added to the column and this was spun prior to adding a second washing buffer. Again this was centrifuged and finally RNase free H<sub>2</sub>O was added to the columns before spinning and the elute was retained and the RNA content measured in a spectrophotometer. RNA was treated with DNase and reverse transcription performed using a first strand cDNA synthesis kit. Briefly, 1  $\mu$ g total RNA was incubated with oligo (dT)<sub>18</sub> primer for 10 min at 65°C and then placed on ice. A reaction mix comprising buffer, 1 mM each deoxynucleotide triphosphate (dNTP), ribonuclease inhibitor and 50 U reverse transcriptase, was added to each tube in a total volume of 50 $\mu$ l and the tubes then incubated at 40°C for 2 h. cDNA was then stored at -70°C.

### **2.31 POLYMERASE CHAIN REACTION (PCR)**

PCR is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. It involves two oligonucleotide primers that

flank the DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. These primers hybridise to opposite strands of the target sequence and are oriented so that DNA synthesis by the polymerase proceeds across the region between the primers. Since the extension products themselves are also complementary to and capable of binding primers, successive cycles of amplification essentially double the amount of the target DNA synthesised in the previous cycle. The result is an exponential accumulation of the specific target fragment.

The polymerase chain reaction was performed by incubating 1-2  $\mu\text{l}$  cDNA samples with 0.25  $\mu\text{l}$  *Taq* DNA polymerase (2u/ $\mu\text{l}$ ) in buffer with 0.2 mM of each dNTP and forward and reverse oligonucleotide primers, making up to 50  $\mu\text{l}$  with RNase-free water. All reagents were kept on ice and the *taq* polymerase was added last. PCR cycling was carried out on a MJ Research PTC-100 thermal cycler for 35 cycles. The annealing temperature varied depending on the product and primers used. Two control tubes were run in parallel, one in which water replaced the RNA and a second omitting reverse transcriptase to ensure there was no genomic DNA contamination. Specific primers were used and these and the resulting product size are shown in the relevant chapters. Primers were designed to span an intron to ensure genomic DNA was not amplified. Primers for the constitutively expressed gene glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) were used to confirm the integrity of the RNA and efficacy of the PCR reaction. After the PCR, the size of product was ascertained using electrophoresis. Products were loaded on a 1%



agarose gel (8  $\mu$ l product : 2  $\mu$ l dye used to visualise loading and dye front on running gel) with ethidium bromide (see Appendix II for recipe) and placed in a tank containing 1 x TBE solution (Tris, boric acid, EDTA, see Appendix II for recipe) and run at 120 V over 45 minutes. Products were visualised using ultraviolet light. Products were purified using the method described in section 2.33 and the identity of all PCR products was confirmed by direct sequencing as described below.

### **2.32 SEQUENCING OF DNA**

The first step in sequencing is to denature the double stranded DNA into single strands. The overall approach of sequencing DNA involves creating sub-fragments of all possible lengths from the DNA we want to sequence, grouping them according to which base they end with. To create these fragments a chain termination method is employed called the dideoxy method. Dideoxy analogues of normal DNA precursors cause premature termination of a growing chain of nucleotides being made by DNA polymerase. This allows generation of fragments of the stretch of DNA we want to sequence and by using four different dideoxy analogs, one for each of the four bases, we can generate four sets of fragments. To identify these, the same DNA primer is used for each reaction but four different fluorescent dyes are used, one for each of the bases. When a sequencing gel is run, a separate colour is obtained for each base. The bands are scanned by a laser beam and the four different dyes fluoresce in different colours. The colour of each band is recorded by a computer which then prints the sequence.

### **2.33 PURIFICATION OF PRODUCTS FOR SEQUENCING**

This was performed for all products prior to sequencing using a Hi-Pure purification kit in order to remove any primer dimers which may affect the sequencing signal. First the amount of product present was measured and made up to 50 $\mu$ l with H<sub>2</sub>O. Binding buffer (250 $\mu$ l) was then added to each sample and the sample then added to a column sitting in a collection tube. Next the samples were centrifuged at 13000g for 1 minute. The elute was discarded and 500 $\mu$ l of washing buffer was added to the column and this was centrifuged for 1 minute at 13000g. These two steps were

repeated using 200µl of washing buffer and then 40µl of elution buffer was added to a column sitting in a clean tube and centrifuged for 1 minute. The elute was then put through the column again and products run on an agarose gel and tested in a spectrophotometer to ascertain purity and quantification.

### **2.34 METHOD FOR SEQUENCING DNA**

For the sequencing reaction, 30-90ng of purified PCR product was added to 4µl of sequencing mix, 4µl of ½ term ( 200mM Tris Hcl, pH 9.0; 5mM MgCl<sub>2</sub>) and 1µl of primer at a concentration of 5pmol/µl. This was then made up to 20µl with H<sub>2</sub>O and the mixture placed in the PCR machine at 96°C for 15 seconds, 50 °C for 25 seconds and 60 °C for 4 minutes for 25 cycles. Once finished the tubes were spun briefly and then 50µl of 96% ethanol and 2µl of 3M sodium acetate were added to each tube. The samples were vortexed thoroughly and left at room temperature for 30 minutes before spinning at 12,000 G at RT for 30 minutes. The supernatant was removed and pellet washed with 100µl of 70% ethanol. This was spun again for 10 minutes and supernatant removed and pellet allowed to dry. This was then resuspended in 4µl formamide: Ethylenediaminetetraacetic acid (EDTA) buffer (5:1 ratio of deionised formamide: 50mM EDTA) and was ready for loading on the sequencing gel.

The sequencing gel was made by mixing 50g urea and 15 ml 40% acrylamide and making up to 80 ml with water. One teaspoon of amberlite was added to this and the mixture was heated gently until the urea dissolved. This was filtered to remove the amberlite and then 10 ml of TBE added. The mixture was made up to 100 ml with water and just prior to pouring 500µl 10% Ammonium persulphate (APS) and 45µl

N,N,N<sup>1</sup>-Tetra-methylethylenediamine (TEMED) were added. The gel was then poured between the glass plates with spacers *in situ* and allowed to set prior to placing in the sequencer along with running buffer and loading with samples. Sequencing was carried out using an Applied Biosystems 373A automated sequencer.

### **2.35 ISOLATION OF RNA BY LASER CAPTURE MICROSCOPY**

The technique of laser capture microscopy (LCM) allows sampling of individual or groups of cells from complex tissues, in such a way that mRNA and/or proteins could be extracted from them and analysed. In the present study the LCM microscope was used with the laser set to the smallest size available (7.5 µm) to allow for sampling of single cells. Briefly, 5µm sections were cut from paraffin wax embedded 19 wk human fetal testis samples and mounted on plain, uncoated, glass slides. Sections were dewaxed in xylene, rehydrated then subjected to immunostaining. Initially frozen sections were also used but resulted in very poor tissue morphology. To visualise the cells within the seminiferous cords an Anti Müllerian Hormone (AMH) polyclonal antibody was used as detailed in Chapter 3 except that the protocol was modified for short, typically 10 min, incubation times at each step to reduce the chance of RNA degradation and RNase inhibitor was included in all the immunohistochemical reagents. After colour development with DAB the sections were dehydrated through graded alcohols and finally xylene. Sections were stored in a vacuum dessicator for at least 30 min prior to capture. Care was taken throughout to avoid RNase contamination of sections and all aqueous solutions were prepared with diethylpyrocarbonate (DEPC) -treated water.

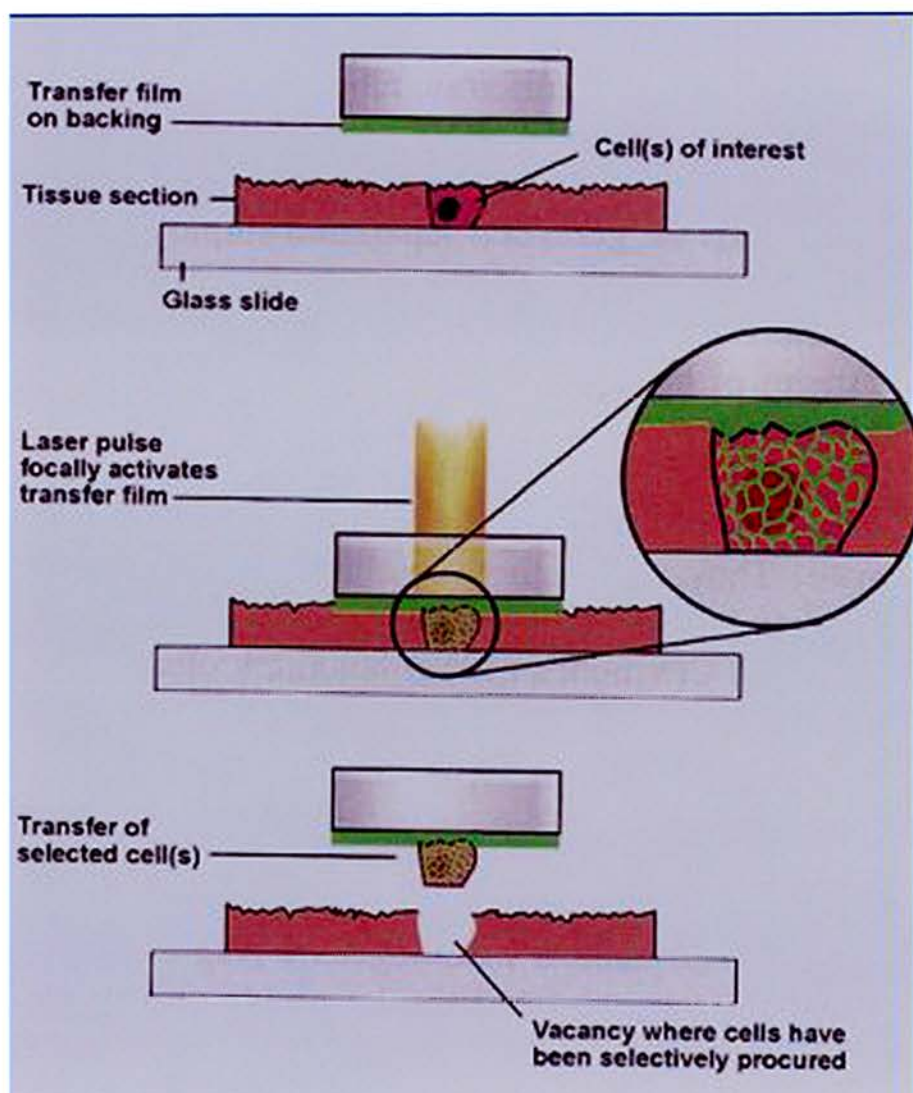
Individual cells were recovered from the stained sections by micro-dissection using the PixCell II LCM system according to the manufacturer's instructions. Each section was overlaid with a thermoplastic membrane mounted on optically transparent caps and cells captured by focal melting of the membrane due to laser activation. The parameters of the laser shot used in this study were: spot size 7.5µm in diameter, power 45mW and duration time 0.5ms (Figure 2.1).

Total RNA was extracted from micro-dissected samples with the Micro RNA Isolation Kit. After incubation with 200µl of denaturing buffer and 1.6µl of β-mercaptoethanol at room temperature for 10 min, the sample was extracted with 20µl of 2M sodium acetate, 220 µl phenol and 60 µl Chloroform:isoamyl alcohol (ratio 25:24:1). The aqueous phase was mixed with 1µl of 10mg/ml carrier glycogen and then precipitated with 200µl of isopropanol. After a 70% ethanol wash then drying in air, the pellet was resuspended in 10µl of RNase free H<sub>2</sub>O. The extracted RNA was reverse transcribed using 10pmol random hexamer primers and 200U of Superscript II reverse transcriptase according to the manufacturer's instructions. Initially, this cDNA was then used in subsequent PCR reactions with primers for c-kit (set 1, Table 3.1) and AMH (set 1, Table 3.1). However, this method failed to successfully amplify products and so was developed further by Dr T Gaskill. This was modified by amplifying the cDNA prior to using nested primers in PCR for c-kit and AMH, as detailed below.

An aliquot of cDNA was amplified using a modified degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) protocol (Kasai *et al.*, 2000) using the

primer UN1, 5'-CCGACTCGAGNNNNNNATGTGG-3' in a total volume of 25 $\mu$ l. 5 $\mu$ l of amplified cDNA was then used in subsequent PCR reactions using primer sets for specific sequences which are detailed in Chapter 3.

**Figure 2.1** Diagram illustrating the sampling of cells from tissue sections by laser capture





### **2.36 *IN SITU* HYBRIDIZATION**

*In situ* hybridization techniques allow specific nucleic acid sequences to be detected in morphologically preserved tissue sections. In combination with immunohistochemistry *in situ* hybridization can relate microscopic topological information to gene activity at the DNA, mRNA and protein level.

### **2.37 GENERATION OF RIBOPROBES FOR *IN SITU* HYBRIDIZATION**

cDNA specific for human NT4 was generated by PCR using oligonucleotides (forward primer: 5'-CTT TCG GGA GTC AGC AGG TGC-3' and reverse primer: 5'-CAG GCA GTG TCA ATT CGA ATC C-3') designed from the human NT4 sequence (Human NT4 sequence Accession no. NM006179). These primers were predicted to amplify a 698 bp product (nucleotides 401-1099). Briefly, RNA was extracted from snap-frozen human fetal ovary using a RNeasy mini kit. RNA was treated with DNase to eliminate any genomic DNA contamination and cDNA synthesized using a first strand cDNA synthesis kit. PCR was then performed at an annealing temperature of 55°C for 35 cycles in 75 mM Tris/HCl (pH 9.0), 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub>, 200 μM each deoxynucleotide-5'-triphosphate, and 0.01% Tween-20 with 25 pmol of each primer and 1.25U AGSGold DNA polymerase in a final volume of 50μl. A clear product of the expected size was obtained which was then subcloned into a TA cloning vector (pGEM-T plasmid) and the resulting clones sequenced to confirm that they were human NT4 cDNA sequence. Riboprobes for *in situ* hybridization were then generated using a PCR strategy to incorporate SP6 or T7 phage promoter sequences into NT4 PCR product.

The pGEM-T plasmid containing human NT4 insert (see above) was used as a template for the reaction. Briefly, for the antisense probe, the NT4 PCR product was amplified using NT4 forward primer (as above) and NT4 reverse primer which had the T7 promotor sequence added to its 5' end (5'-TAA TAC GAC TCA CTA TAG GGA GAC AGG CAG TGT CAA TTC GAA TCC-3'). For the sense probe, product was amplified using the NT4 forward primer linked to the SP6 promotor sequence (5'-ATT TAG GTG ACA CTA TAG AAG TGC TTT CGG GAG TCA GCA GGT GC-3') and NT4 reverse primer (as above). PCR was then performed as describe above. Products were purified using High Pure PCR product purification kit and used directly for transcription with T7 or SP6 RNA polymerase to generate antisense and sense riboprobes, respectively. Probes were labelled with Digoxigenin (DIG) using a commercially available kit. Briefly, DNA templates (300ng) were incubated for 60 min at 37°C with transcription buffer, 10U ribonuclease inhibitor, 0.5 mM each rATP, rCTP, rGTP, 0.33 mM rUTP, 0.17 mM digoxigenin-11-UTP and 30U of appropriate RNA polymerase in a final volume of 20µl. After the addition of 2U DNase I (RNase-free), the probes were incubated at 37°C for an additional 15 min before being purified through Chromaspin columns.

### **2.38 *IN SITU* HYBRIDIZATION METHOD**

Paraffin sections (5µm) were cut over DEPC -treated water and transferred to super frost plus coated slides within a baked glass dish containing ultra pure water at 55°C. The sections were dried overnight at 50°C and then dewaxed in xylene and rehydrated through a graded series of ethanol. All solutions were made up in DEPC water. Slides were immersed in 0.4 M HCl for 20 min, then washed twice in DEPC

water for 5 min. Slides were then treated with 1.5 µg/ml Proteinase K at 37°C for 10 min in 0.1 M Tris/HCl and 0.05 M EDTA (pH 8.0), transferred to 0.2% glycine at 4°C for 10 min, acetylated with 0.25% acetic anhydride in 0.08 M triethanolamine (pH 8.0) for 10 min and washed in 4 x SSC for 5 min. Sections were then incubated with prehybridization buffer at 50°C for 2 hours. The prehybridization buffer was drained and excess dried off and 50 µl hybridisation buffer added containing the probe. This was coverslipped with Gel bond film (hydrophobic side down) and this was incubated overnight at 50°C in a humid box.

Next day, sections were washed in 4 x saline-sodium citrate (SSC) for 10 min, then incubated in RNase A buffer (0.02 mg/ml in 0.01 M Tris/HCl, 0.5 M NaCl and 0.1 M EDTA, pH 8.0) at 37°C for 30 min, washed twice in 2 x SSC for 5 min, transferred to 0.1 x SSC with 30 % formamide at 37°C for 30 min, and washed in TBS, pH 7.4 for 5 min. For detection of DIG labelled probes, the sections were then incubated sequentially at room temperature, with 2 x 5 min washes in TBS between steps, in: 1) 3% H<sub>2</sub>O<sub>2</sub> in TBS for 30 min; 2) normal rabbit serum (NRS, 1:5 dilution in TBS containing 5 drops/ml avidin block) for 30 min; 3) biotin block (8 drops/ml in TBS) for 30 min; 4) Sheep Anti-Digoxigenin (1:100 in NRS/TBS) for 2h; 5) Biotinylated rabbit anti-sheep IgG (1:500 in NRS/TBS) for 30 min; 5) ABCComplex-HRP for 30 min and 6) 3, 3'-diaminobenzidine (DAB) liquid substrate-chromagen system for 2 min approximately. Sections were then counterstained in haematoxylin, dehydrated and coverslip mounted with DPX.

## IMMUNOHISTOCHEMISTRY

### **2.4 IMMUNOHISTOCHEMISTRY: Background and general protocol**

The technique of immunohistochemistry involves localisation of an antigen within a section of tissue by a specific primary antibody. A variety of detection systems can then be used to detect and visualise the specific staining.

Non-specific staining occurs when tissue other than at the site of the desired antigen stains immunopositive and may be due to a variety of causes. The first of these is that the primary antibody may bind non-specifically to epitopes in the tissue. Having a good antibody is essential and the immunohistochemistry results were compared with those obtained by Western Blotting to ensure that results were valid. Secondly, tissues may contain endogenous peroxidase which may catalyse the enzymatic detection thus producing DAB staining at the site of the endogenous enzyme. To avoid this problem, prior to the addition of the primary antibody, tissues were exposed to dilute  $H_2O_2$  to saturate any endogenous peroxidase thus rendering it inactive. Endogenous biotin can also cause a problem by binding to the primary antibody. To overcome this, prior to adding the primary antibody, avidin was applied to the tissue which binds to any endogenous biotin and then adding a biotin which binds to the avidin and this then effectively blocks any endogenous activity. Lastly, secondary antibody may bind to epitopes in the original tissue mimicking those from the species it was raised against as well as to the primary antibody. This was minimised by adding normal serum from the species from which the secondary antibody was raised against prior to the addition of the secondary antibody.

On each staining run the relevant positive control included contained the desired antigen and was therefore expected to stain positively. To act as negative controls, sections of tissue being stained were included which were treated in an identical manner except the primary antibody was omitted. These sections were expected to exhibit minimal, if any staining.

### **General protocol**

Immunohistochemistry was performed on sections of fresh and cultured tissue. Cultured pieces of tissue were fixed in Bouins fluid for 1 hour and fresh tissue was fixed for 5 hours. They were then stored in 70% ethanol prior to routine paraffin embedding.

Sections of tissue (5 $\mu$ m) were mounted on 3-aminopropyl-triethoxysilane (TESPA) coated slides, dewaxed in histoclear and rehydrated through ascending grades of ethanol to distilled water (2 minute washes) then washed in TBS (2 minutes). For some antibodies, antigen retrieval using citrate (0.01M, pH 6.0, pressure cooked for 2.5 minutes) or glycine buffer was employed. The slides were immersed in the boiling buffer in a pressure cooker for 2.5 minutes and then the pressure cooker was removed from the heat and the slides left to stand in the buffer for 10 minutes buffer being washed with tap water followed by a 5 minute wash in TBS. Endogenous peroxidase activity was inhibited by incubation in 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30min. After a wash in water, slides were transferred into TBS for 5 min and blocked for 30 min in blocking serum diluted 1:5 in TBS containing 5% bovine serum albumin. Sections were then blocked with avidin (0.01M; 15min) and biotin (0.001M; 15min)

with washes in TBS in between. The primary antibody, diluted in the appropriate serum, was then added and this was incubated in a humidified atmosphere at 4°C overnight. Optimal antibody concentrations were established in a series of preliminary experiments. The following day, sections were washed and incubated for 30 min with a biotinylated secondary antibody 1:500 in the appropriate diluted serum. Following washes in TBS, sections were incubated with ABC-HRP for 30 minutes. Slides were then rewashed in TBS and bound antibody was visualised using the peroxidase substrate DAB which produces a brown stain. All sections were counterstained with haematoxylin and dehydrated in graded ethanol (2minutes/solution) and histoclear (5 minutes). They were then soaked in Xylene (5 minutes) and mounted with Pertex mounting medium. Images were captured using an Olympus Provis microscope (Olympus Optical Co, London) equipped with a Kodak DCS330 camera (Eastman Kodak).

#### **2.41 Haematoxylin and Eosin stain (H & E stain)**

Tissue sections were dewaxed in histoclear (5 minutes), rehydrated in graded alcohols (absolute ethanol 20 seconds, 95% IMS 20 seconds and 70% IMS 20 seconds) and rinsed in tap water prior to staining in Haematoxylin (See Appendix II for recipe) (5 minutes) . Next, slides were rinsed in 1% acid alcohol (5 seconds), Scotts tap water (40 seconds) then Eosin (See Appendix II for recipe) (5-20 seconds) being rinsed in tap water between each stage. Finally, slides were dehydrated in graded alcohols (70% IMS 20 seconds, 95% IMS 20 seconds and absolute ethanol 20 seconds) steeped in histoclear (5 minutes), then Xylene (5 minutes) and Pertex

mounted. H and E staining was utilised to check the morphology of tissue samples and to count germ cells in time 0 samples in our analysis of cultured tissue.

## **PROTEIN EXTRACTION AND IMMUNOBLOTTING**

### **2.5 WESTERN BLOT**

Western blotting detects the presence of proteins, separated by electrophoresis, using specific antibodies after transfer to a nitro-cellulose membrane. SDS polyacrylamide-gel electrophoresis allows individual polypeptide chains to form a complex with negatively charged molecules of SDS and therefore migrate as a negatively charged SDS-protein complex through a porous gel of polyacrylamide. Since the speed of migration under these conditions is greater the smaller the polypeptide, this can be used to determine the approximate molecular weight of a polypeptide chain as well as the subunit composition of a complex protein. See Appendix II for recipes.

## **2.51 PROTEIN EXTRACTION FROM TISSUE**

Tissue samples which had been stored at  $-70^{\circ}\text{C}$  were kept on ice and then homogenised in extraction buffer at room temperature. The samples were then allowed to stand at room temperature for 1 hour, vortexing every 5 to 10 minutes before being centrifuged at 10,000rpm for 30 minutes at RT. The pellet was discarded and supernatant retained and stored as 100 $\mu\text{l}$  aliquots at  $-70^{\circ}\text{C}$ . The protein was quantified by diluting samples by factors of 10 and 20 and measuring them in a spectrophotometer.

## **2.52 IMMUNOBLOTTING METHOD**

### **PROTEIN SEPARATION**

For recipes of buffers see Appendix II. Samples (20  $\mu\text{g}$  protein) were diluted with an equal volume of reducing loading buffer and boiled for 5 min. These were kept on ice while the gel was being loaded. The combs were removed from a 4-20% gradient Tris-glycine gel and the gel placed in the chamber. The chamber was then filled with Tris-glycine sodium dodecyl sulfate (SDS) running buffer making sure air was dislodged from all the wells. Samples were then loaded into wells in parallel with prestained protein molecular weight markers and run at a constant 100V for  $\approx$  2 hours.

After electrophoresis, the gels were removed from the apparatus and molecular weight markers and dye front were measure and gels immersed in transfer buffer.



### 2.53 BLOTTING

Eight pieces of filter paper and one polyvinylidene fluoride (PDF) membrane were soaked in wet blot transfer buffer for 15 mins. The pads of the transfer apparatus were soaked briefly in buffer and then one laid inside the cassette. The pad was covered with 4 pieces of filter paper and the membrane. The stacking gel was cut off and the remaining piece of gel placed over the membrane. The gel was covered with the remaining filter paper and pad and the cassette closed. This was then placed in the tank filled with buffer and run at a constant voltage of 25V overnight.

The next day, membranes were soaked for 5 min in methanol and washed briefly several times in TBS. They were then blocked for 8 h at room temperature in 0.02 M TBS (pH 7.6) containing 3% w/v BSA and 5% powdered milk. The membranes were washed in TBS with 0.1% Tween-20 (TBST) and then incubate overnight with the primary antibody. Antibodies used were against NT4, TrkB, and p75 NGFR. These were used at dilutions of 1 in 500, 1 in 50 and 1 in 500 respectively (Table 2.2), in TBST with 1% BSA at 4°C. Primary antibody was omitted as a negative control.

The following day, membranes were washed in TBST for three 5 minute washes and then incubated with either anti-rabbit and anti-mouse HRP linked secondary antibodies at 1:4000 in TBST with 1% BSA at room temperature for 1 hour. These were then washed in TBST for 3 washes as before and the bound antibody detected using the enhanced chemiluminescence visualisation system (ECL) according to the manufacturer's instructions.

<b>ANTIBODY</b>	<b>DILUTION</b>
NT4	1:500
TRKB (3)	1:50
P75	1:500
C-KIT	1:750

**Table 2.2** Concentrations of antibody used for Western Blotting

## **EXPLANT CULTURE AND ZYMOGRAPHIC TECHNIQUES**

### **2.6 EXPLANT CULTURE**

Gonads from two fetuses of each sex (ovaries at 12 and 14 weeks gestation, and testes at 17 weeks gestation) were cultured using the initial culture method as described in Section 2.22. The medium comprised MEM $\alpha$  containing 3mg/ml BSA, antibiotics, 2mM glutamine, 2mM pyruvate and ITS. The cultures were maintained at 37°C in a humidified incubator under 5% CO<sub>2</sub> in air. After 48 hours the tissue was removed and fixed in Bouins fluid and the media was collected in a sterile container and frozen at -20°C prior to analysis by zymography. Histological analysis of cultured tissue confirmed that morphology was maintained and the tissue was viable.

## 2.7 ZYMOGRAPHY

The technique of zymography detects activity of the latent and active forms of MMPs. Samples are separated by electrophoresis on a sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) which has a substrate, in this case gelatin, incorporated into it. The gels are then incubated overnight at 37°C and the latent form of the enzyme, which is activated by the SDS, and the active form of the enzyme digest the substrate within the gel at the site corresponding to their molecular weight. The gel is then stained with Coomassie Blue then destained. The sites of enzymatic gelatinase activity are manifest as a clear area of digestion within the gel. The zymograms may then be semi-quantitated by densitometry.

Activities of MMP-2 and MMP-9 in conditioned medium from gonadal cultures were determined using gelatinase zymography (Riley et al 1999a) (See Appendix II for recipes). Human amniotic fluid collected at term during labour was used a positive control, which clearly demonstrates the latent forms of MMP-2 (72 kDa), MMP-9 (92 kDa), a lipocalin-proMMP-9 complex (120 kDa) and dimeric MMP-9 ( $\cong$ 210 kDa).

## ZYMOGRAPHY

### Sample preparation

### Tissue culture incubation medium

- Freeze to -20°C prior to lyophilising samples by freeze drying. A medium sample volume of 1.5 ml is resuspended in 50  $\mu$ l of water.

## **Plate preparation**

- Clean (70% IMS) and assemble plates (8x10 cm) with spacers (0.75 mm) between them.
- Prepare the resolving gel according to the recipe and pour immediately to 1.2 cm below top of plate (fill line), avoiding bubbles and leaks if possible.
- Overlay gel with water saturated sec-Butanol to burst any bubbles and give an even level to top of gel.
- Leave to set (45 minutes, 23°C) and pour off the butanol. Wash with water followed by several washes with Tris Buffer and leave with Tris buffer (Stock B 1:4) overlay to allow polymerisation to occur (minimum of 2 hours, 23°C).
- Pour off buffer, rinse overlay with water and dry with paper. Clean (70% IMS) combs (0.75 mm, 15 well) and position between the plates.

### **Plate preparation**

- Clean (70% IMS) and assemble plates (8x10 cm) with spacers (0.75 mm) between them.
- Prepare the resolving gel according to the recipe and pour immediately to 1.2 cm below top of plate (fill line), avoiding bubbles and leaks if possible.
- Overlay gel with water saturated sec-Butanol to burst any bubbles and give an even level to top of gel.
- Leave to set (45 minutes, 23°C) and pour off the butanol. Wash with water followed by several washes with Tris Buffer and leave with Tris buffer (Stock B 1:4) overlay to allow polymerisation to occur (minimum of 2 hours, 23°C).
- Pour off buffer, rinse overlay with water and dry with paper. Clean (70% IMS) combs (0.75 mm, 15 well) and position between the plates.
- Prepare the stacking gel according to the recipe, apply to the resolving gel as before, remove any bubbles and leave to set (at least 90 minutes, 23°C).

### **Gel electrophoresis**

- Remove the combs from the gels, take gels off the holder and attach to electrophoresis apparatus.
- Place the assembled core in the tank and fill chamber with running buffer (500 mls) to dislodge any trapped air bubbles.
- Load the samples (2.5 µl of the 50 µl of resuspended lyophilised medium, ie: 5%) into the wells, remove any air bubbles and top up the core chamber to

about 3 mm below the top edges of the two plates. For the positive control, 1  $\mu$ l of amniotic fluid at term is loaded.

- Run at a constant voltage, 100 V for 90 minutes at 23°C.
- After electrophoresis measure molecular larkers and dye front.
- Remove gel from plate and immerse in Triton X 100 Wash buffer.

### **Wash, digestion and stain**

- Wash the gels twice (15 minutes/wash, 23°C) in Triton wash buffer then twice (~ 2 minutes/wash, 23°C) in wash buffer.
- Add digestion buffer and incubate at 37°C overnight. After incubation, decant buffer, wash twice in wash buffer (~ 2 minutes/wash, 23°C) and add staining solution (~ 3 hours, 23°C).
- Decant staining solution and replace with destaining solution (~ 30 minutes then 60 minutes, 23°C). Store destained gels in air tight water-filled container until ready to be photographed or scanned.

## **2.71 REVERSE ZYMOGRAPHY**

Reverse zymography detects activity of the TIMPs. In this technique, active MMP incorporated into the gelatin sodium dodecyl sulphate-polyacrylamide gel binds to TIMPs within the samples. When the electrophoresed gels are incubated overnight, the MMPs within the gel become activated and digest the gelatin throughout the gel apart from where it is bound to TIMPs within the sample. After staining, the TIMPs are detected as a stained band on an otherwise clear background. (See Appendix II for recipes). The activities of TIMP were detected by reverse zymography using a commercially available kit with some minor adaptations (Riley et al 1999b).

### **REVERSE ZYMOGRAPHY**

- Method for plate preparation and pouring gels, refer to Zymography protocol

#### **Gel Electrophoresis**

- Assemble apparatus and load samples on 12% gel containing gelatin (1 mg/ml) and a preparation of MMP-2 (conditioned medium from BHK-21 cells which constitutively express MMP-2; University Technologies Inc.) using a minigel apparatus, as in Zymography protocol. The volume loaded for the lyophilised samples is 7.5  $\mu$ l (15% of the 50  $\mu$ l of resuspended lyophilised medium). The standards used are mouse TIMPs-1, -2 and -3, expressed by transfected BHK-21 cells (University Technologies Inc.)
- Run at a constant voltage, 100 V for 90 minutes at 23°C
- After removing gel from apparatus place in container with Rinse Buffer
- Incubate at 23°C with gentle shaking for a 15 mins.
- Change Buffer and incubate with gentle shaking for a further 150 mins.

- Discard Buffer and wash x 2 with deionised water
- Add Digestion Buffer and incubate at 37°C for 17 hrs.
- Discard Buffer, rinse in deionised water x 2.
- Stain and destain gel as in Zymography protocol.

The TIMPs inhibitory activity appeared as dark bands against a lighter background. TIMPs were identified and characterised by comparison with molecular weight markers, control standard solutions containing mouse TIMP-1, TIMP-2 and the glycosylated and unglycosylated forms of TIMP-3 and human amniotic fluid, which contain all TIMP isoforms (Riley et al 1999b). Analysis of samples by PAGE with gelatine substrate omitted demonstrated no significant detectable underlying protein staining at the molecular weights at which TIMP were observed, demonstrating the specificity of the reverse zymography for the detection of TIMP activity.



## **CHAPTER 3**

# **GERM CELL SPECIFIC EXPRESSION OF C-KIT IN THE HUMAN FETAL GONAD**

### 3.1 INTRODUCTION

The germ cells of vertebrate species do not initially form within the genital ridge but originate in the extra embryonic mesoderm of the yolk sac. Primordial germ cells increase by mitosis during migration and become associated with the cells within the gonadal ridges at about 6 weeks (Byskov 1986). During fetal life, male germ cells continue to proliferate until the late second trimester (Hilscher 1991). Within the fetal ovary, following oogonial replication by mitosis, germ cells subsequently enter meiosis only to arrest at diplotene of the first meiotic division (Hilscher 1991), surrounding themselves with granulosa cells, thus forming primordial follicles.

Studies in rodents have highlighted the importance of the C-kit proto-oncogene receptor and its ligand, the kit ligand (stem cell factor) in migration of germ cells from the yolk sac to the developing gonad and their subsequent survival and development (Godin et al 1991; Kierszenbaum et al 2001; Manova et al 1990; Pesce et al 1993). For example, analysis in mice of the effects of mutations of the White Spotting and Steel loci (encoding C-kit and the kit ligand respectively) has allowed the demonstration of the importance of this ligand-receptor pair in multiple stem cell lineages including melanogenesis and haematopoiesis as well as gametogenesis (Ashman 1999; Besmer 1991). C-kit is predominantly expressed by germ cells in the rodent testis although it has also been suggested to be expressed by Leydig cells (Manova et al 1990), whereas Kl is expressed by a wider range of cell types. The presence of a functional C-kit receptor has been implicated in spermatogonial proliferation, survival and adhesion to Sertoli cells (Loveland et al 1997). Vincent et

al have demonstrated expression of C-kit by pachytene spermatocytes and proposed that the C-kit/Kl interaction is essential for meiosis (Vincent et al 1998).

Messenger RNA encoding *C-kit* and *Kl* have been detected in fetal mouse ovaries between embryonic days 8 and 14.5 consistent with a role in germ cell migration and proliferation (reviewed by (Driancourt et al 2000)). In their review Driancourt and colleagues used ovaries from mice in which one copy of the kit gene was replaced by a lac-z reporter construct (Bernex et al 1996) to demonstrate that *C-kit* mRNA is not present in oogonia in fetal ovaries on day 15.5 but is transcribed in oocytes in primordial and growing follicles at high levels. These results are in agreement with the studies by Manova et al (Manova et al 1990) using *in situ* hybridisation. Functional effects of kit ligand/c-kit in the ovary may persist into adult life, for example in the regulation of persistence of meiotic arrest (Horie et al 1991; Ismail et al 1997) and activation of primordial follicle growth (Parrott et al 1999; Yoshida et al 1997).

Studies identifying the sites of expression of C-KIT in the human fetus have been very limited compared with those in rodents and there are inconsistencies between the results so far reported. Horie and co-workers (Horie et al 1993) used specific immunohistochemistry to detect C-KIT protein in frozen sections from a number of human tissues and in their paper immunopositive staining of single sections from a human fetal testis (18 weeks) and human fetal ovary (20 weeks) are shown. However other investigators have suggested that C-KIT is not detectable in the fetal testis after 15 weeks gestation (Rajpert-De Meyts et al 1996). It is notable that in the

study by Rajpert-de-Meyts et al C-KIT remained detectable in intersex testes until later in gestation and other studies from the same group have demonstrated that C-KIT is a marker of carcinoma *in situ* (CIS) a pre-malignant lesion thought to be associated with persistence of fetal-type germ cells in the adult testis (Rajpert-De Meyts et al 1994). Consistent with the suggestion that signalling via c-kit is important in normal male germ cell development and function, alterations C-KIT/KL expression have also been demonstrated in some patients with defective spermatogenesis (Mauduit et al 1999), with reduced expression associated with increased germ cell apoptosis.

As the second trimester is the major time for the regulation of germ cell numbers in the female (Baker 1963) and is a period of continuing testicular development (Wartenberg 1989) we have examined the expression and localisation of C-KIT in the human gonad between 13 and 20 weeks of development. Our studies have demonstrated that C-KIT mRNA and protein are expressed specifically in germ cells of both sexes during this critical period.

## **3.2 MATERIALS AND METHODS**

### **Tissues**

Human fetal gonads were obtained following medical termination of pregnancy (Section 2.21).

A total of 20 specimens were used for this study, divided equally between male and female.

### **Isolation of RNA and synthesis of cDNA from whole tissues**

Total RNA was extracted from snap frozen samples of fetal ovary (13-21 weeks, n=10) and testis (14-19 weeks, n=6) and cDNA synthesis performed (Section 2.3).

### **Isolation of RNA by laser capture microscopy**

RNA was extracted from gonocytes in paraffin wax embedded 19wk human fetal testis samples using an anti-AMH polyclonal antibody to visualise the seminiferous cords (Section 2.35). The extracted RNA was reverse transcribed and an aliquot of cDNA was then amplified using a modified degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) protocol (Section 2.35).

5µl of amplified cDNA was then used in subsequent PCR reactions using primer sets for specific sequences.

## **Amplification of specific cDNAs by polymerase chain reaction (PCR)**

### ***C-KIT***

PCR was performed by incubating either 1µl (whole tissue extracts) or 5µl (samples from LCM) cDNA with *Taq* DNA polymerase in buffer containing 0.2 mM of each dNTP and forward and reverse oligonucleotide primers. Two control tubes were run in parallel, one in which water replaced the RNA and a second omitting reverse transcriptase to ensure there was no genomic DNA contamination. PCR amplification conditions consisted of an initial denaturation step at 95 for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 45 sec.; a final extension period at 72°C for 5 min completed the amplification. Three sets of primers specific for human c-kit (Vandenbark et al 1992) were used, one for cDNA from whole tissue extracts (set 1), the other two following laser capture of samples (sets 2 and 3[nested]) (Table 3.1). All three pairs of primers were designed to span an intron to ensure genomic DNA was not amplified. Set1 amplified a product of 345 bp; set 2 amplified a product of 232 bp; set 3 amplified a product of 138 bp from within the product of primer set 2. Primers for the constitutively expressed gene *GAPDH* were used to confirm the integrity of the RNA and efficacy of the PCR reaction. The identity of all PCR products were confirmed by direct sequencing using an Applied Biosystems 373A automated sequencer.

### **AMH**

Specific primers were used to identify samples containing Sertoli cell mRNAs recovered by LCM. All primers were based on the sequence of human *AMH*

(accession no. NP000470). These primers are shown in Table 3.1 and set 1 amplify a product of 238bp. Set 2 are nested and amplify a product of 117bp from within the product of primer set 1.

<b>Gene</b>	<b>Primer</b>	<b>Sequence (5'-3')</b>	<b>Product size (bp)</b>
<i>C-KIT</i> (1) S1	Forward	AAGGACTTGAGGTTTATTCCT	345
<i>C-KIT</i> (1) S1	Reverse	CTGACG TTCATAATTGAAGTC	345
<i>C-KIT</i> (2) S1	Forward	GTGGTTAAAGGAAACGCTCG	232
<i>C-KIT</i> (2) S1	Reverse	CATACATTCAGCAGGTGCG	232
<i>C-KIT</i> (3) S1	Forward	AAGTGGATGGCACCTGAAAG	138
<i>C-KIT</i> (3) S1	Reverse	GAACTTAGAATCGACCGGCA	138
<i>AMH</i> (1) S2	Forward	TGCAACACCGGTGACAGGCAG	238
<i>AMH</i> (1) S2	Reverse	GCAGCCCAGCCCTCGTCACAG	238
<i>AMH</i> (2) S2	Forward	GCTGCCTTGCCCTCTCTAC	117
<i>AMH</i> (2) S2	Reverse	GAACCTCAGCGAGGGTGTT	117

**Table 3.1** Primers used specific for human *C-KIT* and *AMH*. Human sequences used:

S1 = Accession no. U63834; S2 = Accession no. NP000470

## **Immunohistochemistry**

Immunohistochemistry was performed on paraffin embedded sections of fetal ovaries and testes (Section 2.4) to localise the expression of C-KIT and AMH. A total of 6 ovaries and 6 testes were examined using immunohistochemistry.

### **C-KIT**

Several C-KIT antibodies were used on both fetal ovaries and testes (Table 2.1, Chapter 2). Non-specific binding was eliminated by pre-blocking with normal rabbit serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. C-KIT (1) and C-KIT (2) primary antibodies were added at dilutions of 1: 300 and 1: 30 respectively (in diluted rabbit or swine serum, overnight at 4°C). The secondary rabbit anti-goat and swine anti-rabbit biotinylated IgG antibodies for C-KIT (1) and C-KIT (2) respectively were added at a dilution of 1: 500 (in dilute rabbit or swine serum, for 30 minutes at 23°C).

### **AMH**

Antigen retrieval was performed using citrate buffer (0.01M, pH 6.0, pressure cooked for 2.5 minutes) prior to blocking tissue with 3% H<sub>2</sub>O<sub>2</sub>. Non-specific binding was eliminated by pre-blocking with normal swine serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The rabbit polyclonal primary antibody (Table 2.1 Chapter 2) was added at a dilution of 1: 500 (in diluted swine serum, overnight at 4°C). Secondary swine anti-rabbit biotinylated IgG antibody was added at 1: 500 (in dilute swine serum, for 30 minutes at 23°C).



## **Immunoblotting**

Immunoblotting was performed to detect C-KIT protein in fetal ovaries (n=2) and testes (n=4) (Section 2.5-2.53).

### 3.3 RESULTS

#### Expression of *C-KIT* mRNA

Following RT-PCR a single cDNA (345bp) was amplified from RNA extracted from both fetal ovaries and testes at all gestations examined (13-21 weeks) (Figure 3.1A); although the PCR was not quantitative the amount of mRNA detected appeared higher in samples from testis.

Analysis of cell populations recovered from fixed sections of a week 19 testis by laser capture microscopy showed that at this age expression of *C-KIT* mRNA was confined to the germ cell population (gonocytes) and was not expressed in either Sertoli cells or within the interstitium (Figure 3.1 B-D).

#### Immunohistochemistry

C-KIT protein was detected by immunohistochemistry in all specimens examined, across the gestational range 14-21 weeks (ovary) and 13-19 weeks (testis) (Figures 3.2 and 3.3, representative sections from a total of 6 ovaries and 6 testes). Both anti-C-KIT antibodies tested gave similar staining patterns. In the ovary, positive staining was seen in oogonia and oocytes (Figure 3.2 A-C labelled o), with the great majority of germ cells being stained. The surface epithelium, the ovarian stroma and the pregranulosa cells of primordial follicles were all immunonegative. This was particularly apparent in the 21 week gestation specimen, in which there were a large number of primordial follicles which were not present at earlier gestations (Figure 3.2 C, labelled pf). It was notable that although the C-KIT protein appeared to be concentrated at the cell membrane of the oogonia at earlier gestational ages (Figure

3.2 A, B, arrow o), at 21 weeks the protein was clearly spread throughout the cytoplasm (Figure 3.2C, pf).

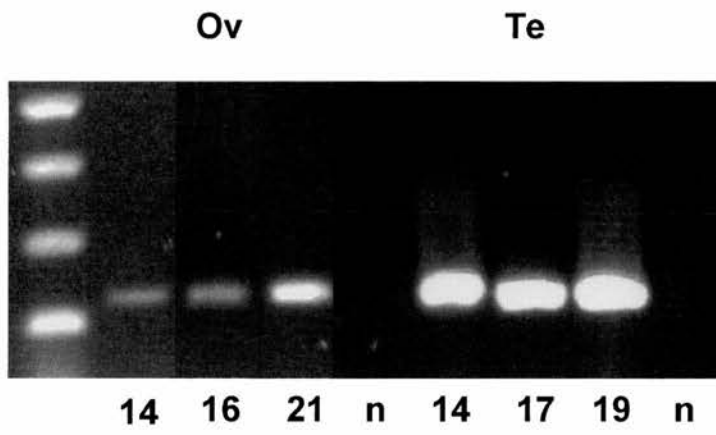
Within the testis, C-KIT protein was localised to gonocytes within the testicular cords (Figure 3.3 A-C) consistent with the cell specific expression of *C-KIT* mRNA. Although most gonocytes were immunopositive at all ages examined (13 to 19 weeks) some immunonegative cells were also present (e.g. 17 weeks, Figure 3.3C). As with the ovary, immunohistochemical staining was concentrated at the germ cell membrane (figure 3.3C arrow g). C-KIT immunoexpression was not detected in the peritubular cells, interstitial cells (figure 3.3C) or the surface epithelium (not shown). Sertoli cells were identified by immunostaining for AMH (Figure 3.3D). No staining was seen on sections of ovary (Figure 3.2D) or testis (Figure 3.3D inset) in which the primary antibody was not included.

**Figure 3.1** Expression of *C-KIT* mRNA in human fetal ovary and testis.

Panel A. RT-PCR analysis of samples extracted from whole ovaries obtained at 14, 16 and 21 weeks, and testis at 14, 17 and 19 weeks as indicated. Lanes labelled 'n' contained samples in which reverse transcriptase was not included.

Panels B, C and D show results of RT-PCR analysis of cell fragments recovered from a week 19 fetal testis by laser capture as described in Materials and Methods. Tissues were immunostained for AMH to identify the positions of Sertoli cells and gonocytes within the seminiferous cords. The mRNA for *C-KIT* (panel B) was detected in gonocytes (G) and whole testis (T) but not in Sertoli cells (S) or the interstitium (In). Likewise AMH (panel C) was only detected in S or T but not in G or In. GAPDH (panel D) was detected in all samples. Lane bl denotes blank in which reverse transcriptase was not included.

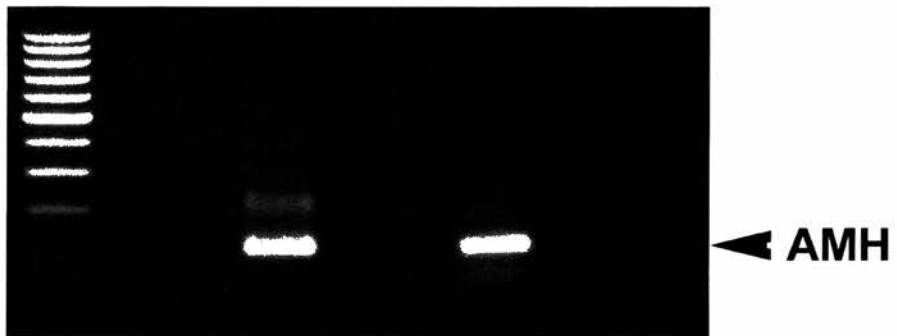
**A**



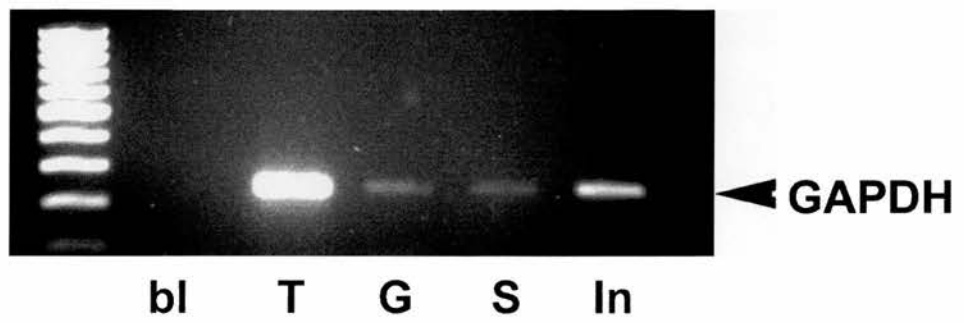
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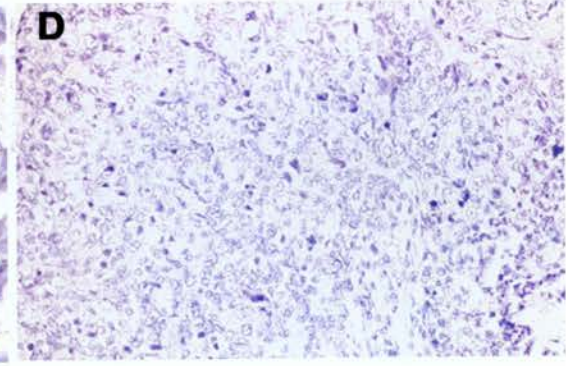
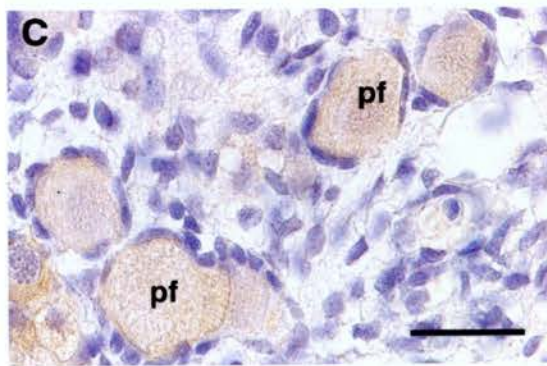
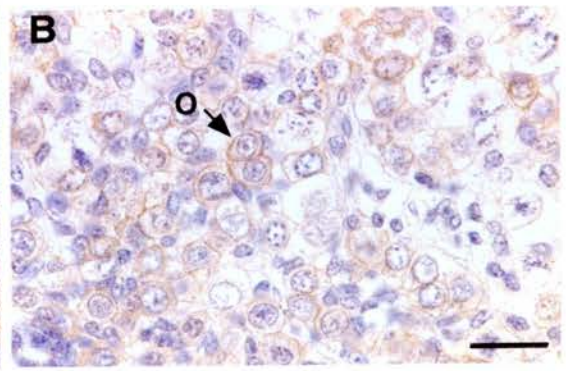
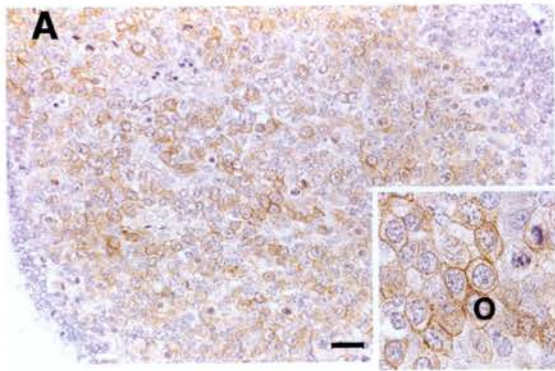
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**D**



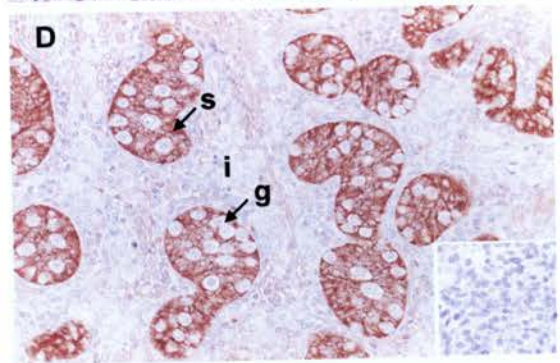
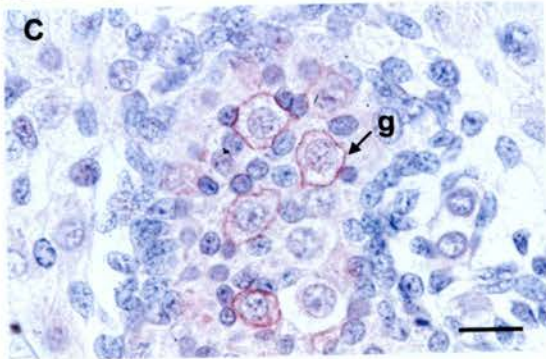
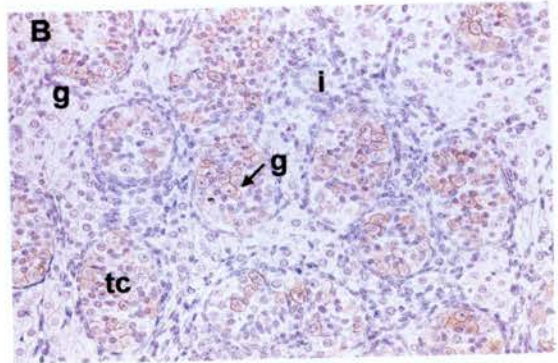
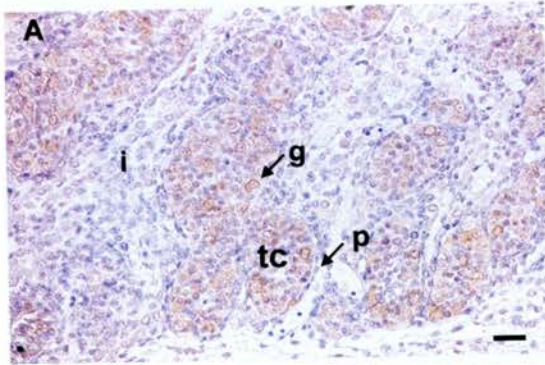
**Figure 3.2** Immunohistochemical localisation of C-KIT in the human fetal ovary (A) 14 week ovary; inset shows oocyte staining for C-KIT (B) 17 week ovary, (C) 21 week ovary, (D) Representative section of fetal ovary omitting anti C-KIT antibody. Staining for C-KIT is brown. **o**, oocyte; **pf**, primordial follicles. Scale bar in A represents 200µm and applies to D; scale bar on B is 50µm and also applies to A inset; scale bar in C represents 25µm



**Figure 3.3** Immunohistochemical localisation of C-KIT (A-C) and AMH (D) proteins to fixed tissue sections from human fetal testis.

(A) 14 week testis, (B) 16 week testis, (C) 17 week testis, (D) 17 week testis, AMH; inset representative section of fetal testis omitting anti c-kit antibody. Staining for c-kit and AMH is brown. **g**, gonocyte; **i**, interstitium; **p**, peritubular cells; **s**, Sertoli cells; **tc**, tubules. Scale bar in A represents 200µm and applies to A, B, D, D inset; scale bar in C represents 25µm.



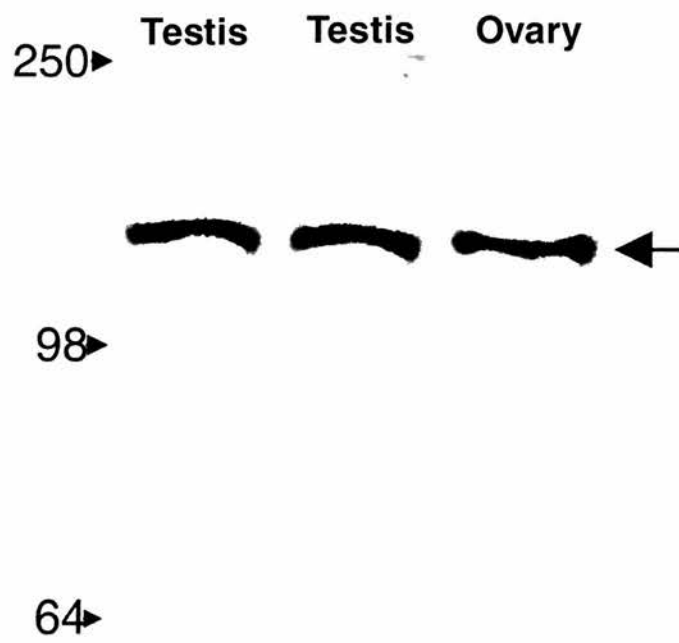


### **Immunoblotting**

The presence of C-KIT protein in both fetal ovary and testis was confirmed by immunoblotting. A prominent 145 kDa band corresponding to the size of the transmembrane receptor protein was detected in samples from gonadal tissues of both sexes (figure 3.4). There was no immunoreactivity in the absence of the primary antibody. The experiment was repeated 3 times with similar results.

**Figure 3.4** Western blot of C-KIT in human fetal ovary and testis.

Total protein samples (10 $\mu$ g) from ovary (14 weeks gestation) and testes (15 and 17 weeks gestation) were separated by SDS-PAGE, transferred to nylon membrane and incubated with anti C-KIT antibodies. Positions of molecular weight markers are indicated, the arrow marks the position of the single protein band which migrated with an apparent molecular size of ~110,000 kDa. No immunoreactive bands were detected in the absence of primary antibody (not shown).



### 3.4 DISCUSSION

The results presented demonstrate unequivocally that the expression of *C-KIT* mRNA and protein occurs in the germ cells of both ovary and testis in the human fetus during the second trimester.

In the rodent ovary expression of the *C-kit* gene has been detected very early in development and is high in proliferating germ cells (Manova et al 1991), however expression stops when oogonia enter meiosis (Manova et al 1990). In the human ovary, entry into meiosis occurs over a wide timescale, being detectable as early as 11 weeks gestation (Gondos et al 1986) although it is believed to be maximal at approximately 20 weeks (Baker 1963). Thus over the range of gestations examined in the present study, many oogonia will continue to proliferate by mitosis while an increasing proportion enter meiosis. The absence of a closely-defined time at which mitosis ceases and meiosis is initiated throughout the ovary is likely to account for the detection of *C-KIT* mRNA in whole tissue samples at all gestations examined up to 21 weeks, at which time many primordial follicles were present. The detection of C-KIT protein in all specimens examined up to and including 21 weeks gestation is consistent with a previous report in sheep in which C-KIT protein but not mRNA was detected in oocytes undergoing meiosis (Tisdall et al 1999). It is possible that the apparent change in intracellular localisation of C-KIT protein from the membrane to the cytoplasm seen in human primordial follicles may be related to this underlying change in gene expression. C-KIT protein has been previously localised to the oocytes of a 20 week gestation fetus (Horie et al 1993) but in that study which was

undertaken on frozen sections it was unclear whether C-KIT was localised to the cytoplasm or cellular membrane.

The range of gestational ages examined in this study covers the period of definitive histogenesis of the ovary, when the finite population of oocytes which will survive within primordial follicles and thus determine reproductive potential is regulated. C-KIT and its ligand, SCF or KL, may have an important role in these processes. Mutations in the genes for these factors result in loss of primordial germ cells (reviewed in Besmer (Besmer et al 1993)), and effects of KL/ C-KIT signalling on germ cell survival and protection from apoptosis have been demonstrated *in vivo* and *in vitro* (Godin et al 1991; Pesce et al 1993; Yee et al 1994). It has been suggested that the pro-survival effects of c-kit in the ovary may be mediated by increased expression of the anti-apoptotic factor Bcl-2 (Tilly 1996). Such studies have also suggested a role for this pathway in regulation of the onset of primordial follicle formation and growth of primary follicles (Yoshida et al 1997). KL/C-KIT may also have a role in later folliculogenesis: both promotion of follicle development and maintenance of arrest of meiosis have been suggested (Horie et al 1991; Ismail et al 1997; Parrott et al 1999). KL appears to have effects on surrounding stromal cells in addition to the oocyte (Parrott et al 2000b). C-KIT is also present in the adult human ovary, in both oocytes and granulosa cells (Tanikawa et al 1998) and has been suggested to have an autocrine role in the ovarian surface epithelium (Parrott et al 2000a). C-KIT expression appeared to be confined to germ cells within the ovary in the present study: no consistent staining of stromal cells was observed.

The technique of laser capture microscopy (LCM) was developed at the National Institutes of Health in the USA to allow sampling of individual, or groups of cells from complex tissues in such a way that mRNA and/or proteins could be extracted from them and analysed (Emmert-Buck et al 1996). The method has recently been applied to the recovery of seminiferous tubules from frozen sections of mouse testis (Suarez-Quian et al 2000). In the present study the LCM microscope was used with the laser set to the smallest size available (7.5  $\mu\text{m}$ ) to allow for sampling of single gonocytes. Recently the size of male germ cells in the human fetal testis (7 to 10 weeks) has been reported as being 9 $\mu\text{m}$  in diameter (Bendsen et al 2001). To enable us to locate individual gonocytes, fixed tissue sections were used and these were stained using a modified immunohistochemical technique. The use of fixed sections meant that only short fragments of cDNA could be identified by RT-PCR (Goldsworthy et al 1999) and we employed a nested PCR strategy to increase signal intensity and specificity. We believe this is the first time LCM methodology has been used to sample individual cell types from the human fetal testis and has allowed us to show that *C-KIT* mRNA is expressed in fetal gonocytes at 19 weeks gestation. In a previous study on human fetal testes C-KIT protein was not detected in the testis beyond 15 weeks gestation using immunohistochemistry (Rajpert-De Meyts et al 1996). In another study presence of C-KIT in the human fetal testis was reported in cells described as spermatogonia in a fetus of 18 weeks gestation (Horie et al 1991). The results we have obtained using specific immunohistochemistry and Western analysis all demonstrate that C-KIT protein is expressed in the fetal germ cells up to and including 19 weeks of gestation. Gonocytes are believed to be the cell of origin of gonadoblastomas (Rajpert-De Meyts et al 1996), and it has been suggested that

prolonged expression of c-kit in germ cells in individuals with intersex conditions may be a component of abnormal germ cell development in such individuals who are at increased risk of testicular neoplasia (Rajpert-De Meyts et al 1996). Following laser capture of cells from a 19 week fetus we failed to detect expression of *C-KIT* mRNA in interstitial cells. Although this result is based on a single stage of development it was in agreement with lack of immunostaining in the interstitium at all ages examined and would not therefore be consistent with findings in the mouse (Manova et al 1990). Studies of mice in which mutations in C-kit or its ligand have been well documented and shown to result in a failure in migration of germ cells into the genital ridge (reviewed by (Besmer et al 1993) however the use of a blocking antibody against C-kit has led to the suggestion that C-kit is important in proliferation of differentiated spermatogonia (Yoshinaga et al 1991) and protection from apoptosis (Packer et al 1995). A role in spermatogonial differentiation rather than proliferation has also been suggested on the basis of experiments involving transplantation of germ cells into testes of *Steel* mice (Ohta et al 2000) and the *C-kit/Kl* genes shown to have a role in meiosis (Vincent et al 1998). Taken together these data reinforce the importance of C-KIT and its ligand in multiple cell lineages both during development and in adulthood.

In conclusion, this study demonstrates conclusively that *C-KIT* mRNA and protein are expressed in oogonia during the transition from rapid proliferation by mitosis to the formation of primordial follicles, and in gonocytes of the developing testis during the second trimester. C-KIT has been demonstrated to be crucial for germ cell



migration, survival and proliferation in the mouse: the present results suggest that C-KIT is likely to be of similar importance in the human.

## **CHAPTER 4**

# **MATRIX METALLOPROTEINASES AND (MMPs) TISSUE INHIBITORS OF METALLOPROTEINASES (TIMPs) IN HUMAN FETAL TESTIS AND OVARY**

## 4.1 INTRODUCTION

The development of the human fetal gonad is a complex process involving dramatic structural changes, the control mechanisms of which remain unclear. In both fetal ovary and testis, the ECM provides both the scaffold to which cells attach and also, by binding to specific cell surface receptors, modulates their function (Woessner, Jr. 1991). Remodelling of the ECM may play an integral role in fetal gonadal development, including cell migration, organisation, differentiation and function.

MMPs are essential for proteolytic degradation of the ECM and the extent of ECM remodelling depends on the ratio of MMP to TIMP (Salamonsen 1996). Regulation of ECM remodelling by MMPs and TIMPs is vital to provide an environment that supports initiation of growth, migration and differentiation by a range of mechanisms (Behrendtsen et al 1997; Giannelli et al 1997; Li et al 2000). These proteins can act from within the matrix and also at the cell surface, where for example MMP-2 and -9 are known to bind to heparin sulphate proteoglycans. They are thus positioned for interaction with cell surface adhesion molecules or receptors and for regulating the turnover of these molecules (Yu et al 2000b).

MMPs and TIMPs also regulate proliferation of a variety of cell types (Edwards et al 1996a) and are involved in the regulation of cytokines and their receptors both directly and indirectly via effects on the ECM. Growth factors bound to ECM are biologically inactive and must be liberated and in some cases activated before binding to receptors (Vu et al 2000), for example MMP-9 proteolytically activates latent transforming growth factor- $\beta$  (TGF- $\beta$ ) (Yu et al 2000a). MMPs may also

control bioavailability by cleaving binding proteins, thus MMP-1 can degrade insulin-like growth factor binding proteins I and II (IGFBP I and II) into fragments with low affinity for insulin growth factor I and II (IGF I and II), thus increasing the bioavailability of IGF (Rajah et al 1995). Regulation of proteolytic degradation of the ECM may therefore provide an important mechanism for controlling growth factor availability and activity thus influencing tissue differentiation during organ development. Secretion of MMPs and TIMPs is under the control of a wide range of cytokines and growth factors (Nagase et al 1999), such as platelet derived growth factor (PDGF) (Johnson et al 1999) and TGF- $\beta$  (Edwards et al 1996b). Sex hormones such as progesterone are also significant in ECM remodelling via inhibition of MMPs-1, -3 and -7 synthesis and stimulation of TIMPs-1 and -2 production in progesterone-dependent tissues (Imada et al 1994; Marbaix et al 1995). Signalling pathways also lead to expression of particular MMP genes as in the case of MMP-1 which is mediated by the MAP kinase pathway (Reunanen et al 1998).

In the rat ovary MMPs are produced by a variety of cell types including mature oocytes, granulosa cells and luteal cells (Bagavandoss 1998; Curry, Jr. et al 2001) and in the bovine ovary MMP-9 and TIMP-1 have been associated with follicular growth (Kaiura et al 2000; McCaffery et al 2000). TIMPs have also been identified in the gonadal tissue of various species including adult human (Curry, Jr. et al 1990). The objective of this study was to establish the secretion and localisation of a range of MMPs and all TIMPs family members to determine their possible role in the development of the fetal testis and ovary.

## **4.2 MATERIALS AND METHODS**

### **Collection of tissue samples**

Gonadal tissue was collected from 21 fetuses after termination of pregnancy (Section 2.21). The gonads were removed and either placed in a sterile petri dish containing  $\alpha$ -MEM prior to culture or were immediately fixed in Bouins fluid for histological analysis.

### **Explant culture**

Gonads from 2 fetuses of each sex (ovaries at 12 and 14 week gestation, and testes at 17 weeks gestation) were cultured for 48 hours (Section 2.6) and the media was collected in a sterile container and frozen at  $-20^{\circ}\text{C}$  prior to analysis by zymography.

### **Detection of gelatinase activities by zymography**

Activities of MMP-2 and MMP-9 were determined using gelatinase zymography (Riley et al 1999a) (Section 2.7, Appendix II).

### **Detection of TIMPs by reverse zymography**

The activity of TIMP-1, -2, -3 and -4 was detected by reverse zymography (Riley et al 1999b) (Section 2.71, Appendix II).

### **Localisation of MMP-1, -2, and -9 and TIMPs by immunohistochemistry**

Immunoreactive MMP-1, -2 and -9, and TIMP-1, -2, -3 and -4 were localised in tissues using immunohistochemistry (Riley et al 1999b) (Section 2.4). The conditions

for individual antibodies are described below. In each case term human fetal membranes were used as a positive control.

### **MMP-1**

Non-specific binding was eliminated by pre-blocking with normal horse serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The mouse monoclonal primary antibody was added at a dilution of 2µg/ml (in diluted horse serum, overnight at 4°C). Secondary horse anti-mouse biotinylated IgG antibody was added at 1: 500 (in dilute horse serum, for 30 minutes at 23°C).

### **MMP-2**

Non-specific binding was eliminated by pre-blocking with normal horse serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The mouse monoclonal primary antibody was added at a dilution of 5µg/ml (in diluted horse serum, overnight at 4°C). Secondary horse anti-mouse biotinylated IgG antibody was added at 1: 500 (in dilute horse serum, for 30 minutes at 23°C).

### **MMP-9**

Non-specific binding was eliminated by pre-blocking with normal horse serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The mouse monoclonal primary antibody was added at a dilution of 10µg/ml (in diluted horse serum, overnight at 4°C). Secondary horse anti-mouse biotinylated IgG antibody was added at 1: 500 (in dilute horse serum, for 30 minutes at 23°C).

### **TIMP-1**

Non-specific binding was eliminated by pre-blocking with normal goat serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The rabbit polyclonal primary antibody was added at a dilution of 2µg/ml (in diluted goat serum, overnight at 4°C). Secondary goat anti-rabbit biotinylated IgG antibody was added at 1: 500 (in dilute goat serum, for 30 minutes at 23°C).

### **TIMP-2**

Non-specific binding was eliminated by pre-blocking with normal goat serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The rabbit polyclonal primary antibody was added at a dilution of 5µg/ml (in diluted goat serum, overnight at 4°C). Secondary goat anti-rabbit biotinylated IgG antibody was added at 1: 500 (in dilute goat serum, for 30 minutes at 23°C).

### **TIMP-3**

Non-specific binding was eliminated by pre-blocking with normal goat serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The rabbit polyclonal primary antibody was added at a dilution of 5µg/ml (in diluted goat serum, overnight at 4°C). Secondary goat anti-rabbit biotinylated IgG antibody was added at 1: 500 (in dilute goat serum, for 30 minutes at 23°C).

### **TIMP-4**

Non-specific binding was eliminated by pre-blocking with normal goat serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C.

The rabbit polyclonal primary antibody was added at a dilution of 5µg/ml (in diluted goat serum, overnight at 4°C). Secondary goat anti-rabbit biotinylated IgG antibody was added at 1: 500 (in dilute goat serum, for 30 minutes at 23°C).

### **Analysis of immunohistochemistry**

Immunostaining of tissue sections was assessed semi-quantitatively for both the ovary and testis using + and - symbols as a measure of the intensity and amount of staining in particular cell types (Table 4.1).



<b>SYMBOL</b>	<b>SCORE</b>
–	No positive staining in any cell of this type
+/-	Some but not most of these cells have stained
+	Pale staining in this cell type
++	Marked staining in this cell type
+++	Intense immunostaining

**Table 4.1** Table showing semi-quantitative scoring of immunohistochemical staining of fetal testes and ovaries.

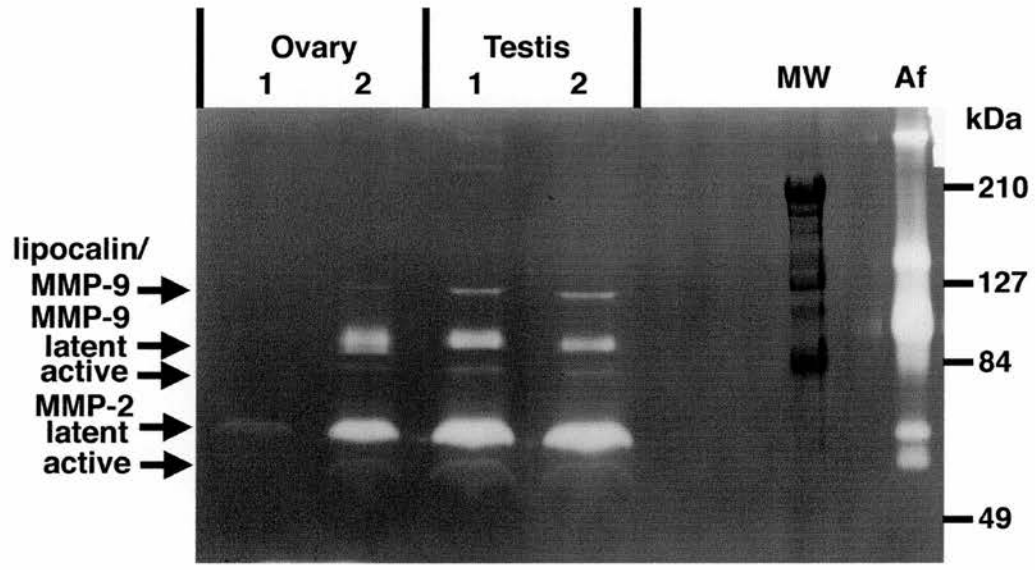
## 4.3 RESULTS

### Secretion of MMPs and TIMPs by the fetal testis

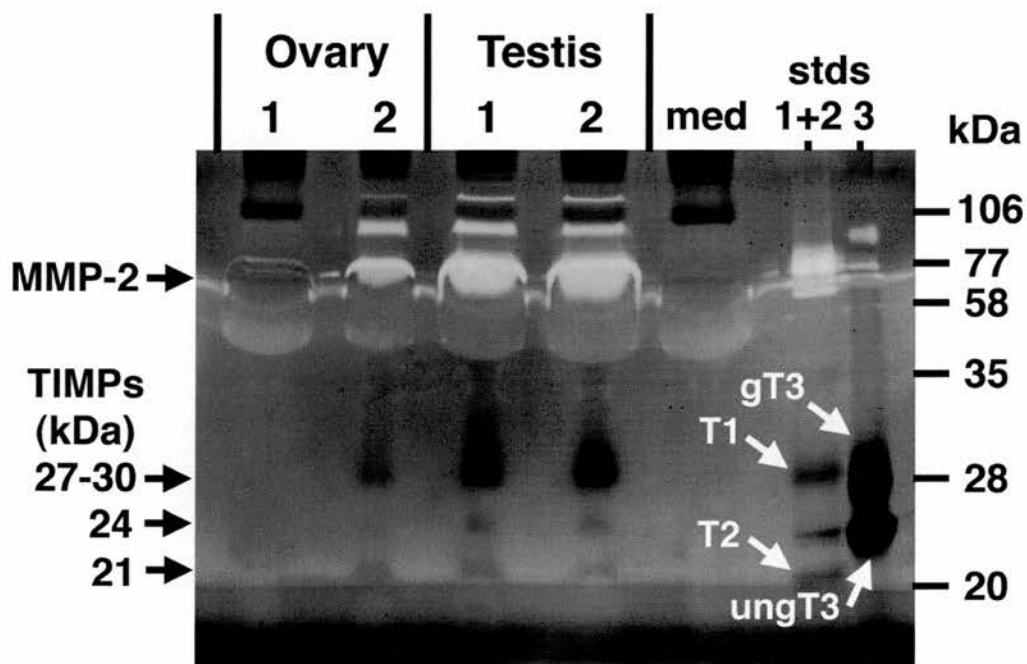
In culture medium conditioned by the fetal testis for 48 h, high levels of gelatinase activity were detected at 120 kDa, 92 kDa, 72 kDa and 66 kDa. These molecular weights correspond to the MMP-9 lipocalin complex, the active form of MMP-9, MMP-2 activated protein in the latent form and the active form of MMP-2 respectively (Figure 4.1). The predominant gelatinase activity detected was of MMP-2, the latent form. MMP-2 and -9 were studied in particular because these MMPs break down collagen IV which is a major component of basement membranes, and so a good candidate and marker for important remodelling events.

The examination of TIMP activity in the testis using reverse zymography showed predominant gelatinase inhibitory activity at 27-30 kDa, which corresponds to the molecular weights of TIMP-1, glycosylated TIMP-3, and TIMP-4 (Figure 4.2). Bands of activity were also present at 24 kDa (corresponding to unglycosylated TIMP-3) and 21 kDa (corresponding to TIMP-2). These bands aligned with standards to TIMP-1, TIMP-2 and glycosylated and unglycosylated TIMP-3. Reverse zymography is unable to distinguish precisely between the TIMP isoforms of 27-30 kDa molecular weight. The presence of these TIMPs of similar molecular weights, including TIMP-4, was therefore confirmed by immunohistochemistry.

**Figure 4.1** Gelatin zymography gel showing gelatinase activity (visualised as lighter bands) in conditioned medium from explant cultures of fetal ovary and testis. Ovary 1, 14 weeks; ovary 2, 12 weeks; testis 1 and 2, 17 weeks. The predominant gelatinase activity is due to MMP-2 (latent form; 72 kDa). Molecular weight markers are as indicated (kDa). A sample of human term amniotic fluid (med) was used as a positive control.



**Figure 4.2** Reverse zymography gel demonstrating the secretion of TIMPs (visualised by darker bands) into culture medium from explant cultures of fetal ovary and testis. Ovary 1, 14 weeks; ovary 2, 12 weeks; testis 1 and 2, 17 weeks. Three predominant bands of TIMP activity are observed at 27-30 kDa, 24 kDa and 21kDa. The standards (stds) of TIMP-1 and TIMP-2 (1 + 2; TIMP-1 as a broad band at 27-30 kDa; TIMP-2 at 21 kDa) and TIMP-3 (3; glycosylated TIMP-3 at 28-30 kDa and unglycosylated TIMP-3 form at 24 kDa) are indicated by arrows. Molecular weight markers are as indicated (kDa).



### **Localisation of MMPs and TIMPs in the fetal testis**

Sections of human testis from ten fetuses between 13 and 19 weeks gestation were used for analysis. Table 4.2 shows the spatial and cellular localisation of MMPs and TIMPs immunoreactivity and relative intensity of staining found and Figure 4.3 shows representative photomicrographs. MMP-1 was found in abundance in the cytoplasm of the interstitial cells and to a lesser extent in the surface epithelium (Figure 4.3A). MMP-1 was also distributed among some of the peritubular cells and within the tubules, particularly in Sertoli cells. MMP-2 was predominantly localised to the interstitium of the testis and also found within the cytoplasm of some of the tubular cells, being more prevalent in gonocytes than Sertoli cells (Figure 4.3B). It was also present in the surface epithelium but absent in the peritubular cells. MMP-9 was present chiefly within the tubules and was also present in some of the interstitial cells and surface epithelium (Figure 4.3C). Like MMP-2, MMP-9 was not observed in the peritubular cells.

TIMPs-1, -2, -3 and -4 were also immunolocalised within the fetal testis. TIMP-1 was localised to the cytoplasm of the interstitial cells and also within the tubules, staining in particular Sertoli cells (Figure 4.3D). TIMP-2 was predominantly localised to the interstitium, with very little within the tubules and no immunoreactivity in the peritubular cells or surface epithelium (Figure 4.3E). Staining for TIMP-3 was very intense within the interstitium and was also present although to a lesser degree in some of the peritubular cells (Figure 4.3F). There was no TIMP-3 immunostaining within the tubules and surface epithelium. TIMP-4 was present mainly in the interstitial cells but there was also strong positive staining

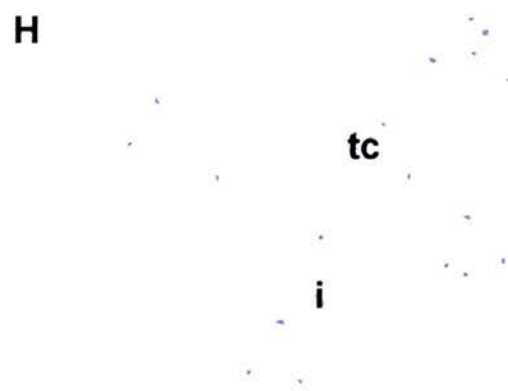
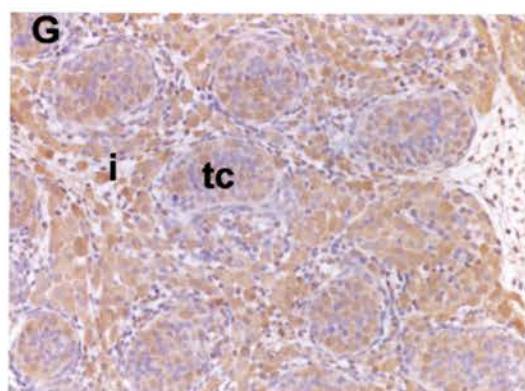
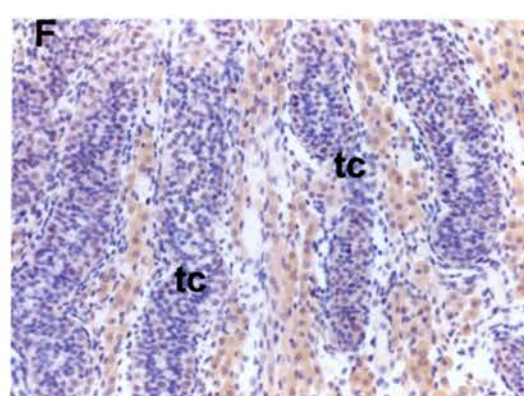
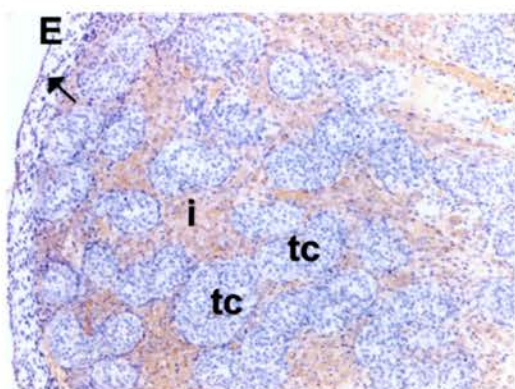
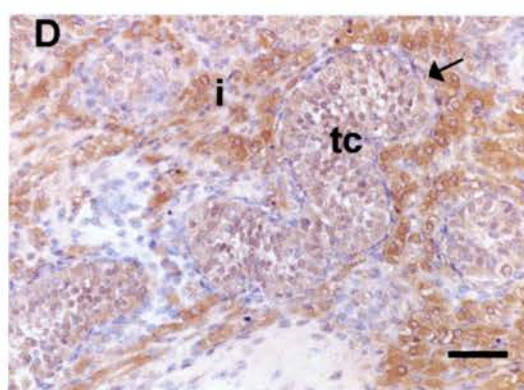
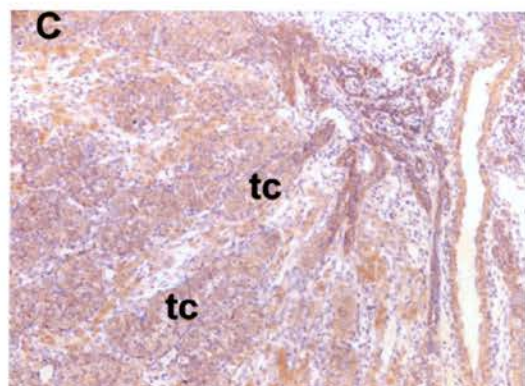
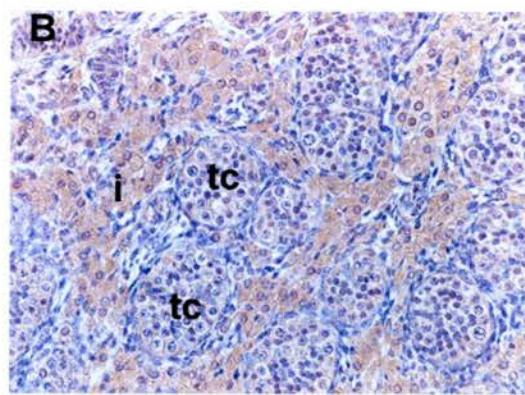
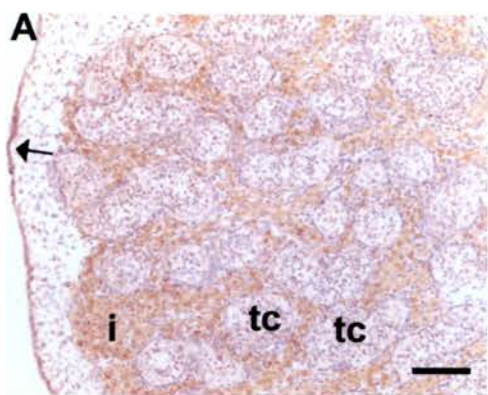
within the Sertoli cell and gonocyte cytoplasm (Figure 4.3G). It was absent from both the peritubular cells and the surface epithelium. Immunostaining was also observed in the vascular endothelium for MMPs-1, -2, -9 and TIMPs-1, -2, -3 and -4 (Table 4.2). No major changes were seen in immunostaining for any MMP or TIMP examined over the gestational range examined. Sections incubated without primary antibody showed no non-specific staining (Figure 4.3H).



	Peritubular cells	Gonocytes	Sertoli Cells	Interstitialium	Surface epithelium	Blood Vessels
MMP-1	+/-	+	++	+++	+	+
MMP-2	-	+	+/-	++	+/-	+
MMP-9	-	++	++	++	-	+
TIMP-1	+/-	+	++	++	+/-	+
TIMP-2	-	+/-	+/-	++	-	+
TIMP-3	+/-	-	-	+++	-	+
TIMP-4	-	++	++	++	-	+

**Table 4.2** Semi-quantitative analysis of cellular and spatial localisation of MMP-1, -2, -9 and TIMPs -1, -2, -3 and -4 using immunohistochemistry in the fetal testis between 13 and 19 weeks gestation.

**Figure 4.3** Localisation of MMP-1, -2, -9 and TIMPs -1, -2, -3 and -4 in human fetal testis. **(A)** Shows MMP-1 in fetal testis at 13 wks. Arrow denotes surface epithelium. **(B)** MMP-2 in 15 wk. fetal testis **(C)** MMP-9 in 17 wk. fetal testis **(D)** TIMP-1 in 19 wk. fetal testis. Arrow indicates peritubular cells. **(E)** TIMP-2 in 13 wk. fetal testis. The arrow denotes the surface epithelium. **(F)** TIMP-3 in 15 wk. fetal testis **(G)** TIMP-4 in 15 wk. fetal testis **(H)** Representative section of fetal testis omitting primary antibody. **i**, interstitium; **tc**, tubules. Scale bar in **A** represents 100  $\mu$ m and applies to panels **B**, **C** and **E**; scale bar in **D** represents 50  $\mu$ m and applies to panels **F-H**.



### **Secretion of MMPs and TIMPs by the fetal ovary**

Analysis using zymography of culture medium conditioned by the fetal ovary for 48 h, demonstrated in the two samples examined that the predominant gelatinase activity was identified at 72 kDa molecular weight, corresponding to the latent form of MMP-2 (Figure 4.1). The active form of MMP-2, which has a molecular weight of 66 kDa, was also detected but at a lower level. Gelatinase activity was also detectable at 92 kDa, corresponding to latent MMP-9 and a less intense band of activity was observed at 120 kDa, corresponding to the MMP-9 lipocalin complex. In one sample, MMP-2 (latent and active) and MMP-9 were identified in much smaller amounts.

A broad spectrum of TIMP activity was detected in culture medium conditioned by the fetal ovary for 48 h by reverse zymography (Figure 4.2). Three bands of gelatinase inhibitory activity were present at 27-30 kDa (corresponding to the molecular weight of TIMP-1, glycosylated TIMP-3 and TIMP-4), 24 kDa (corresponding to unglycosylated TIMP-3) and 21 kDa (corresponding to TIMP-2). As with zymography, one of the samples showed very low levels of TIMPs activity.

### **Localisation of MMPs and TIMPs in the fetal ovary**

Sections of fetal ovaries from 11 fetuses between 13 and 21 weeks gestation were used for analysis. Table 4.3 and Figure 4.4 describe and demonstrate the spatial and cellular localisation of MMPs and TIMPs and relative intensity of immunostaining

found. As with the fetal testis, no major or systematic variation in immunostaining was seen for any MMP or TIMP examined with increasing gestational age.

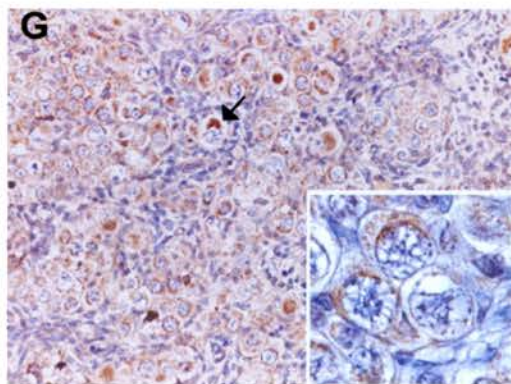
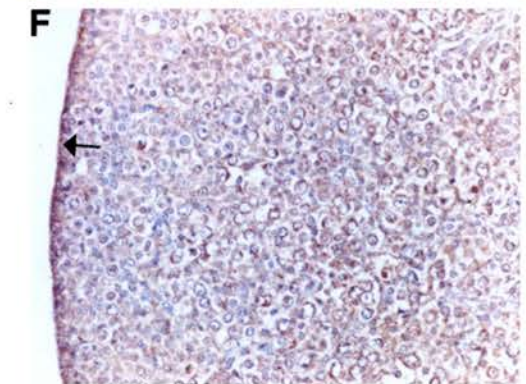
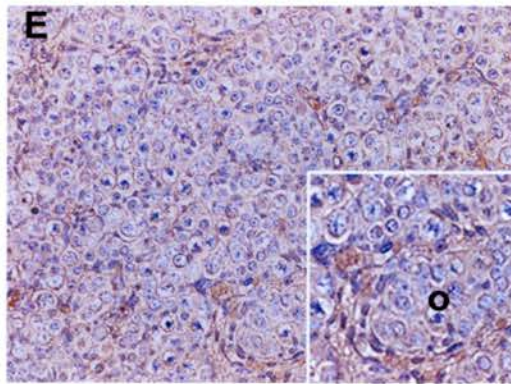
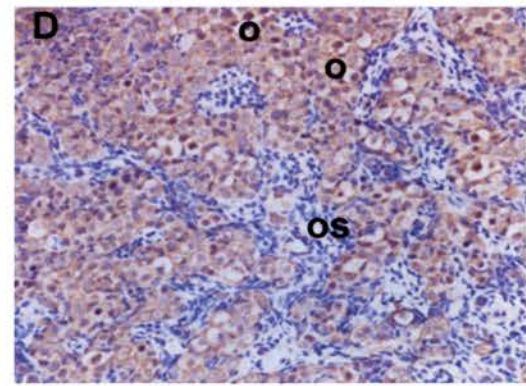
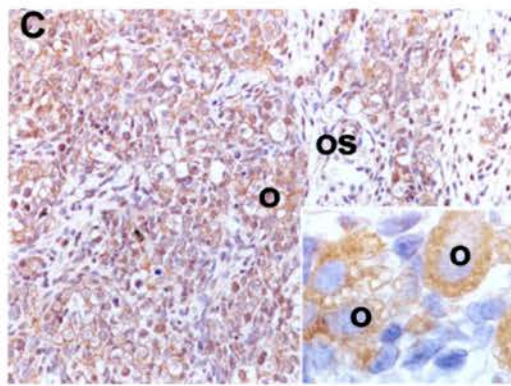
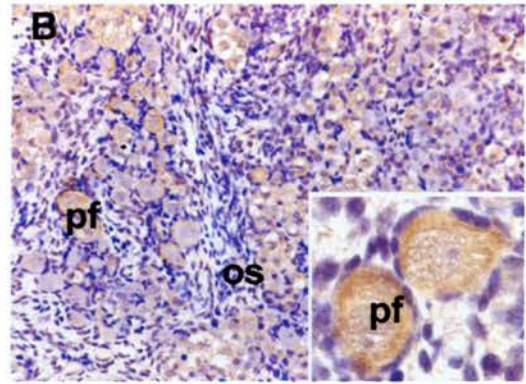
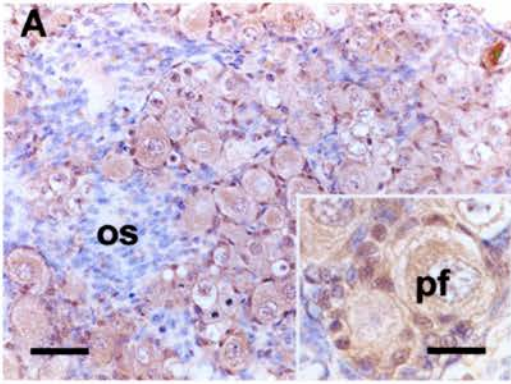
MMP-1 was abundantly present in the cytoplasm of the oocytes throughout the ovarian cortex and was also localised to some of the cells of the ovarian stroma and the surface epithelium, although this immunoreactivity was less intense (Figure 4.4A). MMP-2 was predominantly found in the oocyte cytoplasm and was also present in some of the cells of the surface epithelium but was absent from the ovarian stroma (Figure 4.4B). A similar staining pattern in the oocyte cytoplasm and surface epithelium was found with MMP-9 (Figure 4.4C).

All four members of the TIMP family were also localised within the fetal ovary. TIMP-1 was found chiefly in the cytoplasm of the oocytes but was also present in the surface epithelium (Figure 4.4D). TIMP-2 was weakly associated with the oocyte cytoplasm but was more localised to the ovarian stroma and was absent from the surface epithelium (Figure 4.4E). TIMP-3 was widely distributed, immunostaining some of the oocyte cytoplasm, ovarian stroma and cells of the surface epithelium (Figure 4.4F). Immunostaining for the TIMP-4 antibody was restricted to the oocyte cytoplasm (Figure 4.4G). MMPs-1, -2, -9 and TIMPs-1, -2, -3 and -4 were also localised to the vascular endothelium (Table 4.3). No immunostaining was observed in the negative control (Figure 4.4H).

	Oocyte cytoplasm	Ovarian Stroma	Surface epithelium	Blood Vessels
MMP-1	++	+/-	+/-	+
MMP-2	++	-	+/-	+
MMP-9	++	-	+/-	+
TIMP-1	+++	-	+/-	+
TIMP-2	+/-	+/-	-	+
TIMP-3	+/-	+/-	+/-	+
TIMP-4	++	-	-	+

**Table 4.3** Semi-quantitative analysis of cellular and spatial localisation of MMP-1, -2, -9 and TIMPs -1, -2, -3 and -4 by immunohistochemistry in the fetal ovary between 13 and 21 weeks gestation.

**Figure 4.4** Localisation of MMP-1, -2, -9 and TIMPs -1, -2, -3 and -4 in human fetal ovary **(A)** MMP-1 in fetal ovary at 21 wks. Inset demonstrates primordial follicles stained with MMP-1 in same ovary **(B)** MMP-2 in fetal ovary at 21 wks. Inset shows primordial follicles with staining for MMP-2 in oocyte cytoplasm in same ovary. **(C)** MMP-9 in fetal ovary at 13 wks. Inset shows oocytes stained with MMP-9 in 16 wk ovary **(D)** TIMP-1 in 15 wk. fetal ovary **(E)** TIMP-2 in 17 wk. fetal ovary. Inset shows oocytes and rete ovarii staining with TIMP-2 in same ovary. **(F)** TIMP-3 in 13 wk. fetal ovary. Arrow denotes surface epithelium. **(G)** TIMP-4 in 15 wk. fetal ovary. Arrow denotes primordial follicle. Inset shows oocyte staining for TIMP-4 in same ovary. **(H)** Representative section of fetal ovary omitting primary antibody. **os**, ovarian stroma; **pf**, primordial follicle; **o**, oocyte. Scale bar in A represents 50  $\mu$ m and applies to panels **B-H** and scale bar in **A inset** represents 12  $\mu$ m and applies to insets in **B, C, E** and **G**.





#### 4.4 Discussion

This study demonstrates the presence of MMP-1, -2, -9 and all four TIMP family members in the human fetal ovary and testis during mid-gestation. MMP-2 appears the predominant gelatinase matrix metalloproteinase secreted by the gonads, being most abundant in its latent form. All of the TIMP family were secreted by both the testis and ovary. These data therefore indicate the likely involvement of MMPs and TIMPs during this time of gonadal development.

Using zymographic techniques, we are unable to comment on absolute amounts of MMPs and TIMPs present as they are not quantitative but they do allow relative intensities of activity to be established. However, one of the ovarian samples analysed (at 14 weeks gestation) appeared to be producing much lower levels of MMPs and TIMPs than the other samples tested. This may be due to a change in secretion levels over gestation or to a delay in receiving the tissue after termination. Immunolocalisation does not specifically identify the cellular site of production of these TIMPs and MMPs as they are secreted and may bind to the ECM or directly to the cell, however it is likely that the MMPs and TIMPs have been directly secreted at these sites to mediate specific functions (Vu et al 2000). The drugs used to induce termination included mifepristone and a prostaglandin E1 analogue. Mifepristone is a potent anti-progestin and anti-glucocorticoid, which also has anti-oestrogenic effects (Teutsch et al 1994). Although concentrations of mifepristone reaching the chorionic villi are low compared with those in serum and decidua (Wang et al 1994) it may have some effect on the fetal gonadal tissue, although the nature and extent of any such effects are currently unknown. Exogenous prostaglandin E1 is also used to

induce uterine contractions and promote cervical dilatation at termination. Prostaglandins are involved in many aspects of normal ovarian function and it is uncertain whether these concentrations used in the termination procedure might cross the placenta to the fetus and then have an effect on the fetal gonadal tissue (Greystoke et al 2000).

Both MMPs and TIMPs are likely to be involved in the tissue remodelling that accompanies the rapid growth, differentiation and structural changes of the fetal gonads in the second trimester. Interactions between MMPs and TIMPs are probably important in controlling both fibrillar collagen remodelling by MMP-1, an important structural matrix component and also collagen IV, by MMP-2 and -9, a major component of basement membranes. Thus, cells can be permitted to grow, differentiate and undergo mitosis. Cell migration may also be allowed (Giannelli et al 1997), as seen for instance in the formation of primordial follicles in the ovary that occurs at this time in development and the movement of gonocytes from a central location within the testicular tubule to lying adjacent to the basement membrane. MMPs and TIMPs influence many cellular functions (Salamonsen 1996; Vu et al 2000) and may play other roles within the gonads. The interaction between a cell and its surrounding matrix, for instance via integrins and focal adherins, is a vital regulator of cell function (Brooks et al 1996) (Giancotti 1997; Steffensen et al 1998) and studies on roles of MMPs at the cell surface have shown that they can stimulate cell proliferation through interaction with cytokines (Edwards et al 1996a). MMPs-1, -2 and -9 bind to heparin sulphate proteoglycans on the cell surface (Fisher et al 1994; Yu et al 2000b) which may prevent diffusion of the MMP and confer a

high degree of local control for tissue remodelling, cell matrix interactions and local modulation of cytokine shedding or degradation. The binding of MMP-2 and -9 to heparin sulphate proteoglycan therefore may have an effect on processes involving excessive tissue breakdown, such as angiogenesis. As we have observed vascular staining in both testis and ovary for MMP-1, -2 and -9 and all TIMPs, it is likely that these proteins are involved in angiogenesis within the gonadal tissue (Yu et al 2000b). MMP-1 may also have another role in angiogenesis as it enhances smooth muscle cell migration within the vessel wall by degrading collagen to gelatin, leaving this available for the action of gelatinases such as MMP-2 (Pilcher et al 1997).

MMPs and TIMPs may also regulate cell cycle progression or death (Boudreau et al 1996). TIMP-3 induces apoptosis of colon carcinoma cells (Smith et al 1997), and in mammary cells inhibition of MMP activity rescues cell apoptosis (Schedin et al 2000). The number of germ cells within the ovary reaches a peak at approximately 20 weeks gestation (Baker et al 1974) with a parallel though lesser increase in the number of atretic cells. The rapid increase and subsequent loss of germ cell numbers at this time is likely to have a major impact on the compliment of primordial follicles. The regulatory mechanisms involved are fundamental to the determination of reproductive lifespan but are poorly understood. The presence of MMP-1, -2, -9, TIMP-1 and -4 in the oocytes suggests that they may potentially regulate survival signals and so possibly affecting cell proliferation. MMPs also regulate growth factor activity by cleaving the proteins that bind them, including IGF I and II. MMP-1 and -2 both can degrade IGFBPs I and II allowing IGF I and II to become active (Rajah et al 1995; Vu et al 2000). IGF I and II, its receptor and binding proteins I and II are

expressed in the human ovary and testis and have been implicated germ cell development (Zhou et al 1993a; Zhou et al 1993b). In addition, TIMPs also stimulate proliferation directly in other cell systems (Hayakawa et al 1994) and MMP activity and its control by TIMPs regulates activation of cytokines such as tumour necrosis factor  $\alpha$  (TNF  $\alpha$ ) (McGeehan et al 1994) at the cell matrix interface. The TIMP-1-procathepsin L complex has been previously suggested to be a potent activator of steroidogenesis in the rat testis, and was secreted by Sertoli cells (Boujrad et al 1995). The present data suggest that TIMP-1 is mainly localised to the Sertoli cells within the tubules, thus it may play a similar steroidogenic role in the human fetal testis.

In conclusion, this study demonstrates that MMPs and TIMPs are secreted by the human fetal gonad during mid gestation and are localised with discrete cellular and spatial distributions within the fetal testicular and ovarian tissue. These results suggest that MMPs and TIMPs are involved in ECM remodelling at this time and may also play a role in paracrine regulation including germ cell proliferation by regulating growth factor availability and action, and possibly also steroidogenesis in the testis. This study provides a basis from which to work towards further assessment of the functions of MMPs and TIMPs within the human fetal gonad during this period of structural change and development.

**CHAPTER 5**

**EXPRESSION OF NEUROTROPHINS AND  
THEIR RECEPTORS IN THE HUMAN FETAL  
OVARY AND THEIR EFFECT ON GERM CELL  
SURVIVAL**

## 5.1 INTRODUCTION

Mammalian ovarian development is characterised by a sequential pattern of mitotic proliferation of oogonia, initiation then arrest of meiosis, and primordial follicle formation. As reproductive lifespan is determined by the continuing presence of primordial follicles, the regulation of germ cell proliferation and survival in the form of primordial follicles is central to reproductive potential. Waves of atresia of oogonia have been identified at particular stages of development (Baker 1963; Speed 1988) but the factors and pathways involved are largely unknown, particularly in the human. The neurotrophin family are known to have a critical role in cell proliferation, migration, differentiation and survival within the nervous system (Davies 1994; Henderson et al 1993; Snider 1994). However they also have important functions in non neuronal tissue (Brill et al 1995; Dissen et al 1995; Mitsiadis et al 1995; Ojeda et al 1992; Sariola et al 1991; Wheeler et al 1992; Yaar et al 1991) and have been demonstrated in the mammalian ovary (Berkemeier et al 1991; Dissen et al 1995; Ernfors et al 1990b; Ibanez et al 1992; Klein et al 1989; Lara et al 1990; Timmusk et al 1993). There they appear to be involved in follicular formation (Dissen et al 1995; Dissen et al 2001) and ovulation (Dissen et al 1996; Mayerhofer et al 1996). More recently, neurotrophins have been shown to promote germ cell survival in the mouse ovary (Spears 2003). There is therefore increasing evidence that the various neurotrophins may have specific roles in ovarian development and function, but no data are currently available on their presence in the normal human ovary. We have therefore investigated the presence and distribution of the neurotrophins and their receptors in the developing human ovary during the period leading up to the formation of primordial follicles. Following this, we

examined the effect blocking neurotrophin action in organ culture had on germ cell survival and proliferation.

## **5.2 MATERIALS AND METHODS**

### **Tissue samples**

Human fetal ovaries aged between 13 and 21 weeks of gestation were obtained following medical termination of pregnancy (Section 2.21).

### **Isolation of RNA and RT-PCR**

Total RNA was extracted from snap frozen samples of fetal ovary and cDNA synthesised (Section 2.3). RT-PCR was performed (Section 2.31) using primers specific for human neurotrophins and their receptors. All products were purified (Section 2.33) and their identities confirmed by direct sequencing (Section 2.34).

### **Amplification of specific neurotrophins and receptors by PCR**

#### ***NGF***

Specific primers were used to identify human *NGF* and are detailed in Table 5.1. The PCR programme used was as follows: samples were denatured at 95°C for 2 minutes; samples were then heated to 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds for 35 cycles. A final extension step at 72°C for 15 minutes completed the programme and amplified a PCR product of 167 bp.

#### ***BDNF***

Specific primers were used to identify human *BDNF* and are detailed in Table 5.1. The PCR programme used was as follows: samples were denatured at 95°C for 2 minutes; samples were then heated to 95°C for 30 seconds, 50°C for 30 seconds, and



72°C for 45 seconds for 35 cycles. A final extension step at 72°C for 15 minutes completed the programme and amplified a PCR product of 222 bp.

### ***NT3***

Specific primers were used to identify human *NT3* and are detailed in Table 5.1. The PCR programme used was as follows: samples were denatured at 95°C for 2 minutes; samples were then heated to 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds for 35 cycles. A final extension step at 72°C for 15 minutes completed the programme and amplified a PCR product of 334 bp.

### ***NT4***

Specific primers were used to identify human *NT4* and are detailed in Table 5.1. The PCR programme used was as follows: samples were denatured at 95°C for 2 minutes; samples were then heated to 95°C for 30 seconds, 57°C for 30 seconds, and 68°C for 45 seconds for 35 cycles. A final extension step at 68°C for 15 minutes completed the programme and amplified a PCR product of 400 bp.

### ***TRKA***

Specific primers were used to identify human *TRKA* and are detailed in Table 5.1. The PCR programme used was as follows: samples were denatured at 95°C for 2 minutes; samples were then heated to 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds for 35 cycles. A final extension step at 72°C for 15 minutes completed the programme and amplified a PCR product of 229/247 bp.

### ***TRKB***

Specific primers were used to identify full-length human *TRKB* and are detailed in Table 5.1. The PCR programme used was as follows: samples were denatured at 95°C for 2 minutes; samples were then heated to 95°C for 30 seconds, 57°C for 30 seconds, and 68°C for 45 seconds for 35 cycles. A final extension step at 68°C for 15 minutes completed the programme and amplified a PCR product of 525 bp.

### ***trTRKB***

Specific primers were used to identify human *trTRKB* and are detailed in Table 5.1. The PCR programme used was as follows: samples were denatured at 95°C for 2 minutes; samples were then heated to 95°C for 30 seconds, 57°C for 30 seconds, and 68°C for 45 seconds for 35 cycles. A final extension step at 68°C for 15 minutes completed the programme and amplified a PCR product of 430 bp.

### ***TRKC***

Specific primers were used to identify human *TRKC* and are detailed in Table 5.1. The PCR programme used was as follows: samples were denatured at 95°C for 2 minutes; samples were then heated to 95°C for 30 seconds, 57°C for 30 seconds, and 68°C for 45 seconds for 35 cycles. A final extension step at 68°C for 15 minutes completed the programme and amplified a PCR product of 228/204 bp and an extra band of 300bp. This band persisted despite changing the PCR conditions so a gel extraction technique was used to confirm the separate sequences for each band.

Samples were run on a 1.5% agarose gel and the resulting 3 bands cut out. GenElute Agarose Spin Columns were used to extract the DNA. Briefly, the columns were

prewashed with 100 µl of H<sub>2</sub>O and centrifuged for 5 – 10 seconds at maximum speed. The elute was discarded and a gel slice loaded onto the column and centrifuged at maximum speed for 10 seconds. The DNA yield was measured using a spectrophotometer and then products were directly sequenced using the method described.

### ***P75***

Specific primers were used to identify human *P75* and are detailed in Table 5.1. The PCR programme used was as follows: samples were denatured at 95°C for 2 minutes; samples were then heated to 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds for 35 cycles. A final extension step at 72°C for 15 minutes completed the programme and amplified a PCR product of 489 bp.

**Table 5.1** Table listing the sequences for specific primers for human neurotrophins and their receptors. Human sequences used: S3 = Accession no. NM002506; S4 = Accession no. X91251; S5 = Accession no. NM002527; S6 = Accession no. NM006179; S7 = Accession no. NM002529; S8 = Accession no. NM006180; S9 = Accession no. AF508964; S10 = Accession no. NM002530; S11 = Accession no. NM002507

<b>Gene</b>	<b>Primer</b>	<b>Sequence (5'-3')</b>	<b>Product size (bp)</b>
<i>NGF</i> S3	Forward	TAAAAAGCGGCGACTCCGTT	167
<i>NGF</i> S3	Reverse	ATTCGCCCTGTGGAAGATG	167
<i>BDNF</i> S4	Forward	AACAATAAGGACGCAGCATT	222
<i>BDNF</i> S4	Reverse	TGCAGTCTTTTTGTCTGCCG	222
<i>NT3</i> S5	Forward	TACGCGGAGCATAAGAGTCAC	334
<i>NT3</i> S5	Reverse	GGCACACACACAGGACGTGTC	334
<i>NT4</i> S6	Forward	CTTTCGGGAGTCAGCAGGTGC	400
<i>NT4</i> S6	Reverse	CAGGCAGTGTC AATTCGAATCC	400
<i>TRKA</i> S7	Forward	TCTTCACTGAGTTCCTGGAG	229/247- spliced variants
<i>TRKA</i> S7	Reverse	TTCTCCACCGGGTCTCCAGA	229/247- spliced variants
<i>TRKB</i> S8	Forward	CATGTTACCAATCACACGGAGTACC	525
<i>TRKB</i> S8	Reverse	GGATCTTGGTCATTCCAATAATGAC	525
<i>trTRKB</i> S9	Forward	CATGTTACCAATCACACGGAGTA	430
<i>trTRKB</i> S9	Reverse	CCATCCAGTGGGATCTTATGAAA	430
<i>TRKC</i> S10	Forward	CATCCATGTGGAATACTACC	228/204 - spliced variants
<i>TRKC</i> S10	Reverse	TGGGTCACAGTGATAGGAGG	228/204 - spliced variants
<i>P75</i> S11	Forward	CTGGACAGCGTGACGTTCTCC	489
<i>P75</i> S11	Reverse	CTGCCACCGTGCTGGCTATGA	489

### ***In situ* hybridization for *NT4***

Riboprobes were generated using a PCR strategy to incorporate SP6 or T7 phage promoter sequences into *NT4* PCR product (Section 2.37). In situ hybridisation was then performed for *NT4* (Section 2.38).

### **Immunohistochemistry**

Immunohistochemistry was performed on paraffin embedded sections (Section 2.4) to localise the expression of NGF, NT3, NT4, the receptors TRKB and P75 and BrdU.

### **NGF**

Non-specific binding was eliminated by pre-blocking with normal swine serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The rabbit polyclonal primary antibody was added at 1: 30 (in diluted swine serum, overnight at 4°C) and secondary swine anti-rabbit biotinylated IgG antibody added at 1: 500 (in dilute swine serum, for 30 minutes at 23°C). A NGF peptide block was used as a negative control. The peptide block (concentration 200µg/ml ) was added to the antibody in a ratio of 10:1. This solution of peptide and antibody was then diluted in swine serum to a concentration of 1:30 and incubated overnight at 4 degrees.

### **NT3**

Non-specific binding was eliminated by pre-blocking with normal swine serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The rabbit polyclonal primary antibody was added at 1: 50 (in diluted swine serum, overnight at 4°C) and secondary swine anti-rabbit biotinylated IgG antibody added at 1: 500 (in dilute swine serum, for 30 minutes at 23°C).

### **NT4**

Non-specific binding was eliminated by pre-blocking with normal swine serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The rabbit polyclonal primary antibody was added at 1: 100 (in diluted swine serum, overnight at 4°C) and secondary swine anti-rabbit biotinylated IgG antibody added at 1: 500 (in dilute swine serum, for 30 minutes at 23°C).

### **TRKB**

Several TRKB antibodies were used on both fetal ovaries and testes. After dewaxing and rehydrating, antigen retrieval was carried out. The tissue was immersed in boiling 0.01M citrate buffer pH 6.0 for 2.5 mins and then left to stand in the buffer for 10 minutes. Non-specific binding was eliminated by pre-blocking with normal swine serum in the case of TRKB (1) and TRKB (3) and donkey serum in the case of TRKB (2) (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. Primary antibodies were added at serial dilutions of 1: 25, 1:50 and 1:100 (in diluted donkey or swine serum, overnight at 4°C). The secondary swine anti-rabbit and donkey anti-chicken biotinylated IgG antibodies for TRKB (1) and (3) and

TRKB (2) respectively were added at a dilution of 1: 500 (in dilute donkey or swine serum, for 30 minutes at 23°C).

### **P75**

After dewaxing and rehydrating, antigen retrieval was carried out. The tissue was immersed in boiling 0.01M citrate buffer pH 6.0 for 5 mins and then left to stand in the buffer for 20 minutes. Non-specific binding was eliminated by pre-blocking with normal rabbit serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The mouse monoclonal primary antibody was added at dilutions of 1: 25 to ovarian tissue and 1:50 to testicular tissue (in diluted rabbit serum, overnight at 4°C). Secondary rabbit anti-mouse biotinylated IgG antibody was added at 1: 500 (in dilute rabbit serum, for 60 minutes at 23°C).

### **BrdU**

After dewaxing and rehydrating, antigen retrieval was carried out. The tissue was immersed in boiling 0.01M citrate buffer pH 6.0 for 2.5 mins and then left to stand in the buffer for 10 minutes. Non-specific binding was eliminated by pre-blocking with normal rabbit serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at 23°C. The mouse monoclonal primary antibody was added at a dilution of 1: 30 to sections of cultured tissue (in diluted rabbit serum, overnight at 4°C). Secondary rabbit anti-mouse biotinylated IgG antibody was added at 1: 500 (in dilute rabbit serum, for 60 minutes at 23°C).



## **Immunoblotting**

Protein was extracted from snap frozen fetal ovaries and immunoblotting performed for NT4, TRKB and P75 (Sections 2.51-2.53).

## **Ovary Culture**

Ovaries from 5 fetuses ranging from 13 to 16 weeks gestation were dissected free of adherent tissues using sterile technique, bisected longitudinally and then cut into slices ~0.5mm thick. Samples of fresh tissue were fixed for histological analysis. The remaining tissue fragments were cultured for 48 hours in (i) control media, (ii) control media + basic fibroblastic growth factor (bFGF) (iii) media containing K252a, the neurotrophin tyrosine kinase receptor inhibitor (Section 2.23), and (iv) media containing K252a + bFGF. At the end of the culture period, tissue was fixed for 1 hr in Bouins fluid and then transferred to 70% ethanol before embedding in paraffin wax for histological analysis. The tissue was sectioned at 5  $\mu$ m thickness and using immunohistochemistry, stained for BrdU .

## **Analysis of cultured tissue**

Sections of uncultured control and cultured tissue were analysed to investigate the effects of culture and of K252a and bFGF on the number and proliferation of germ cells present (Section 2.24).

## 5.3 Results

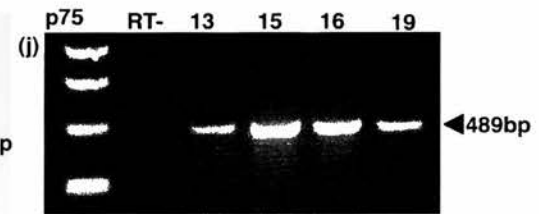
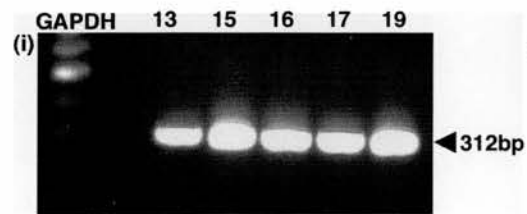
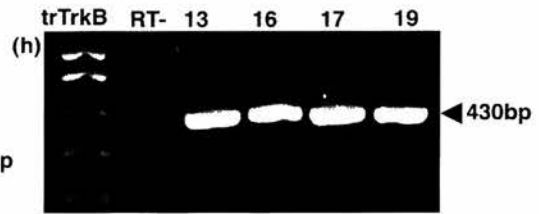
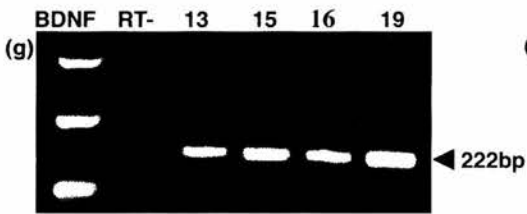
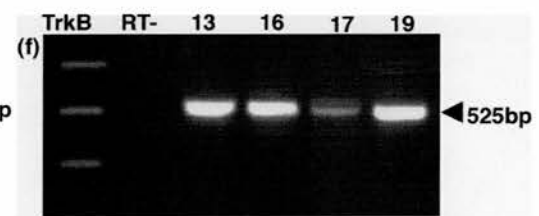
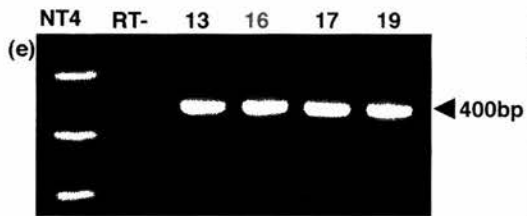
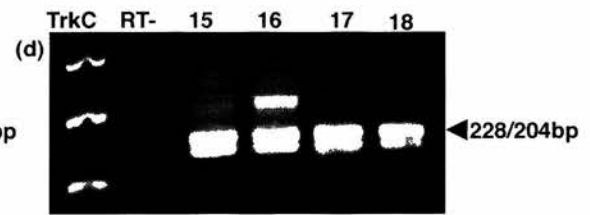
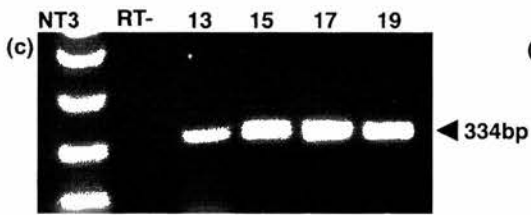
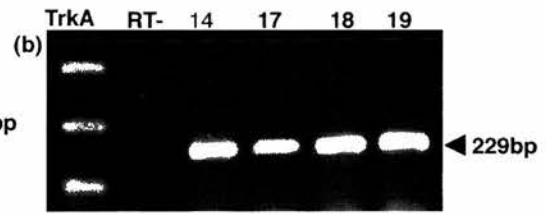
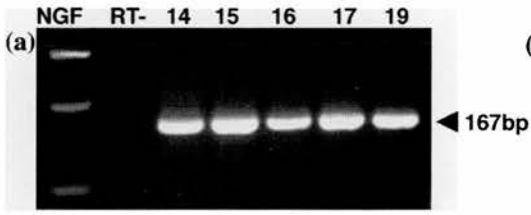
### RT-PCR

After RT-PCR, cDNA was amplified from RNA extracted from fetal ovaries for each of the neurotrophins, *NGF*, *NT3*, *NT4*, and *BDNF* (Fig. 5.1). cDNA for each of the receptors, *TRKA*, full-length *TRKB*, the truncated form of *TRKB*, *TRKC* and *P75*, were also identified (Fig. 5.1). Positive results were found at all gestations examined for each of these neurotrophins and their receptors over the range 13–21 wk. Products of 228 and 204 bp were detected for *TRKC* (Fig. 5.1D), representing spliced variants of the gene (Labouyrie et al 1999), both confirmed by direct sequencing. In some samples an additional PCR product of 300 bp was detected; sequence analysis showed that it was unrelated to the tyrosine kinase receptor family.

### *In situ* hybridization for *NT4*

Hybridization histochemistry demonstrated that the germ cells were the predominant site of expression of *NT4* mRNA within the ovary before the formation of primordial follicles (Fig. 5.2 A and B). The pattern of expression clearly demarcated the ovarian stroma, within which some cells showed *NT4* mRNA expression, from the more uniformly and intensely stained clusters of oogonia (Fig. 5.2A). No marked change in the level or pattern of expression was detected before the formation of primordial follicles. However, at that stage of development, intense staining was detected in the flattened granulosa cells surrounding the enlarged oocytes (Fig. 5.2C), in which *NT4* expression was reduced, but still present. Less mature oogonia, situated more peripherally in the ovary, continued to express *NT4* mRNA as in the earlier gestations examined. The cells of the ovarian surface epithelium did not express *NT4* mRNA.

**Figure 5.1** Expression of mRNA for neurotrophins and their receptors in human fetal ovary. RT-PCR analysis of samples extracted from whole ovaries obtained from 13 to 21 weeks gestation, as indicated above each panel. Panels **(a)-(h)** and **(j)** show mRNA expression for various neurotrophins and their receptors as labelled. In panel **(d)** products of 228 and 204 bp size were detected for TrkC representing spliced variants of the gene and in some samples an additional PCR product of 300 bp was detected: sequence analysis showed that it was unrelated to the tyrosine kinase receptor family. Panel **(i)** demonstrates GAPDH expression in these samples. Lanes marked RT- contained samples in which the reverse transcriptase was not included.



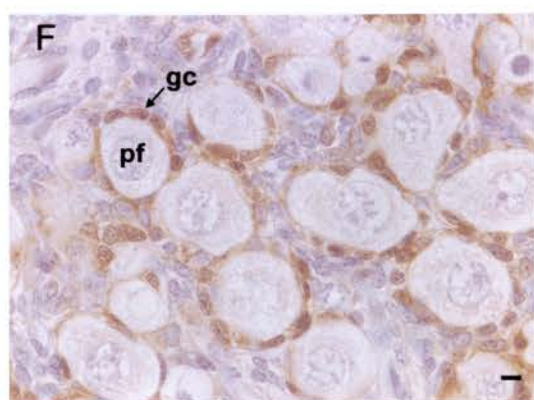
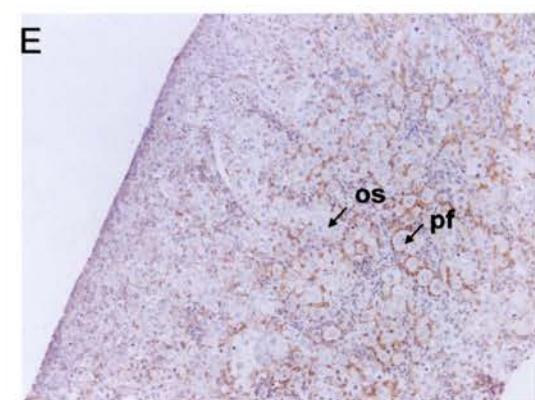
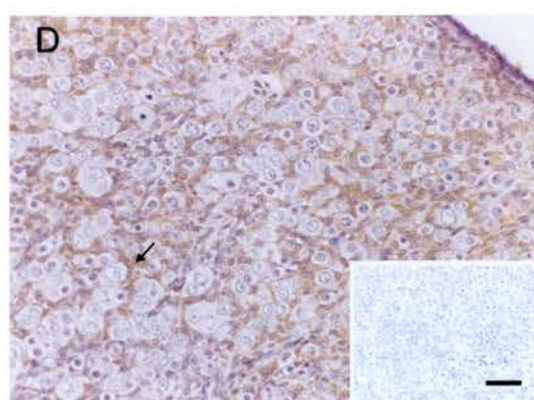
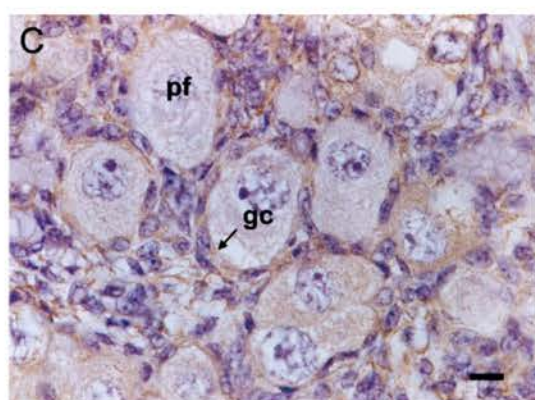
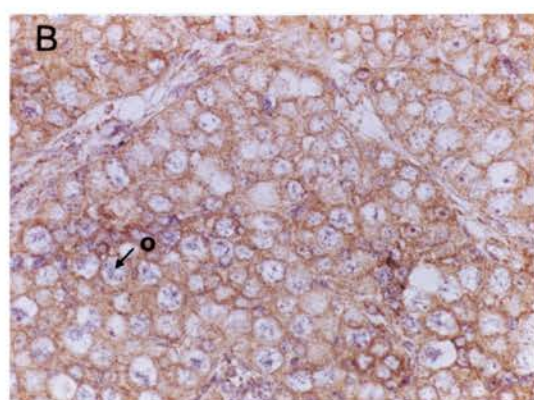
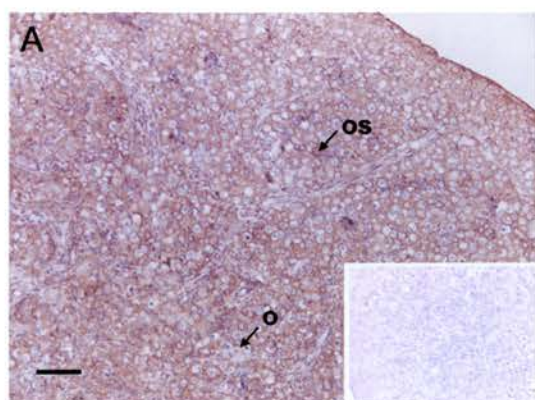
### **Immunohistochemical localization of NT4, TrkB, and P75**

Expression of NGF, NT3, NT4, TrkB, and P75 proteins was detected by immunohistochemistry in all specimens examined across the gestational range 13–21 wks. Oogonia showed weak cytoplasmic immunostaining for NT4 at all gestations (Fig. 5.2 D–F), but marked staining was detected in epithelioid cells among and immediately surrounding the nests of germ cells, consistent with early differentiation of pregranulosa cells (Fig. 5.2D). This pattern strikingly outlined the germ cells, both individually and in clusters. Generally, cells of the ovarian stroma showed only slight staining, although individual cells at the edge of the stroma, thus in close proximity to oogonial clusters, showed more marked staining (Fig. 5.2D). The most intense expression of NT4 protein was, however, seen in the cytoplasm of flattened granulosa cells of primordial follicles at later gestations. This pattern of staining very clearly demarcated formed primordial follicles predominantly located in the medullary region of the ovary from the more peripheral oogonia; thus, a medullary/cortical gradient was observed (Fig. 5.2 E and F). The pattern of distribution of NT4 protein therefore differed from that of *NT4* mRNA at earlier gestations, but was similar once primordial follicles were formed. No staining of the ovarian surface epithelium was detected. NGF protein was found in both stromal cells and oogonia (Fig 5.3A) throughout the gestations examined. The ovarian epithelium also expressed NGF. NT3 was localised to stromal cells and clearly seen in the cell streams (Fig 5.3C). At later gestations granulosa cells were also found to express NT3 (Fig 5.3D). Using an antibody specific to full-length TRKB, the protein was immunolocalized to the cytoplasm of ovarian germ cells across the gestational range examined (Fig. 5.4A and B). In particular, clear staining of the cytoplasm of

oocytes in primordial follicles was observed (Fig. 5.4B), with faint staining of the cytoplasm of pregranulosa cells.

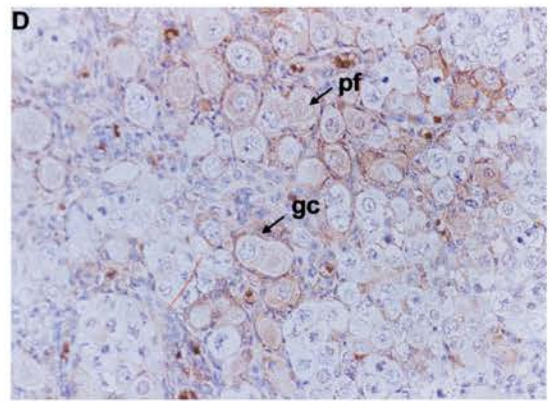
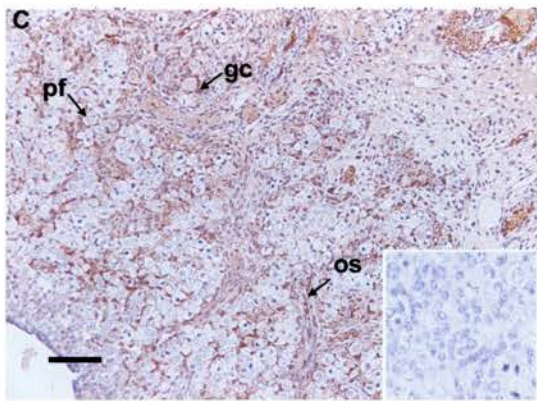
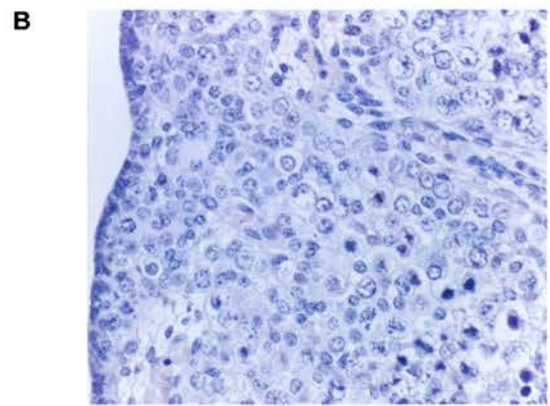
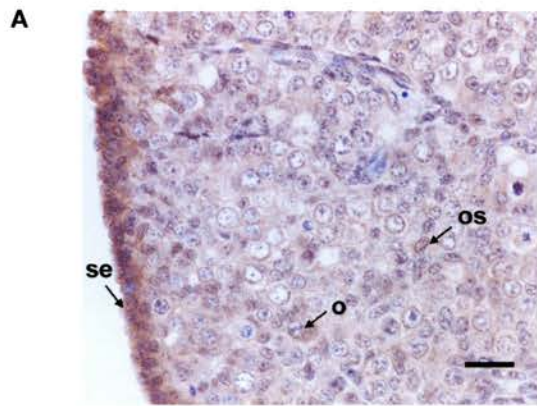
The distribution of expression of P75 protein was very different. P75 was predominantly localized to cells of the ovarian stroma at all gestations examined (Fig. 5.4C and D), thus clearly demonstrating the branching pattern of the stroma from the medulla toward the surface of the ovary, surrounding, but not becoming intermixed with, the clusters of oogonia. P75 immunostaining was not detected in germ cells at any gestation examined. In contrast to the expression of NT4, the flattened granulosa cells of primordial follicles clearly did not express P75, although it was present in the immediately adjacent cells of the ovarian stroma (Fig. 5.4C and D). P75 was not expressed by the ovarian surface epithelium.

**Figure 5.2** *In situ* hybridisation and immunolocalisation of NT4 in the human fetal ovary: **(A)** localisation of *NT4* mRNA expression in a 16 week ovary (antisense probe). Inset shows results for sense RNA probe; **(B)** *NT4* mRNA expression in 16 week ovary at higher magnification; **(C)** *NT4* mRNA localisation in 21 week gestation ovary. Immunohistochemistry: **(D)** 13 week ovary stained for NT4, arrow indicates positively stained stromal cells; inset representative section of fetal ovary omitting primary antibody **(E)** 21 week ovary stained for NT4; **(F)** 21 week ovary at higher magnification, stained for NT4. Positive staining in all panels is brown, and sections are counterstained with haematoxylin. Labels: Oo, oogonia; o, oocyte; pf, primordial follicle; gc, granulosa cell; os, ovarian stroma. Scale bar in A represents 1000 microns and applies to E; scale bar in D insert represents 250 microns and applies to D and A insert; scale bar in C represents 50 microns and applies to B; scale bar in F represents 100 microns.

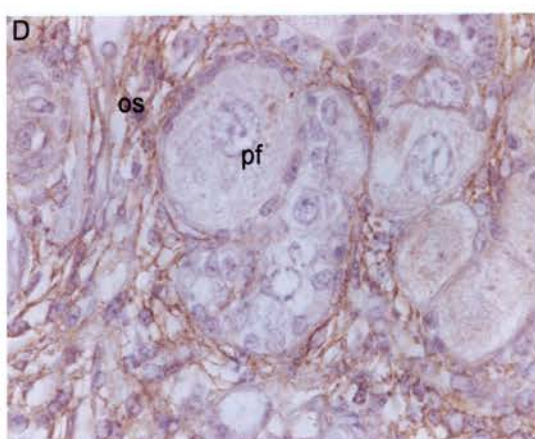
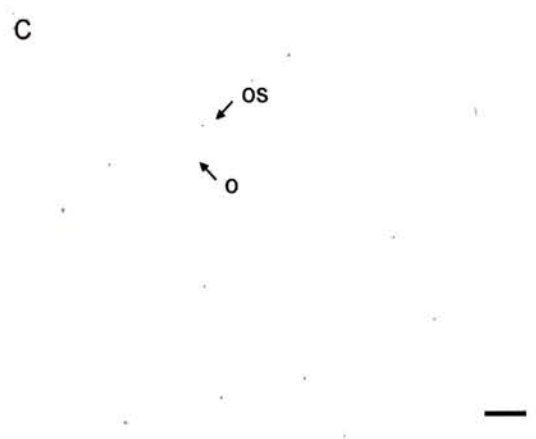
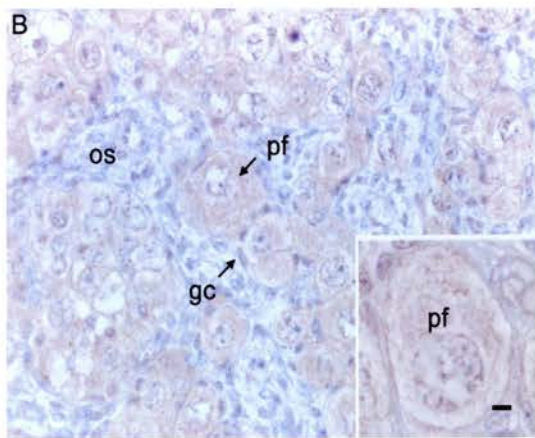
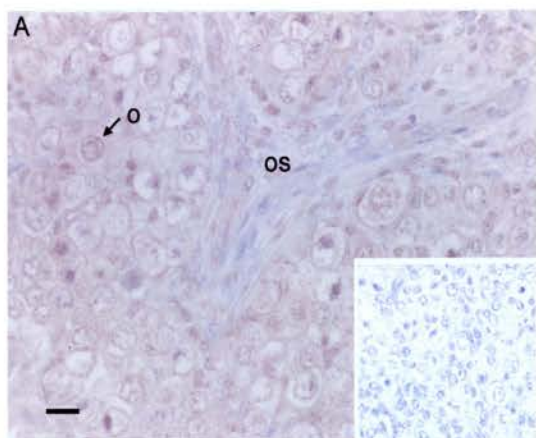




**Figure 5.3** Immunohistochemical localisation of NGF and NT3 in the human fetal ovary: **(A)** 14 week ovary stained for NGF; **(B)** 14 week ovary treated with peptide block against NGF **(C)** 16 week ovary stained for NT3, inset negative control for NT3; **(D)** 21 week ovary stained for NT3 at higher magnification. Positive staining in all panels is brown, and sections are counterstained with haematoxylin. Labels: o, oocyte; pf, primordial follicle; gc, granulosa cell; os, ovarian stroma; se, surface epithelium. Scale bar in A represents 250  $\mu\text{m}$  and applies to B, C inset and D. Scale bar in C represents 500  $\mu\text{m}$ .



**Figure 5.4** Immunohistochemical localisation of TRKB and P75 in the human fetal ovary **(A)** 18 week ovary stained for TRKB, A inset shows representative section of fetal ovary omitting primary antibody; **(B)** 21 week ovary stained for TRKB, inset shows same ovary at higher magnification; **(C)** 17 week ovary stained for P75; **(D)** 21 week ovary stained for P75 at higher magnification; Positive staining in all panels is brown, and sections are counterstained with haematoxylin. Labels: o, oocyte; pf, primordial follicle; gc, granulosa cell; os, ovarian stroma. Scale bar in A represents 250 microns and applies to B and A inset; scale bar in B inset represents 100 microns and applies to D; scale bar in C represents 1000 microns.

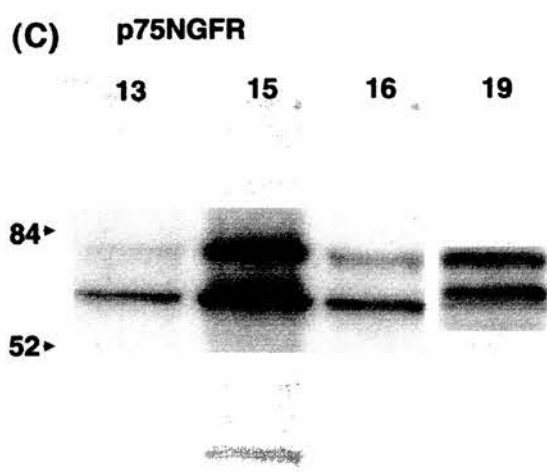
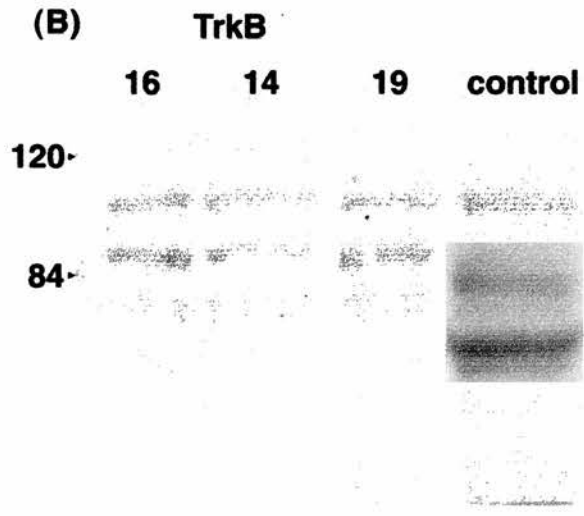
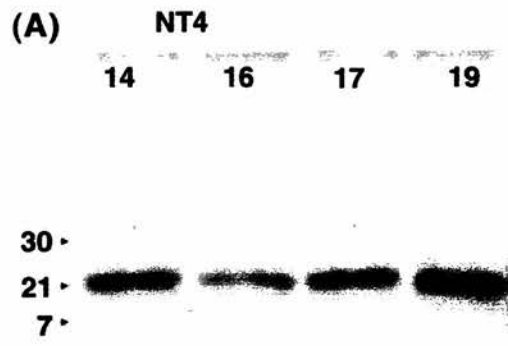


## **Immunoblotting**

The presence of NT4, TRKB, and P75 proteins in the fetal ovary was confirmed by immunoblotting. NT4 protein expression was detected as a single band of 21 kDa (Fig. 5.5A), also observed in the positive control (rat cerebral cortex). Two immunoreactive bands of 110 and 95 kDa were observed for TRKB (Fig. 5.5B). As the antibody specifically detects full-length TRKB, these bands may represent variously glycosylated forms of the full-length molecule (Klein et al 1990), rather than the truncated form. A band of 110 kDa was also detected in the positive control of rat cerebral cortex. Differences in lower mol wt forms may reflect differences in the pattern of glycosylation between the two tissues (Fig. 5.5B). A prominent 75-kDa band representing the P75 protein was observed (Fig.5.5C). An additional 65-kDa band was also detected, representing another form of the molecule as detected by others, (Russo et al 1999; Scharfmann et al 1994). A similar band was detected in the positive control tissue. The intensity of expression was comparable among the samples used (13–21 wk gestation) for the three proteins, although insufficient samples were available for rigorous quantitative analysis. A negative control was also performed for all three proteins by omitting the primary antibody, and in all cases immunoreactivity was abolished.

**Figure 5.5** Western blot of NT4, TRKB and P75 NGFR in human fetal ovary.

Total protein extracts (20 $\mu$ g) from whole ovaries at 13-21 weeks gestation as indicated were separated by SDS-PAGE, transferred to PVDF membrane and incubated with anti-NT4, -TRKB and -P75 antibodies. **(A)** NT4: a protein band which migrated with apparent molecular size of 21 kDa size is indicated. **(B)** TRKB: protein bands of apparent molecular size 110 kDa and 95 kDa are indicated, representing two forms of full length TRKB. **(C)** P75: protein band of apparent molecular size 75 kDa, and an additional band of approximately 65 kDa. Control tissue was rat cerebral cortex in each case. Positions of molecular weight markers are indicated. No immunoreactive bands were detected in the absence of primary antibody.



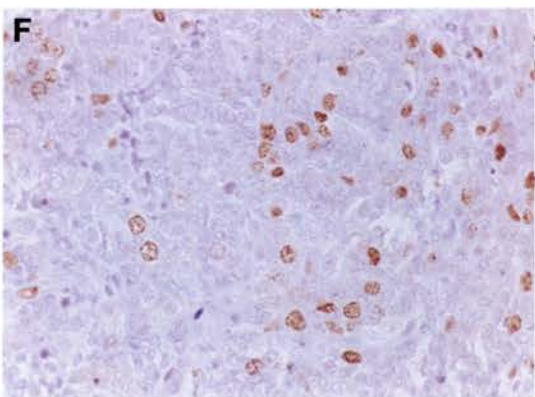
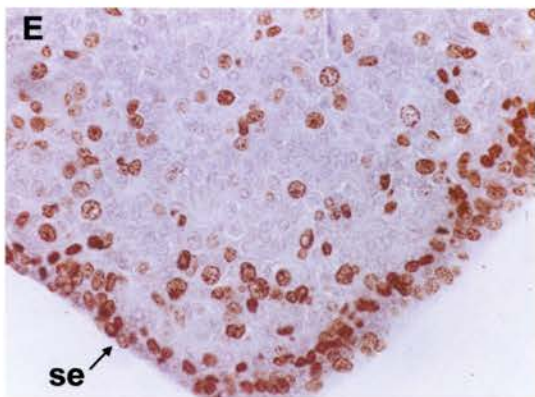
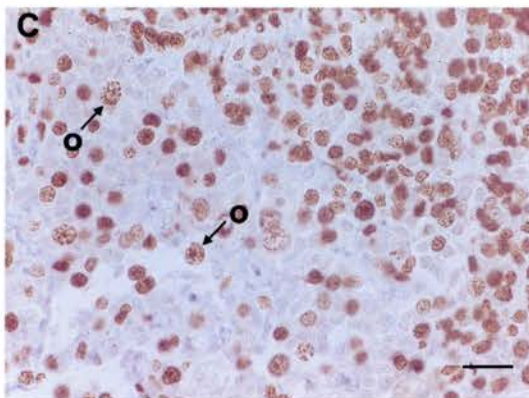
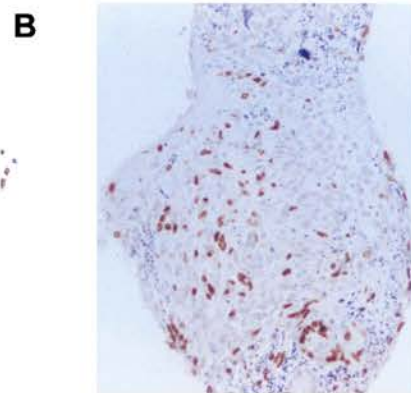
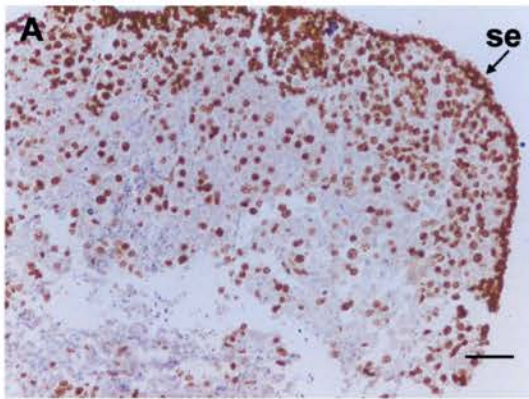
## Ovarian culture

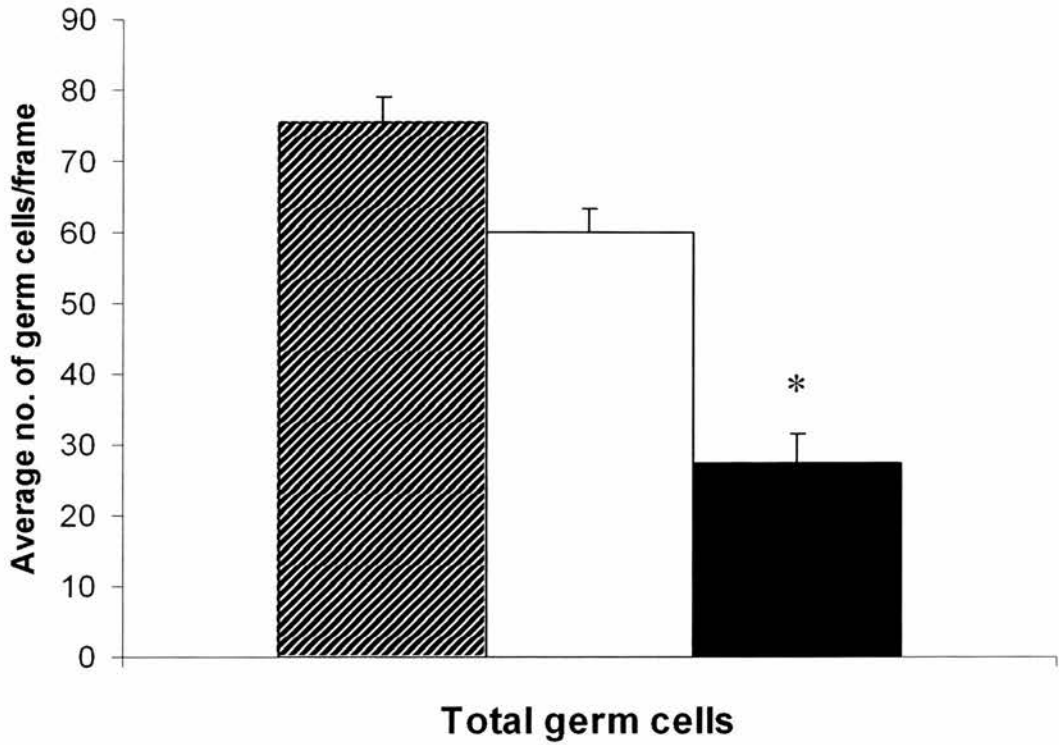
After 48 hours of culture there was a small decrease in germ cell number in comparison to the time 0 samples which was not significant ( $p=0.13$ ) (Fig 5.7). K252a treatment resulted in a marked decrease in germ cell number from  $59.9\pm 3.4$  to  $27.6 \pm 4.2$  per grid ( $p=0.01$ ) (Figs 5.6, 5.7). Consistent with this 54% decrease in germ cells, there was a significant fall in the number of proliferating germ cells (ie: BrdU positive cells) in samples treated with K252a in comparison to the control samples (Figs 5.6, 5.8). Proliferating germ cell numbers fell by 66% between the two groups, from  $12.8\pm 2.3$  to  $4.3\pm 1.5$  per grid ( $p=0.001$ ).

In the 3 of the 5 experiments we added FGF to some of the tissue during culture to act as a control for any effect of K252a on other tyrosine kinase receptors. A very small fall in germ cell number was seen in tissue cultured in FGF compared to control tissue (Fig 5.6, Fig 5.9) ( $p=0.5$ ) and a small increase was noted in germ cell number between tissue treated with K252a and that with K252a and FGF (Figs 5.6, 5.9) ( $p=0.7$ ). Similarly there was little effect of FGF on cell proliferation (Figs 5.6, 5.10). There was small increase of 11% in the total number of germ cells in tissue cultured with FGF compared to tissue cultured in control media alone ( $p=0.6$ ). In contrast there was a small decrease in the number of proliferating germ cells in tissue cultured in K252a and FGF compared to that treated with K252a alone, from  $3.87\pm 2.76$  to  $3.04\pm 0.96$ . This did not reach statistical significance ( $p=0.7$ ). Although not quantified, some stromal cell and particularly surface epithelial cell proliferation was noted in the cultures (Fig 5.6).

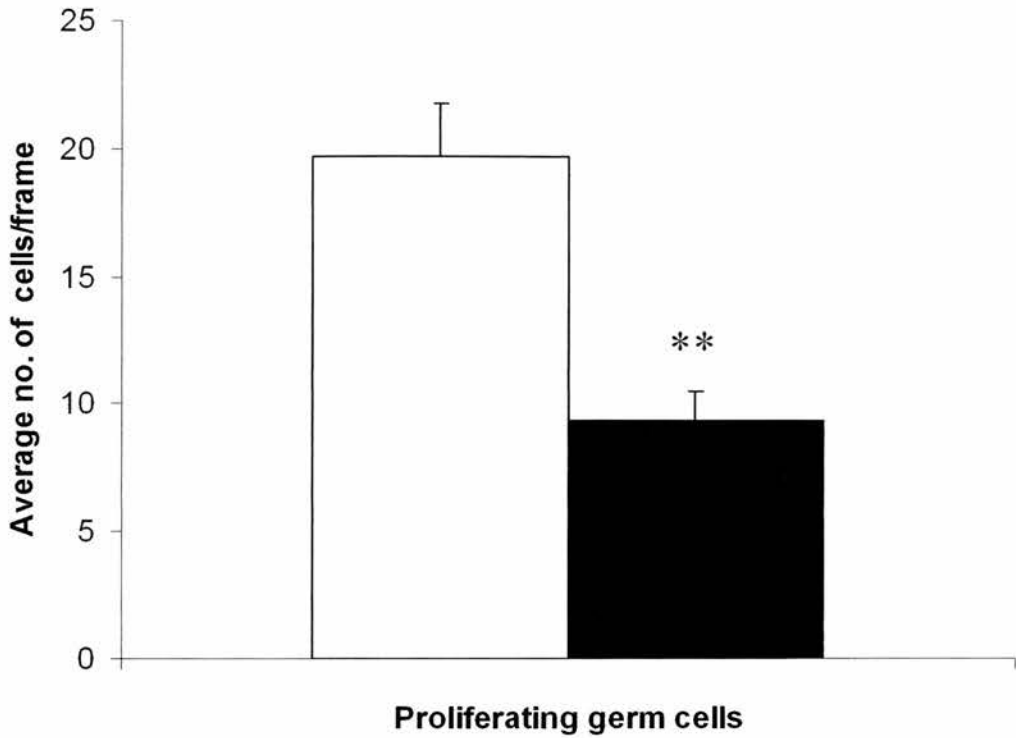


**Figure 5.6.** Immunohistochemical staining for BrdU of a 13 weeks human fetal ovary, marking proliferating cells **(A)** control sample; **(B)** sample cultured in K252a; **(C)** control sample at higher magnification; **(D)** sample cultured in K252a at higher magnification; **(E)** control sample cultured with FGF; **(F)** sample cultured in K252a and FGF. Positive staining in all panels is brown, and sections are counterstained with haematoxylin. Labels: o, oocyte; se, surface epithelium. Scale bar in A represents 500  $\mu\text{m}$  and applies to B; scale bar in C represents 250  $\mu\text{m}$  and applies to D, E and F.

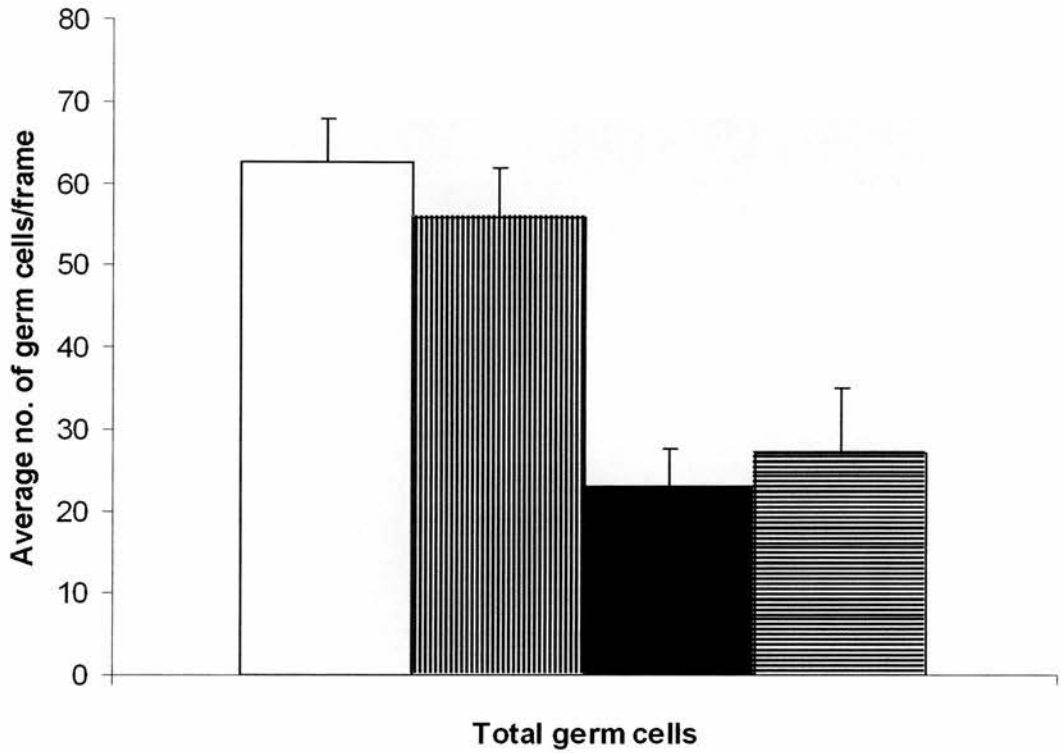




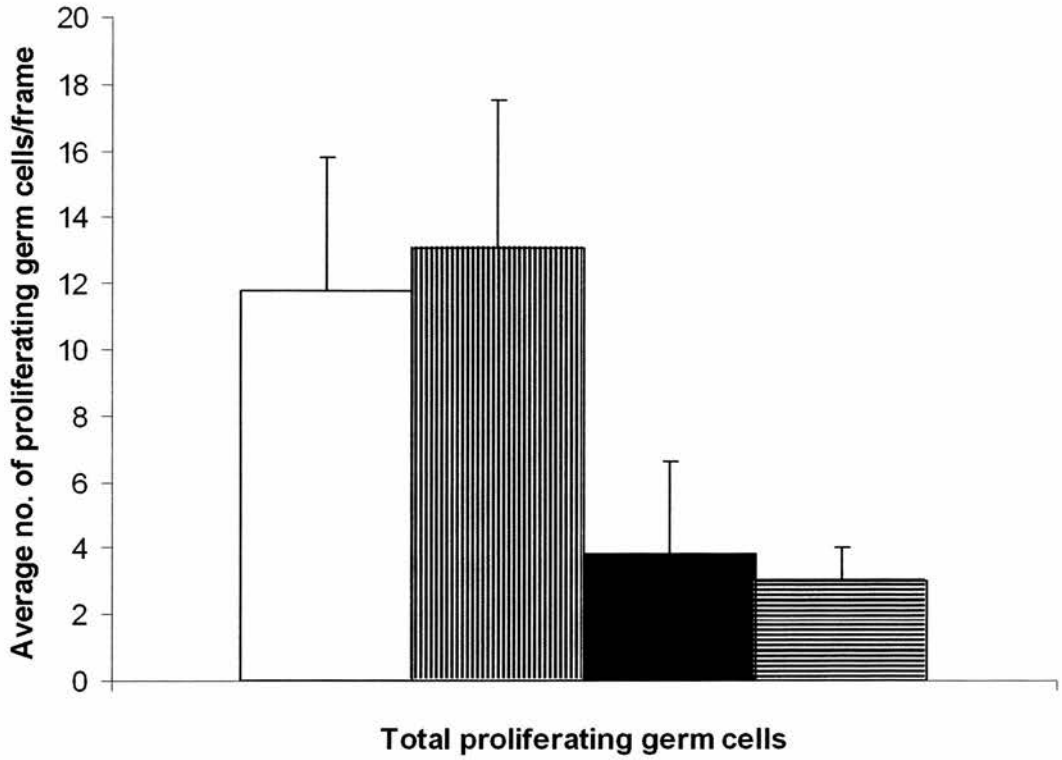
**Figure 5.7** Number of germ cells present per frame in the human fetal ovaries (13-16 wks.) in fresh and cultured tissue. The shaded columns represent uncultured, time 0 samples, the white columns represent control samples cultured for 48 hours and the black columns represent tissue treated in culture with K252a neurotrophin receptor inhibitor. Mean  $\pm$ sem, n=5, \*  $p < 0.05$  vs control 48 hr cultures.



**Figure 5.8** Number of proliferating (BrdU immunopositive) germ cells present per frame in the human fetal ovary (13-16 wks.) after 48 hours of culture. The white columns represent control samples and the black columns represent tissue treated with K252a neurotrophin receptor inhibitor. Mean  $\pm$ sem, n=5. \*\* p < 0.005 vs control cultures.



**Figure 5.9** Number of germ cells present per frame in the human fetal ovaries (13-16 wks.) in cultured tissue. The white column represents control samples cultured for 48 hours; the vertically shaded column represents tissue cultured in control media and FGF; the black column represents tissue cultured in K252a; the horizontally shaded column represents tissue cultured in K252a and FGF. Mean  $\pm$ sem, n=3.



**Figure 5.10** Number of proliferating germ cells present per frame in the human fetal ovaries (13-16 wks.) in cultured tissue. The white column represents control samples cultured for 48 hours; the vertically shaded column represents tissue cultured in control media and FGF; the black column represents tissue cultured in K252a; the horizontally shaded column represents tissue cultured in K252a and FGF. Mean  $\pm$ sem, n=3.

## 5.4 Discussion

The present results demonstrate the expression of all neurotrophins and both high affinity TRK receptors and the non selective P75 receptor within the developing human ovary. In addition, blockade of high-affinity neurotrophin receptors using K252a markedly decreases germ cell survival and proliferation. These data therefore indicate that neurotrophins may play a major role in ovarian development and germ cell regulation in the human, as has been suggested in the rodent.

Localization of *NT4* mRNA confirmed that the germ cells are the predominant site of expression of this neurotrophin before the formation of primordial follicles. Expression was, however, developmentally regulated, thus after the formation of primordial follicles, the predominant site of expression of *NT4* mRNA and protein was the flattened granulosa cell, whereas its cognate high affinity receptor TRKB protein was localized to the oocyte. These data therefore indicate the involvement of neurotrophins in this crucial step in ovarian development, *i.e.* in the formation of the essential structures of the ovary.

Tissue specimens were obtained after medical termination of pregnancy, induced by administration of the antigestogen mifepristone and PGE<sub>1</sub>. It is possible that the administration of these drugs, directly or indirectly, might have affected the expression of neurotrophins in the fetal ovary, in which there is some expression of steroidogenic enzymes at these gestations (Voutilainen et al 1986). Hypoxia has also been demonstrated to modify expression of neurotrophins and their receptors in several tissues (Hiltunen et al 2001; Kokaia et al 1998). Although the results presented here are in broad agreement with the limited data obtained in the

developing rodent ovary (see below), it would be appropriate to corroborate the present data in specimens obtained by other methods.

Neurotrophins, specifically NGF, were originally identified on the basis of their role in the regulation of neuronal survival (Levi-Montalcini et al 1968).

Trk-mediated cell survival requires protein synthesis, and neurotrophins are predominantly believed to act by suppression of apoptosis. Neurotrophins may also regulate cell migration (Behar et al 1997) and differentiation (Polak et al 1993). In addition to these prodevelopment roles, neurotrophins can promote cell death. This had been suggested to be a function of the p75 receptor when present without the coexistence of Trk receptors, whereas in their presence p75 promotes cell survival (Casaccia-Bonnel et al 1999). Differential effects of p75 activation on Trk receptor autophosphorylation dependent on both ligand and Trk subtype have been previously described (Vesa et al 2000). It has also been demonstrated that the immature form of NGF, termed proNGF has a greater affinity for p75 than the mature form of NGF (Lee et al 2001). There is thus the potential for complex interplay between the different receptor pathways.

There are no previous data on the presence of neurotrophins in the human ovary, but their expression has been demonstrated in the rat ovary (Dissen et al 1995; Dissen et al 2001; Timmusk et al 1993). The immediate postnatal period in the rat is the time of primordial follicle formation, and increased expression of NT4 and its high affinity receptor TrkB, but not other neurotrophins/Trk receptors, was demonstrated at that time (Dissen et al 1995). NT4 expression has been localized to the germ cell in both rodent and *Xenopus* (Dissen et al 1995). (Ibanez et al 1992) The present results



are therefore in broad agreement with the limited previous data in other species. NT4 protein was not, however, predominantly localized to the germ cells. The main site of localization was granulosa cells surrounding oocytes in primordial follicles and, at earlier developmental stages, in epithelioid stromal cells within clusters of oogonia. It appears likely that these cells are the precursors of the granulosa cells. This is similar to the pattern of development demonstrated in the rodent, where these cells are derived from the cells of the rete ovarii, which, in turn, derive from the mesonephros (reviewed in (Byskov 1986). The rete ovarii is also recognized to be of central importance in the regulation of the onset of meiosis (Byskov 1974) and of the cortical/medullary pattern in the rodent ovary (Byskov et al 1997; Hirshfield et al 1995). The neurotrophins may therefore be involved in the regulation of development of the oocyte in the human ovary at a number of levels.

A central component of the mechanism of action of neurotrophins in the nervous system is that they are produced and released by the target cell in proportion to the final innervation density, taken up by the innervating neuron, and transported to the nucleus where they mediate their pro-survival effects (Davies et al 1987; Korsching et al 1983). The predominant localization of *NT4* mRNA and protein in different cell types before primordial follicle formation is consistent with a similar pattern of secretion/uptake by germ cells and somatic cells, respectively, suggesting that NT4 is involved in the intercellular communication between these cell types. The change in pattern of expression of *NT4* mRNA, being low in the oocyte and high in the granulosa cell after primordial follicle formation, indicates that the signals mediated by this pathway may vary according to the developmental status of the germ cell/somatic cell.

Both NGF and NT3 proteins were localised within the human fetal ovary and although we did not attempt to localise it, BDNF was detected. NGF appeared to be expressed in both somatic and germ cells throughout the second trimester. This is similar to the distribution in the rat ovary (Dissen et al 1995). NT3 had a more selective distribution as it was expressed exclusively in the stromal cells and later the granulosa cell populations. The localisation of NT3 suggests it may be involved in the formation and development of primordial follicles and its presence in the stromal cells suggests a possible paracrine effect on the germ cell population. NGF is likely to be involved in somatic cell proliferation as seen in the developing rat ovary (Dissen et al 2001) which in turn affects germ cell survival (Romero et al 2002). NGF levels drop in the rodent at the time of follicular assembly (Dissen et al 1995), suggesting it is involved in earlier ovarian histogenesis and it is possible it has a similar role in the human.

The localization of TRKB to the germ cells suggests that these are a major site of NT4 action, whereas P75 was localized to the ovarian stroma and was not present in the granulosa cells of primordial follicles. The distribution of P75 in the human fetal ovary is therefore very similar to that in the neonatal rat ovary (Dissen et al 1995). The differential distribution of the several receptors may contribute to the regulation of neurotrophin signalling in the immediate environment of the forming primordial follicle. The localization of other TRK receptors has not been investigated. In the newborn rat, *TrkB* mRNA appeared to be localized to presumptive pregranulosa cells (Dissen et al 1995). Whether this is a species difference or reflects a particular developmental stage is unclear at present. Developmental changes in the requirement for specific neurotrophins during neuronal differentiation and survival have been

described (Davies 1996). It has long been recognized that oocytes must interact with somatic cells to form primordial follicles and survive (McLaren 1991). The intermingling of NT4-immunopositive epithelioid cells within the clusters of oogonia may indicate a crucial role for oogonal-derived neurotrophins in determining germ cell survival by enhancing the movement and differentiation of somatic cells and thus promoting primordial follicle development.

Three Trk receptors with high affinity for specific neurotrophins have been identified as well as the low affinity p75 receptor (Barbacid 1994; Chao 1994). Truncated isoforms of TrkB and TrkC resulting from alternative splicing have also been described (Barbacid 1994; Klein et al 1990; Strohmaier et al 1996). The present results demonstrate that mRNA for both full-length and truncated isoforms of *TRKB* is expressed in the human fetal ovary. The truncated isoforms of the Trk receptors lack the intracellular, tyrosine kinase domain, but retain the ligand-binding domain, and may be found in both neuronal and nonneuronal cell types, although the truncated form may be more abundant in the latter (Klein et al 1990; Wetmore et al 1995). Changing patterns of expression of full-length vs. truncated forms have been demonstrated during tissue repair in certain regions of the nervous system (Beck et al 1993). TrkB-truncated isoforms have recently been suggested to be involved in neurotrophin endocytosis (Alderson et al 2000) and in regulation of specific patterns of dendritic growth distinct from those mediated by the full-length isoform (Yacoubian et al 2000). Conversely, several neurotrophins and their receptors may require to be coexpressed by specific cell types (Fan et al 2000; Miller et al 2000). The relevance of Trk splice variants to ovarian development and function remains to be investigated.

In this study K252a was used to investigate the functional activity of neurotrophin signalling in ovarian tissue. K252a is an indole carbazole, widely used as a potent and selective inhibitor of the intracellular protein kinase domain of Trk receptors without affecting other serine/threonine kinases at the concentrations used here (Koizumi et al 1988; Levine et al 2000b; Tapley et al 1992). K252a blocks the activity of all high-affinity Trk receptors, so we cannot interpretate which neurotrophin(s) and receptor(s) might be involved in the effects observed. bFGF acts as a survival and mitogenic factor for germ cells (Dissel-Emiliani et al 1996; Han et al 1993; Lynch et al 2000) and although FGF acts via tyrosine kinase receptors, their actions do not appear to be inhibited by K252a. Treating ovarian tissue with K252a resulted in a decrease in germ cell number of 54% and a 66% fall in germ cell proliferation. There was no significant effect of adding bFGF to the culture, indicating that bFGF receptors are not inhibited by K252a. This data agrees with the effect of Trk receptor inhibition in the mouse and neonatal rat ovaries, where it has been shown to reduce oocytes survival and primordial follicle development (Spears 2003). Although from this data we cannot identify specific neurotrophins or receptors responsible for this effect, it seems that NT4 and TrkB are likely candidates, as indicated by the *TrkB* *-/-* mice (Spears 2003).

Although the present study does not comprehensively localize within the ovary all identified members of the neurotrophin family and their receptors, the differential localization of NGF, NT3, NT4, TRKB and P75 may indicate multiple roles for neurotrophin signalling. Indeed, neurotrophins have been implicated in several processes in the rodent ovary. Increased *NT4/TrkB* mRNA expression was associated with primordial follicle formation (Dissen et al 1995), whereas increased *NGF/TrkA*

expression was induced by the LH surge during first ovulation (Dissen et al 1996) and is involved in the regulation of intercellular gap junction integrity between thecal cells (Mayerhofer et al 1996). Increased NGF production within the ovary resulted in disruption of oestrous cyclicity (Dissen et al 2000). It therefore appears that individual neurotrophin/receptor pathways are involved in the regulation of specific intraovarian processes. Direct evidence for the importance of neurotrophins in ovarian development is scanty. Recent studies of mice with an inactivating mutation of *TrkB* are consistent with this pathway having an important role in primordial follicle formation (Spears 2003), although transgenic *NT4* knockout mice appear to be normally fertile (Liu et al 1995). The ovaries of neonatal *NGF*-knockout mice also show reduced proliferation of mesenchymal cells and reduced primordial follicle growth, suggesting that both cell populations are targets for NGF action (Dissen et al 2001), consistent with our localisation of NGF. Mice carrying a null mutation of the *p75* gene appear to have normal numbers of ovarian follicles, but this receptor may be involved in the reduction in mesenchymal cell proliferation found in the *NGF*-knockout mouse (Dissen et al 2001), as this is the site of expression of *p75*. Later stages of ovarian development in neurotrophin/*Trk* knockout models have not been studied in detail, as they are generally nonviable (Snider 1994).

These results therefore demonstrate the presence of neurotrophins and their receptors in the developing human ovary. Differential patterns of expression between cell types and developmental changes, particularly associated with primordial follicle formation, suggest multiple roles for them in the regulation of germ cell and somatic cell proliferation, survival, and differentiation. The effect of neurotrophin blockade

has revealed that neurotrophins are essential for germ cell survival and proliferation in the human fetal ovary.

**CHAPTER 6**

**EXPRESSION OF NEUROTROPHINS AND**

**THEIR**

**RECEPTORS IN THE HUMAN FETAL TESTIS**

**AND THEIR REGULATION OF GERM CELL**

**NUMBER**

## 6.1 Introduction

Development of the human fetal testis involves differentiation, maturation and proliferation of several cell types. The first sign of male gonadal development is the appearance of Sertoli cells at 6-7 weeks gestation (Byskov 1986), initiated by the expression of the testis-determining gene *Sry* (Tilman et al 2002). Following migration from the yolk sac to the nephrogonadoblastic ridge, PGCs associate with Sertoli cells to form the seminiferous cords. The peritubular cell population originates from the mesonephros. While their migration is also determined by *Sry* and is central to cord formation (Buehr et al 1993), the factors mediating these crucial intercellular signals are poorly understood (Capel et al 1999; Tilman et al 1999). At the same time there is considerable proliferation of germ cells and Leydig cells (Bendsen et al 2003; Hilscher 1991; Murray et al 2000; Voutilainen 1992). This period of development is believed to be central to the establishment of adult testicular function and is also the point at which the cellular abnormalities that later manifest as testicular malignancies may arise (Sharpe 2001). This study investigates the possibility that neurotrophins may be involved in these intercellular interactions in the developing human testis.

A number of paracrine factors controlling migration, differentiation and proliferation of PGCs and Sertoli cells have been described in the rodent, including members of the transforming growth factor  $\beta$  (TGF $\beta$ ) family (Cupp et al 1999; Dissel-Emiliani et al 1996; Levine et al 2000a; Olaso et al 1998). Neurotrophins are members of the nerve growth factor family and related to TGF $\beta$ . They regulate neuronal survival and differentiation in nervous tissue (Levi-Montalcini 1987) (Snider 1994) and their



effects are mediated via Trk and p75 receptors. Neurotrophins may also function in non-neuronal tissues (Sariola 2001), including the developing gonads of both sexes. For example several members of the neurotrophin family and their receptors have been identified in the developing rodent testis (Campagnolo et al 2001; Cupp et al 2000; Levine et al 2000b; Russo et al 1999) and roles in testicular cord formation (Levine *et al.* 2000b) have been suggested in addition to later involvement in the function and interaction of germ cells and Sertoli cells (Djakiew et al 1994; Lonnerberg et al 1992; Onoda et al 1991). Preliminary data suggest that members of the neurotrophin family and their receptors may also be expressed in the developing human testis (Cupp et al 2000). Recent studies have shown that NT4 and its cognate high-affinity receptor TrkB may be of particular importance in the interaction between germ cells and somatic cells in the developing ovary of both rodent and human (Anderson et al 2002; Dissen et al 1995; Spears 2003). In order to determine whether neurotrophins could influence development and maturation of the human fetal testis we have examined the cell-specific expression and distribution of several members of the neurotrophin family and their receptors during the second trimester, with particular emphasis on NT4 and TrkB.

## 6.2 MATERIALS AND METHODS

### Tissues

Human fetal testes (13-19 weeks gestation) were obtained following medical termination of pregnancy (Section 2.21). A total of 15 specimens were used for this study (13 weeks, 2; 14 weeks, 3; 15 weeks, 2; 16 weeks, 2; 17 weeks, 4; 19 weeks, 2). Testes were dissected free, and either fixed for immunohistochemical analysis or snap frozen and stored at  $-70^{\circ}\text{C}$ . Fixation was carried out in Bouins for 5 hours, followed by transfer to 70% ethanol prior to processing into paraffin using standard methods.

### Isolation of RNA and RT-PCR

Total RNA was extracted from snap frozen samples of fetal testis and RT-PCR performed using primers specific for human neurotrophins and their receptors (Table 6.1) (Sections 2.3, 2.31, 5.2). All products were purified (Section 2.33) and their identities confirmed by direct sequencing (Sections 2.34).

### *In situ* hybridization for *NT4*

Riboprobes were generated using a PCR strategy to incorporate SP6 or T7 phage promoter sequences into *NT4* PCR product (Section 2.37). *In situ* hybridisation was then performed for *NT4* (Section 2.38).

## **Immunohistochemistry**

Immunohistochemistry was performed on paraffin embedded sections (Section 2.4) to localise the expression of NGF, NT3, NT4, the receptors TRKB and P75, and BrdU (Section 5.2).

**Table 6.1** Sequence of primers used for detection of neurotrophins and their receptors. Human sequences used: S3 = Accession no. NM002506; S4 = Accession no. X91251; S5 = Accession no. NM002527; S6 = Accession no. NM006179; S7 = Accession no. NM002529; S8 = Accession no. NM006180; S9 = Accession no. AF508964; S10 = Accession no. NM002530; S11 = Accession no. NM002507

<b>Gene</b>	<b>Primer</b>	<b>Sequence (5'-3')</b>	<b>Product size (bp)</b>
<i>NGF</i> S3	Forward	TAAAAAGCGGCGACTCCGTT	167
<i>NGF</i> S3	Reverse	ATTCGCCCTGTGGAAGATG	
<i>BDNF</i> S4	Forward	AACAATAAGGACGCAGCATT	222
<i>BDNF</i> S4	Reverse	TGCAGTCTTTTTGTCTGCCG	
<i>NT3</i> S5	Forward	TACGCGGAGCATAAGAGTCAC	334
<i>NT3</i> S5	Reverse	GGCACACACACAGGACGTGTC	
<i>NT4</i> S6	Forward	CTTTCGGGAGTCAGCAGGTGC	400
<i>NT4</i> S6	Reverse	CAGGCAGTGTC AATTCGAATCC	
<i>TRKA</i> S7	Forward	TCTTCACTGAGTTCCTGGAG	229/247- spliced variants
<i>TRKA</i> S7	Reverse	TTCTCCACCGGGTCTCCAGA	
<i>TRKB</i> S8	Forward	CATGTTACCAATCACACGGAGTACC	525
<i>TRKB</i> S8	Reverse	GGATCTTGGTCATTCCAATAATGAC	
<i>trTRKB</i> S9	Forward	CATGTTACCAATCACACGGAGTA	430
<i>trTRKB</i> S9	Reverse	CCATCCAGTGGGATCTTATGAAA	
<i>TRKC</i> S10	Forward	CATCCATGTGGAATACTACC	228/204 - spliced variants
<i>TRKC</i> S10	Reverse	TGGGTCACAGTGATAGGAGG	
<i>P75</i> S11	Forward	CTGGACAGCGTGACGTTCTCC	489
<i>P75</i> S11	Reverse	CTGCCACCGTGCTGGCTATGA	

### **Immunoblotting**

Protein was extracted from snap frozen fetal testes and immunoblotting performed for NT4, TRKB and P75 (Sections 2.5-2.53).

### **Testis Culture**

Testes from 3 fetuses of 14, 16 and 19 weeks gestation were dissected free of adherent tissues using sterile technique, bisected longitudinally and then cut into slices ~0.5mm thick. Samples of fresh tissue were fixed for histological analysis. The remaining tissue fragments were cultured for 48 hours in control media and media containing K252a, the neurotrophin tyrosine kinase receptor inhibitor (Section 2.23). At the end of the culture period, tissue was fixed for 1 hr in Bouins fluid and then transferred to 70% ethanol before embedding in paraffin wax for histological analysis. The tissue was sectioned at 5  $\mu$ m thickness and using immunohistochemistry, stained for BrdU.

### **Analysis of cultured tissue**

Sections of uncultured control and cultured tissue were analysed to investigate the effects of culture and of K252a on the number and proliferation of gonocytes, Sertoli cells and peritubular cells present (Section 2.24).

### 6.3 Results

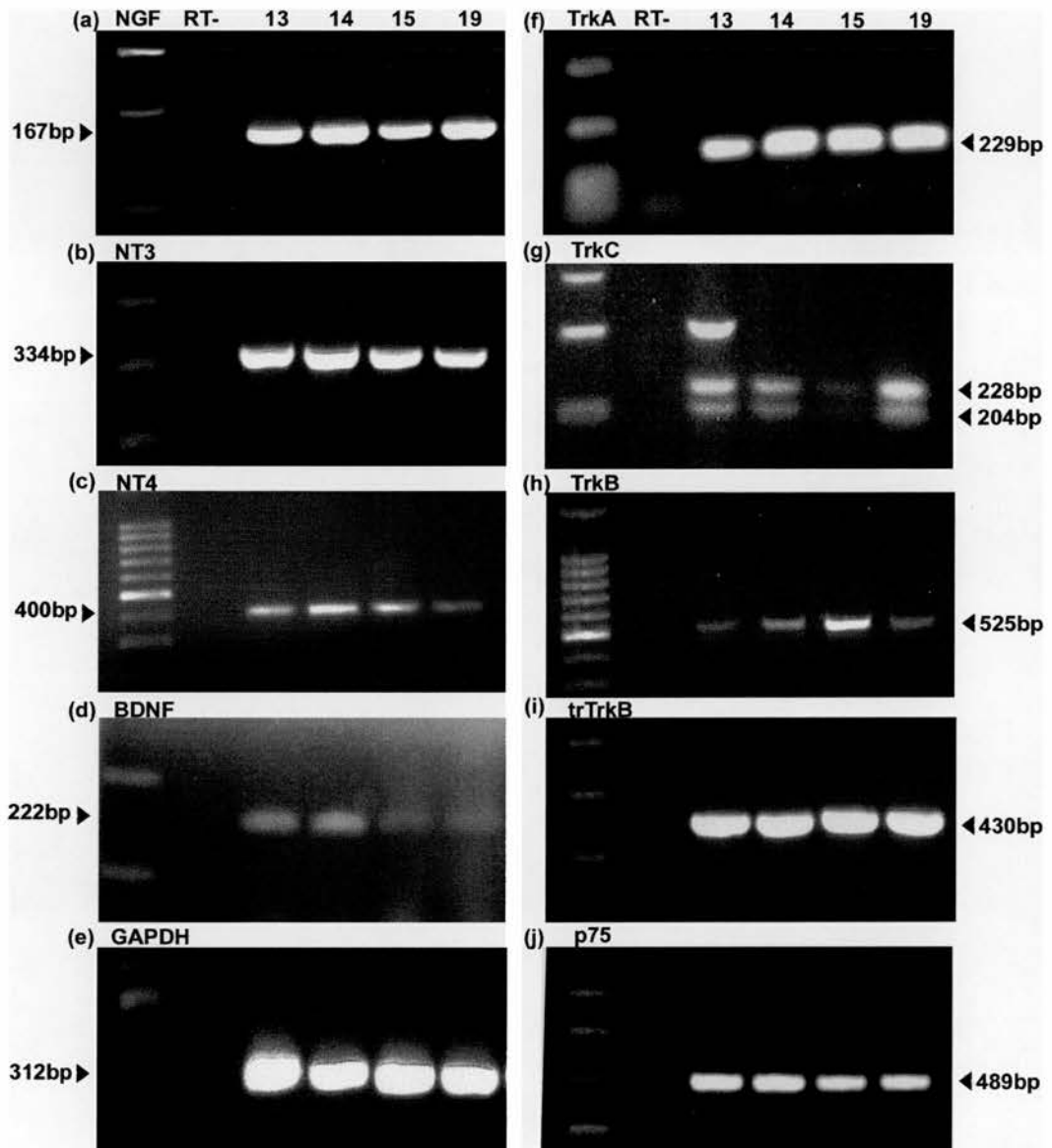
#### Expression of mRNAs for neurotrophins and their receptors

Expression of mRNAs was detected by RT-PCR using RNA extracted from fetal testes (Figure 6.1). RT-PCR for the constitutively expressed gene *GAPDH* was used to confirm the integrity of the RNA. Human fetal brain and placenta were used as positive controls. mRNA for the neurotrophins *NGF* (a), *NT3* (b), *NT4* (c) and *BDNF* (d), and the receptors *TRKA* (f), *TRKB* (h), *TRKC* (g) and *P75* (j) was detected in all specimens at all gestations examined. Both full-length and the truncated forms of *TRKB* were detected (h and i). Products of 228 and 204 bp size were detected for *TRKC* (g), representing spliced variants of the gene (Labouyrie et al 1999), confirmed by sequencing. In some samples an additional PCR product of 300 bp was noted and the sequence found to be unrelated to the tyrosine kinase receptor family.

**Figure 6.1.** Expression of mRNA for neurotrophins and their receptors in human fetal testis.

RT-PCR analysis of samples extracted from whole testes obtained from 13–19 wk gestation, as indicated above each panel. (a–d and f–j), mRNA expression for the neurotrophins and their receptors as indicated. Products of 228 and 204 bp were detected for *TRKC* (panel g) representing spliced variants of the gene, and in some samples an additional PCR product of 300 bp was detected: sequence analysis showed that it was unrelated to the tyrosine kinase receptor family. (e) *GAPDH* expression. Lanes marked RT- contained samples in which the reverse transcriptase was not included.





### ***In situ* hybridisation for NT4**

Cell specific patterns of expression of *NT4* were further investigated by non-radioactive *in situ* hybridisation on fixed tissue sections. *NT4* mRNA expression was predominantly localised to the peritubular cells (Figure 6.2A and B). *NT4* mRNA was also detected in Sertoli cells but no mRNA appeared to be expressed in gonocytes (Figure 6.2B). A low level of expression was detected in the interstitium with the exception of the endothelial cells of small blood vessels which showed more clearly positive expression. There was no apparent change in the pattern or intensity of expression of *NT4* mRNA over the gestational range examined (14-19 wk). Tissue sections incubated with the sense riboprobe showed no staining (Figure 6.2A inset) confirming the specificity of the probe.

### **Immunohistochemical localisation of neurotrophins and receptors**

Expression of NT4, NGF, NT3 and P75 proteins were detected by immunohistochemistry in all specimens examined across the gestational range 13-19 wk. Expression of NT4 was confirmed by immunolocalisation of the protein to the cytoplasm of the peritubular cells and Sertoli cells within the seminiferous tubules; gonocytes were immunonegative (Figure 6.2C and D). Very little immunostaining was noted in the interstitial cells, but endothelial cells within small blood vessels were immunopositive for NT4. The most intense immunopositive reaction for NGF was in Sertoli cells (Figure 6.3A) although some gonocytes also appeared immunopositive. Weak staining was noted in the interstitial cells (Figure 6.3A). NT3 protein was also immunolocalised mainly to the Sertoli cells with some interstitial

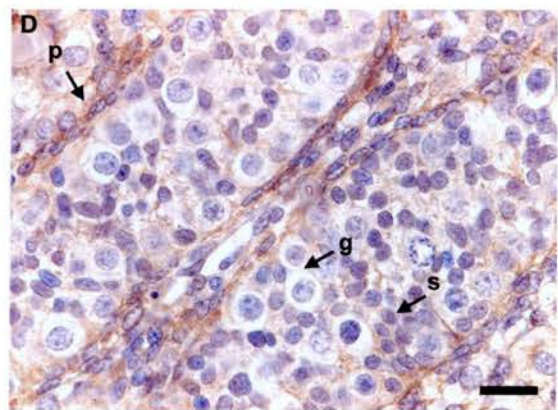
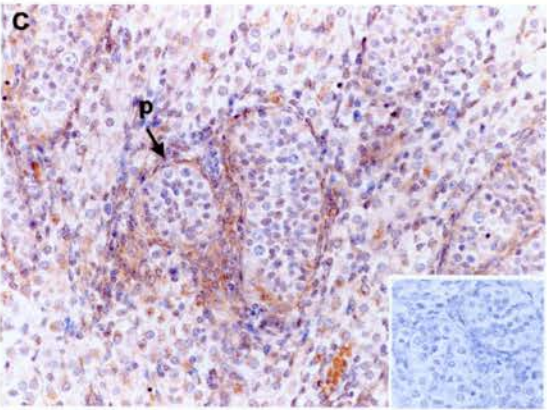
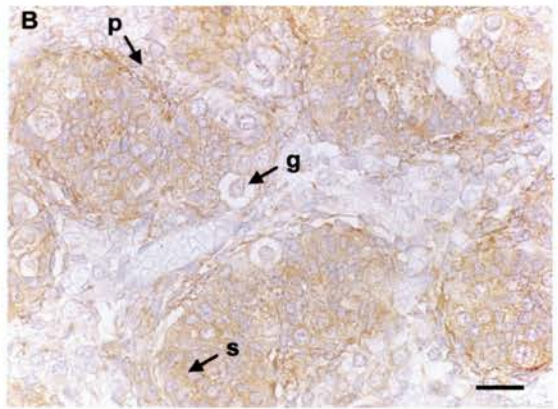
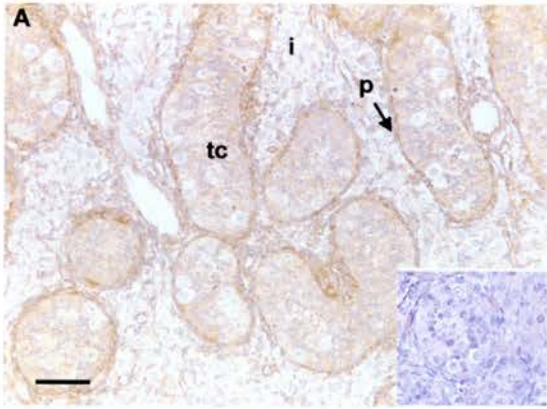
staining (Figure 6.3B and C). Very few gonocytes stained positively for NT3 and no protein was detected in the peritubular cells.

Immunolocalisation of P75 was most intense in the peritubular cell compartment (Figure 6.3D and E) but it was also present in the interstitial cells. Neither Sertoli cells nor gonocytes showed any P75 immunoreactivity. Despite the use of three different antibodies, specific staining for TRKB receptor protein was not detectable. No change in pattern or intensity of staining for any of the proteins detected was noted over the gestational range examined. Control experiments conducted by omitting the application of the primary antibody or using a specific blocking peptide in the case of NGF showed no staining (Figure 6.3A inset and F).

**Figure 6.2** *In situ* hybridization and immunohistochemical localization of *NT4* in human fetal testis.

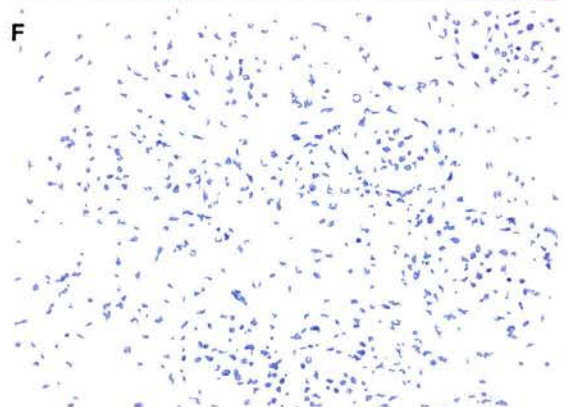
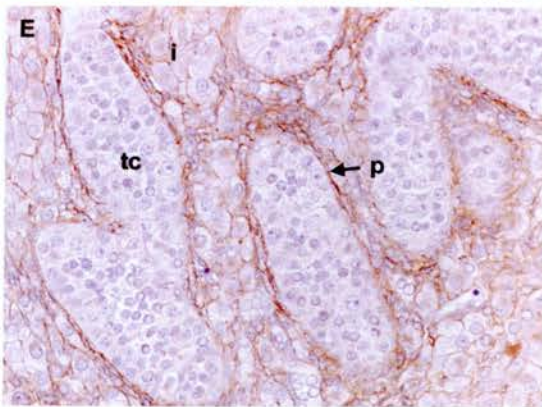
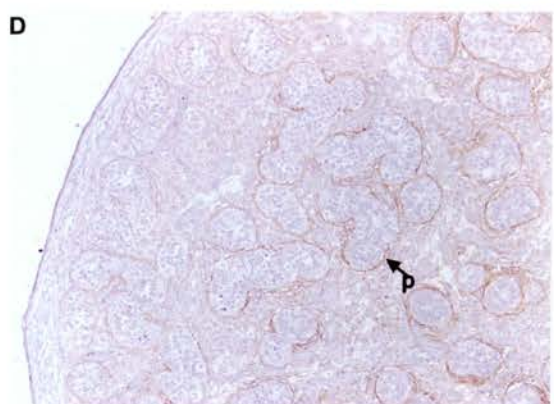
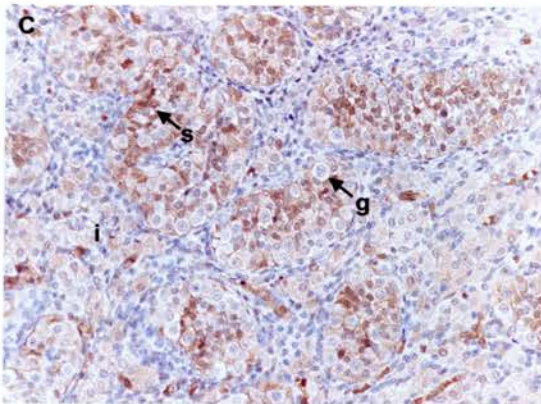
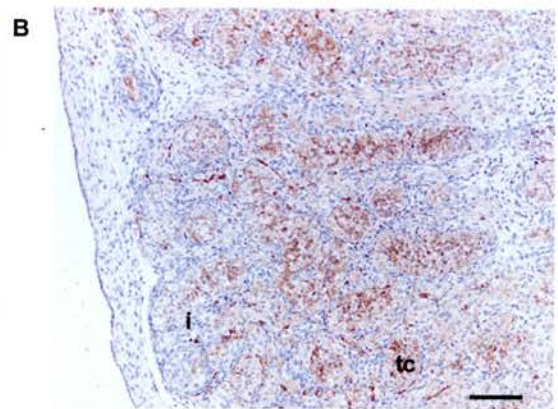
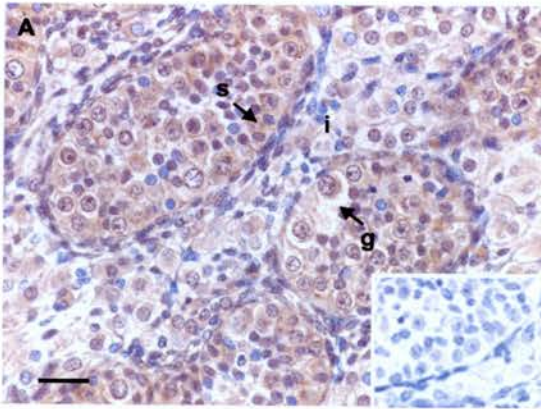
*In situ* hybridization: (A) localisation of *NT4* mRNA expression in a 19 wk testis (antisense probe). Inset shows results for sense RNA probe; (B) *NT4* mRNA expression in 16 wk testis at higher magnification.

Immunohistochemistry: C, *NT4* in 17 wk testis; D, *NT4* in 13 wk testis at higher magnification. C inset shows representative section of fetal testis omitting primary antibody. Positive staining is brown, and sections are counterstained with haematoxylin. g, gonocyte; s, Sertoli cell; p, peritubular cell; i, interstitium; tc, testicular cord. The scale bar in A represents 500  $\mu\text{m}$ . The scale bar in B represents 250  $\mu\text{m}$  and applies to A inset, C and C inset. The scale bar in D represents 50  $\mu\text{m}$ .



**Figure 6.3** Immunohistochemical localisation of NGF, NT3 and P75 in human fetal testis.

(A) 15 wk testis stained for NGF, inset shows section of testis treated with peptide block for NGF antibody; (B) 17 wk testis stained for NT3; (C) 17 week testis stained for NT3 at higher magnification; (D) 19 wk testis stained for p75; (E) 17 wk testis stained for p75 at higher magnification; (F) A representative section of fetal testis omitting primary antibody. Positive staining in all panels is brown, and sections are counterstained with hematoxylin. g, gonocyte; s, Sertoli cell; p, peritubular cell; i, interstitium; tc, testicular cord. The scale bar in A represents 250  $\mu$ m and also applies to A inset, C, E and F. The scale bar in B represents 500  $\mu$ m and applies to D.



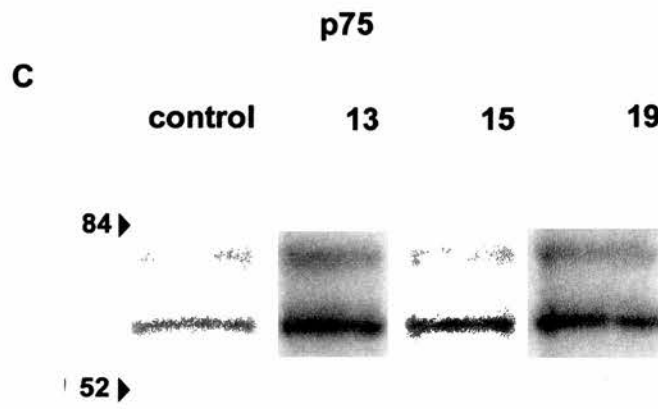
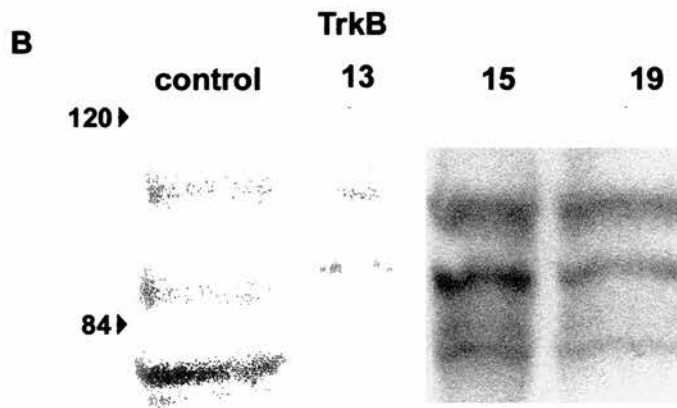
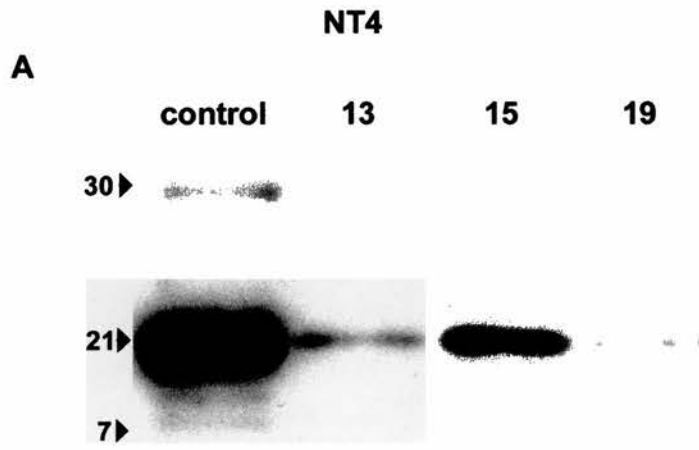
## **Immunoblotting**

The presence of NT4, TRKB and P75 proteins in the fetal testis was confirmed by immunoblotting; a positive control of rat cerebral cortex was run on all gels (Figure 6.4). The immunoblot of NT4 protein expression detected a single band of 21 kDa molecular mass in all samples of fetal testis (Figure 6.4A). For detection of TRKB, the antibody used was directed against the full-length protein. Two immunoreactive bands of 110 kDa and 80 kDa molecular masses were detected (Figure 6.4B). These bands are reported to represent the glycosylated and unglycosylated forms of the TRKB protein respectively. Two immunoreactive bands were also detected for the P75 receptor, at 75kDa and 65 kDa in all samples of fetal testis (Figure 6.4C). In all cases the sizes of the proteins detected were identical to those in the positive control tissue. These immunoblots were repeated three times using different samples: no consistent changes with gestation were noted. A negative control was also performed for all three proteins by omitting the primary antibody and in all cases immunoreactivity was abolished.



**Figure 6.4** Western blot of NT4, TRKB, and P75 in human fetal testis.

Total protein extracts (20  $\mu$ g) from whole testes at 13–19 wk gestation, as indicated, were separated by SDS-PAGE, transferred to PDF membrane and incubated with anti-NT4, -TRKB, and -P75 antibodies. A, NT4, a protein band that migrated with an apparent molecular size of 21 kDa is indicated. B, TRKB, protein bands with apparent molecular sizes of 110 and 95 kDa are indicated, representing two forms of full-length TRKB. C, p75, a protein band with an apparent molecular size of 75 kDa and an additional band of approximately 65 kDa. Control tissue was rat cerebral cortex in each case. The positions of molecular mass markers are indicated. No immunoreactive bands were detected in the absence of primary antibody.



## Testis culture

After 48 hr of culture there was a small increase in the number of gonocytes in comparison to the time 0 samples ( $p=0.02$ ) (Figure 6.5A) and a decrease in the number of interstitial cells ( $p=0.05$ ). The numbers of Sertoli cells and peritubular cells did not change significantly during this period of culture. Consistent with this increase in gonocyte number an average of 12% of that cell type were immunostained with BrdU, compared to less than 2% of Sertoli cells and peritubular cells.

K252a treatment resulted in a consistent decrease in the number of gonocytes present in the tissue ( $p < 0.01$ , Figure 6.5B-D). There was also a significant fall in the number of peritubular cells ( $p=0.006$ ), and a small but statistically significant ( $p=0.04$ ) reduction in the number of Sertoli cells (Figure 6.5B). In contrast there was an increase in the number of interstitial cells, but this did not reach statistical significance. The number of proliferating (i.e. BrdU immunopositive) gonocytes was reduced by an average of 58% from  $2.4 \pm 0.6$  to  $0.9 \pm 0.2$  per grid (Figure 6.5C and D), very similar to the reduction in the number of gonocytes (54%). The number of proliferating peritubular cells was also reduced, by a mean of 71% from  $1.9 \pm 0.8$  to  $0.5 \pm 0.1$  per grid, compared to a reduction in the number of peritubular cells of 30%. There was thus a mean fall of 26% in the proportion of gonocytes that were BrdU immunopositive, compared to a fall of 71% for peritubular cells. However these decreases did not reach statistical significance. There was also a reduction in the number of proliferating Sertoli cells from  $0.8 \pm 0.5$  to  $0.3 \pm 0.0$  per grid, which again did not reach statistical significance.

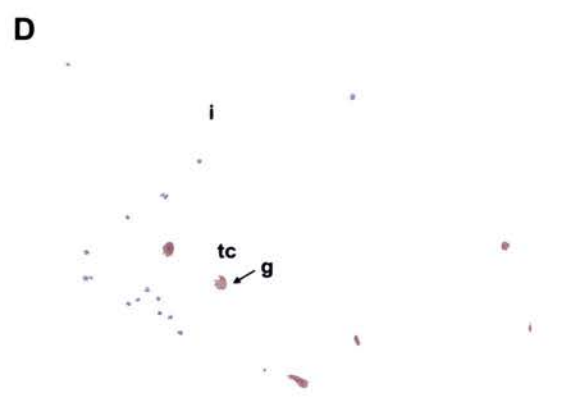
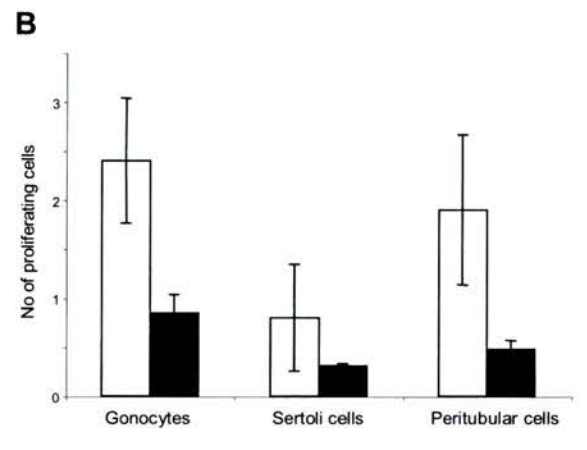
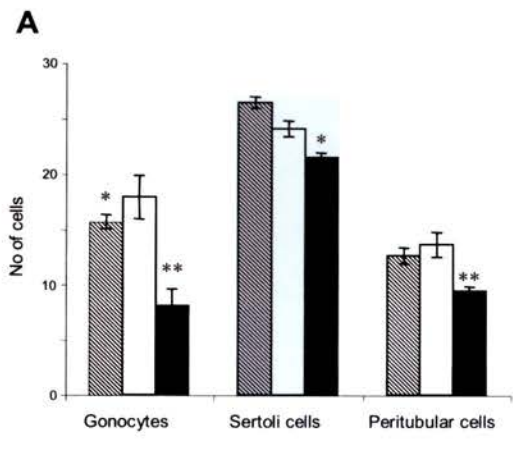
Comparable results were obtained in the 3 experiments, without clear evidence of a change with increasing gestational age.

**Figure 6.5** Testis tissue culture data

**A.** Number of each cell type present per frame in the human fetal testis in fresh and cultured tissue. The shaded columns represent uncultured, time 0 samples, the white columns represent control samples cultured for 48 hours and the black columns represent tissue treated in culture with K252a neurotrophin receptor inhibitor. Mean  $\pm$ sem, n=3 \* p < 0.05 and \*\* p < 0.001 vs control 48 hr cultures.

**B** Number of proliferating (BrdU immunopositive) cells of each type present per frame in the human fetal testis after 48 hours of culture. The white columns represent control samples and the black columns represent tissue treated with K252a neurotrophin receptor inhibitor. Mean  $\pm$ sem, n=3.

**C and D** Sections of a 19 week fetal testis stained using immunohistochemistry for Brdu. C shows tissue cultured for 48 hours in control media; D shows tissue cultured for 48 hours with K252a neurotrophin receptor inhibitor. Positive staining is brown. g, gonocytes; s, Sertoli cell; p, peritubular cell; tc, testicular cord; i, interstitium. The scale bar in C represents 250  $\mu$ m and also applies to D.



## 6.4 Discussion

The gestational age range examined in this study follows the period of testicular cord differentiation and is a time of gonocyte and Sertoli cell proliferation and active steroidogenesis (Bendsen et al 2003; Byskov 1986; Hilscher 1991; Murray et al 2000; Voutilainen 1992). These data demonstrate the gene expression and presence of neurotrophins and their receptors in the human fetal testis at this time, and that blockade of high-affinity neurotrophin receptors reduced gonocyte and peritubular cell survival and proliferation while having little effect on Sertoli or interstitial cells. The striking localisation of expression of NT4 (at both mRNA and protein levels) and the p75 neurotrophin receptor to the peritubular cells may indicate that these cells are central to the functions of neurotrophins in the developing testis. The formation of cords is crucial to Sertoli cell and Leydig cell differentiation and inhibition of germ cell meiosis (Tilman et al 2002) and is thus not only the main morphological feature distinguishing the developing testis from the ovary, but is also of paramount functional importance. These features of normal development have in the mouse been clearly linked to Sry expression (Capel et al 1999) which also induces stimulation of cell migration from the mesonephros into the gonad inducing testis cord formation (Tilman et al 1999). Peritubular cells originate in the mesonephros (Buehr et al 1993), and their precursors have been demonstrated to express the p75 receptor (Campagnolo et al 2001). Trk receptor knock-out models also provide evidence for the involvement of neurotrophins in the cell migration associated with testicular cord formation (Cupp et al 2002), supporting earlier studies using K252a treatment of organ cultures (Levine et al 2000b). The present results

therefore support the hypothesis that neurotrophin expression in the human testis is important in the regulation of normal development.

Neurotrophins are small, secreted proteins related to the TGF $\beta$  superfamily, originally identified on the basis of their role in the regulation of neuronal survival. However, neurotrophins may also regulate cell migration (Behar et al 1997) and differentiation (Polak et al 1993). In addition to signalling through high-affinity Trk receptors, neurotrophins also signal through the low-affinity p75 receptor and the pattern of expression of the different receptor types influences the response (Barker et al 1994; Casaccia-Bonofil et al 1999). Further complex interaction is suggested by the demonstration that the immature form of NGF, termed proNGF has a greater affinity for p75 than the mature form of NGF (Lee et al 2001). Thus neurotrophin signalling is a complex interaction between the ligands and the Trk/p75 receptor complement present. In addition to the full length *TRKB* we have identified mRNA for truncated *TRKB* in the developing testis. The truncated form lacks the tyrosine kinase domain and is predominantly expressed in non-neuronal cells (Klein et al 1990; Wetmore et al 1995). Although the function of truncated receptors remains elusive, it has been postulated that these isoforms have a role as cell adhesion molecules acting as a selective barrier preventing the diffusion of neurotrophin and promoting elimination by internalisation (Biffo et al 1995; Zhou et al 1997).

There are increasing data regarding the expression of neurotrophins and their receptors in the developing rodent testis, but very little data in the human. In the rodent testis expression has been detected from very early in development (Cupp et



al 2000; Levine et al 2000b). The present data suggest a cell-specific distribution of the neurotrophins although some cell types, such as Sertoli cells, expressed more than one neurotrophin. Expression of multiple neurotrophins may be required for normal development of some neuronal cell types (Fan et al 2000). We were unable to localise TRKB using immunohistochemistry although using RT-PCR and Western analysis expression of both full length and truncated *TRKB* mRNA and the full-length protein was demonstrated. Previous reports of the cellular localisation of p75 in the rat (Campagnolo et al 2001; Levine et al 2000b; Russo et al 1999) are in agreement with our findings in the human, being predominantly localised to the peritubular cells. This pattern of expression shows some parallels with that seen in the rodent and human fetal ovary (Dissen et al 1995) (Anderson et al 2002) where P75 is localised to the stromal cells surrounding clusters of replicating germ cells.

Functional roles for the neurotrophins in morphological sex determination, cell migration and testicular cord formation have been suggested (Levine et al 2000b; Russo et al 1999). Treatment of organ cultures of E13 rat testis with K252a inhibited cord formation (Levine et al 2000b). In the present studies we have also used K252a to investigate the functional activity of neurotrophin signalling in the developing human testis. K252a is an indole carbazole, widely used as a potent and selective inhibitor of the intracellular protein kinase domain of Trk receptors without affecting other serine/threonine kinases at the concentrations used here (Koizumi et al 1988; Levine et al 2000b; Tapley et al 1992). K252a blocks the activity of all high-affinity Trk receptors, so these data do not permit interpretation in terms of which neurotrophin(s) and receptor(s) might be involved in the effects observed. While the

investigation of selective blockade of specific Trk receptors has been attempted in the investigation of many models including the developing testis (Levine et al 2000b), the effects are generally much less marked than those of K252a consistent with considerable redundancy in neurotrophin signalling, as also demonstrated by the phenotypes of knock-out animals (Snider 1994). In this culture system, the integrity of the tissue was well maintained and cell proliferation was detected. This was noted to be at a much higher rate in gonocytes than in other cell types. The major effect of K252a on the testicular cords was on gonocyte survival with relatively little effect on Sertoli cells. Sertoli cells were the primary site of expression of NGF and NT3, and although the site of expression of TRK receptors was not demonstrated in the present study, TrkA has been previously reported to be expressed by rat Sertoli cells between E16 and P0 (Cupp et al 2000). These results suggest that neurotrophins are critical for gonocyte survival and replication, but their effects may be mediated indirectly via Sertoli cells. This is the classic pattern of Sertoli cell/germ cell interaction with paracrine signalling between these two cell populations, most clearly exemplified by the mediation of the effects of FSH and androgen. It is also apparent that the relative number of gonocytes and Sertoli cells is maintained during early testicular development despite a 10-fold increase in the number of gonocytes between 6 and 9 weeks gestation (Bendsen et al 2003): the present data suggest that neurotrophin signalling may contribute to the regulation of this ratio. Selective gonocyte loss has not previously been reported as a result of neurotrophin blockade but cord formation is certainly affected, inevitably involving gonocyte survival (Levine et al 2000b). These data may be compared to the recent demonstration that neurotrophins are involved in germ cell survival in the human fetal ovary at a comparable stage of

development (Anderson et al 2002; Spears 2003), although in the ovary it appears that germ cells are a direct site of neurotrophin action. While that may also be the case in the testis, direct evidence is thus far lacking.

While K252a resulted in a marked loss of gonocytes, there was also a reduction in the number of peritubular cells. There was also a striking reduction in gonocyte and peritubular cell proliferation. As the fall in the number of BrdU immunopositive peritubular cells was much greater than the fall in the total number of that cell type (71% vs 30%) whereas the comparable figures for gonocytes were similar (58% vs 54%), these data may suggest a specific effect of neurotrophin on peritubular cell proliferation in addition to survival, consistent with those cells being the major site of expression of NT4 and the P75 receptor. The importance of neurotrophins and their receptors in rat testicular cord formation (Cupp et al 2000; Levine et al 2000b) may also reflect a major site of action on the peritubular cell population. As the p75 receptor may promote apoptosis in the absence of Trk receptor signalling (Casaccia-Bonofil et al 1999; Chao et al 1995) relatively unopposed p75 signalling may contribute to the decrease in peritubular cell proliferation and survival in the presence of K252a.

We have recently demonstrated the expression of oestrogen receptor (ER)  $\beta$  within the human fetal testis (Gaskell et al 2003). Sertoli and peritubular cells were demonstrated to express both ER $\beta$  1 and 2 isoforms, whereas gonocytes expressed only ER $\beta$ 2, indicating that Sertoli and peritubular cells in particular may be sites of estrogen action. Oestrogen and neurotrophins interact in neural development

selectively enhancing neuronal growth and development (Simerly 2002), and *ER* mRNA may be co-expressed with neurotrophins (Miranda et al 1994; Toran-Allerand 1996). Some of these effects of oestrogen may be mediated by the ER $\beta$  rather than the classical ER $\alpha$  (Toran-Allerand et al 1999). The expression of both neurotrophins and their receptors and ER $\beta$  in Sertoli cells and peritubular cells suggest that these pathways may interact in a wide variety of cell types.

In conclusion, this study demonstrates that neurotrophins and their receptors are expressed in the developing human testis during the second trimester. Neurotrophins have been demonstrated to be crucial for cellular migration, germ cell survival and proliferation (Campagnolo et al 2001; Cupp et al 1999; Cupp et al 2000; Cupp et al 2002; Levine et al 2000b; Russo et al 1999) in the rodent testis: the present results suggest that they are likely to be of similar importance in the human with a major role in the regulation of proliferation and survival of germ cells and peritubular cells.

**CHAPTER 7**  
**GENERAL CONCLUSION**

## 7.0 GENERAL CONCLUSION

This thesis provides support for the hypothesis that the various factors studied, namely c-kit receptor, neurotrophins and their receptors and MMPs and TIMPs, play an essential role in the development and differentiation of the human fetal testis and ovary during the second trimester.

### 7.1 C-kit receptor and ligand

The studies in Chapter 3 investigating the role of C-kit receptor and its ligand in human fetal gonadal development demonstrated expression of these factors in both ovary and testis at all gestational ages examined. The expression of *C-KIT* mRNA and protein is germ cell specific in human fetal gonads and suggests an important role for the C-KIT/KL signalling system in germ cell proliferation and survival in the developing human gonad. There is evidence that mutations in the genes for these factors result in loss of primordial germ cells (Besmer et al 1993) and that these factors are important for germ cell survival and protection from apoptosis, possibly via the increased expression of Bcl-2 (Pesce et al 1993; Tilly 1996; Yee et al 1994). The present results support this evidence and also that of studies in animal models showing that C-kit is important for folliculogenesis in the ovary (Horie et al 1991; Ismail et al 1997; Parrott et al 1999) and may play a role in spermatogonial proliferation, germ cell migration and protection from apoptosis (Besmer et al 1993; Packer et al 1995; Yoshinaga et al 1991). This has furthered our limited knowledge of the factors controlling germ cell development in human fetal gonads and has clarified conflicting reports on the existence of C-KIT in germ cells in the human fetal testis. Further work could be done to block C-KIT/KL signalling *in vitro* to

examine the resultant effect on germ cell survival and proliferation as has been done in mice. In addition to evaluating proliferation using BrdU staining, a method for detecting apoptotic cells such as TUNEL could be employed. If possible, studying earlier gestations could be helpful regarding the role C-KIT may play in germ cell migration in the human.

## **7.2 MMPs and TIMPs in human fetal testis and ovary**

The studies described in Chapter 4 investigated the role of MMPs and TIMPs in the development of the human fetal gonads. These demonstrated that MMPs -1, -2 -9 and all TIMP family members are secreted by the developing fetal human ovary and testis and are localised to specific cell and tissue sites, MMP-2 being the most abundant gelatinase. These findings support the hypothesis that MMPs and TIMPs are involved in ECM remodelling during fetal gonadal development. The localisation of these factors in the vasculature within the gonads suggests a role in angiogenesis thereby correlating with findings in other tissues (Pilcher et al 1997; Yu et al 2000b). MMP and TIMP expression in the fetal gonads in the second trimester also corroborates with their possible role regulating germ cell proliferation by modifying growth factor availability and action and also activating steroidogenesis in the testis (Boujrad et al 1995; Edwards et al 1996a). In summary, MMPs and TIMPs are likely to play a role in ECM remodelling during fetal gonadal development and also in the cell and matrix interactions that control a range of cellular functions. This provides a basis from which to work towards further assessment of the functions of MMPs and TIMPs within the human fetal gonad. Further studies investigating the inhibition and/or promotion of MMP and TIMP activity on gonadal tissue *in vitro* should be

carried out, for example investigating the effect of TIMP-1 on TNF $\alpha$  secretion. Further work should also be done regarding the action of TIMP-1 on steroidogenesis as this could be quantitatively measured after organ culture.

### **7.3 The role of neurotrophins in human fetal ovarian and testicular development**

These studies in Chapters 5 and 6 have demonstrated the presence of neurotrophins and their receptors in the developing human ovary and testis during the second trimester. This was a novel finding in the ovary and clarified previous studies investigating their presence in the testis. Following this, neurotrophin action was blocked in organ culture and the resulting effect on germ cell survival and proliferation was examined.

In both ovary and testis this markedly decreased germ cell survival and also produced a significant decrease in oocyte proliferation. There was a significant decrease also noted in peritubular cell proliferation and survival in the testis. These results support our hypothesis that neurotrophins are present in the human fetal gonads and have an essential role to play in cell proliferation and survival, in particular in the regulation of germ cell number. These findings correlate with the expression of neurotrophins and receptors in rodent gonads (Dissen et al 1995) (Timmusk et al 1993) (Campagnolo et al 2001; Cupp et al 2000; Dissen et al 2001; Levine et al 2000b; Russo et al 1999) and also on germ cell number in the ovary (Spears 2003). Previous studies have suggested neurotrophin involvement in rat



testicular cord formation (Cupp et al 2000; Levine et al 2000b; Russo et al 1999) indicating similar action on gonocytes and peritubular cells.

The information generated from these studies should be extended by adding neurotrophins to organ culture in an attempt to identify the roles of individual neurotrophic factors. Alternatively, it may be feasible to block individual Trk receptors using chimeric fusion proteins. Neurotrophin effects on steroidogenesis in the testis should also be investigated as testosterone production could be measured. From organ culture, the effects on factors such as TGF $\alpha$  and TGF $\beta$  could be assessed and also the effect of manipulation of neurotrophins on the expression of ER $\alpha$  and ER $\beta$  in fetal ovaries. After studying these factors *in vitro* the research should be continued in animal models *in vivo*. This could provide a possible clinical application for neurotrophins, in manipulating germ cell number and hence ovarian follicular development and spermatogenesis.

The main limitation throughout this research was the relative scarcity of tissue samples. In addition to this was the unpredictability of the timing of the arrival of samples and therefore inevitably some delay in processing them. By building a bank of frozen and fixed tissue samples over a longer period of time it should be possible to advance these studies and gain further knowledge concerning the role of these factors in human gonadal development.

In summary, this research has demonstrated that the various factors studied in the preceding chapters are unequivocally present in the human fetal ovaries and testis

during the mid-trimester. These results alone imply essential roles in gonadal tissue proliferation and development. The discovery that neurotrophins are required for germ cell and peritubular cell development furthers our understanding as to the processes affecting the regulation of development of human gonadal tissue. To date, very little research into human fetal gonadal development has been carried out due to the scarcity and difficulty obtaining tissue and most information regarding this subject has been extrapolated from animal models. By carrying out these studies, the mechanisms of development during this time period in the human have been clarified and factors directly contributing to germ cell and peritubular cell development have been identified. This forms a base for further research looking at ways of manipulating these factors *in vitro*. From this conditions for *in vitro* maturation of gonadal tissue may be optimised and thereby the lifespan of the tissue may be altered, offering therapeutic applications for this research.

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## APPENDIX I

### Materials

The sources of all the reagents used for the experiments described in the preceding chapters are detailed below.

TISSUE CULTURE MATERIALS –	SOURCE
<b>GENERAL</b>	
24 well culture plates	Costar Ltd., High Wycombe, UK
Millicell CM filters	Millipore, Bedford MA
Minimal essential medium alpha (MEM $\alpha$ )	Gibco, Paisley, UK
Bovine serum albumin (BSA), Fraction V, electrophoresis grade	Sigma, Poole Dorset
Penicillin	Sigma, Poole Dorset
Streptomycin sulphate	Sigma, Poole Dorset
Amphotericin	Sigma, Poole Dorset
ITS	Sigma, Poole Dorset
Glutamine	Sigma, Poole Dorset
Pyruvate	Sigma, Poole Dorset
Trk kinase inhibitor, K252a, Nocardiosis sp. Acts as a potent inhibitor (IC <sub>50</sub> = 3 nM) of the tyrosine protein kinase activity of the <i>TrkA</i> as well as the kinase activity of its transforming alleles, the <i>Trk</i> oncogenes, and of the related neurotrophin receptors <i>TrkB</i> and <i>TrkC</i> . Induces apoptosis and cell cycle arrest by inhibiting Cdk1 and Cdc25c.	Calbiochem, Nottingham, UK
Basic Fibroblast growth factor (bFGF) Human. ED <sub>50</sub> = 100-250 pg/ml	Gift from Amgen Inc, California
Dimethylsulfoxide (DMSO)	Sigma, Poole Dorset
Bromodeoxyuridine (BrdU)	Sigma, Poole Dorset
Bouins fluid	Triangle Biomedical Sciences, Lancashire
Stereologer Microscope	Systems Planning and Analysis Inc, Alexandria, VA, USA

<b>MOLECULAR BIOLOGY GENERAL</b>	<b>SOURCE</b>
0.2 ml eppendorf tubes	Ambion, Huntingdon, UK
Deoxynucleotide triphosphate	Roche Diagnostics, Lewes, UK
<i>Taq</i> DNA polymerase	AGS Gold, Hybaid, Ashford, UK
Complete buffer	AGS Gold, Hybaid, Ashford, UK
TBE buffer	See Appendix 1 for recipe
Agarose	Sigma, Poole Dorset
dNTPs	Boehringer Mannheim, Lewes, UK
Ethidium Bromide	Sigma, Poole Dorset
Gel tank	Biorad, CA, USA
DNA Hi Pure Purification kit	Boehringer Mannheim, Lewes, UK
GenElute Agarose Spin Columns	Sigma, Poole Dorset
Primer sequences	Sigma Genosys

<b>ISOLATION OF RNA, SYNTHESIS AND AMPLIFICATION OF cDNA</b>	<b>SOURCE</b>
RNeasy mini kit	Quiagen, Crawley, UK
DNAase	Gibco, Paisley, UK
First strand cDNA synthesis kit	Roche Diagnostics, Lewes, UK
Deoxynucleotide triphosphate	Roche Diagnostics, Lewes, UK
<i>Taq</i> DNA polymerase	AGS Gold, Hybaid, Ashford, UK

<b>GENERATION OF RIBOPROBES FOR <i>IN SITU</i> HYBRIDIZATION</b>	<b>SOURCE</b>
Tris/HCl	See Appendix II for recipe
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	See Appendix II for recipe
pGEM-T Vector	Promega
MgCl <sub>2</sub>	See Appendix II for recipe
Tween-20	Sigma, Poole, Dorset
RNeasy mini kit	Qiagen, Crawley, UK
DNase	Invitrogen Ltd, Paisley, UK
First strand cDNA synthesis kit	Roche Diagnostics, Lewes, UK
High Pure PCR product purification kit	Roche Diagnostics, Lewes, UK
Digoxigenin labelling kit	Maxiscript, Ambion
RNA Polymerase	Maxiscript, Ambion
Ribonuclease inhibitor	Promega
DNase I	Sigma, Poole, Dorset
Chromaspin columns (DEPC-100),	Clontech, Palo Alto, CA, USA



<b>IN SITU HYBRIDIZATION</b>	<b>SOURCE</b>
Super frost plus coated slides	Menzel-Glaser, Germany
Gel bond film	FMC Bioproducts, Maine, USA
Proteinase K	Sigma, Poole, Dorset
EDTA	ICN Biochemicals Inc., Aurora, Ohio
Glycine	Sigma, Poole, Dorset
Acetic anhydride	Sigma, Poole, Dorset
0.08 M triethanolamine	Sigma, Poole, Dorset
Prehybridization buffer	See Appendix II for recipe
Hybridization buffer	See Appendix II for recipe
SSC	See Appendix II for recipe
RNase A	Sigma, Poole, Dorset
TBS	See Appendix II for recipe
Formamide	Sigma, Poole, Dorset
Hydrogen Peroxidase	Sigma, Poole, Dorset
Normal swine/rabbit serum (NSS/NRS)	Diagnostics Scotland, Carlisle, UK
Avidin block	Vector, Peterborough, UK
Biotin block	Vector, Peterborough, UK
Sheep Anti-Digoxigenin	Roche Diagnostics, Lewes, UK
Biotinylated rabbit anti-sheep IgG	Vector, Peterborough, UK
Avidin-Biotin Complex-Horse radish peroxidase	Dako, Cambridge, UK
DAB liquid substrate-chromagen system	Dako, Cambridge, UK

<b>SEQUENCING</b>	<b>SOURCE</b>
ABI 377 Sequencer	Perkin Elmer
Big Dye Terminators	Perkin Elmer
Sequencing reaction mix	Applied Biosystems Ltd, Cheshire
½ Term	See Appendix II for recipe
Formamide	Sigma, Poole, Dorset
EDTA	ICN Biochemicals Inc., Aurora, Ohio
Urea	Sigma, Poole, Dorset
Acrylamide	Northumbria Biologicals Ltd., Cramlington UK
Amberlite	Sigma, Poole, Dorset
Filter unit	Nalgene
TEMED	Biorad, CA, USA
APS	Biorad, CA, USA
TBE	See Appendix II for recipe
Running Buffer	See Appendix II for recipe
DNA Hi Pure Purification kit	Boehringer Mannheim, Lewes, UK

<b>LASER CAPTURE MICROSCOPY</b>	<b>SOURCE</b>
PixCell II LCM system	Arcturus Engineering Inc., Mountain View, CA
B-mercaptoethanol	Sigma, Poole, Dorset
Micro RNA Isolation Kit	Stratagene, La Jolla
AMH	
Rabbit polyclonal antibody	Gift from Dr. R. Rey
Chloroform:isoamyl	Sigma, Poole, Dorset
Phenol	Sigma, Poole, Dorset
Sodium Acetate	Sigma, Poole, Dorset
Glycogen	Sigma, Poole, Dorset
Isopropanol	Sigma, Poole, Dorset
Superscript II (Gibco BRL) reverse transcriptase	Gibco, Paisley, UK

<b>IMMUNOHISTOCHEMISTRY</b>	<b>SOURCE</b>
TESPA coated slides	Sigma, Poole, Dorset
Histoclear	National Diagnostics, Atlanta, Georgia, USA
Hydrogen Peroxide (H <sub>2</sub> O <sub>2</sub> )	Merck Ltd (BDH), Lutterworth, UK
Methanol	Merck Ltd (BDH), Lutterworth, UK
Tris base	Sigma, Poole, Dorset
Tris-buffered saline (TBS)	See Appendix II for recipe
Blocking serum	Diagnostics Scotland, Carlisle, UK
Glycine buffer	See Appendix II for recipe
Citrate buffer	See Appendix II for recipe
Bovine serum albumin, Fraction V, electrophoresis grade	Sigma, Poole, Dorset
Avidin block	Vector, Peterborough, UK
Biotin block	Vector, Peterborough, UK
Biotinylated secondary antibodies	Dako, Cambridge, UK
Biotinylated anti chicken secondary antibody	Jackson Laboratories, Bar Harbor, Maine, USA
Avidin-Biotin Complex-Horse radish peroxidase	Dako, Cambridge, UK
DAB liquid substrate-chromagen system	Dako, Cambridge, UK
Haematoxylin	See Appendix II for recipe
Eosin	See Appendix II for recipe

<b>SPECIFIC IMMUNOHISTOCHEMISTRY</b>	<b>SOURCE</b>
<b>NGF</b>	
Rabbit polyclonal antibody	Santa Cruz, CA, USA
NGF peptide block	Santa Cruz, CA, USA
<b>NT3</b>	
Rabbit polyclonal antibody	Santa Cruz, CA, USA
<b>NT4</b>	
Rabbit polyclonal antibody	Santa Cruz, CA, USA
<b>TrkB (1)</b>	
Rabbit polyclonal antibody	Santa Cruz, CA, USA
<b>TrkB (2)</b>	
Chicken polyclonal antibody	Promega, Southampton, UK
<b>TrkB(3)</b>	
Rabbit polyclonal antibody	Oncogene, San Diego, CA, USA
<b>p75</b>	
Mouse monoclonal antibody	Neomarkers, Fremont, Ca, USA
<b>Brdu</b>	
Mouse monoclonal antibody	Roche Diagnostics, Lewes, UK
<b>c-kit (1)</b>	
Goat polyclonal antibody	Santa Cruz, CA, USA
<b>c-kit (2)</b>	
Rabbit polyclonal antibody	Dako, Cambridge, UK
<b>AMH</b>	
Rabbit polyclonal antibody	Gift from Dr. R. Rey, Buenos Aires, Argentina
<b>MMP-1</b>	
Mouse monoclonal antibody	Chemicon International Inc., Harrow, UK
<b>MMP-2</b>	
Mouse monoclonal antibody	Calbiochem, Nottingham, UK
<b>MMP-9</b>	
Mouse monoclonal antibody	Insight, Biotechnology, Wembley, Middlesex, UK
<b>TIMP-1</b>	
Rabbit polyclonal antibody	Sigma, Poole, Dorset
<b>TIMP-2</b>	
Rabbit polyclonal antibody	Triple Point Biologics, Forest Grove, OR, USA
<b>TIMP-3</b>	
Rabbit polyclonal antibody	Sigma, Poole, Dorset
<b>TIMP-4</b>	
Rabbit polyclonal antibody	Chemicon International Inc., Harrow, UK

<b>WESTERN BLOT AND PROTEIN EXTRACTION</b>	<b>SOURCE</b>
Protein extraction buffer	See Appendix II for recipe
Gel tank	Gibco, Paisley, UK
Homogenisation/denaturing buffer	See Appendix II for recipe
Reducing loading buffer	See Appendix II for recipe
Tris-glycine gel	Novex, Invitrogen, The Netherlands
Tris-glycine SDS running buffer	Novex, Invitrogen, The Netherlands
Molecular weight markers	Biorad, CA, USA
PDF membrane	Amersham Life Science, Buckinghamshire, UK
Wet blot apparatus	Biorad, CA, USA
Wet blot buffer	See Appendix II for recipe
BSA	Sigma, Poole, Dorset
Tween-20	Sigma, Poole, Dorset
<b>NT4</b>	
Rabbit polyclonal antibody	Santa Cruz, USA
<b>TrkB</b>	
Rabbit polyclonal antibody	Oncogene, San Diego, CA, USA
<b>p75</b>	
Mouse monoclonal antibody	Neomarkers, Ca, USA
Anti-rabbit HRP linked secondary antibody	Amersham Life Science, Buckinghamshire, UK
Anti-mouse HRP linked secondary antibody	Amersham Life Science, Buckinghamshire, UK
ECL Reagent	Amersham Life Science, Buckinghamshire, UK

<b>ZYMOGRAPHY</b>	<b>SOURCE</b>
Minigel apparatus	Biorad, Hemel Hempstead, Herts, UK
Stacking gel buffer (Stock B)	See Appendix II for recipe
Stock wash buffer X 10	See Appendix II for recipe
Resolving gel	See Appendix II for recipe
Stacking gel	See Appendix II for recipe
Triton-X-100 Wash	See Appendix II for recipe
Digestion buffer	See Appendix II for recipe
Destaining solution	See Appendix II for recipe
Staining solution	See Appendix II for recipe
Butanol	Sigma, Poole, Dorset, UK

<b>REVERSE ZYMOGRAPHY</b>	<b>SOURCE</b>
Reverse zymography kit	University Technologies Inc., Calgary, Canada
BHK-21 cells	University Technologies Inc., Calgary, Canada
Wash buffer/Rinse buffer	See Appendix II for recipe
Incubation buffer/Digestion buffer	See Appendix II for recipe
All other reagents/solutions as for Zymography	See Appendix II for recipe

## APPENDIX II

All chemicals were from Sigma-Aldrich, Poole, UK unless otherwise stated

### IMMUNOHISTOCHEMISTRY SOLUTIONS

CITRATE BUFFER (0.1M)

**pH 6.0**

42.02 g Citric Acid (monohydrate)

1900mls distilled water

Add conc. NaOH to solution to pH 5.5

Make up to 2 litres and pH to 6.0

Use as 0.01M, diluting in distilled water

GLYCINE/0.1%EDTA (0.5M)

**pH 3.5**

Glycine 75.07g

1900mls distilled water

Add conc. HCl to solution to pH 4.0. Make up to 2 litres and pH to 3.5

Add 2g EDTA

Use as 0.05M, diluting in distilled water

## HAEMATOXYLIN (Harris')

HAEMATOXYLIN	3 litres
Haematoxylin	15 g
Absolute alcohol	150 mls
Potassium Alum *	300 g
Distilled Water	3 litres
Mercuric Oxide	7.5 g
Glacial Acetic Acid	120 mls

Haematoxylin is dissolved in Absolute Alcohol and added to the Alum, which has been dissolved in warm water. Bring to the boil and add Mercuric Oxide slowly. Cool by plunging in ice. Acetic Acid \*\* is added after filtering into staining dish

\* Potassium Alum – Aluminium Potassium Sulphate

\*\* 4 mls Acetic Acid per 100 mls Haematoxylin

## EOSIN

1% solution in water	1500 mls (15 g)
1% solution in Methanol	500 mls (5 g)

Mix 3 parts (1500 mls) aqueous solution with one part (500 mls) spirit solution. Filter and add 1 ml Formaldehyde to prevent bacterial growth. Add sprinkle of Calcium Chloride.



## **IN-SITU HYBRIDISATION SOLUTIONS**

### **PREHYBRIDISATION BUFFER**

#### Contents per ml

50% deionised formamide

4 x SSC

1 x Denhardts solution

10 mM dithiotreitol (DTT)

125 µg Salmon Testes DNA

125 µg yeast tRNA

### **HYBRIDISATION BUFFER**

#### Contents per ml

50% deionised formamide

4 x SSC

1 x Denhardts solution

10 mM Dithiotreitol (DTT)

125 µg Salmon Testes DNA

125 µg yeast tRNA

10% dextran sulphate

2µl appropriate riboprobe per 50µl hybridization buffer

**TRIS BUFFERED SALINE (TBS)**

0.05M Tris

0.85% NaCl pH 7.4

**1N HCL**

36 mls conc HCL

9964 mls H<sub>2</sub>O

**PROTEINASE K BUFFER**

100 mls 1M Tris, pH 8.0

100 mls 0.5 M EDTA pH 8.0

800 mls H<sub>2</sub>O

ICN Biochemicals Inc., Ohio

**TRIETHYLAMINE (TEA) BUFFER**

14.9g Triethanolamine

800 mls H<sub>2</sub>O, pH to 8.0 using NaOH,

make up to 1 litre with H<sub>2</sub>O

**5 X RNase BUFFER**

100 mls 1M Tris

20 mls 0.5M EDTA, pH 8.0

1000 mls 5M NaCl

880 mls H<sub>2</sub>O

ICN Biochemicals Inc, Ohio

**20 X SSC**

175.3g NaCl

88.2g citric acid

800 mls H<sub>2</sub>O pH to 7.0 then make up to 1 litre with H<sub>2</sub>O

**20 X STE**

17.5g NaCl

10 mls 0.5M pH 7.4

1 ml 0.5M EDTA

ICN Biochemicals Inc, Ohio

pH to 7.4, make up to 100 mls with H<sub>2</sub>O

**3M Na ACETATE**

24.6g Na-Acetate

100 mls H<sub>2</sub>O pH to 6.0 or 7.0 with acetic acid

**0.1 SSC/30% FORMAMIDE**

1.75 mls 20x SSC

105 mls formamide

H<sub>2</sub>O to 350 mls

## **TEA BUFFER / ACETIC ANHYDRIDE**

850µl acetic anhydride added To 350 mls TEA just

**before** use.

## **PROTEIN EXTRACTION BUFFER**

1.67mls 0.375M Tris **pH 6.8**

6.33mls dH<sub>2</sub>O

1ml glycerol

1ml 10% SDS

BioRad, Hemel Hempstead, UK

1 protease inhibitor tablet

Boehringer, Lewes, UK

Mix all ingredients adding the protease inhibitor last and leave it to dissolve.

Use immediately or store at -20 °C.

## **WESTERN BLOT SOLUTIONS**

### **HOMOGENISATION BUFFER**

58 mM Tris **pH 6.8**

1% SDS

1% glycerol

### **REDUCING LOADING BUFFER**

187 mM Tris **pH 6.8**

2% SDS

2%  $\beta$  mercaptoethanol

1% sucrose

0.01% bromophenol blue

BioRad, Hemel Hempstead, UK

dH<sub>2</sub>O

Adjust pH with 5 N HCl

Store in aliquots at -20°C

### **WET BLOT TRANSFER BUFFER**

0.02M Tris

0.2M glycine

20% methanol

Merck Ltd, Lutterworth, UK

Add dH<sub>2</sub>O to make a volume of 2.5L.

Use for a maximum of 3 times and then discard.

### **TBS**

0.02M Tris, 1.5M NaCl **pH 7.6**

4.85g Tris

17.53g NaCl

Make up to 2 litres with dH<sub>2</sub>O and pH to 7.6.

Adjust pH with 5 N HCl.

## **BLOCKING SOLUTION**

0.02 M TBS (pH 7.6)

3% w/v BSA

5% powdered milk

## **PRIMARY ANTIBODY SOLUTION**

0.02 M TBS pH 7.6

0.1% Tween-20

1% BSA

## **GEL FOR PCR ELECTROPHORESIS**

### **For minigel:**

1 % AGAROSE GEL

50 mls TAE

0.5 g agarose

Mix in conical flask and heat for 2 minutes in

Microwave until agarose dissolved. Allow to

cool for a few minutes and then add 10  $\mu$ l

ethidium bromide. Pour gel into gel cast with comb

in situ and allow to set.

**For 150ml gel:**

1 % AGAROSE GEL

150 mls TAE

1.5 g agarose

30 µl ethidium bromide

Method as above

**SOLUTIONS FOR SEQUENCING**

**20X SSC**

To make up 1 litre:

175.3g NaCl, 3M

88.2g Citric Acid

800 ml Deionised Water

Make up and check pH using pH paper.

Adjust pH using NaOH (5M) or HCl.

### **10X TBE**

To make up 1 litre:

108g Tris Base

55g Boric Acid

3.77g EDTA

ICN Biochemicals Inc, Ohio

Add deionised water to a final volume of

1 litre.

For sequencing, pH to **8.4**.

### **10X Running Buffer**

To make up 1 litre:

41.8g MOPS

3.72g Sodium Acetate

4.1g EDTA

ICN Biochemicals Inc, Ohio

Add deionised water to 800 ml before adjusting

pH to 7.0 and then make to final volume.

### **½ TERM**

200mM Tris HCl

**pH 9.0**

5mM MgCl<sub>2</sub>



## ZYMOGRAPHY

### SAMPLE APPLICATION BUFFER

10 mls 20 % v/v glycerol

1g 2 % w/v SDS

BioRad, Hemel Hempstead, UK

20 mg 0.04 % w/v bromophenol blue

BioRad, Hemel Hempstead, UK

Dissolve in 50 mls Elgastat x 1 purified H<sub>2</sub>O

Store in 1 ml aliquots at 5°C

### RUNNING TANK BUFFER X 10

**pH 8.3**

1.9 M glycine

0.25 M Tris

1 % SDS

BioRad, Hemel Hempstead, UK

Dissolve in 1 litre Elgastat x 1 purified H<sub>2</sub>O.

Dilute 1:10 with Elgastat x 1 purified H<sub>2</sub>O

for use.

### RESOLVING GEL BUFFER: STOCK A

**pH 8.8**

1.5 M Tris

Dissolve in ~50 mls Elgastat x 1 purified H<sub>2</sub>O.

pH with 5 N HCL.

Make up volume to 100 mls with Elgastat x 1 purified H<sub>2</sub>O.

**STACKING GEL BUFFER: STOCK B****pH 6.8**

0.5 M Tris

Dissolve in ~50 mls Elgastat x 1 purified H<sub>2</sub>O.

pH with 5 N HCL.

Make up volume to 100 mls with Elgastat x 1 purified H<sub>2</sub>O.**STOCK WASH BUFFER X 10 (TBS X 10)**

0.5 M Tris

1.5 M NaCl

Dissolve in ~750 mls Elgastat x 1 purified H<sub>2</sub>O,  
adjust pH with 5 N HCL.Make up volume to 1 litre with Elgastat x 1 purified H<sub>2</sub>O.Dilute 1:10 with Elgastat x 1 purified H<sub>2</sub>O for use (TBS).**RESOLVING GEL (LOWER GEL)****7.5% Acrylamide/Bis**2.35 mls Elgastat x1 purified H<sub>2</sub>O1.0 ml gelatin 10 mg/ml Elgastat x1 purified H<sub>2</sub>O

Bovine Skin Type III Bloom 225

2.5 mls Stock A Buffer

100 µl 10% SDS

BioRad, Hemel Hempstead, UK

50 µl 10% ammonium persulphate

4.0 mls acrylamide/bis

acrylamide/bis-acrylamide 37.5:1 (30% solution) BioRad, Hemel Hempstead, UK

Mix by gently swirling. Degas via water

Pressure for 5-10 minutes.

5 µl TEMED

Add TEMED, swirl to mix gently.

### **STACKING GEL (UPPER GEL)**

3.05 mls Elgastat x1 purified H<sub>2</sub>O

**4 % Acrylamide/Bis**

1.25 mls stock B buffer

50 µl 10% SDS

BioRad, Hemel Hempstead, UK

50 µl 10% ammonium persulphate

0.65 mls acrylamide/bis

BioRad, Hemel Hempstead, UK

Mix by gentle swirling. Degas by vacuum

for 5 – 10 minutes.

5 µl TEMED

Swirl to mix gently.

### **TRITON-X-100 WASH**

2.5% Triton-X-100 in TBS 1:10

Merck Ltd, Lutterworth, UK

**DIGESTION BUFFER****pH 7.6**

50 mM Tris

0.2 M NaCl

5mM CaCl<sub>2</sub>

Merck Ltd, Lutterworth, UK

1μM ZnCl<sub>2</sub>

Merck Ltd, Lutterworth, UK

0.02 % Brij-35

Dissolve in ~750 mls Elgastat x1 purified

H<sub>2</sub>O, adjust pH with 5N HCL.

Make up to 1 litre with Elgastat x1 purified

H<sub>2</sub>O. Store at 5°C.**DESTAINING SOLUTION**

30 % Methanol

Merck Ltd, Lutterworth, UK

10 % glacial acetic acid

Merck Ltd, Lutterworth, UK

750 mls Elgastat x1 purified

H<sub>2</sub>O**STAINING SOLUTION**

0.5 % w/v Coomassie brilliant blue R250

BioRad, Hemel Hempstead, UK

in destaining solution.

## REVERSE ZYMOGRAPHY

### RESOLVING GEL (LOWER GEL)

**12 % Acrylamide/Bis**

1.69 mls Elgastat x1 purified H<sub>2</sub>O

1.0 ml gelatin 10 mg/ml Elgastat x1 purified H<sub>2</sub>O      Bovine Skin Type III Bloom 225

2.5 mls Stock A Buffer

100 µl 10% SDS

BioRad, Hemel Hempstead, UK

50 µl 10% ammonium persulphate

4.0 mls acrylamide/bis

BioRad, Hemel Hempstead, UK

0.66 mls Solution A

Mix by gentle swirling.

Degas via water pressure/dissector

vacuum for 10 minutes.

5 µl TEMED

Swirl to mix gently.

### STACKING GEL (UPPER GEL)

2.89 mls Elgastat x1 purified H<sub>2</sub>O

**5% Acrylamide/Bis**

1.25 mls stock B buffer

50 µl 10% SDS

BioRad, Hemel Hempstead, UK

50 µl 10% ammonium persulphate

0.812 mls acrylamide/bis

BioRad, Hemel Hempstead, UK

Mix by gentle swirling. Degas by vacuum

for 5 – 10 minutes.

5  $\mu$ l TEMED

Swirl to mix gently.

**WASH BUFFER/RINSE BUFFER**

**pH 7.5**

6.05 g 50 mM Tris

735 g 5mM CaCl<sub>2</sub>

Merck Ltd, Lutterworth, UK

25 mls 2.5 % Triton-X-100

Merck Ltd, Lutterworth, UK

Dissolve in ~750 mls Elgastat x1 purified

H<sub>2</sub>O, adjust pH with 5N HCL.

Add 25 mls Triton-X-100, allow to dissolve completely ~ 1 – 1.5 hours on magnetic stirrer.

Make up to 1 litre with Elgastat x1 purified H<sub>2</sub>O.

**INCUBATION/DIGESTION BUFFER**

As rinse buffer but without the Triton-X-100.

Recipes for other reagents refer to Zymography protocol.

## APPENDIX III

### PRESENTATIONS

#### Oral

Robinson LLL. Regulation of human fetal ovarian development. Invited speaker at Gynaecological Visiting Society (Edinburgh) October 2003

Robinson LLL, Wood KL, Spears N, Anderson RA. A role for neurotrophins in human ovarian development. Presented at the Edinburgh Obstetrical Society Registrars' Evening (Edinburgh) November 2001.

Robinson LLL, Wood KL, Spears N, Anderson RA. A role for neurotrophins in human ovarian development. Presented at the Munro Kerr Summer Meeting (Edinburgh) June 2001.

Robinson LLL, Fulton N, Brooks J, Spears N, Anderson RA. Development of the human fetal gonad. Invited lecture at a Satellite Symposium for the Animal Science Annual Meeting (Aberdeen) September 2000.

Robinson LLL, Fulton N, Brooks J, Spears N, Anderson RA. Identification of neurotrophins and their receptors in human fetal gonads. Presented at the Munro Kerr Summer Meeting (Edinburgh) June 2000.

**Poster**

Robinson LLL, Sznajder NA, Riley SC, Anderson RA. Identification and localisation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in human fetal gonads. Presented at the Society for Gynaecological Investigation (Toronto) March 2001.

Robinson LLL, Sznajder NA, Riley SC, Anderson RA. Identification and localisation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in human fetal gonads. Presented at the Scottish Consultants in Obstetrics and Gynaecology Millennium Meeting (Pitlochry) December 2000.

Robinson LLL, Fulton N, Brooks J, Spears N, Anderson RA. Identification of neurotrophins and their receptors in human fetal gonads. Presented at the Society for the Study of Fertility (Edinburgh) August 2000.



## APPENDIX IV

### PUBLICATIONS

# The Human Fetal Testis Is a Site of Expression of Neurotrophins and Their Receptors: Regulation of the Germ Cell and Peritubular Cell Population

LYNNE L. L. ROBINSON, JULIE TOWNSEND, AND RICHARD A. ANDERSON

Medical Research Council Human Reproductive Sciences Unit, Centre for Reproductive Biology, Edinburgh EH16 4SB, United Kingdom

In the fetal testis, organization of the tissue into two compartments consisting of cords containing Sertoli and germ cells surrounded by peritubular cells and of other cells within the interstitium is essential for subsequent function. Neurotrophins (NTs) act as survival and differentiation factors in the nervous system and have been detected in the developing rodent testis. Expression of mRNA for nerve growth factor; NTs 3 and 4 and brain-derived neurotrophic factor; the high-affinity receptors TrkA, TrkB, and TrkC; and the low-affinity p75 receptor were detected in the human testis between 14 and 19 wk gestation. NT4 mRNA and protein were predomi-

nantly localized to the peritubular cells. These cells were also the site of expression of p75. By contrast, nerve growth factor and NT3 were mainly expressed in Sertoli and interstitial cells. Treatment of testis organ cultures with the Trk-specific kinase inhibitor K252a resulted in a marked decrease in both gonocyte and peritubular cell number and proliferation with little effect on Sertoli cells. These data demonstrate the expression of NTs and their receptors in the human fetal testis during the second trimester and indicate possible roles in the regulation of proliferation and survival of germ cells and peritubular cells. (*J Clin Endocrinol Metab* 88: 3943–3951, 2003)

DEVELOPMENT OF THE human fetal testis involves differentiation, maturation, and proliferation of several cell types. The first sign of male gonadal development is the appearance of Sertoli cells at 6–7 wk gestation (1), initiated by the expression of the testis-determining gene *Sry* (2). After their migration from the yolk sac to the nephrogonadoblastic ridge, primordial germ cells associate with Sertoli cells to form the seminiferous cords. The peritubular cell population originates from the mesonephros. Peritubular cell migration is also determined by *Sry* and is central to cord formation (3–5); however, the factors mediating these crucial intercellular signals are poorly understood. At the same time, there is considerable proliferation of germ cells and Leydig cells (6–9). This period of development is believed to be central to the establishment of adult testicular function and is also the point at which the cellular abnormalities that later manifest as testicular malignancies may arise (10). This study investigates the possibility that neurotrophins may be involved in these intercellular interactions in the developing human testis.

A number of paracrine factors controlling migration, differentiation, and proliferation of primordial germ cells and Sertoli cells, including members of the TGF $\beta$  family, have been described in the rodent (11–14). Neurotrophins are members of the nerve growth factor (NGF) family and are related to TGF $\beta$ . They regulate neuronal survival and differentiation in nervous tissue (15, 16) and include NGF,

brain-derived neurotrophic factor (BDNF), neurotrophin (NT) 3, NT4 (also known as NT5), and NT6 (16). Many of the effects of the neurotrophins are mediated via high-affinity tyrosine kinase (Trk) receptors, which have specificity for the various neurotrophins (17). Three members of the Trk receptor family have been described: TrkA, the receptor for NGF; TrkB, the receptor for BDNF and NT4; and TrkC, the receptor for NT3. Truncated forms of the TrkB and TrkC receptors, which lack the intracellular tyrosine kinase domains, have also been described, although their function is unknown (16–18). All neurotrophins are also recognized by a more widely expressed receptor known as p75, which is a member of the TNF receptor family (19).

Neurotrophins also may function in nonneuronal tissues (20), including the developing gonads of both sexes. For example, several members of the neurotrophin family and their receptors have been identified in the developing rodent testis (21–24), and roles in testicular cord formation (23) have been suggested in addition to later involvement in the function and interaction of germ cells and Sertoli cells (25–27). Preliminary data suggest that members of the neurotrophin family and their receptors may also be expressed in the developing human testis (24). Recent studies have shown that NT4 and its cognate high-affinity receptor TrkB may be of particular importance in the interaction between germ cells and somatic cells in the developing ovary of both rodent and human (28, 29). To determine whether neurotrophins could influence development and maturation of the human fetal testis, we have examined the cell-specific expression and distribution of several members of the neurotrophin family and their receptors during the second trimester, with particular emphasis on NT4 and TrkB.

Abbreviations: BDNF, Brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; ER, estrogen receptor; HRP, horseradish peroxidase; NGF, nerve growth factor; NRS, normal rabbit serum; NT, neurotrophin; SSC, sodium saline citrate; TBS, Tris-buffered saline; Trk, high-affinity tyrosine kinase.

## Materials and Methods

### Tissues

Human fetal testes (13–19 wk gestation) were obtained after medical termination of pregnancy. Women gave consent according to national guidelines (30), and the study was approved by the Lothian Paediatrics/Reproductive Medicine Research Ethics Sub-Committee. Termination of pregnancy was induced by treatment with mifepristone (200 mg orally), followed by 1 mg prostaglandin E1 analog (Gemprost, Beacon Pharmaceuticals, Tunbridge Wells, UK) administered every 3 h per vaginam. None of the terminations were for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Gestational age was determined by ultrasound examination before termination and confirmed by subsequent direct measurement of foot length. A total of 15 specimens were used for this study (13 wk, two specimens; 14 wk, three; 15 wk, two; 16 wk, two; 17 wk, four; and 19 wk, two specimens).

Testes were dissected free and either fixed for immunohistochemical analysis or snap frozen and stored at  $-70^{\circ}\text{C}$ . Fixation was carried out in Bouin's fixative for 5 h, followed by transfer to 70% ethanol, before processing into paraffin, using standard methods.

### Isolation of RNA and RT-PCR

Total RNA was extracted from snap-frozen samples of fetal testis using the RNeasy mini kit (Quiagen, Crawley, UK). RNA was treated with DNase (Life Technologies, Inc., Paisley, UK), and reverse transcription was performed using a first-strand cDNA synthesis kit (Roche Diagnostics, Lewes, UK) on aliquots containing 1  $\mu\text{g}$ . Subsequently, PCR was performed as described previously (29). Two control tubes were run in parallel, one in which water replaced the RNA and a second omitting reverse transcriptase to ensure there was no genomic DNA contamination. Primers specific for human neurotrophins and their receptors were used and designed to span an intron in the case of the Trks, to prevent amplification of any contaminating DNA (Table 1). Primers for the constitutively expressed gene *GAPDH* were used to confirm the integrity of the RNA and efficacy of the PCR reaction. The identity of all PCR products was confirmed by direct sequencing using an Applied Biosystems (Foster City, CA) 373A automated sequencer.

### In situ hybridization for NT4

Riboprobes were generated using a PCR strategy to incorporate SP6 or T7 phage promoter sequences into NT4 PCR product (29). Probes were labeled with digoxigenin using a commercially available kit (Maxiscript, Ambion Inc., Huntingdon, UK). Briefly, DNA templates (300 ng) were incubated for 60 min at 37 C with transcription buffer; 10 U ribonuclease inhibitor; 0.5 mM each of rATP, rCTP, rGTP; 0.33 mM

rUTP; 0.17 mM digoxigenin-11-UTP; and 30 U of appropriate RNA polymerase in a final volume of 20  $\mu\text{l}$ . After the addition of 2 U DNase I (RNAase-free), the probes were incubated at 37 C for an additional 15 min before being purified through Chromaspin columns (DEPC-100, Clontech, Palo Alto, CA).

*In situ* hybridization was carried out as described previously (29). Briefly, 5- $\mu\text{m}$  sections were cut and mounted before immersion in 0.4 M HCl for 20 min. Slides were then washed and treated with 1.5  $\mu\text{g}/\text{ml}$  proteinase K at 37 C for 10 min in 0.1 M Tris/HCl and 0.05 M EDTA (pH 8.0), transferred to 0.2% glycine at 4 C for 10 min, acetylated with 0.25% acetic anhydride in 0.08 M triethanolamine (pH 8.0) for 10 min, and washed in 4 $\times$  sodium saline citrate (SSC) for 5 min. Sections were incubated with prehybridization buffer at 50 C for 2 h and then hybridized at 50 C overnight with hybridization buffer.

On the next day, sections were washed in 4 $\times$  SSC for 10 min, then incubated in RNAase A at 37 C for 30 min, washed twice in 2 $\times$  SSC for 5 min, transferred to 0.1 $\times$  SSC with 30% formamide at 37 C for 30 min, and washed in Tris-buffered saline (TBS, pH 7.4) for 5 min. For detection of digoxigenin, labeled sections were then incubated sequentially at room temperature, with 2 $\times$  5 min washes in TBS between steps, in: 1) 3%  $\text{H}_2\text{O}_2$  in TBS for 30 min; 2) normal rabbit serum (NRS), 1:5 dilution in TBS containing 5 drops/ml avidin block (Vector Laboratories, Peterborough, UK) for 30 min; 3) biotin block (Vector Laboratories) 8 drops/ml in TBS for 30 min; 4) sheep antidigoxigenin (1:100 in NRS/TBS; Roche Diagnostics) for 2 h; 5) biotinylated rabbit antishsheep IgG (1:500 in NRS/TBS; Vector Laboratories) for 30 min; 6) ABCComplex-horseradish peroxidase (HRP) (DAKO Corp., Cambridge, UK) for 30 min; and 7) diaminobenzidine liquid substrate-chromagen system (DAKO Corp.) for approximately 2 min. Sections were then counterstained in hematoxylin, dehydrated, and coverslip mounted with Pertex mounting medium (Cellpath, Newtown, UK).

### Immunohistochemistry

Immunohistochemistry was performed to localize the expression of NGF, NT3, NT4, the receptors TrkB and p75, and bromodeoxyuridine (BrdU), as described previously (29). Briefly, 5- $\mu\text{m}$  sections were mounted, dewaxed, and rehydrated. Antigen retrieval was used for TrkB only by immersion of slides in boiling 0.01 M citrate buffer (pH 6.0) for 2.5 min, then leaving them to stand in the buffer for 10 min. In all cases, endogenous peroxidase activity was inhibited by incubation in 3%  $\text{H}_2\text{O}_2$  in methanol for 30 min. After a wash in water, slides were transferred into TBS [0.05 M Tris and 0.85% NaCl (pH 7.4)] for 5 min and blocked for 30 min in the appropriate diluted serum. Sections were then blocked with avidin (0.01 M, 15 min) and biotin (0.001 M, 15 min) (both from Vector Laboratories), with washes in TBS in between. The following primary antibodies were used: NGF, NT4, NT3 (all rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), p75 (mouse monoclonal; Neomarkers, Fremont, CA), anti-BrdU (mouse monoclonal; Roche Diagnostics). These were applied at dilutions of 1:30, 1:100, 1:50, 1:25, and 1:30, respectively. Three antibodies against TrkB were used [rabbit polyclonal (Oncogene, San Diego, CA), rabbit polyclonal (Santa Cruz Biotechnology), and chicken polyclonal (Promega, Southampton, UK)] in serial dilutions of 1:25, 1:50, and 1:100 in the appropriate serum at 4 C overnight.

Sections were then washed and incubated for 30 min with a biotinylated secondary antibody diluted 1:500 in the appropriate serum. For NGF, NT4, and NT3, a biotinylated antirabbit secondary antibody was used; for p75 and anti-BrdU (both DAKO Corp.), biotinylated antimouse was applied; and for TrkB, both biotinylated antirabbit (DAKO Corp.) and antichick (Jackson Laboratories, Bar Harbor, ME) secondary antibodies were used. After washes in TBS, sections were incubated with avidin biotin HRP-linked complex (DAKO Corp.) according to the manufacturer's instructions, and bound antibody was visualized using 3,3'-diaminobenzidine tetra-hydrochloride (DAKO Corp.). Primary antibodies were omitted as negative controls, except for NGF, in which the primary antibody was preabsorbed with the blocking peptide (Santa Cruz Biotechnology).

All sections were counterstained with hematoxylin, dehydrated, mounted, and visualized by light microscopy. Images were captured using an Olympus Provis microscope (Olympus Optical Co., London, UK) equipped with a Kodak DCS330 camera (Eastman Kodak, Roch-

**TABLE 1.** Sequence of primers used for detection of neurotrophins and their receptors

Gene	Primer	Sequence (5'–3')	Product size (bp)
NGF	Forward	TAAAAAGCGGCGACTCCGTT	167
	Reverse	ATTCGCCCCCTGTGGAAGATG	
BDNF	Forward	AACAATAAGGACGCAGCATT	222
	Reverse	TGCAGTCTTTTGTCTGCCG	
NT3	Forward	TACGCGGAGCATAAGAGTCAC	334
	Reverse	GGCACACACACAGGACGTGTC	
NT4	Forward	CTTTCGGGAGTCAGCAGGTGC	400
	Reverse	CAGGCAGTGTCAATTCGAATCC	
TrkA	Forward	TCTTCACTGAGTTCCTGGAG	229/247 <sup>a</sup>
	Reverse	TTCTCCACCGGGTCTCCAGA	
TrkB	Forward	CATGTTACCAATCACACGGAGTACC	525
	Reverse	GGATCTTGGTCATTCCAATAATGAC	
TrkB	Forward	CATGTTACCAATCACACGGAGTA	430
	Reverse	CCATCCAGTGGGATCTTATGAAA	
TrkC	Forward	CATCCATGTGGAATACTACC	228/204 <sup>a</sup>
	Reverse	TGGGTCACAGTGATAGGAGG	
p75	Forward	CTGGACAGCGTGACGTTCTCC	489
	Reverse	CTGCCACCGTGGCTATGA	

<sup>a</sup> Spliced variants.

ester, NY) and assembled using Photoshop 6.0 (Adobe, Mountain View, CA).

### Immunoblotting

Immunoblotting was carried out according to the protocol described previously (29). Briefly, fetal testes were homogenized in denaturing buffer, and samples (20  $\mu$ g protein) were diluted with an equal volume of reducing loading buffer and boiled for 5 min. Proteins were separated by SDS-PAGE on a 4–20% gradient Tris-glycine gel using Tris-glycine SDS running buffer (both from Novex, Invitrogen, Amsterdam, The Netherlands) in parallel with prestained protein molecular weight markers (Bio-Rad, Hercules, CA) and blotted onto polyvinylidene difluoride membranes (Amersham Life Sciences, Buckinghamshire, UK) overnight using a wet-blot apparatus (Bio-Rad).

Thereafter, membranes were soaked for 5 min in methanol and washed briefly several times in TBS. They were then blocked for 8 h at room temperature in 0.02 M TBS (pH7.6) containing 3% weight/volume BSA (Sigma) and 5% powdered milk. Membranes were washed in TBS with 0.1% Tween 20, and then incubated for overnight with the primary antibody. Antibodies to NT4 (rabbit polyclonal; Santa Cruz Biotechnology), full-length TrkB (rabbit polyclonal; Oncogene), and p75 (mouse monoclonal; Neomarkers) were applied at dilutions of 1:500, 1:50, and 1:500, respectively, in TBS with 0.1% Tween 20 and 1% BSA at 4 C. Primary antibody was omitted as a negative control. Bound antibody was detected using either antirabbit or antimouse HRP-linked secondary antibodies (1:4000; Amersham Life Sciences) and the enhanced chemiluminescence visualization system (Amersham Life Sciences) according to the manufacturer's instructions.

### Testis culture

Testes from three fetuses of 14, 16, and 19 wk gestation were dissected free of adherent tissues using sterile technique, bisected longitudinally, and then cut into slices approximately 0.5 mm thick under a dissecting microscope. Samples of fresh tissue were fixed for histological analysis. The remaining tissue fragments were cultured on 0.4  $\mu$ m pore Millicell CM filters (Millipore, Bedford MA) in a 24-well plate (Transwell, Costar, High Wycombe, UK). To each well 0.4 ml of medium was added, enough to just cover the tissue fragments. Any remaining wells were partially filled with medium to maintain humidity in the culture vessel.

The medium comprised MEM $\alpha$  (Life Technologies, Inc., Paisley, UK) containing 3 mg/ml BSA, antibiotics (100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin sulfate, and 0.125  $\mu$ g/ml amphotericin), 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, and 5  $\mu$ g/ml sodium selenite, 2 mmol/liter glutamine, and 2 mmol/liter pyruvate (all chemicals supplied by Sigma). To some wells the Trk inhibitor K252a (Calbiochem, Nottingham, UK) was added, at a concentration of 100 nmol/liter. Dimethylsulfoxide was used as the solvent for K252a and was added to control wells at the appropriate dilution. The cultures were maintained at 37 C in a humidified incubator, under 5% CO<sub>2</sub> in air for 48 h. After 24 h, the medium was removed and replaced with fresh medium containing 30  $\mu$ mol/liter BrdU (Sigma) to label proliferating cells. At the end of the culture period, tissue fragments were fixed for 1 h in Bouin's fluid, and then transferred to 70% ethanol before embedding in paraffin wax for histological analysis. The tissue was sectioned at 5  $\mu$ m thickness and immunostained for BrdU, using the protocol described above.

Sections of uncultured control and cultured tissue were analyzed to investigate the effects of culture and of K252a on the number and proliferation of the various cell types present. Analysis was carried out blind using the Area Fraction Probe in the Stereologer software program (Systems Planning and Analysis Inc., Alexandria, VA), as described previously (31). Tissue was serially sectioned, and sections greater than 20  $\mu$ m apart were counted, ensuring the same cells were not counted more than once. The counting was performed using a 121-point grid in the eyepiece of the microscope. Only cells whose nuclei lay beneath the intersections on the grid were counted. The number of germ cells, Sertoli cells, peritubular cells, and interstitial cells present per randomly chosen grid were recorded, with the number of each cell type that was immunostained for BrdU. Between 25 and 73 grids were counted for each testis piece, as determined by the program. The number of points lying outside the tissue in any grid was also recorded and the total cell numbers counted were corrected for this. The average number of each cell type

present per grid was calculated for each experimental condition, and data were analyzed using paired *t* tests.

## Results

### Expression of mRNAs for neurotrophins and their receptors

Expression of mRNAs was detected by RT-PCR using RNA extracted from fetal testes (Fig. 1). RT-PCR for the constitutively expressed gene *GAPDH* was used to confirm the integrity of the RNA. Human fetal brain and placenta were used as positive controls (data not shown). mRNA for the neurotrophins NGF (Fig. 1a), NT3 (Fig. 1b), NT4 (Fig. 1c), and BDNF (Fig. 1d) and the receptors TrkA (Fig. 1f), TrkB (Fig. 1h), TrkC (Fig. 1g), and p75 (Fig. 1j) was detected in all specimens at all gestations examined. Both full-length and the truncated forms of TrkB were detected (Fig. 1 h and i). Products of 228- and 204-bp size were detected for TrkC (Fig. 1 g), representing spliced variants of the gene (32) confirmed by sequencing. In some samples at all gestations an additional PCR product of 300 bp was noted and the sequence found to be unrelated to the tyrosine kinase receptor family.

### In situ hybridization for NT4

Cell-specific patterns of expression of NT4 were further investigated by nonradioactive *in situ* hybridization on fixed tissue sections. NT4 mRNA expression was predominantly localized to the peritubular cells (Fig. 2, A and B). NT4 mRNA was also detected in Sertoli cells but no mRNA appeared to be expressed in gonocytes (Fig. 2B). A low level of expression was detected in the interstitium, with the exception of the endothelial cells of small blood vessels, which showed more clearly positive expression. There was no apparent change in the pattern or intensity of expression of NT4 mRNA over the gestational range examined (14–19 wk). Tissue sections incubated with the sense riboprobe showed no staining (Fig. 2A, inset) confirming the specificity of the probe.

### Immunohistochemical localization of neurotrophins and receptors

Expression of NT4, NGF, NT3, and p75 proteins was detected by immunohistochemistry in all specimens examined across the gestational range of 14–19 wk. Expression of NT4 was confirmed by immunolocalization of the protein to the cytoplasm of the peritubular cells and Sertoli cells within the seminiferous tubules; gonocytes were immunonegative (Fig. 2, C and D). Very little immunostaining was noted in the interstitial cells, but endothelial cells within small blood vessels were immunopositive for NT4 (data not shown). The most intense immunopositive reaction for NGF was in Sertoli cells (Fig. 2E), although some gonocytes also appeared immunopositive. Weak staining was noted in the interstitial cells (Fig. 2E). NT3 protein was also immunolocalized mainly to the Sertoli cells, with some interstitial staining (Fig. 2F). Very few gonocytes stained positively for NT3, and no protein was detected in the peritubular cells.

Immunolocalization of p75 was most intense in the peritubular cell compartment (Fig. 2, G and H), but it was also present in the interstitial cells. Neither Sertoli cells nor gonocytes showed any p75 immunoreactivity. Despite the use of

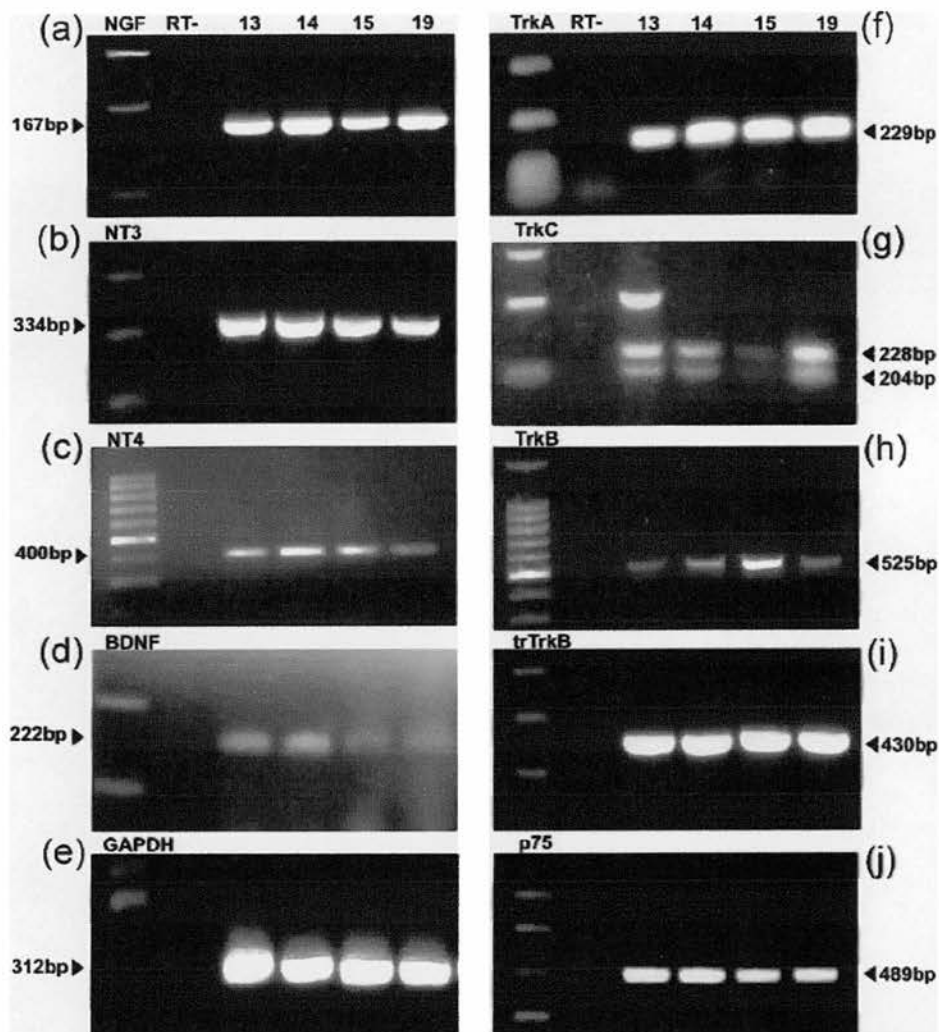


FIG. 1. Expression of mRNA for neurotrophins and their receptors in human fetal testis. RT-PCR analysis of mRNA expression of the neurotrophins and their receptors, as shown above each panel, in samples extracted from whole testes from 13–19 wk gestation. Products of 228 and 204 bp were detected for TrkC (g), representing spliced variants of the gene, and in some samples an additional PCR product of 300 bp was detected. e, *GAPDH* expression is shown. Lanes marked RT- contained samples in which the reverse transcriptase was not included.

three different antibodies, specific staining for TrkB receptor protein was not detectable.

No change in pattern or intensity of staining for any of the proteins detected was noted over the gestational range examined. Control experiments conducted by omitting the application of the primary antibody or using a specific blocking peptide, in the case of NGF, showed no staining (Fig. 2, C and E, insets).

#### Immunoblotting

The presence of NT4, TrkB, and p75 proteins in the fetal testis was confirmed by immunoblotting; a positive control of rat cerebral cortex was run on all gels (Fig. 3). The immunoblot of NT4 protein expression detected a single band of 21 kDa molecular mass in all samples of fetal testis (Fig. 3A). For detection of TrkB, the antibody used was directed against the full-length protein. Two immunoreactive bands of 110 kDa and 80 kDa molecular mass were detected (Fig. 3B). These bands are reported to represent the glycosylated and unglycosylated forms, respectively, of the TrkB protein (33). Two immunoreactive bands were also detected for the

p75 receptor, at 75 kDa and 65 kDa, in all samples of fetal testis (Fig. 3C). In all cases, the sizes of the proteins detected were identical to those in the positive control tissue. These immunoblots were repeated three times using different samples; no consistent changes with gestation were noted. A negative control was also performed for all three proteins by omitting the primary antibody, and in all cases immunoreactivity was abolished.

#### Testis culture

After 48 h of culture, there was a small increase in the number of gonocytes in comparison with the time 0 samples ( $P = 0.02$ ) (Fig. 4A) and a decrease in the number of interstitial cells ( $P = 0.05$ ). The numbers of Sertoli cells and peritubular cells did not change significantly during this period of culture. Consistent with this increase in gonocyte number, an average of 12% of gonocytes were immunostained with BrdU, compared with less than 2% of Sertoli cells and peritubular cells.

K252a treatment resulted in a consistent decrease in the number of gonocytes present in the tissue ( $P < 0.01$ ) (Fig. 4).

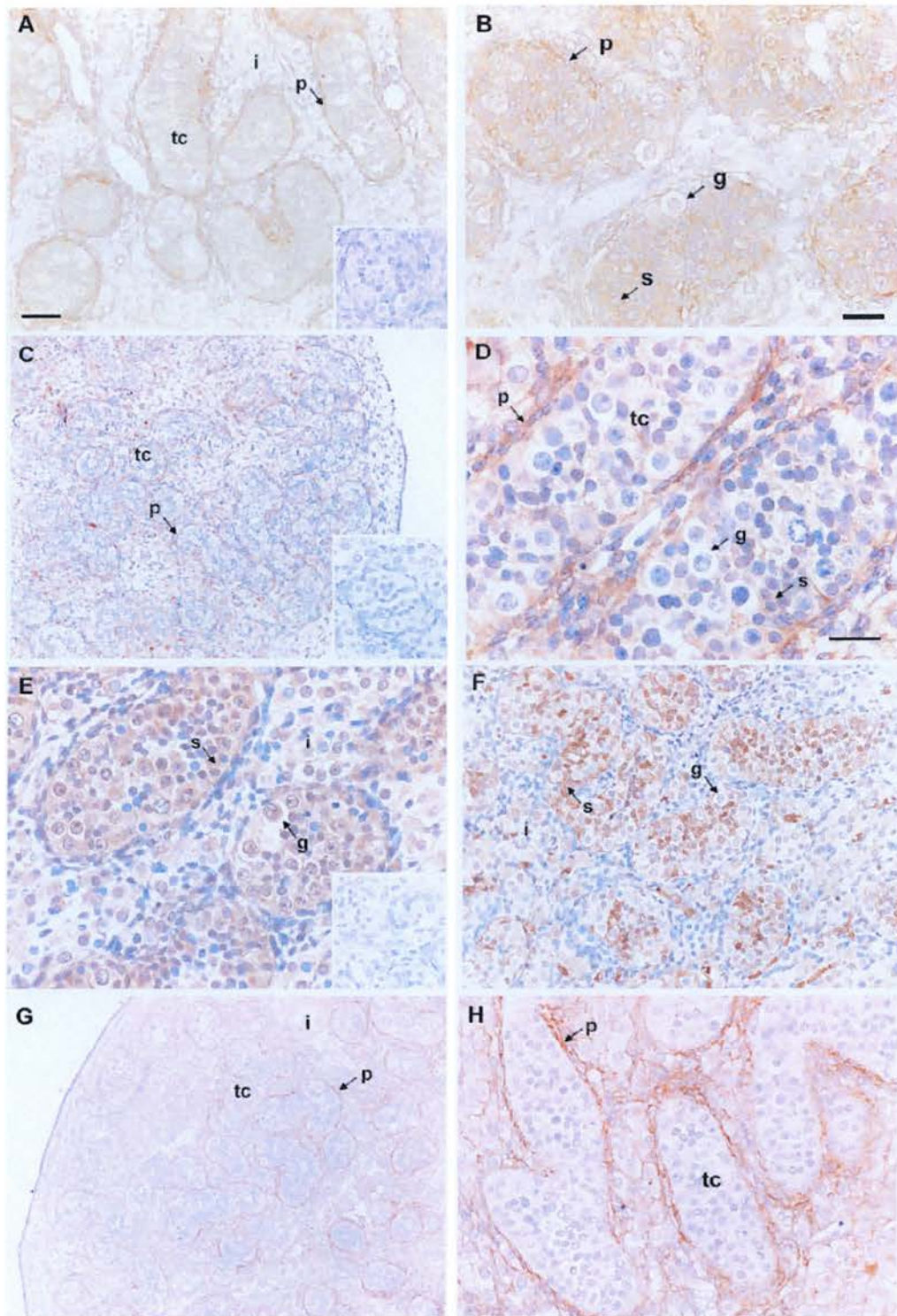


FIG. 2. *In situ* hybridization and immunohistochemical localization of neurotrophins and their receptors in human fetal testis. *In situ* hybridization: A, localization of NT4 mRNA expression in a 19-wk testis (antisense probe). *Inset* shows results for sense RNA probe; B, NT4 mRNA expression in 16-wk testis at higher magnification. Immunohistochemistry: C, NT4 in 17-wk testis; D, NT4 in 13-wk testis at higher magnification; E, 15-wk testis stained for NGF; F, 17-wk testis stained for NT3; G, 19-wk testis stained for p75; H, 17-wk testis stained for p75 at higher magnification. *Insets* in A, C, and E show negative controls. Positive staining in all panels is brown, and sections are counterstained with hematoxylin. g, Gonocyte; s, Sertoli cell; p, peritubular cell; i, interstitium; tc, testicular cord. Scale bars: A, C, and G, 500  $\mu$ m (magnification,  $\times$ 200); B, E, F, and H, 250  $\mu$ m (magnification,  $\times$ 400); D, 100  $\mu$ m (magnification,  $\times$ 1000); *insets*, magnification,  $\times$ 400.

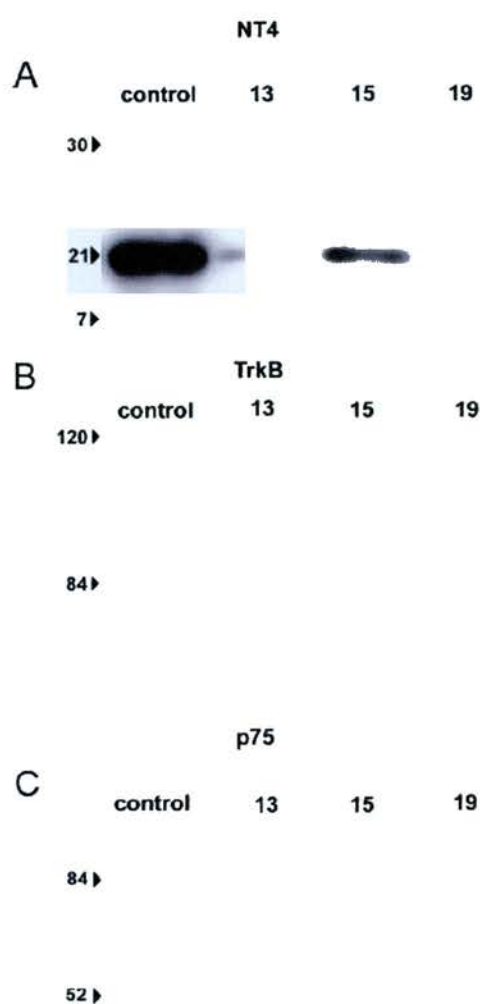


FIG. 3. Western blot of NT4, TrkB, and p75 in human fetal testis. Total protein extracts (20  $\mu$ g) from whole testes at 13–19-wk gestation, as shown, were separated by SDS-PAGE, transferred to PDF membrane, and incubated with anti-NT4, -TrkB, and -p75 antibodies. A, NT4, a protein band that migrated with an apparent molecular size of 21 kDa. B, TrkB protein bands with apparent molecular sizes of 110 and 95 kDa, representing two forms of full-length TrkB. C, p75, a protein band with an apparent molecular size of 75 kDa and an additional band of approximately 65 kDa. Control tissue was rat cerebral cortex in each case. The positions of molecular mass markers are indicated. No immunoreactive bands were detected in the absence of primary antibody (data not shown).

There was also a significant fall in the number of peritubular cells ( $P = 0.006$ ) and a small but statistically significant ( $P = 0.04$ ) reduction in the number of Sertoli cells (Fig. 4A). In contrast, there was an increase in the number of interstitial cells, but this did not reach statistical significance. The number of proliferating (*i.e.* BrdU immunopositive) gonocytes was reduced by an average of 58%, from  $2.4 \pm 0.6$  to  $0.9 \pm 0.2$  per grid (Fig. 4, B–D), very similar to the reduction in the

number of gonocytes (54%). The number of proliferating peritubular cells was also reduced, by a mean of 71%, from  $1.9 \pm 0.8$  to  $0.5 \pm 0.1$  per grid, compared with a 30% reduction in the number of peritubular cells. Thus, there was a mean fall of 26% in the proportion of gonocytes that were BrdU immunopositive, compared with a fall of 71% for peritubular cells. However these decreases did not reach statistical significance. There was also a reduction in the number of proliferating Sertoli cells, from  $0.8 \pm 0.5$  to  $0.3 \pm 0.0$  per grid, which again did not reach statistical significance.

## Discussion

The gestational age range examined in this study follows the period of testicular cord differentiation and is a time of gonocyte and Sertoli cell proliferation, and active steroidogenesis (1, 6–9). These data demonstrate the gene expression and presence of neurotrophins and their receptors in the human fetal testis at this time. Data also show that blockade of high-affinity neurotrophin receptors reduced gonocyte and peritubular cell survival and proliferation while having little effect on Sertoli or interstitial cells. The striking localization of expression of NT4 (at both mRNA and protein levels) and the p75 neurotrophin receptor to the peritubular cells may indicate that these cells are central to the functions of neurotrophins in the developing testis. The formation of cords is crucial to Sertoli- and Leydig-cell differentiation and inhibition of germ cell meiosis (2) and, thus, is not only the main morphological feature distinguishing the developing testis from the ovary but is also of paramount functional importance. In the mouse these features of normal development have been clearly linked to the *Sry* expression (4), which also induces stimulation of cell migration from the mesonephros into the gonad, and thus testis cord formation (5). Peritubular cells originate in the mesonephros (3), and their precursors have been demonstrated to express the p75 receptor (22). Trk receptor knock-out models also provide evidence for the involvement of neurotrophins in the cell migration associated with testicular cord formation (34), supporting earlier studies using K252a treatment of organ cultures (23). The present results, therefore, support the hypothesis that neurotrophin expression in the human testis is important in the regulation of normal development.

Neurotrophins are small, secreted proteins related to the TGF $\beta$  superfamily, originally identified on the basis of their role in the regulation of neuronal survival. However, neurotrophins also may regulate cell migration (35) and differentiation (36). In addition to signaling through high-affinity Trk receptors, neurotrophins also signal through the p75 receptor, and the pattern of expression of the different receptor types influences the response (37, 38). Further interaction is suggested by the demonstration that the immature form of NGF, termed proNGF, has a greater affinity for p75 than the mature form of NGF (39). Thus, neurotrophin signaling is a complex interaction between the ligands and the Trk/p75 receptor complement present. In addition to the full-length TrkB, we have identified mRNA for truncated TrkB in the developing testis. The truncated form lacks the tyrosine kinase domain and is predominantly expressed in nonneuronal cells (33, 40). Although the function of trun-

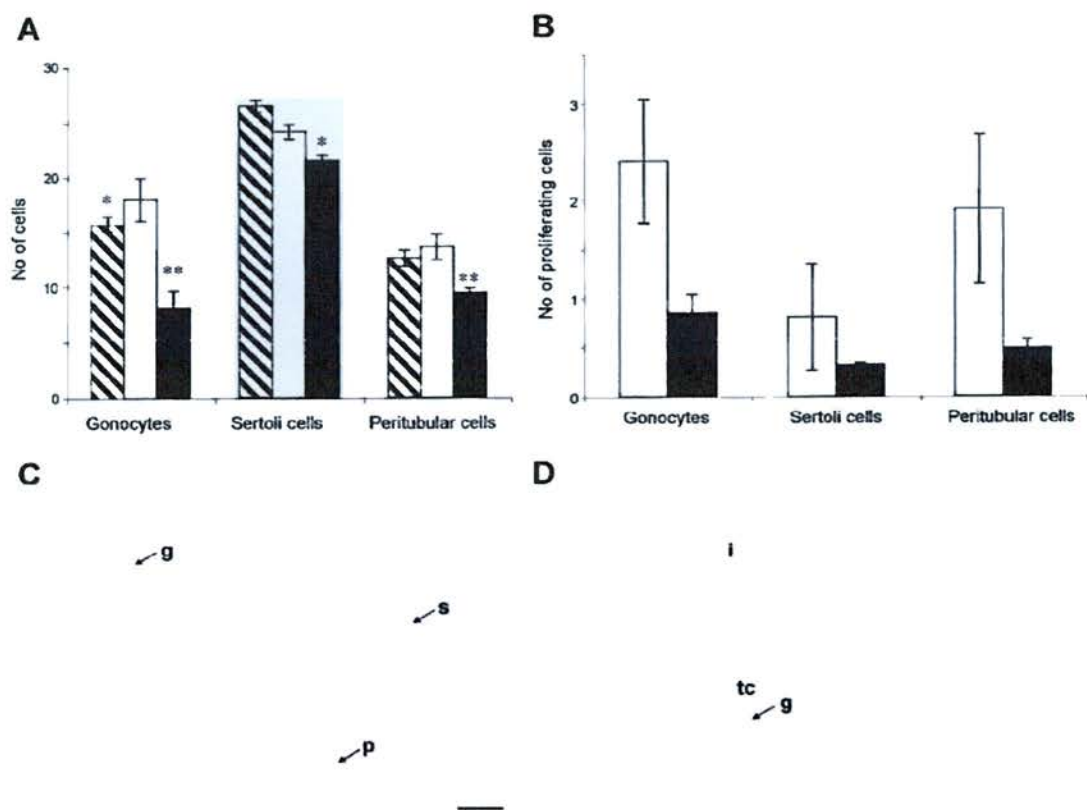


FIG. 4. Testis tissue culture data. A, Number of each cell type present per 121-point grid in the human fetal testis, in fresh and cultured tissue. Tissue from testes of 14, 16, and 19 wk gestation was used in three separate experiments.  $\square$ , Uncultured, time 0 samples;  $\square$ , control samples cultured for 48 h;  $\blacksquare$ , tissue treated in culture with K252a neurotrophin receptor inhibitor. Mean  $\pm$  SEM; \*,  $P < 0.05$  vs. control 48 h cultures. B, Number of proliferating (BrdU immunopositive) cells of each type present per grid in the human fetal testis after 48 h of culture.  $\square$ , Control samples;  $\blacksquare$ , tissue treated with K252a neurotrophin receptor inhibitor. Mean  $\pm$  SEM; n = 3. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  vs. control cultures. C and D, Sections of a 19-wk fetal testis stained using immunohistochemistry for BrdU. C, Tissue cultured for 48 h in control medium; bar, 250  $\mu$ m. D, tissue cultured for 48 h with K252a neurotrophin receptor inhibitor; bar, 250  $\mu$ m. Positive staining is brown. g, Gonocyte; s, Sertoli cell; p, peritubular cell; tc, testicular cord; i, interstitium.

cated receptors remains elusive, it has been postulated that these isoforms have a role as cell adhesion molecules acting as a selective barrier preventing the diffusion of neurotrophin and promoting elimination by internalization (41, 42).

There are increasing data regarding the expression of neurotrophins and their receptors in the developing rodent testis but very little data in the human. In the rodent, testis expression has been detected from very early in development (21, 23). The present data suggest a cell-specific distribution of the neurotrophins, although some cell types, such as Sertoli cells, expressed more than one neurotrophin. Expression of multiple neurotrophins may be required for normal development of some neuronal cell types (43). We were unable to localize TrkB using immunohistochemistry although, using RT-PCR and Western blotting analysis, expression of both full-length and truncated TrkB mRNA and the full-length protein was demonstrated. Previous reports of predominant localization of p75 in the peritubular cells in the rat (22–24) are in agreement with our findings in the human. This pattern of expression shows some parallels with that seen in the rodent and human fetal ovary (28, 29) in which

p75 is localized to the stromal cells surrounding clusters of replicating germ cells.

Functional roles for the neurotrophins in morphological sex determination, cell migration, and testicular cord formation have been suggested (23, 24). Treatment of organ cultures of E13 rat testis with K252a inhibited cord formation (23). In the present studies, we have also used K252a to investigate the functional activity of neurotrophin signaling in the developing human testis. K252a is an indole carbazole, widely used as a potent and selective inhibitor of the intracellular protein kinase domain of Trk receptors without affecting other serine/threonine kinases at the concentrations used here (23, 44, 45). K252a blocks the activity of all high-affinity Trk receptors, so these data do not permit interpretation in terms of which neurotrophin(s) and receptor(s) might be involved in the effects observed. Although the investigation of selective blockade of specific Trk receptors has been attempted in the investigation of many models, including the developing testis (23), the effects are generally much less marked than those of K252a, consistent with considerable redundancy in neurotrophin signaling, as also



demonstrated by the phenotypes of knockout animals (16). In this culture system, the integrity of the tissue was well maintained, and cell proliferation was detected. This was noted to be at a much higher rate in gonocytes than in other cell types. The major effect of K252a on the testicular cords was on gonocyte survival, with relatively little effect on Sertoli cells. Sertoli cells were the primary site of expression of NGF and NT3, and although the site of expression of Trk receptors was not demonstrated in the present study, TrkA has been previously reported to be expressed by rat Sertoli cells between E16 and P0 (21). These results suggest that neurotrophins are critical for gonocyte survival and replication, but their effects may be mediated indirectly via Sertoli cells. This is the classic pattern of Sertoli cell/germ cell interaction, with paracrine signaling between these two cell populations, most clearly exemplified by the mediation of the effects of FSH and androgen. It is also apparent that the relative number of gonocytes and Sertoli cells is maintained during early testicular development despite a 10-fold increase in the number of gonocytes between 6 and 9 wk gestation (6); the present data suggest that neurotrophin signaling may contribute to the regulation of this ratio. Selective gonocyte loss has not previously been reported as a result of neurotrophin blockade, but cord formation is certainly affected, inevitably involving gonocyte survival (23). These data may be compared with the recent demonstration that neurotrophins are involved in germ-cell survival in the human fetal ovary at a comparable stage of development (29), although in the ovary it appears that germ cells are a direct site of neurotrophin action. Although that may also be the case in the testis, direct evidence is thus far lacking.

Whereas K252a resulted in a marked loss of gonocytes, there was also a reduction in the number of peritubular cells. There was also a striking reduction in gonocyte and peritubular-cell proliferation. Because the fall in the number of BrdU immunopositive peritubular cells was much greater than the fall in the total number of that cell type (71% vs. 30%), whereas the comparable figures for gonocytes were similar (58% vs. 54%), these data may suggest a specific effect of neurotrophin on peritubular-cell proliferation in addition to survival, consistent with those cells being the major site of expression of NT4 and the p75 receptor. The importance of neurotrophins and their receptors in rat testicular cord formation (21, 23) may also reflect a major site of action on the peritubular cell population. Because the p75 receptor may promote apoptosis in the absence of Trk receptor signaling (37, 46), relatively unopposed p75 signaling may contribute to the decrease in peritubular cell proliferation and survival in the presence of K252a.

We have recently demonstrated the expression of estrogen receptor (ER)  $\beta$  within the human fetal testis (47). Sertoli and peritubular cells were demonstrated to express both ER $\beta$  1 and 2 isoforms, whereas gonocytes expressed only ER $\beta$ 2, indicating that Sertoli and peritubular cells in particular may be sites of estrogen action. Estrogen and neurotrophins interact in neural development, selectively enhancing neuronal growth and development (48), and ER mRNA may be co-expressed with neurotrophins (49, 50). Some of these effects of estrogen may be mediated by ER other than the classical ER $\alpha$  (51). The expression of both neurotrophins and their

receptors and ER $\beta$  in Sertoli cells and peritubular cells suggest that these pathways may interact in a wide variety of cell types.

In conclusion, this study demonstrates that neurotrophins and their receptors are expressed in the developing human testis during the second trimester. Neurotrophins have been demonstrated to be crucial for cellular migration, germ-cell survival, and proliferation (21–24, 34) in the rodent testis; and the present results suggest that they are likely to be of similar importance in the human, with a major role in the regulation of proliferation and survival of germ cells and peritubular cells.

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# The role of neurotrophin receptors in female germ-cell survival in mouse and human

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## Summary

During mammalian ovary formation, the production of ovarian follicles is accompanied by an enormous loss of germ cells. It is not known how this loss is regulated. We have investigated the role of the Trk tyrosine kinase receptors, primarily TrkB, in this process. The ovaries of *TrkB*<sup>-/-</sup> and *TrkC*<sup>-/-</sup> mice with a mixed (129Sv × C57BL/6) genetic background were examined shortly after birth. Around 50% of *TrkB*<sup>-/-</sup> mice had grossly abnormal ovaries that contained greatly reduced numbers of follicles. No defects were found in the ovaries of *TrkC*<sup>-/-</sup> mice. Congenic *TrkB*<sup>-/-</sup> mice were generated on 129Sv and C57BL/6 backgrounds: whereas the former had a mixed ovarian phenotype similar to that of the original colony of mice, the ovaries of all offspring of the C57BL/6 congenic line contained reduced numbers of follicles. RT-PCR showed that mRNA encoding TrkB and its two ligands, neurotrophin 4 (NT4) and brain-derived neurotrophic factor (BDNF), were present throughout the period of

follicle formation in the mouse. In situ hybridisation showed that *TrkB* was expressed primarily in the germ cells before and after follicle formation. Mouse neonatal and fetal ovaries and human fetal ovaries were cultured in the presence of K252a, a potent inhibitor of all Trk receptors. In mice, K252a inhibited the survival of germ cells in newly formed (primordial) follicles. This effect was rescued by the addition of basic fibroblast growth factor (bFGF) to the culture medium. Combined addition of both BDNF and NT4 blocking antibodies lowered germ-cell survival, indicating that these TrkB ligands are required in this process. The results indicate that signalling through TrkB is an important component of the mechanism that regulates the early survival of female germ cells.

Key words: Trk, Oogonia, Oocyte, Survival, Human, Mouse, Neurotrophin

## Introduction

Female embryonic mice produce tens of thousands of germ cells as the ovary forms. Shortly before birth, germ cells (termed oogonia at this stage) stop mitosis, initiate meiosis (which is halted at the diplotene stage) and associate closely with somatic pregranulosa cells to form primordial follicles. There is now a finite supply of female germ cells (now termed oocytes), which cannot be replenished if lost. Concurrent with these processes, there is a massive wave of cell death that results in the death of 80-90% of oocytes in mice and humans (Brambell, 1927; Baker, 1963; Hirshfield, 1991). The same general pattern of oocyte loss occurs in all mammals. In mice, this wave of oocyte death is most pronounced around the time of birth, when follicle formation is at its peak; in humans, it occurs at around five months of gestation. This process is vital to the reproductive potential of all female mammals because their reproductive lifespan is determined by the supply of primordial follicles, but its regulation is not understood.

Primordial follicles consist of an oocyte surrounded by flattened granulosa cells, and are considered to be at a 'resting' stage of development. Follicles can remain at this stage

throughout the reproductive lifespan of a female. The first sign of further development of the primordial follicle is the rounding up of granulosa cells. When follicles contain primarily rounded granulosa cells, they are considered to have entered the growth phase and are termed 'primary follicles'. This process first occurs shortly after birth in the mouse.

The neurotrophins are a small family of closely related peptide factors. Nerve growth factor (NGF) was the first to be discovered; BDNF, NT3, NT4 and NT6 have since been identified (Snider, 1994). The neurotrophins act on both high and low affinity cell-surface receptors. Many of the effects of the neurotrophins on cell survival and neuronal growth are mediated by high affinity glycoprotein tyrosine receptor kinases, or Trk receptors. Trk receptors consist of an extracellular domain, which contains the neurotrophin-binding site, a short transmembrane segment, and an intracellular domain that encodes a tyrosine kinase. The neurotrophins bind selectively to the high affinity Trk receptors, which form homodimers and autophosphorylate to trigger the intracellular cascade (Segal and Greenberg, 1996). There are three members of the Trk receptor family: TrkA, the receptor for NGF; TrkB,

the receptor for BDNF and NT4; and TrkC, the receptor for NT3. The functions of truncated forms of the TrkB and TrkC receptors, which lack the intracellular tyrosine kinase domains (Klein et al., 1990; Dechant, 2001), are unclear. In addition to the Trk receptors, all neurotrophins bind with relatively equal, low affinity to a membrane receptor known as p75, a member of the tumour necrosis receptor superfamily. The p75 receptor lacks tyrosine kinase activity, but it does appear to have signalling capabilities. It might modulate cellular responses to the neurotrophins by enhancing the sensitivity of the Trk receptors (Hantzopoulos et al., 1994), whereas in the absence of Trk receptors it can induce cell death (Friedman, 2000).

The neurotrophins are implicated in a variety of developmental processes at numerous neural sites. Their best-known roles are in the regulation of cell survival. Thus, neurons that contain one or more of the Trk receptors might require the presence of sufficient concentrations of the appropriate neurotrophin(s) for their continued survival. They might also be involved in the regulation of neuronal differentiation, growth and migration (Ghosh and Greenberg, 1995; Segal and Greenberg, 1996).

All three Trk receptors are expressed around the time of follicle formation in rats and humans (Disson et al., 1995; Anderson et al., 2002). In rats, expression of *TrkB* mRNA increases sharply and *TrkA* mRNA decreases abruptly during the period of follicle formation whereas *TrkC* remains constant throughout. Expression of *NT4* mRNA increases concomitantly with that of its ligand *TrkB*. In humans, the expression pattern of *NT4* mRNA changes as follicles start to form, with expression, which is predominantly in oogonia before follicle formation, switching predominantly to the somatic pregranulosa cells around the time of follicle formation (Anderson et al., 2002). Thus, the location of *NT4* mRNA production moves from the germ cell to the somatic cell just as germ cells undergo the massive wave of apoptosis. Together, this indicates the possible involvement of TrkB signalling in regulating germ-cell survival as follicles form.

Here, we report evidence that TrkB plays an important role in the survival of germ cells in mouse and human ovaries around the time of follicle formation. We have examined the ovaries of transgenic mice with a mutation in the catalytic domain of the TrkB and TrkC receptors (Klein et al., 1993; Klein et al., 1994), and show the results of culturing fetal and neonatal mouse ovaries and fetal human ovaries in the presence of (1) K252a, a potent inhibitor of the Trk receptors, and (2) blocking antibodies against NT4 and BDNF.

## Materials and methods

### Animals

Mice were housed in an environmentally controlled room on a 14-hour light, 10-hour dark photoperiod. Animals were provided with food and water ad libitum, and kept in accordance with UK legal requirements. Transgenic mice had a mutation in the catalytic domain of either the TrkB (Klein et al., 1993) or TrkC (Klein et al., 1994) receptor. Heterozygous pairs were bred to provide *TrkB*<sup>+/+</sup> and *TrkB*<sup>-/-</sup> offspring, and *TrkC*<sup>+/+</sup> and *TrkC*<sup>-/-</sup> offspring. Offspring were earmarked for identification and DNA prepared from the material from the ear punches for subsequent genotyping.

### Genotyping transgenic mice

For *TrkB*<sup>+/+</sup> × *TrkB*<sup>-/-</sup> offspring, sense primer 5'-TCGCGTAAA-

GACGGAACATGATCC and antisense primer 5'-AGACCATGATGAGTGGGTGCGCC were used to amplify a 900 bp *TrkB*<sup>-/-</sup> band, and sense primer 5'-CCAGCCTCTGAGCCCAGAAAGC and antisense primer 5'-GCTGAAGGACGCAGCGACAAT were used to amplify a *TrkB*<sup>-/-</sup> band of ~450 bp. PCR reactions for the *TrkB*<sup>-/-</sup> band and the *TrkB*<sup>-/-</sup> band were performed separately. For *TrkC*<sup>-/-</sup> × *TrkC*<sup>-/-</sup> offspring, we used the sense primer 5'-CTGAAGTCACTGGCTA-GAGTCTGGG together with antisense primers for *TrkC*<sup>-/-</sup> (5'-GTC-CCATCTTGCTTACCCTGAGG) and *TrkC*<sup>-/-</sup> (5'-CCAGCCTCTGAGCCCAGAAAGC), which amplified 400 bp and 500 bp bands, respectively (Schimmang et al., 1995). PCR reactions for the *TrkC*<sup>-/-</sup> band and the *TrkC*<sup>-/-</sup> band were performed simultaneously.

### Human fetal ovaries

Human fetal ovaries were obtained following medical termination of pregnancy. Women gave written consent according to national guidelines (Polkinghorne, 1989) and the study was approved by the Lothian Paediatrics/Reproductive Medicine Research Ethics SubCommittee. Termination of pregnancy was induced by treatment with mifepristone (200 mg orally) followed 48-hours later by prostaglandin E1 analogue (Gemeprost, Beacon Pharmaceuticals, Tunbridge Wells, UK) 1 mg 3-hourly per vaginam. None of the terminations were for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Gestational age was determined by ultrasound examination prior to termination and confirmed by subsequent direct measurement of foot length.

### Mouse ovary cultures

Neonatal C57BL/6 × CBA/Ca mice were killed by decapitation. Fetal ovaries were obtained from pregnant females killed by cervical dislocation 16.5 days after mating. Ovaries were removed aseptically and placed in watch glasses containing Liebovitz L-15 dissecting medium (Gibco-BRL, Renfrew, UK) supplemented with 0.3% (w/v) bovine serum albumin (BSA) (Fraction V, Sigma, Poole, UK). Tissue surrounding the ovaries was removed using sterile needles. Freshly dissected newborn ovaries were bisected using a sterile scalpel blade. In the first cultures with K252a alone (Calbiochem, Nottingham, UK) ovaries were subsequently halved again using fine-gauged needles. In the cultures with K252a and bFGF (R&D Systems, Abington, UK) and in those with the anti-NT4 and anti-BDNF blocking antibodies (Sigma), ovary halves were used because the quarter ovaries used in the earlier experiments were more difficult to handle and process for histological analysis. Whole ovaries were used with embryonic day 16.5 (E16.5) mice, as it was not possible to cut the E16.5 ovaries cleanly because of fragility of the tissue. Tissue was either fixed in Bouin's for analysis (uncultured control) or cultured. Culture pieces were placed on a polycarbonate membrane on the base of a 96-well plate (Iwaki, Japan). Wells contained 100 µl of pregassed medium overlaid with 100 µl of silicone fluid (Gibco-BRL). Ovarian pieces were cultured in α-MEM (Gibco-BRL) supplemented with ascorbic acid (28 µM) and 0.3% (w/v) BSA, with additions as detailed below. The tissue was cultured in a humidified incubator (5% CO<sub>2</sub>, 37°C). Half of the used medium (50 µl) was exchanged for fresh medium every other day for the duration of the culture period. Upon fixation, ovarian pieces were washed in PBS containing polyvinyl pyrrolidone (3 mg ml<sup>-1</sup>) to remove any medium and fixed for 1.5-2 hrs in Bouin's solution.

### Human ovary cultures

Ovaries were dissected free of adherent tissues using sterile technique, bisected longitudinally and cut into ~0.5 mm-thick slices. Samples of fresh tissue were fixed for histological analysis. The remaining tissue fragments were cultured on 0.4 µm pore Millicell CM filters (Millipore, Bedford, MA, USA) in a 24-well plate (Transwell, Costar, High Wycombe, UK). Medium (0.4 ml) was added to each well to just cover the tissue fragments. Any remaining wells were partially filled with medium to maintain humidity in the culture vessel. The

medium comprised  $\alpha$  MEM containing 3 mg ml<sup>-1</sup> BSA, 100 IU ml<sup>-1</sup> penicillin, 100  $\mu$ g ml<sup>-1</sup> streptomycin sulphate, 0.125  $\mu$ g ml<sup>-1</sup> amphotericin, 5  $\mu$ g ml<sup>-1</sup> insulin, 5  $\mu$ g ml<sup>-1</sup> transferrin, 5  $\mu$ g ml<sup>-1</sup> sodium selenite, 2 mM glutamine and 2 mM pyruvate (all chemicals supplied by Sigma). Ovaries were cultured in the presence or absence of K252a. Because K252a was reconstituted in dimethylsulfoxide (DMSO), the equivalent amount of DMSO was added to control wells. The cultures were maintained at 37°C in a humidified incubator under 5% CO<sub>2</sub> in air for 48 hours and fixed for histological analysis at the end of the culture period.

#### Histological assessment of mouse ovaries

After fixation in Bouin's, ovaries or ovarian pieces were embedded in wax and 5  $\mu$ m sections cut. Every third (cultured ovarian piece) or fifth (in vivo ovaries) section was analysed. Individual images were captured using the Leica Q5001W digital imaging microscope (Leica Microsystems, Milton Keynes, UK) using a 40 $\times$  objective. Healthy oocytes containing a visible germinal vesicle were counted. In addition, in the experiment in which NT4 and BDNF activity was inhibited, a count was made of dead and dying oocytes. In some culture experiments, the maximum and minimum diameters of each oocyte were measured. All analyses were carried out blind.

#### Histological assessment of human ovaries

Sections of tissue were analysed to determine the density of germ cells in the ovary. Analysis was carried out blind using the Area Fraction Probe in the Stereologer software programme (Systems Planning and Analysis Inc, Alexandria, VA, USA) as previously described (Sharpe et al., 2002). A 121-point graticule was used to count the number of germ cells within a frame: only cells whose nuclei lay beneath the intersections on the grid were counted. Between 18 and 42 frames were used on each ovary piece, as determined by the programme. Tissue sections were at least 20  $\mu$ m apart to ensure that no cell was counted twice. Data are presented as number of germ cells per frame.

#### RT-PCR

Ovaries were dissected from E16.5, postnatal day 0 (P0) and P4 C57BL/6  $\times$  CBA/Ca F1 mice, frozen in liquid nitrogen and mRNA subsequently extracted using a Quickprep micro mRNA purification kit (Pharmacia, St. Albans, UK). Brain tissue was collected from mice at P0. cDNA was prepared from mRNA using random primers (Promega, Southampton, UK). Separate PCR reactions were then carried out for cyclophilin, TrkB, NT4 and BDNF. Two separate TrkB reactions were carried out. The first set of primers were to the tyrosine kinase domain of the gene and, thus, recognised only full length transcripts of TrkB, the second were to the ligand-binding domain and recognised both full-length and truncated TrkB receptors. The following primers were used:

cyclophilin, 5'-CCAGGGTGGTGACTTTACAC-3' (forward), 5'-CGGAAATGGTGATCTTCTTG-3' (reverse);

TrkB (full length), 5'-ATGGCAGAGGGTAACCC-3' (forward), 5'-CTCTCTGGAGGCATCCAT-3' (reverse) (Singh et al., 1997);

TrkB (full length and truncated), 5'-CTCCGTGTGATTGGTAA-CATG-3' (forward), 5'-AGTCCAGACACTCAGGATTTGGAC-3' (reverse) (Anderson et al., 2002);

NT4, 5'-CCCTGCGTCAGTACTTCTTCGAGAC-3' (forward), 5'-CTGGACGTCAGGCACGGCCTGTTC-3' (reverse) (Botchkarev et al., 1999); and

BDNF, 5'-GTGAGAAGCTTGATGACCATCC-3' (forward), 5'-AACAGAATCCACTATCTTCCC-3' (reverse) (Botchkarev et al., 1999).

#### In situ hybridisation

Ovaries from E16.5 and P4 C57BL/6  $\times$  CBA/Ca F1 mice were fixed for 30 minutes in freshly-made 4% paraformaldehyde/PBS and embedded in wax. Sections (6  $\mu$ m) were cut and mounted on TESPA-coated slides. Slides were then dewaxed, treated in proteinase K (20

$\mu$ g ml<sup>-1</sup> for 2 minutes at 37°C) and hybridised with digoxigenin-labelled riboprobes. The probe, which was cloned into pBluescript (Stratagene, La Jolla, CA, USA), has been described previously (Klein et al., 1990) and recognised both truncated and full length TrkB. For antisense probes, plasmids were digested with *Eco*RI (Roche, Lewes, UK) and transcribed in vitro with T7 (Roche). For sense probes, plasmids were digested with *Xho*I (Roche) and transcribed in vitro with T3 (Roche). Probes were labelled with digoxigenin using a DIG RNA-Labeling Mix (Roche) and then cleaned with 70% ethanol. The probe was revealed with an anti-digoxigenin alkaline phosphatase antibody (Roche) (100  $\mu$ l made up to 50 ml with ddH<sub>2</sub>O and left overnight at 4°C). Colour detection was carried out the following day in nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indyl phosphate, toluidine salt (Roche), with levamisole (Vector, Peterborough, UK). Slides were counterstained with nuclear fast red (Vector).

#### Immunocytochemistry

Ovaries were fixed in Bouin's fixative for 1 hour then transferred to a 70% ethanol/eosin solution and embedded in wax. Sections (5  $\mu$ m) were cut and mounted on electrostatically charged slides (BDH Laboratory Supplies), dried overnight in a 60°C oven and dewaxed. Endogenous peroxidases were quenched with a 3% hydrogen peroxidase solution in methanol for 30 minutes at room temperature. Immunocytochemistry was performed as described (Anderson et al., 2002). Briefly, slides were blocked with 20% normal donkey serum (NDS; Diagnostics Scotland, Carlisle, UK) in TBS containing 5% BSA and 8 drops avidin solution per ml (Avidin/Biotin Blocking Kit, Vector) for 30 minutes at room temperature. Slides were blocked using biotin from the same kit in the same way as avidin. Chicken IgY primary antibody specific to full-length TrkB was diluted 1/10 in TBS/BSA/NDS, applied to the slides and incubated overnight at 4°C (Anti-TrkB In pAb, Promega, UK). Biotinylated donkey anti-chicken IGY secondary antibody was diluted 1/500 in TBS/BSA/NDS, applied to the slides and incubated at room temperature for 30 minutes, with avidin biotin horseradish peroxidase linked complex (DAKO) applied according to the manufacturers instructions. Bound antibody was visualised using 3,3'-diaminobenzidine tetrahydrochloride (DAKO). Sections were counterstained with haematoxylin.

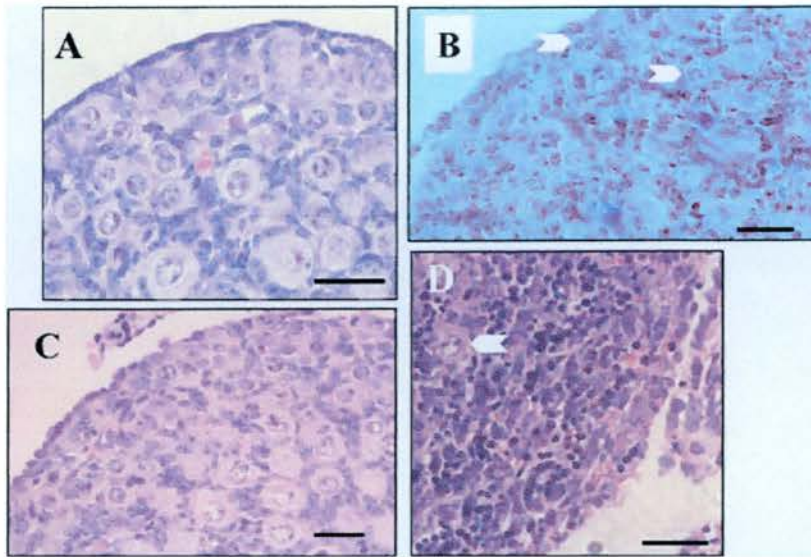
#### Statistics

Data from mouse in vivo ovary counts were analysed with Mann-Whitney U tests. Total counts of mouse and human cultured ovaries were analysed with probability values (*P*) of differences in oocyte numbers determined by analysis of variance: where appropriate, paired comparisons were made using Student's *t*-test. Where the data did not have a normal distribution, a Kruskal-Wallis test was used. The Kolmogorov Smirnov test was used to compare differences in proportions of oocytes with varying diameters in the cultured mouse ovaries.

## Results

### Transgenic mice on mixed genetic background

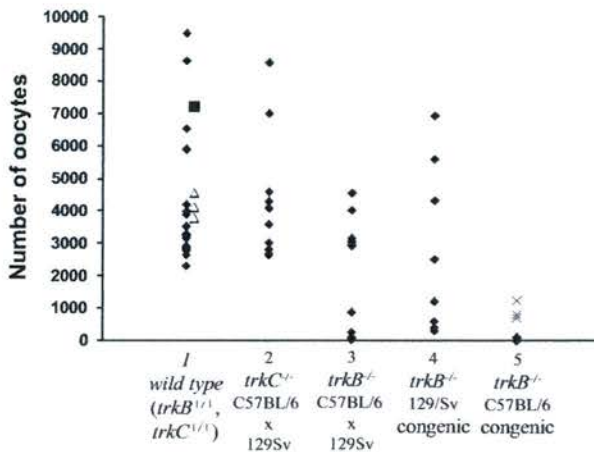
Ovaries were examined and the total number of oocytes estimated in 23 *TrkB*<sup>+/+</sup>, *TrkC*<sup>+/+</sup> mice (P4-6), 11 *TrkB*<sup>-/-</sup> mice (P4-5) and 10 *TrkC*<sup>-/-</sup> mice (P6), all on a mixed C57BL/6  $\times$  129/Sv genetic background. At this age, normal ovaries are full of follicles at the primordial and primary stage (Fig. 1A). In ~50% of *TrkB*<sup>-/-</sup> animals, the ovaries were grossly abnormal (Fig. 1B) and contained substantially fewer oocytes (<1000) than those of the wild-type animals (>2000) (Fig. 2). Sometimes, the follicles contained only a few granulosa cells or dark oocytes with abnormal looking nuclei. In all cases, the ovaries contained



**Fig. 1.** Sections of ovaries from wild-type and mutant mice at P4-5 stained with haematoxylin and eosin. Ovaries from 50% of *TrkB*<sup>-/-</sup> mice from a mixed genetic background and all mice from the congenic C57BL/6 background have grossly abnormal ovaries that contain greatly reduced numbers of follicles. (A) *TrkB*<sup>+/+</sup>, *TrkC*<sup>+/+</sup> ovary, containing many follicles. (B) *TrkB*<sup>-/-</sup> ovary on C57BL/6 × 129Sv background with only few follicles remaining (arrowheads). (C) Unaffected *TrkB*<sup>-/-</sup> ovary on C57BL/6 × 129Sv background, which contains as many follicles as (A). (D) Ovary from fourth generation C57BL/6 congenic *TrkB*<sup>-/-</sup> mouse, which contains large areas of cell death and only a few follicles (arrowhead). Scale bars: 20 μm.

a large number of dark, apoptotic-looking granulosa cells and the ovary sections had a fuzzy appearance (Fig. 1B,D). This phenotype was not seen in the ~100 ovaries from wild-type mice that we have examined to date, including the 23 *TrkB*<sup>+/+</sup>, *TrkC*<sup>+/+</sup> ovaries analysed in this experiment (Fig. 1A, Fig. 2). The ovaries

of the remaining 50% of the *TrkB*<sup>-/-</sup> mice obtained in this experiment appeared normal (Fig. 1C, Fig. 2) and contained the expected number of follicles. The ovaries of all *TrkC*<sup>-/-</sup> mice looked normal and contained oocyte numbers comparable with those of the *TrkB*<sup>+/+</sup>, *TrkC*<sup>+/+</sup> mice (Fig. 2).



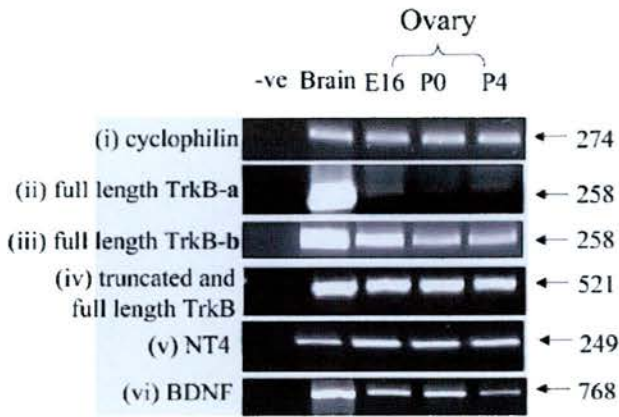
**Fig. 2.** Ovaries of *TrkC*<sup>-/-</sup> mice contain normal numbers of follicles but those of *TrkB*<sup>-/-</sup> mice contain few follicles. This phenomenon is found in ~50% of *TrkB*<sup>-/-</sup> mice on either the mixed genetic background or the 129/Sv congenic line, but all ovaries in the C57BL/6 congenic line are affected. Scattergram of number of oocytes in ovaries of *TrkB*<sup>+/+</sup>, *TrkC*<sup>+/+</sup> mice (column 1); *TrkC*<sup>-/-</sup> mice (column 2); *TrkB*<sup>-/-</sup> mice from the original mixed genetic background colony (column 3); *TrkB*<sup>-/-</sup> mice from the 129/Sv congenic colony (column 4); and *TrkB*<sup>-/-</sup> mice from the C57BL/6 colony (column 5). In column 1, *TrkB*<sup>+/+</sup>, *TrkC*<sup>+/+</sup> mouse on C57BL/6 background is represented with a filled square and those on 129/Sv background are represented with open triangles, other mice are of the mixed genetic background. In column 5, filled diamonds represent mice from the seventh generation of the C57BL/6 congenic line of *TrkB*<sup>-/-</sup> mice and crosses are mice from the fourth generation.

### Congenic strains of *TrkB*<sup>-/-</sup> mice

The mixed genetic background of the mice in the original colony was a likely explanation of the variable effect of the *TrkB*<sup>-/-</sup> mutation on ovarian development described above. To test this, congenic strains of *TrkB*<sup>-/-</sup> mice were generated, based on either C57BL/6 or 129/Sv inbred strains. Breeding pairs were set up at the fourth (C57BL/6 and 129Sv) and seventh (C57BL/6) generation to generate *TrkB*<sup>-/-</sup> offspring for analysis. Examination of all the offspring collected from 10 pairs of fourth generation matings showed that a total of five out of 225 C57BL/6 pups were *TrkB*<sup>-/-</sup> (four of these were female), in contrast with 19 out of 207 129/Sv pups (nine of which were female). This indicates that C57BL/6 *TrkB*<sup>-/-</sup> mice are more severely affected in general (although the 5 C57BL/6 *TrkB*<sup>-/-</sup> mice that survived to P4 did not appear different from the *TrkB*<sup>-/-</sup> pups on a mixed or 129/Sv background). Fig. 2 shows the number of oocytes in ovaries of the C57BL/6 and 129/Sv congenic strain *TrkB*<sup>-/-</sup> females at P4. Of the six *TrkB*<sup>-/-</sup> female mice obtained in the C57BL/6 line, all had severely affected ovaries with large apoptotic-looking areas (Fig. 1D) and few follicles (Fig. 2). By contrast, the nine female 129/Sv *TrkB*<sup>-/-</sup> mice were similar to the original mixed-background mice, with some ovaries containing normal numbers of oocytes and others with areas of cell death and depleted stores of oocytes (Fig. 2). The Mann-Whitney test showed that there were significantly fewer oocytes in the ovaries of C57BL/6 *TrkB*<sup>-/-</sup> mice than in wild-type ovaries ( $P < 0.0001$ ).

### Expression of *TrkB*, NT4 and BDNF mRNA and protein

Ovaries were obtained from female mice at E16.5, P0 and P4. RT-PCR showed that *TrkB* (both full length and truncated),



*NT4* and *BDNF* were expressed in mice at E16.5 (prior to the start of follicle formation), at P0 (in the middle of follicle formation) and at P4 (when follicle formation is complete) (Fig. 3). Full-length *TrkB* was present at very low levels at all times, compared to expression in a similar amount of brain

Fig. 3. *TrkB* (full length and truncated), *NT4* and *BDNF* are expressed in mouse ovaries at E16, P0 and P4. Full-length *TrkB* is expressed at very low levels in all ovary samples. Each RT-PCR reaction was carried out on P0 brain and on ovary at E16.5, P0 and P4. RT-PCR was carried out for: (1) cyclophilin; (2) full-length *TrkB* using equivalent amounts of mRNA (in terms of cyclophilin expression); (3) full-length *TrkB* receptor with eight times more E16 ovary mRNA than brain mRNA and five times more P0 and P4 ovary mRNA than brain mRNA (in terms of cyclophilin expression); (4) *TrkB* receptor, both truncated and full length, using equivalent amounts of mRNA (in terms of cyclophilin expression); (5) *NT4*, using equivalent amounts of mRNA (in terms of cyclophilin expression); and (6) *BDNF* using equivalent amounts of mRNA (in terms of cyclophilin expression).

tissue (as determined by equivalent expression of cyclophilin) (Fig. 3, lane 2), but when more ovary tissue was used in the reaction, the presence of the full length form in the ovary was seen clearly (Fig. 3 lane 3). By contrast, the PCR reaction that detected both full-length and truncated forms of *TrkB* showed expression at high levels in brain and in ovaries at all ages (Fig. 3, lane 4). Thus, truncated *TrkB* was expressed in the ovary at a much higher level than full-length *TrkB*.

In situ hybridisation using a probe that recognised both full-length and truncated forms of *TrkB* showed that expression was primarily confined to the germ cells, in oogonia at E16.5 and oocytes at P4 (Fig. 4A-D). This probe was checked by in situ hybridisation using E19 and P0 brain, which showed the same expression pattern as described previously (Ringstedt et al., 1993). Immunocytochemistry was then carried out using an antibody specific for full-length *TrkB*. This showed a similar pattern of ovarian expression, but *TrkB* protein was much more abundant in P0 oocytes than in E16 oogonia (Fig. 4E-H).

#### Effect of K252a on newborn mouse ovaries

Ovarian quarters from P0 mice were either fixed immediately or cultured with medium containing K252a (0, 50 and 100 nM). At P0,

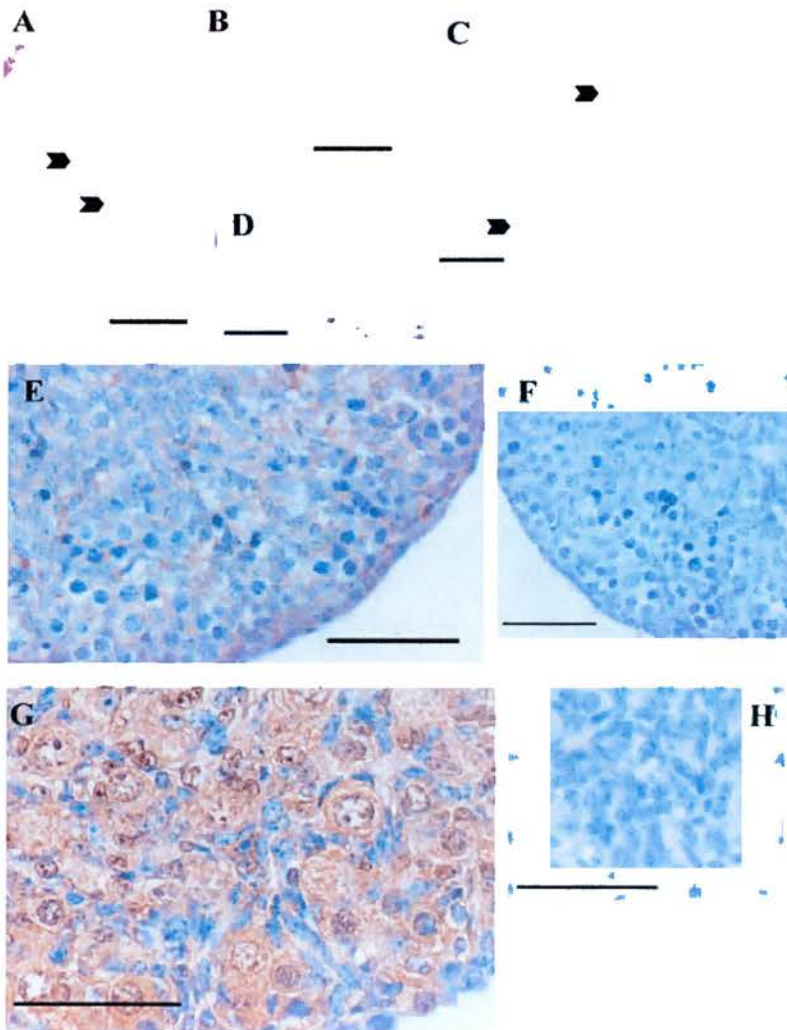


Fig. 4. *TrkB* is expressed in germ cells of ovaries both before and after follicle formation in the mouse. In situ hybridisation uses a probe that recognises truncated and full-length forms of *TrkB*, whereas immunocytochemistry is specific for full length *TrkB*. (A-D) In situ hybridisation. (A) Using antisense probe (staining is primarily in oogonia, arrowheads) and (B) sense probe on E16.5 ovaries. (C) Antisense probe (staining is primarily in oocytes, arrowheads) and (D) sense probe on P4 ovaries (D). (E-H) Immunocytochemistry. (E) On E16 ovary with primary antibody present and (F) negative control for E16 ovary with no primary antibody. There is little full-length *TrkB* present at E16, but some expression can be seen in oogonia. (G) P0 ovary with primary antibody present and (H) P0 ovary with no primary antibody (full-length *TrkB* is strongly expressed in oocytes). Scale bars: 20  $\mu$ m in A-D; 50  $\mu$ m in E-H.

ovaries contained primordial follicles. The development of follicles from the primordial to the primary, growing stage was supported in culture. This is shown in Fig. 5A,B and was confirmed by measurements of oocyte diameter (Fig. 6B,C). Ovaries from P0 mice cultured for 7 days in the presence of K252a exhibited areas of extensive cell death with few follicles present at the end of the culture period (Fig. 5C). There was a dose-dependent decrease in the total number of follicles in K252a-treated ovaries, compared to untreated cultured ovaries. The decrease in follicle number in ovaries cultured in the presence of 50 nM K252a was not statistically significant ( $P=0.08$ ), but there was a highly significant decrease in the presence of 100 nM K252a ( $P<0.005$ ) (Fig. 6A). Fig. 6C shows the proportion of oocytes at different diameters in control and K252a-treated cultured ovaries. Although >85% of oocytes were lost when ovaries were cultured in the presence of 100 nM K252a (Fig. 6A), the surviving oocytes had a similar distribution in diameter to those in ovaries cultured in control medium (Kolmogorov Smirnov test showed no significant difference in distribution). Thus, oocytes that survived in the presence of K252a grew to similar diameters to those in control cultures. K252a, therefore, inhibits the survival of follicles, but does not affect the growth of follicles that do survive.

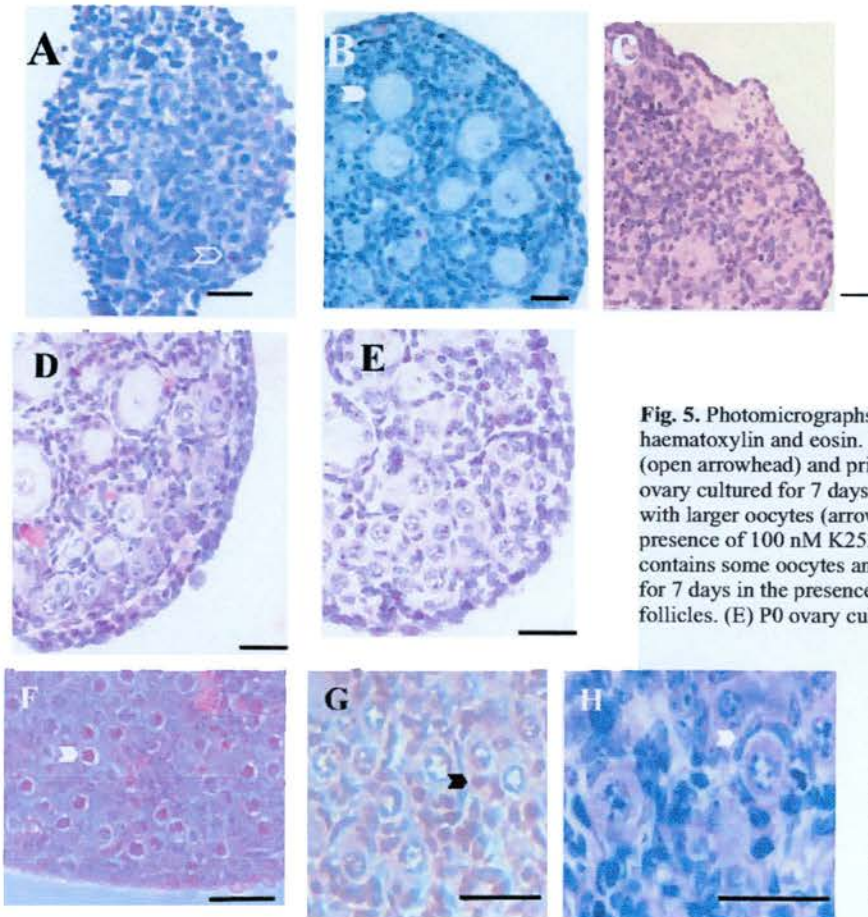
### Rescue of cultured ovaries with bFGF

Previous studies have shown that, at the doses used here,

K252a blocks Trk receptors but not other tyrosine kinase receptors, including those for bFGF (Tapley et al., 1992). This was confirmed in our study by culturing P0 ovaries in media containing: (1) 100 nM K252a; (2) 40 ng ml<sup>-1</sup> bFGF; and (3) 100 nM K252a and 40 ng ml<sup>-1</sup> bFGF. bFGF acts via a non-Trk tyrosine kinase receptor (Wert and Palfrey, 2000) and has been shown to stimulate primordial follicle development (Nilsson et al., 2001). Ovaries cultured in the presence of bFGF with or without the addition of K252a looked healthy and contained many follicles (Fig. 5D,E). Fig. 7 shows the total number of oocytes in the different treatment groups. The increase in oocyte numbers in ovaries cultured in bFGF alone was not significant. Ovaries cultured in 100 nM K252a showed a large reduction in oocyte survival, with significantly fewer oocytes present at the end of the culture period ( $P<0.05$ ). bFGF rescued follicles from the effect of K252a: there was no difference between the number of oocytes in untreated ovaries and in ovaries cultured in the presence of K252a and bFGF. K252a did not, therefore, block the function of all tyrosine kinase receptors nonspecifically.

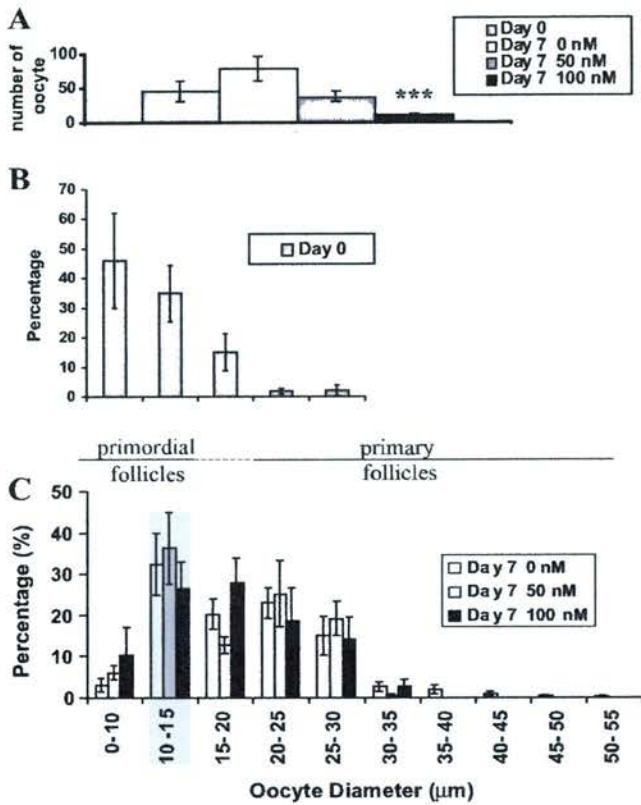
### Inhibition of BDNF and NT4 activity

P0 ovaries were cultured for 7 days in control medium or in medium containing (1) 10 µg ml<sup>-1</sup> anti-BDNF antibody, (2) 10 µg ml<sup>-1</sup> anti-NT4 antibody, and (3) 10 µg ml<sup>-1</sup> each of anti-BDNF and of anti-NT4 antibody. The addition of either anti-BDNF or anti-NT4 antibodies alone had no significant effect on oocyte survival, but when added in combination, oocyte survival was lowered significantly (Fig. 8A;  $P<0.05$ ). Examination of the cultured ovaries showed that all treated ovaries, but not control ovaries, had large areas around the edge that contained no healthy oocytes (Fig. 9). The density of dying and dead oocytes was significantly higher in all three treatment groups than in control ovaries (Fig. 8B;  $P<0.05$  in all cases).



**Fig. 5.** Photomicrographs of sections of ovarian pieces stained with haematoxylin and eosin. (A) Uncultured ovary at P0 containing oogonia (open arrowhead) and primordial follicles (closed arrowhead). (B) P0 ovary cultured for 7 days, which contains many growing, primary follicles with larger oocytes (arrowhead). (C) P0 ovary cultured for 7 days in the presence of 100 nM K252a (which inhibits oocyte survival). The ovary contains some oocytes and large areas of cell death. (D) P0 ovary cultured for 7 days in the presence of 40 ng ml<sup>-1</sup> bFGF contains many healthy follicles. (E) P0 ovary cultured for 7 days in the presence of 100 nM K252a and 40 ng ml<sup>-1</sup> bFGF. The ovary has been rescued from the effect of K252a and contains many healthy follicles. (F) Uncultured ovary from E16.5 mouse containing oogonia (arrowhead) but no follicles. (G) E16.5 ovary cultured in control medium for 4 days contains many follicles, all of which are at the primary, growing stage with rounded granulosa cells (arrowhead). (H) Ovary at P0, an equivalent age for the cultured ovary shown in G, with follicles at the primordial, resting stage with flattened granulosa cells (arrowhead). Scale bars: 20 µm.

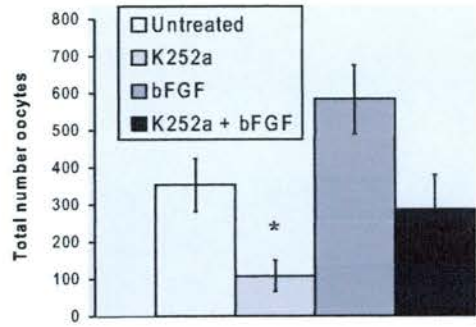




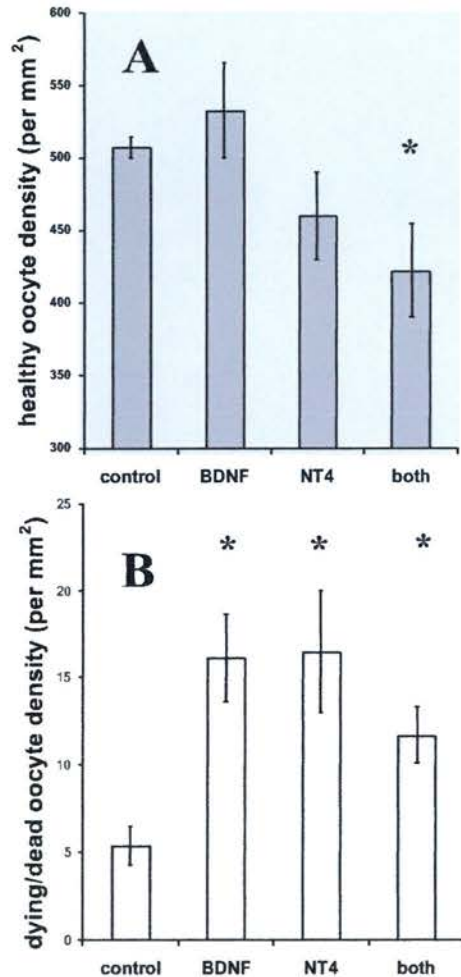
**Fig. 6.** K252a reduces oocyte survival in newborn mouse ovaries in culture, but does not affect the distribution of oocyte diameters in surviving follicles. (A) The total number of follicles in uncultured day 0 ovaries and ovaries cultured in 0 nM, 50 nM and 100 nM K252a. Asterisks indicate significant difference compared with control culture (D7 0 nM),  $P < 0.005$ . (B) The proportion of follicles containing oocytes of various diameters in uncultured day 0 ovaries. (C) The proportion of follicles containing oocytes of various diameters in ovaries cultured in 0 nM, 50 nM and 100 nM K252a. Line between B and C shows the follicle stage that corresponds to different diameters of oocytes.

**Culture of E16.5 ovaries**

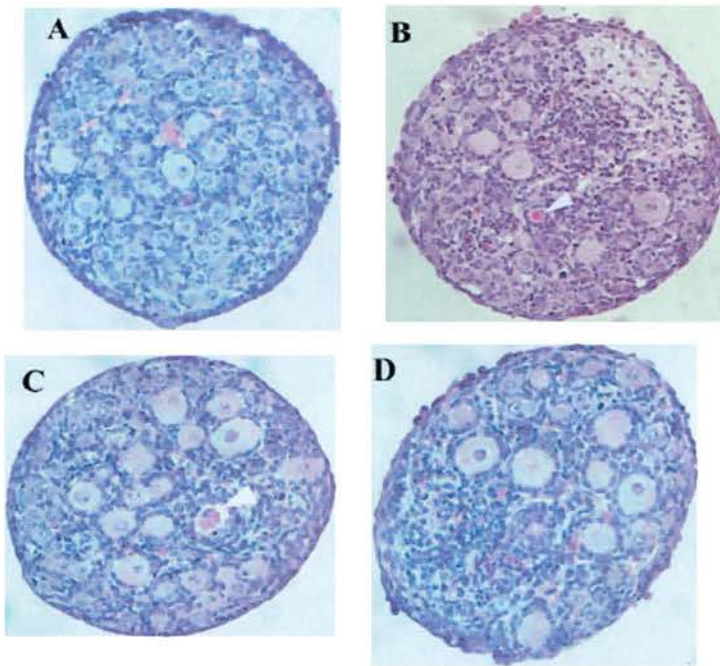
At the start of the culture period, E16.5 ovaries contained oogonia but no primordial follicles (Fig. 5F). After culture for 4 days follicles formed, with many oocyte-enclosed follicles present by the end of the culture period (Fig. 5G). Examination of sections showed that cultured E16.5 ovaries appeared not to form follicles in the normal manner. This was confirmed by examination of an ovary from a P0 animal in vivo and of an E16.5 ovary cultured in control medium for 4 days. At P0, ovaries contain both oogonia and primordial follicles (and occasional primary follicles) (Fig. 5H). E16.5 ovaries that were cultured for 4 days (the in vitro equivalent of P0 ovaries) contained oogonia and follicles by the end of the culture period. However, in marked contrast to the situation in vivo, these follicles were virtually all at the primary stage, with rounded granulosa cells (Fig. 5G). Fig. 10A shows the results of a detailed comparison of in vivo and cultured ovaries. Both ovaries contained similar percentages of oogonia and oocytes.



**Fig. 7.** bFGF rescues cultured newborn mouse ovaries from the effect of K252a. Histogram of the total number of follicles in ovaries cultured in control conditions and in 100 nM K252a, 50 ng ml<sup>-1</sup> bFGF, and K252a plus bFGF. Asterisk indicates significant difference compared with untreated control culture,  $P < 0.05$ .



**Fig. 8.** Blocking the effects of BDNF and NT4 in culture decreases germ-cell survival and increases germ-cell death. (A,B) The density of healthy oocytes (A), and dying and dead oocytes (B) in ovaries cultured in control medium or in medium containing 10 µg ml<sup>-1</sup> anti-BDNF, 10 µg ml<sup>-1</sup> anti-NT4, and 10 µg ml<sup>-1</sup> each of anti-BDNF and of anti-NT4. Asterisk indicates significant difference compared to untreated control culture,  $P < 0.05$ .



**Fig. 9.** Photomicrographs of sections of ovarian pieces stained with haematoxylin and eosin. (A-D) Ovaries were cultured for 7 days in control medium (A), medium containing  $10 \mu\text{g ml}^{-1}$  anti-BDNF (B), medium containing  $10 \mu\text{g ml}^{-1}$  anti-NT4 (C) and medium containing  $10 \mu\text{g ml}^{-1}$  each of anti-BDNF and of anti-NT4 (D). Areas around the edge of ovaries cultured in the presence of antibodies had few if any germ cells. These ovaries also often contained dying/dead oocytes (white arrowheads).

Oocytes were then further classified into those contained in primordial and primary follicles. This further classification was only possible where granulosa cells were clearly visible around the oocytes and clearly part of that follicle. This was not possible in ~65% of both in vivo and in vitro ovaries and these oocytes were excluded from further analysis. The oocytes from the in vivo ovary were predominantly at the primordial stage but those in the cultured ovary were virtually all at the primary stage. E16.5 ovaries were also examined after 1, 2 and 3 days in culture in control medium (results not shown). Examination of sections showed that in virtually no instances were primordial follicles found. It appears, therefore, that when ovaries containing only oogonia are cultured, follicles form directly at the primary (growing) stage, and completely bypass the primordial (resting) stage of development that occurs in vivo. This culture system was then used to examine the survival of primary follicles in culture.

#### Effect of K252a on E16.5 ovaries

E16.5 ovaries were cultured in medium containing 0 nM or 100 nM K252a for four days. There was no difference in oocyte number between ovaries cultured in the presence of K252a and those cultured in the absence of K252a (Fig. 10B). The way in which follicles formed in E16.5 cultured ovaries, bypassing the primordial follicle stage, might explain the lack of effect of K252a. K252a appears to affect follicles specifically at the primordial stage, which is represented in

P0 cultured ovaries but not in E16.5 cultured ovaries.

#### Culture of fetal human ovaries in the presence of a Trk receptor blocker

Ovaries from five fetuses, ranging from 13 to 16 weeks of gestation, were cultured in 0 nM or 100 nM K252a. At these ages, ovaries contained oogonia only (Fig. 11A). Even in control medium, follicles did not form but oogonia survived (Fig. 11B). In ovaries cultured in 0 nM K252a, a mean of 78% of oogonia survived after 48 hours in culture, whereas only 36% survived when ovaries were cultured in the presence of 100 nM K252a ( $P=0.01$ ; Fig. 11C,D).

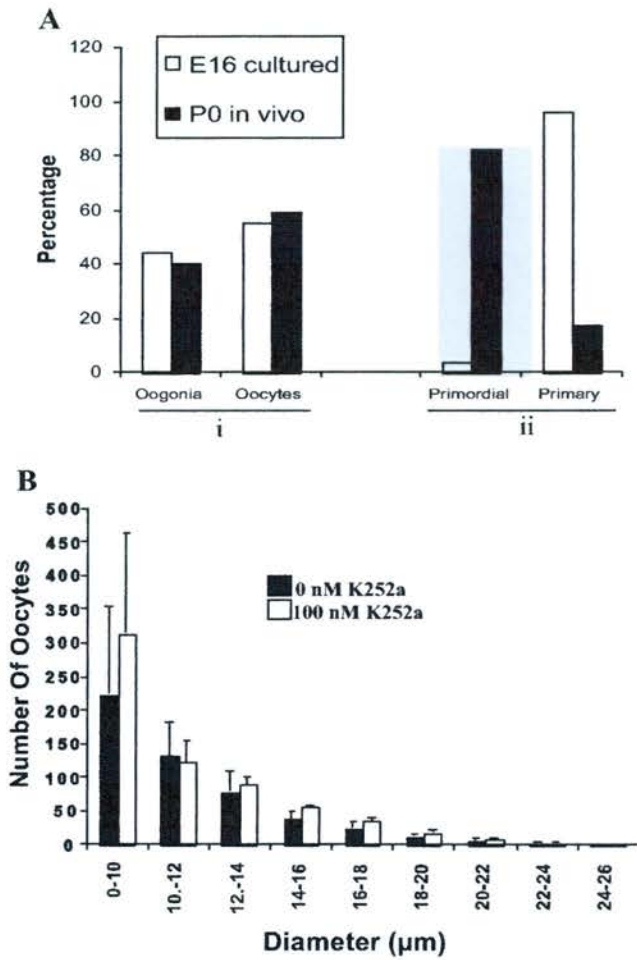
#### Discussion

The factors that determine whether oocytes survive or die as follicles form are unknown. We have investigated the possible role of the Trk receptors in this process by examining *TrkB*<sup>-/-</sup> and *TrkC*<sup>-/-</sup> mice, and interfering with the action of Trk receptors and their ligands in cultures of mouse and human ovaries.

In the original mixed-genetic background colony of *TrkB*<sup>-/-</sup> mice (C57BL/6 × 129/Sv), ~50% of ovaries contained reduced populations of oocytes, whereas the remainder appeared normal. Examination of congenic strains of *TrkB*<sup>-/-</sup> mice based on either 129/Sv or C57BL/6 lines of mice showed that, as with the original colony, ~50% of the ovaries of the 129/Sv congenic *TrkB*<sup>-/-</sup> mice had a normal complement of follicles, but that ovaries of all *TrkB*<sup>-/-</sup> C57BL/6 congenic mice had greatly reduced numbers of follicles. By contrast, the ovaries of all *TrkC*<sup>-/-</sup> mice were normal. These findings indicate that TrkB is an important factor in oocyte survival. The fact that, on certain backgrounds, its loss does not always have a significant effect indicates that other factors are also likely to be involved in oocyte survival. The efficacy of these factors might vary with background, being low in C57BL/6 and high in 129/Sv mice. Similar background effects have been found in the development of transgenic  $\alpha_3$  Connexin mice (Gong et al., 1999).

*TrkB*<sup>-/-</sup> offspring have retarded development in general and die within the first 10 days of birth. The paucity of oocytes in *TrkB*<sup>-/-</sup> mice was not caused by this general retardation because 50% of mice on the original mixed background and of the 129/Sv congenic mice had normal ovaries but retarded general development. In addition, *TrkC*<sup>-/-</sup> mice exhibited similarly retarded development but their ovaries were unaffected.

Ovaries were cultured either prior to (human), or during and shortly after (mouse) follicle formation, in the presence or absence of K252a. K252a is an indole carbazole and a potent, specific inhibitor of the intracellular protein-kinase domain of the Trk receptors (Tapley et al., 1992). K252a dramatically inhibited oocyte survival in newborn mouse ovaries in culture, inducing a loss of 85% of germ cells. In other systems, K252a is reported to block the activity of Trk receptors but not other tyrosine kinase receptors at the doses used here (Tapley et al.,



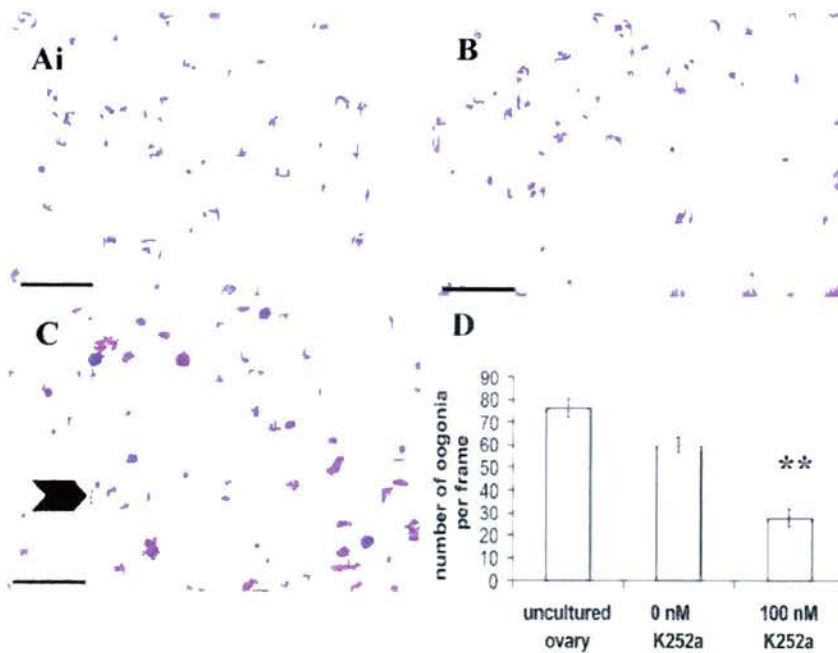
**Fig. 10.** Very few primordial follicles form in E16.5 fetal ovaries in culture and K252a has no effect on oocyte survival in this culture system. (Ai) The proportion of oogonia and oocytes in E16.5 ovaries cultured for four days and in P0 ovary without culture. (Aii) Further classification of oocytes into those contained in primordial and primary follicles, in E16.5 ovaries cultured for four days and in P0 ovary without culture. (B) The diameters of oocytes in E16.5 ovaries cultured in the presence or absence of 100 nM K252a.

1992). Evidence for this in the ovary was obtained by adding bFGF, which acts via a non-Trk tyrosine kinase receptor, to cultures containing K252a. bFGF rescued ovaries from the effects of K252a, indicating that K252a did not block bFGF receptors. These findings also demonstrate that ovarian cells are responsive to bFGF. It is conceivable that bFGF is another survival factor for oocytes acting in concert with neurotrophins.

RT-PCR showed that mRNA encoding NT4 and BDNF are both present throughout the period of follicle formation in the mouse. We have already shown this is the case in humans, where production of NT4 mRNA moves from a germ-cell to a somatic-cell location as follicles form (Anderson et al., 2002). Although NT4 expression increases in rat follicles as they start to form (Disson et al., 1995), the fertility of NT4<sup>-/-</sup> mice appears normal (Conover et al., 1995). It is not possible to examine the fertility of BDNF<sup>-/-</sup> mice because, like TrkB<sup>-/-</sup> mice, they die shortly after birth (Conover et al., 1995).

The combined inhibition of NT4 and BDNF activities in culture using blocking antibodies lowered oocyte survival, but blocking either ligand alone had no detectable effect. Dead and dying oocytes were rare in control ovaries, but significantly increased in all treated ovaries, irrespective of whether blocking antibodies were added singly or in combination. The lack of a significant effect of blocking either BDNF or NT4 alone on oocyte survival indicates that the ligands are able to

compensate for each other to a large extent. The combined addition of anti-BDNF and anti-NT4 was less effective than the addition of K252a, probably because the antibodies were less able to penetrate the tissue. This would explain the variation in the extent of oocyte loss from region to region within the treated



**Fig. 11.** Effect of K252a on human ovaries in culture. (A-C) Photomicrographs of human fetal ovaries at 13-weeks gestation. (A) Uncultured ovary containing oogonia. (B) Ovary cultured in the absence of K252a with many oogonia surviving. (C) Ovary cultured in the presence of 100 nM K252a with few oogonia surviving (arrowhead). Scale bars: 20 µm. (D) Density of germ cells in uncultured ovaries, and ovaries cultured in the presence of 0 nM and 100 nM K252a. The number of oogonia was quantified by random stage microscopy. Data are the number of oogonia (mean±s.e.m.) per 121-point grid. A total of 18-42 grids per treatment were counted in each of five experiments. Asterisks indicate significant difference compared with control culture (0 nM K252a),  $P < 0.01$ .

ovaries. A less likely explanation is that other, as yet unidentified, ligand(s) are involved.

Newborn ovary cultures supported development of follicles from the primordial to the primary stage. By contrast, culture of E16.5 mouse ovaries supported formation of follicles from oogonia such that follicles formed directly at the primary stage, bypassing the primordial stage of development. K252a had no effect in this system, indicating that Trk receptors do not play a role in the survival of primary follicles. We conclude, therefore, that K252a inhibits the survival of primordial follicles in newborn ovary cultures. Thus, Trk receptors appear to play a role in the survival of follicles at the primordial but not the primary stage of development. Although we found no indication that neurotrophins affect primary follicle survival, they do appear to influence follicle function at that stage: *NGF*<sup>-/-</sup> mice have fewer follicles leaving the primordial follicle pool and undergoing growth initiation (Disson et al., 2001), whereas early postnatal rat ovaries cultured with NGF have increased numbers of follicle stimulating hormone receptors (Romero et al., 2002).

The neurotrophins and their receptors also play a role in later ovarian function. TrkA and NGF are involved in the regulation of ovulation (Disson et al., 1996) and BDNF might be involved in oocyte maturation in antral follicles (Seifer et al., 2002). In addition, there is recent evidence that neurotrophins play a role in testis development. *Trk1*<sup>-/-</sup> and *TrkC*<sup>-/-</sup> male foetuses have reduced numbers of germ cells and impaired seminiferous tubule development compared to wild-type mice (Cupp et al., 2002). Similarly, in human fetal testes, Trk-receptor signalling is involved in the regulation of germ cells and peritubular cells (Robinson et al., 2003).

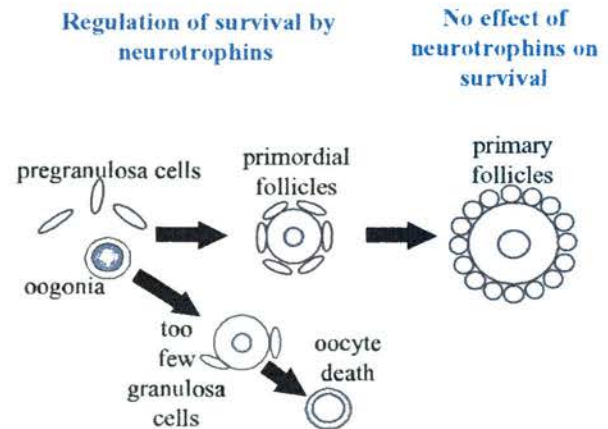
TrkB was present in mouse ovaries throughout the period of follicle formation, with mRNA (both truncated and full length) and protein (full length only) located primarily in the germ cells. Immunocytochemistry of full-length TrkB was strongest in the oocytes of P0 ovaries. It is likely that the effect of K252a on germ-cell survival shown here is caused by inhibition of TrkB function. It is less likely that it results from either inhibition of TrkC receptors, given the absence of any ovarian phenotype in *TrkC*<sup>-/-</sup> mice, or inhibition of TrkA receptors, because expression of these virtually disappears as follicles form in the rodent (Disson et al., 1995). Our data do not exclude a role of TrkA and TrkC in oocyte survival, because these receptors might become more important in the absence of TrkB signalling. This could be investigated in double mutants.

Truncated *TrkB* mRNA appeared to be more abundant than full-length *TrkB* mRNA in ovaries from E16.5 to P4. This pattern appears to be common in non-neuronal tissues (Wetmore and Olson, 1995) but the reason is unclear. The truncated receptors lack a tyrosine-kinase domain, and their effects are not well understood. However, the effects of mutations and antagonists on follicle survival described here are caused by interference with the low abundance, full-length Trk receptors. *TrkB*<sup>-/-</sup> mice have a mutation in the tyrosine-kinase domain. Whereas full length TrkB is not expressed in these mice, examining the head of newborn mice showed that levels of truncated TrkB receptors remained unchanged (Klein et al., 1993). Similarly, K252a blocks the tyrosine-kinase function of the Trk receptors, but does not interfere with potential activity of Trk receptors outwith the tyrosine-kinase

domain. The role of the truncated receptors in the ovary remains to be determined.

It is unclear why some follicles survive in *TrkB*<sup>-/-</sup> mice. In only two instances, both in C57BL/6 congenic mice, were ovaries with no follicles found. In all other cases, some follicles survived. Lundy et al. (Lundy et al., 1999) showed that primordial follicles contain a variable number of somatic cells, with the number of granulosa cells in sheep primordial follicles ranging from three to 52. It has been suggested that oocytes contained in primordial follicles require a particular number of associated granulosa cells for their continued survival (Sawyer et al., 2002). It is possible that an oocyte needs sufficient granulosa cells to sequester sufficient neurotrophins for its continued survival. Surviving oocytes in ovaries of *TrkB*<sup>-/-</sup> mice would, therefore, be the ones that have a sufficient number of granulosa cells attached (Fig. 12). In these instances, the increased numbers of granulosa cells might produce an increased amount of either neurotrophins (which might signal through other Trk receptors) or other factors that might compensate for a lack of neurotrophin signalling (possibly bFGF).

Culture of human ovaries used foetuses with gestational ages ranging from 13 to 17 weeks. At these ages germ cells are proliferating, before primordial follicle formation. Primordial follicles first appear in the human ovary from approximately 19-weeks gestation. Lack of tissue and the slowness of human development with respect to that of rodents meant that we could not do experiments at the equivalent time to that of follicle formation in the mouse. The experiments on human ovaries examined the effect of K252a on prefollicular germ cells (oogonia) only. Human foetal ovaries cultured in the presence of K252a showed a decrease in oogonia, with K252a causing the loss of ~50% of oogonia over a 48-hour period. This indicates that Trk signalling might affect oogonial survival.



**Fig. 12.** The possible effect of neurotrophins on folliculogenesis. Oogonia and pregranulosa cells associate into primordial follicles, with flattened granulosa cells, (a 'resting' stage of development). Oocytes with too few granulosa cells die at this point. Surviving oocytes are contained in follicles that have enough granulosa cells to provide the oocyte with sufficient neurotrophins. Follicles that leave the primordial stage and enter the primary stage exhibit rounding up of granulosa cells and undergo oocyte growth. Trk receptors have no effect on primary follicle survival.

In conclusion, the data demonstrate that TrkB and its ligands are present in mouse ovaries as follicles form, as we have previously shown in human ovaries (Anderson et al., 2002). Our studies of mutant mice indicate that TrkB plays an important role in oocyte survival. Culture of mouse and human ovaries with a potent inhibitor of all Trk receptors, K252a, decreases germ-cell survival. This effect appears to be specific to oogonia and primordial follicles and does not occur in primary follicles. Blocking NT4 and BDNF also decreased germ-cell survival. Together, our results point to a role of the TrkB receptor and its ligands in the regulation of germ-cell survival at the oogonal and primordial follicle stage in mammalian ovaries. This pathway is therefore crucial to the determination of female reproductive lifespan.

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# Neurotrophins and Their Receptors Are Expressed in the Human Fetal Ovary

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Mammalian ovarian development is characterized by a sequential pattern of mitotic proliferation of oogonia, initiation then arrest of meiosis, and primordial follicle formation. The factors regulating these processes are poorly understood. The neurotrophins are survival and differentiation factors in the nervous system, acting via high affinity receptors of the *trk* protooncogene family and the low affinity p75 nerve growth factor receptor, and have also been described in the rodent ovary, where changes in NT4/TrkB gene expression have been detected at the time of primordial follicle formation. There are no data on neurotrophin expression in the normal human ovary. We have investigated the expression and localization of neurotrophins and their receptors in the midtrimester human fetal ovary (13–21 wk gestation). Expression of mRNA for neurotrophins and their receptors was detected by RT-PCR. Clusters of oogonia were found to be the predominant site of NT4 mRNA expression using *in situ* hybridization. However, at

later gestations granulosa cells of primordial follicles showed increased expression, with lesser expression in the enclosed oocytes. NT4 protein was also localized to the granulosa cells by immunohistochemistry and at earlier developmental stages to epithelioid cells, which were mingled with clusters of oogonia not expressing NT4. TrkB receptor protein was localized by immunohistochemistry to germ cells at all gestations examined. The p75 nerve growth factor receptor protein was exclusively expressed in the ovarian stroma. These data demonstrate the expression of neurotrophins and their receptors within the human fetal ovary. Developmental changes in the pattern of expression of NT4 around the time of primordial follicle formation suggest that neurotrophins may be involved in signaling between somatic cells and germ cells at this crucial stage of ovarian development. (*J Clin Endocrinol Metab* 87: 890–897, 2002)

THE DEVELOPMENT of the mammalian ovary is initiated after migration of primordial germ cells from the extraembryonic mesoderm of the yolk sac to the nephrogonadoblastic ridge (1). The germ cells proliferate both during and after migration, and associate with mesonephric cells (2). This results in nests of syncytial germ cells surrounded by a meshwork of the mesonephric-derived ovarian stroma or cell streams (3). The germ cells subsequently enter meiosis only to arrest at diplotene of the first meiotic division and at this time point become surrounded by somatic cells, thus forming primordial follicles (4). This process, allowing communication between oocyte and somatic cell, is crucial for the survival of both cell types (5). As reproductive life span is determined by the continuing presence of primordial follicles, the regulation of germ cell proliferation and survival in the form of primordial follicles is central to reproductive potential. Waves of atresia of oogonia have been identified at particular stages of development (6, 7), but the factors and pathways involved are largely unknown, particularly in the human.

The neurotrophins are a family of related growth factors that are of major importance in the regulation of neuronal survival and differentiation. Nerve growth factor (NGF) is the founder member (8) and several other neurotrophins have been identified, including brain-derived neurotrophic factor and neurotrophins 3 and 4 (NT3 and NT4, the latter also called

NT5) (9). The biological actions of the neurotrophins are mediated by tyrosine kinase receptors encoded by the *trk* protooncogene family, known as TrkA, TrkB, and TrkC (10). Truncated isoforms of Trk receptors lacking the intracellular tyrosine kinase domain have also been described (10–12). In addition, all neurotrophins are recognized by a more widely expressed low affinity receptor known as p75 nerve growth factor receptor (NGFR), which is a member of the tumor necrosis receptor superfamily (13). Neurotrophins may also have roles in nonneuronal systems (14, 15), and several members of the neurotrophin family and their receptors have been demonstrated in the ovary (16–22). NT4 appears to be expressed by oocytes in both the rat and *Xenopus* (20, 22), and the expression of NT4 and its cognate high affinity receptor TrkB, but not other neurotrophins/receptors, was found to be increased at the time of primordial follicle formation in the neonatal rat ovary (22). Conversely, expression of NGF and its high affinity receptor Trk A were reduced at this time. Follicular development was reduced in a NGF-null mutant model (23), although the number of primordial follicles was maintained. A specific and marked increase in NGF and TrkA receptor expression has also been demonstrated at the time of ovulation and suggested to mediate thecal cell differentiation at that time (24, 25).

There is therefore increasing evidence that the various neurotrophins may have specific roles in ovarian development and function, but no data are currently available on their presence in the normal human ovary. We have therefore investigated the presence and distribution of the neurotrophins and their receptors in the developing human ovary

Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; NGF, nerve growth factor; NGFR, nerve growth factor receptor; NI, neurotrophin.

during the period leading up to the formation of primordial follicles.

## Materials and Methods

### Tissue samples

Human fetal ovaries were obtained after medical termination of pregnancy. Women gave written consent according to national guidelines (26), and the study was approved by the Lothian Pediatrics/Reproductive Medicine research ethics subcommittee. Termination of pregnancy was induced by treatment with mifepristone (200 mg, orally), followed by PGE<sub>1</sub> analog (Gemprost, Beacon Pharmaceutical, Lunbridge Wells, UK; 1 mg every 3 h, per vaginam). None of the terminations was for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Gestational age was determined by ultrasound examination before termination and was confirmed by subsequent direct measurement of foot length. A total of 20 specimens were used for this study.

Ovaries were dissected free and either fixed for immunohistochemical analysis or snap-frozen and stored at -70°C. Fixation was carried out in Bouin's for 5 h, followed by transfer to 70% ethanol before processing into paraffin using standard methods.

### Isolation of RNA and synthesis and amplification of cDNA

Total RNA was extracted from snap-frozen samples of fetal ovary (13–21 wk) using the RNeasy mini kit (QIAGEN, Crawley, UK). RNA was treated with deoxyribonuclease (Life Technologies, Inc., Paisley, UK), RI was performed using a first strand cDNA synthesis kit (Roche, Lewes, UK), and PCR was performed as previously described (27). Briefly, 1 µg total RNA was incubated with oligo(deoxyribonucleic)<sub>18</sub> primer for 10 min at 65°C and then placed on ice. A reaction mix comprising buffer, 1 mM each of dNTP, ribonuclease inhibitor, and 50 U reverse transcriptase was added to each tube in a total volume of 50 µl, and the tubes were then incubated at 40°C for 2 h. Subsequently, PCR was performed by incubating 1-µl cDNA samples with *Taq* DNA polymerase (AGS Gold, Hybaid, Ashford, UK) in buffer with 0.2 mM of each dNTP and forward and reverse oligonucleotide primers. Two control tubes were run in parallel, one in which water replaced the RNA and a second omitting reverse transcriptase to ensure that there was no genomic DNA contamination. Positive control samples (rat brain) were also run in parallel (not shown). PCR amplification was carried out for 35 cycles. Primers specific for human neurotrophins and their receptors were used (Table 1), designed to span an intron to ensure that genomic DNA was not amplified. Primers for the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to confirm the integrity of the RNA and the efficacy of the PCR reaction. The identities of all PCR products were confirmed by direct sequencing

using a PE Applied Biosystems 373A automated sequencer (Foster City, CA).

### In situ hybridization for NT4

Riboprobes were generated using a PCR strategy to incorporate SP6 or T7 phage promoter sequences into N14 PCR product. Briefly, for the antisense probe, the N14 PCR product was amplified using N14 forward primer (5'-CTTTCGGGAGTCCAGGAGG-3') and N14 reverse primer, which had the T7 promoter sequence added to its 5'-end (5'-TAAATACGACACACATAGGGAGACAGGCAGTGCATTCGAATCC-3'). For the sense probe, product was amplified using the N14 forward primer linked to the SP6 promoter sequence (5'-ATTAGGTGACACATAGAAAGTGCATTCGGGAGTCCAGGAGG-3') and N14 reverse primer (5'-CAGGCAGTGCATTCGAATCC-3'). pGEM-1 plasmid DNA containing human N14 insert (nucleotides 401–1099) was used as a template. Primers specific to human N14 were used to make the plasmid insert (Table 1). After PCR, products were purified (High Pure PCR product purification kit, Roche) and used directly for transcription with T7 or SP6 RNA polymerase to generate antisense and sense riboprobes, respectively. Probes were labeled with digoxigenin using a commercially available kit (Maxiscript, Ambion, Inc., Huntingdon, UK), incubated with ribonuclease-free deoxyribonuclease I and purified through Chromaspin columns (DEPC-100, CLONTECH Laboratories, Inc., Palo Alto, CA).

Sections (5 µm) were cut over diethylpyrocarbonate-treated water and transferred to SuperFrost Plus-coated slides (BDM, Poole, UK), dewaxed in xylene, and rehydrated through a graded series of ethanol. Slides were then treated with 1.5 µg/ml proteinase K at 37°C for 10 min. Sections were incubated with prehybridization buffer, then hybridized at 50°C overnight with hybridization buffer containing 2 µl appropriate riboprobe/50 µl hybridization buffer. Sections were then incubated in ribonuclease A and blocked with avidin and biotin (Vector Laboratories, Inc., Peterborough, UK) before detection of digoxigenin label using sheep antidigoxigenin (1:100 in normal rat serum/Iris-buffered saline, Roche), biotinylated rabbit anti-sheep IgG (1:500 in normal rat serum/Iris-buffered saline, Vector Laboratories, Inc.), avidin-biotin horseradish peroxidase-linked complex (DAKO Corp., Copenhagen, Denmark), and diaminobenzidine liquid substrate-chromagen system (DAKO Corp.). Sections were then counterstained in hematoxylin, dehydrated, and mounted with DPX.

### Immunohistochemistry

Immunohistochemistry was performed as previously described (27). Sections (5 µm) were mounted on IESPA (Sigma, Poole, UK)-coated slides, dewaxed, and rehydrated. After inhibition of endogenous per-

TABLE 1. Sequence of primers used for detection of neurotrophins and their receptors

Gene	Primer	Sequence (5'-3')	Product size (bp)
NGF	Forward	TAAAAACCCCCACTCCCTT	167
NGF	Reverse	ATTCCTCCCTCTCCACATC	167
BDNF	Forward	AACAATAACCACCCACCATT	222
BDNF	Reverse	TCCACTCTTTTCTCTCCCC	222
NT3	Forward	TACCCCCACCATAAAGACTCAC	334
NT3	Reverse	CCCACACACACACCACCTCTC	334
NT4	Forward	CTTTCGGGAGTCCAGGAGG	400
NT4	Reverse	CACCCACTCTCAATTCGAATCC	400
NT4 (plasmid insert)	Forward	TCTTTCGGGAGTCCAGGAGG	698
NT4 (plasmid insert)	Reverse	CCCCTCCCTCCACACTCT	698
TrkA	Forward	TCTTCACTCACTTCTCCAC	247
TrkA	Reverse	TTCTCCACCCCTCTCCACA	247
TrkB	Forward	ACTCCACACACTCACCATTCTAC	522
TrkB	Reverse	CTCCCTCTCATTCCTAACATC	522
trTrkB	Forward	CATCTTACCAATCACACCCTACTA	430
trTrkB	Reverse	CCATCCACTCCCATCTTATGAAA	430
TrkC	Forward	CATCCATCTCCAATACTACC	228/204-spliced variants
TrkC	Reverse	TCCCTCACACTCATAACCACC	228/204-spliced variants
p75 NGFR	Forward	CTCCACACCCCTCACCTTCTCC	489
p75 NGFR	Reverse	CTCCACCCCTCCTCCCTATCA	489

oxidase activity by incubation in 3% H<sub>2</sub>O<sub>2</sub> in methanol, sections were blocked in normal swine, donkey, or rabbit serum (Diagnostics Scotland, Carlisle, UK) for N14, TrkB, and p75 NGFR detection, respectively, and with avidin and biotin (both from Vector Laboratories, Inc.). The following primary antibodies were used: N14 (rabbit polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), TrkB (chicken polyclonal, Promega Corp., Southampton, UK), p75 NGFR (mouse monoclonal, Neomarkers, Fremont, CA). These were applied at dilutions of 1:100, 1:25, and 1:25, respectively, in the appropriate serum at 4°C overnight. Sections were then washed and incubated with appropriate biotinylated secondary antibody diluted 1:500 (N14 and p75 NGFR, Diagnostics Scotland, TrkB, The Jackson Laboratory, Bar Harbor, ME). Sections were incubated with avidin-biotin horseradish peroxidase-linked complex (DAKO Corp.) according to the manufacturer's instructions. Bound antibody was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAKO Corp.). Primary antibodies were omitted as negative controls.

All sections were counterstained with hematoxylin, dehydrated, mounted, and visualized by light microscopy. Images were captured using an Provis microscope (Olympus Corp., London, UK) equipped with a Kodak DCS330 camera (Eastman Kodak Co., Rochester, NY) and assembled using Photoshop 5 (Adobe, Becton Dickinson, Inc., Mountain View, CA).

### Immunoblotting

Fetal ovaries were homogenized in denaturing buffer containing 1% SDS. Samples (20 µg protein) were diluted with an equal volume of reducing loading buffer [187 mM Tris (pH 6.8), 2% SDS, 2% β-mercaptoethanol, 1% sucrose, and 0.01% bromophenol blue] and boiled for 5 min. Proteins were separated by SDS-PAGE on a 4–20% gradient Tris-glycine gel (Novex, Invitrogen, Paisley, UK) in parallel with prestained protein mol wt markers (Bio-Rad Laboratories, Inc., Richmond, CA) and blotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were blocked in 3% (wt/vol) BSA (Sigma) and 5% powdered milk and then incubated overnight with the primary antibody. Antibodies to N14 (rabbit polyclonal, Santa Cruz Biotechnology, Inc.), full-length TrkB (rabbit polyclonal, Oncogene, Cambridge, MA), and p75 NGFR (mouse monoclonal, Neomarkers) were used at dilutions of 1:500, 1:50, and 1:500, respectively. Primary antibody was omitted as a negative control. Bound antibody was detected using horseradish peroxidase-linked secondary antibodies (1:4000; Amersham Pharmacia Biotech) and the enhanced chemiluminescence visualization system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

## Results

### RT-PCR

After RT-PCR, cDNA was amplified from RNA extracted from fetal ovaries for each of the neurotrophins, NGF, NT3, NT4, and brain-derived neurotrophic factor (Fig. 1). cDNA for each of the receptors, TrkA, full-length TrkB, the truncated form of TrkB, TrkC and p75 NGFR, were also identified (Fig. 1). Positive results were found at all gestations examined for each of these neurotrophins and their receptors over the range 13–21 wk. Products of 228 and 204 bp were detected for TrkC (Fig. 1D), representing spliced variants of the gene (28), both confirmed by direct sequencing. In some samples an additional PCR product of 300 bp was detected; sequence analysis showed that it was unrelated to the tyrosine kinase receptor family.

### In situ hybridization for NT4

Hybridization histochemistry demonstrated that the germ cells were the predominant site of expression of NT4 mRNA within the ovary before the formation of primordial follicles (Fig. 2, A and B). The pattern of expression clearly demar-

cated the ovarian stroma, within which some cells showed NT4 mRNA expression, from the more uniformly and intensely stained clusters of oogonia (Fig. 2A). No marked change in the level or pattern of expression was detected before the formation of primordial follicles. However, at that stage of development, intense staining was detected in the flattened granulosa cells surrounding the enlarged oocytes (Fig. 2C), in which NT4 expression was reduced, but still present. Less mature oogonia, situated more peripherally in the ovary, continued to express NT4 mRNA as in the earlier gestations examined. The cells of the ovarian surface epithelium did not express NT4 mRNA.

### Immunohistochemical localization of NT4, TrkB, and p75 NGFR

Expression of NT4, TrkB, and p75 NGFR proteins was detected by immunohistochemistry in all specimens examined across the gestational range 13–21 wk. Oogonia showed weak cytoplasmic immunostaining for NT4 at all gestations (Fig. 2, D–F), but marked staining was detected in epithelioid cells among and immediately surrounding the nests of germ cells, consistent with early differentiation of pregranulosa cells (Fig. 2D). This pattern strikingly outlined the germ cells, both individually and in clusters. Generally, cells of the ovarian stroma showed only slight staining, although individual cells at the edge of the stroma, thus in close proximity to oogonial clusters, showed more marked staining (arrow, Fig. 2D). The most intense expression of NT4 protein was, however, seen in the cytoplasm of flattened granulosa cells of primordial follicles at later gestations. This pattern of staining very clearly demarcated formed primordial follicles predominantly located in the medullary region of the ovary from the more peripheral oogonia; thus, a medullary/cortical gradient was observed (Fig. 2, E and F). The pattern of distribution of NT4 protein therefore differed from that of NT4 mRNA at earlier gestations, but was similar once primordial follicles were formed. No staining of the ovarian surface epithelium was detected. Using an antibody specific to full-length TrkB, the protein was immunolocalized to the cytoplasm of ovarian germ cells across the gestational range examined (Fig. 2, G and H). In particular, clear staining of the cytoplasm of oocytes in primordial follicles was observed (Fig. 2H), with faint staining of the cytoplasm of pregranulosa cells.

The distribution of expression of p75 NGFR protein was very different. p75 NGFR was predominantly localized to cells of the ovarian stroma at all gestations examined (Fig. 2, I–K), thus clearly demonstrating the branching pattern of the stroma from the medulla toward the surface of the ovary, surrounding, but not becoming intermixed with, the clusters of oogonia. p75 NGFR immunostaining was not detected in germ cells at any gestation examined. In contrast to the expression of NT4, the flattened granulosa cells of primordial follicles clearly did not express p75 NGFR, although it was present in the immediately adjacent cells of the ovarian stroma (Fig. 2K). p75 NGFR was not expressed by the ovarian surface epithelium.



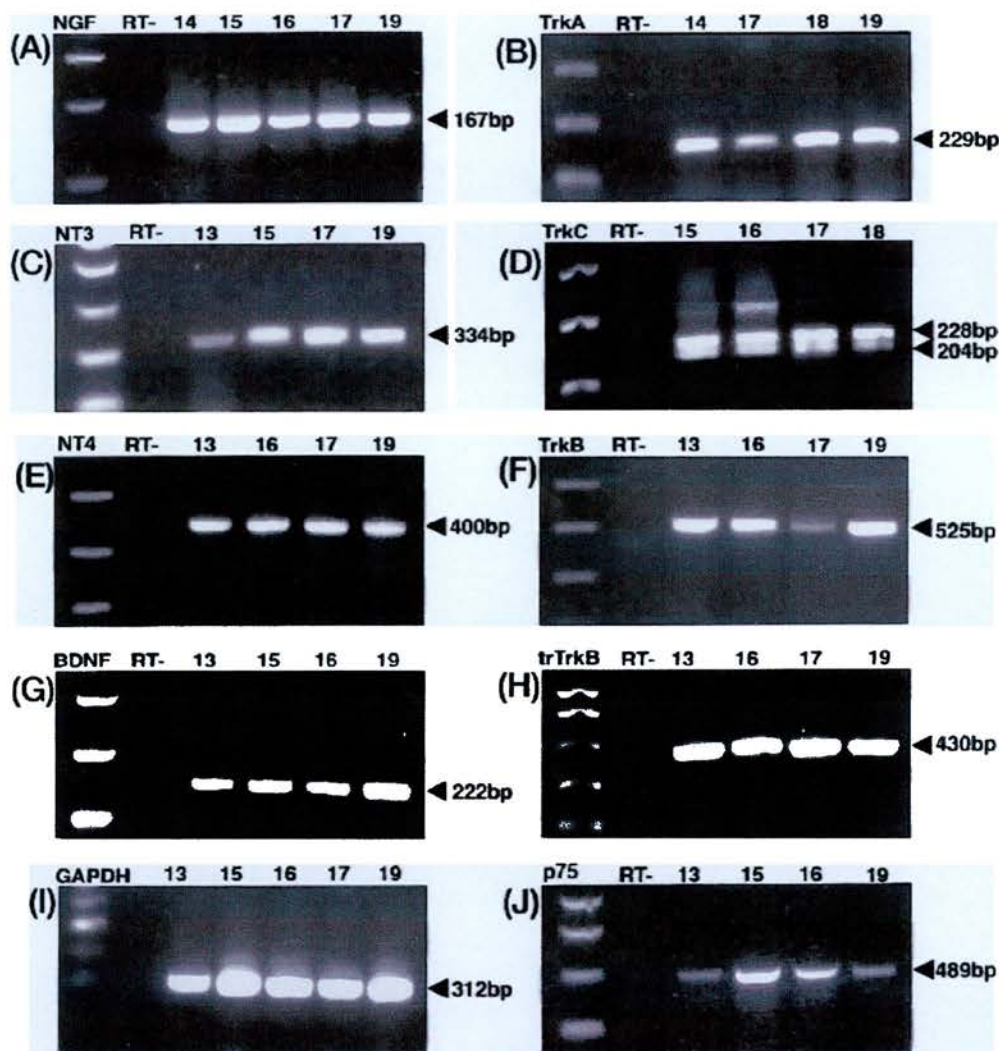


FIG. 1. Expression of mRNA for neurotrophins and their receptors in human fetal ovary. RT-PCR analysis of samples extracted from whole ovaries obtained from 13–21 wk gestation, as indicated *above* each panel. A–H and J, mRNA expression for various neurotrophins and their receptors as labeled. D, Products of 228 and 204 bp were detected for TrkC representing spliced variants of the gene, and in some samples an additional PCR product of 300 bp was detected; sequence analysis showed that it was unrelated to the tyrosine kinase receptor family. I, GAPDH expression in these samples. Lanes marked RT– contained samples in which the reverse transcriptase was not included.

#### Immunoblotting

The presence of NT4, TrkB, and p75 NGFR proteins in the fetal ovary was confirmed by immunoblotting. NT4 protein expression was detected as a single band of 21 kDa (Fig. 3A), also observed in the positive control (rat cerebral cortex). Two immunoreactive bands of 110 and 95 kDa were observed for TrkB (Fig. 3B). As the antibody specifically detects full-length TrkB, these bands may represent variously glycosylated forms of the full-length molecule (11) rather than the truncated form. A band of 110 kDa was also detected in the positive control of rat cerebral cortex. Differences in lower mol wt forms may reflect differences in the pattern of glycosylation between the two tissues (Fig. 3B). A prominent 75-kDa band representing the p75 NGFR protein was observed (Fig. 3C). An additional 65-kDa band was also de-

tected, representing another form of the molecule as detected by others (29, 30). A similar band was detected in the positive control tissue. The intensity of expression was comparable among the samples used (13–21 wk gestation) for the three proteins, although insufficient samples were available for rigorous quantitative analysis. A negative control was also performed for all three proteins by omitting the primary antibody, and in all cases immunoreactivity was abolished (data not shown).

#### Discussion

The present results demonstrate the expression of neurotrophin mRNA and protein within the developing human ovary. Expression of both the high affinity Trk receptors and the low affinity nonselective p75 NGFR receptor was also

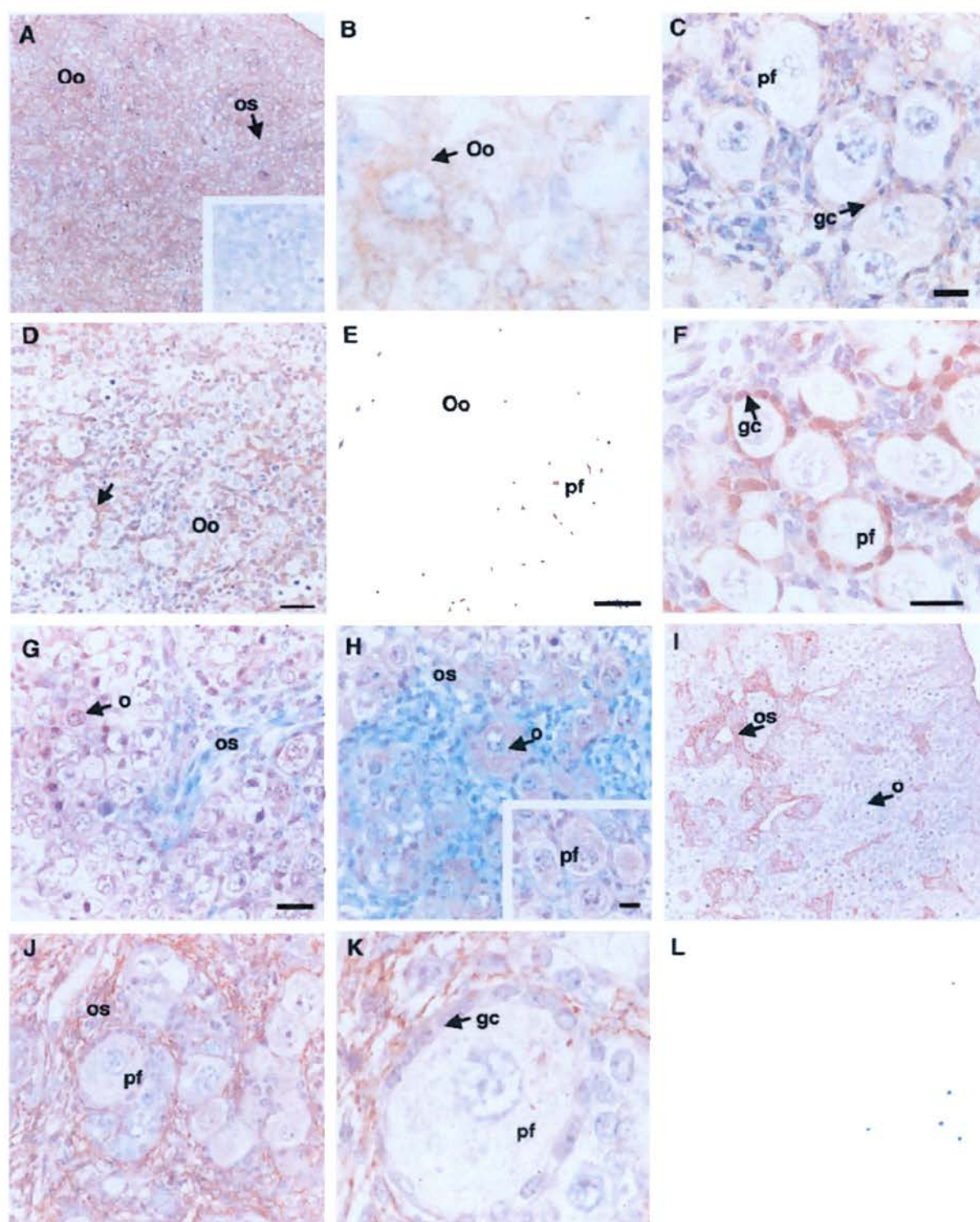


FIG. 2. *In situ* hybridization and immunohistochemical localization of neurotrophins and their receptors in human fetal ovary. *In situ* hybridization: A, localization of NT4 mRNA expression in a 16 wk ovary (antisense probe; *inset* shows results for sense RNA probe). B, NT4 mRNA expression in 16 wk ovary at higher magnification. C, NT4 mRNA localization in 21 wk gestation ovary. Immunohistochemistry: D, 13 wk ovary stained for NT4. The *arrow* indicates positively stained stromal cells. E, 21 wk ovary stained for NT4; F, 21 wk ovary at higher magnification. G, 18 wk ovary stained for TrkB; H, 21 wk ovary stained for TrkB (*inset* shows same ovary at higher magnification); I, 17 wk

demonstrated. Localization of NT4 mRNA confirmed that the germ cells are the predominant site of expression of this neurotrophin before the formation of primordial follicles. Expression was, however, developmentally regulated, thus after the formation of primordial follicles, the predominant site of expression of NT4 mRNA and protein was the flattened granulosa cell, whereas its cognate high affinity receptor TrkB was localized to the oocyte using immunohistochemistry. These data therefore indicate the involvement of neurotrophins in this crucial step in ovarian development, *i.e.* in the formation of the essential structures of the ovary.

Tissue specimens were obtained after medical termination of pregnancy, induced by administration of the antigestogen mifepristone and PGE<sub>1</sub>. It is possible that the administration of these drugs, directly or indirectly, might have affected the expression of neurotrophins in the fetal ovary, in which there is some expression of steroidogenic enzymes at these gestations (31). Hypoxia has also been demonstrated to modify expression of neurotrophins and their receptors in several tissues (32, 33). Although the results presented here are in broad agreement with the limited data obtained in the developing rodent ovary (see below), it would be appropriate to corroborate the present data in specimens obtained by other methods.

Neurotrophins, specifically NGF, were originally identified on the basis of their role in the regulation of neuronal survival (8). They are small, secreted proteins related to the TGF $\beta$  superfamily. The neurotrophin hypothesis holds that developing axons require target-derived factors for their survival, and that these factors are produced in limited quantity, resulting in competition between in-growing axons and selective survival (34). Trk-mediated cell survival requires protein synthesis, and neurotrophins are predominantly believed to act by suppression of apoptosis. Neurotrophins may also regulate cell migration (35) and differentiation (15). In addition to these prodevelopmental roles, neurotrophins can promote cell death. This had been suggested to be a function of the p75 NGFR receptor when present without the coexistence of Trk receptors, whereas in their presence p75 NGFR promotes cell survival (36). Differential effects of p75 NGFR activation on Trk receptor autophosphorylation dependent on both ligand and Trk subtype have been previously described (37). There is thus the potential for complex interplay between the different receptor pathways.

There are no previous data on the presence of neurotrophins in the human ovary, but their expression has been demonstrated in the rat ovary (21–23). The immediate postnatal period in the rat is the time of primordial follicle formation, and increased expression of NT4 and its high affinity receptor TrkB, but not other neurotrophins/Trk receptors, was demonstrated at that time (22). NT4 expression has been localized to the germ cell in both rodent and *Xenopus* (20, 22). The present results are therefore in broad agreement with the

limited previous data in other species. NT4 protein was not, however, predominantly localized to the germ cells. The main site of localization was granulosa cells surrounding oocytes in primordial follicles and, at earlier developmental stages, in epithelioid stromal cells within clusters of oogonia. It appears likely that these cells are the precursors of the granulosa cells. This is similar to the pattern of development demonstrated in the rodent, where these cells are derived from the cells of the rete ovarii, which, in turn, derive from the mesonephros (reviewed in Ref. 2). The rete ovarii is also recognized to be of central importance in the regulation of the onset of meiosis (38) and of the cortical/medullary pattern in the rodent ovary (39, 40). The neurotrophins may therefore be involved in the regulation of development of the oocyte in the human ovary at a number of levels.

A central component of the mechanism of action of neurotrophins in the nervous system is that they are produced and released by the target cell in proportion to the final innervation density, taken up by the innervating neuron, and transported to the nucleus where they mediate their pro-survival effects (41, 42). The predominant localization of NT4 mRNA and protein in different cell types before primordial follicle formation is consistent with a similar pattern of secretion/uptake by germ cells and somatic cells, respectively, suggesting that NT4 is involved in the intercellular communication between these cell types. The change in pattern of expression of NT4 mRNA, being low in the oocyte and high in the granulosa cell after primordial follicle formation, indicates that the signals mediated by this pathway may vary according to the developmental status of the germ cell/somatic cell. The localization of TrkB to the germ cells suggests that these are a major site of NT4 action, whereas p75 NGFR was localized to the ovarian stroma and was not present in the granulosa cells of primordial follicles. The distribution of p75 NGFR in the human fetal ovary is therefore very similar to that in the neonatal rat ovary (22). The differential distribution of the several receptors may contribute to the regulation of neurotrophin signaling in the immediate environment of the forming primordial follicle. The localization of other Trk receptors has not been investigated. In the newborn rat, TrkB mRNA appeared to be localized to presumptive pregranulosa cells (22). Whether this is a species difference or reflects a particular developmental stage is unclear at present. Developmental changes in the requirement for specific neurotrophins during neuronal differentiation and survival have been described (34). It has long been recognized that oocytes must interact with somatic cells to form primordial follicles and survive (5). The intermingling of NT4-immunopositive epithelioid cells within the clusters of oogonia may indicate a crucial role for oogonia-derived neurotrophins in determining germ cell survival by enhancing the movement and differentiation of somatic cells and thus promoting primordial follicle development.

ovary stained for p75 NGFR; J, 21 wk ovary stained for p75 NGFR; K, 21 wk ovary stained for p75 NGFR at higher magnification; L, representative section of fetal ovary omitting primary antibody. Positive staining in all panels is brown, and sections are counterstained with hematoxylin. Oo, Oogonia; o, oocyte; pf, primordial follicle; gc, granulosa cell; os, ovarian stroma. The scale bar in D represents 250  $\mu$ m and applies to J, L, and A inset; the scale bar in E represents 1000  $\mu$ m and applies to I and A; the scale bar in F represents 100  $\mu$ m and applies to K; the scale bar in G represents 250  $\mu$ m and applies to H; the scale bar in H inset represents 100  $\mu$ m. The scale bar in C represents 50  $\mu$ m and applies to B.

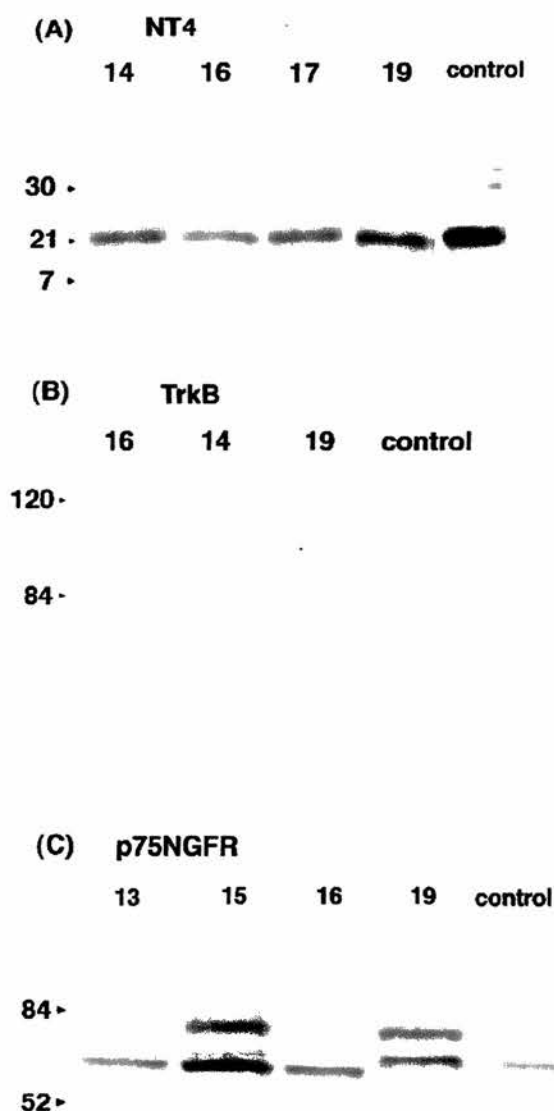


FIG. 3. Western blot of NT4, TrkB, and p75 NGFR in human fetal ovary. Total protein extracts (20  $\mu$ g) from whole ovaries at 13–21 wk gestation, as indicated, were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and incubated with anti-NT4, -TrkB, and -p75 NGFR antibodies. A, NT4, a protein band that migrated with an apparent molecular size of 21 kDa is indicated. B, TrkB, protein bands with an apparent molecular size of 110 and 95 kDa are indicated, representing two forms of full-length TrkB. C, p75 NGFR, a protein band with an apparent molecular size of 75 kDa and an additional band of approximately 65 kDa. Control tissue was rat cerebral cortex in each case. The positions of molecular mass markers are indicated. No immunoreactive bands were detected in the absence of primary antibody (data not shown).

Three Trk receptors with high affinity for specific neurotrophins have been identified as well as the low affinity p75 NGFR receptor (10, 13). Truncated isoforms of TrkB and

TrkC resulting from alternative splicing have also been described (10–12). The present results demonstrate that mRNA for both full-length and truncated isoforms of TrkB is expressed in the human fetal ovary. The truncated isoforms of the Trk receptors lack the intracellular, tyrosine kinase domain, but retain the ligand-binding domain, and may be found in both neuronal and nonneuronal cell types, although the truncated form may be more abundant in the latter (11, 43). Changing patterns of expression of full-length *vs.* truncated forms have been demonstrated during tissue repair in certain regions of the nervous system (44). TrkB-truncated isoforms have recently been suggested to be involved in neurotrophin endocytosis (45) and in regulation of specific patterns of dendritic growth distinct from those mediated by the full-length isoform (46). Conversely, several neurotrophins and their receptors may require to be coexpressed by specific cell types (47, 48). The relevance of Trk splice variants to ovarian development and function remains to be investigated.

Although the present study does not comprehensively localize within the ovary all identified members of the neurotrophin family and their receptors, the differential localization of TrkB, p75 NGFR, and NT4 may indicate multiple roles for neurotrophin signaling. Indeed, neurotrophins have been implicated in several processes in the rodent ovary. Increased NT4/TrkB mRNA expression was associated with primordial follicle formation (22), whereas increased NGF/TrkA expression was induced by the LH surge during first ovulation (24) and is involved in the regulation of intercellular gap junction integrity between thecal cells (25). Increased NGF production within the ovary resulted in disruption of estrous cyclicity (49). It therefore appears that individual neurotrophin/receptor pathways are involved in the regulation of specific intraovarian processes. Direct evidence for the importance of neurotrophins in ovarian development is scanty. *In vitro* incubation of neonatal rat ovary with the nonselective Trk receptor antagonist K-252a reduced the number of primordial follicles present (22). Recent studies of mice with an inactivating mutation of TrkB are consistent with this pathway having an important role in primordial follicle formation (50), although transgenic NT4 knockout mice appear to be normally fertile (51). The ovaries of neonatal NGF-knockout mice also show reduced proliferation of mesenchymal cells and reduced primordial follicle growth, suggesting that both cell populations are targets for NGF action (23). Mice carrying a null mutation of the p75 NGFR gene appear to have normal numbers of ovarian follicles, but this receptor may be involved in the reduction in mesenchymal cell proliferation found in the NGF-knockout mouse (23), as this is the site of expression of the p75NGFR. Later stages of ovarian development in neurotrophin/Trk knockout models have not been studied in detail, as they are generally nonviable (52).

These results therefore demonstrate the presence of neurotrophins and their receptors in the developing human ovary. Differential patterns of expression between cell types and developmental changes, particularly associated with primordial follicle formation, suggest multiple roles for them in the regulation of germ cell and somatic cell proliferation, survival, and differentiation.

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# Germ cell specific expression of c-kit in the human fetal gonad

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**The proto-oncogene receptor, c-kit, and its ligand have been demonstrated to be essential to the processes of germ cell migration, proliferation and survival in the rodent. The aim of the present study was to investigate the expression of *c-kit* mRNA and protein in human fetal ovary and testis across the gestational period 13–21 weeks. In the ovary, this crucial period of development spans the transition from oogonial replication by mitosis to primordial follicle formation. In the testis, germ cells (gonocytes) are mitotically active. Expression of *c-kit* mRNA was demonstrated by reverse transcription–polymerase chain reaction (RT–PCR) in both ovary and testis at all gestational ages examined. Testicular germ cell specific expression of *c-kit* mRNA was confirmed by RT–PCR using specific cell types recovered by laser capture microscopy. The expression of c-kit protein by both male and female germ cells was demonstrated by immunohistochemistry at all gestational ages examined, and was confirmed by immunoblotting. In both, c-kit was localized to the cell membrane except in oocytes within primordial follicles where it was localized to the cytoplasm. These data demonstrate that the expression of *c-kit* mRNA and protein is germ cell specific in human fetal gonads and are consistent with an important role for the c-kit/kit ligand signalling system in germ cell proliferation and survival in the developing human gonad.**

*Key words:* c-kit/gonocyte/human/oocyte/laser capture microscopy

## Introduction

In the human the genital ridge destined to develop into either a testis or an ovary appears as a thickening of the intermediate mesoderm at 4 weeks and remains identical in males and females until the seventh week. At 8 weeks the testis and ovary can be distinguished morphologically from each other and the testis can be seen to contain testis cords containing Sertoli cells surrounded by an interstitium which includes Leydig cells (Gilbert, 1997). Germ cells of vertebrate species do not initially form within the genital ridge but originate in the extraembryonic mesoderm of the yolk sac. Primordial germ cells increase by mitosis during migration and become associated with the cells within the gonadal ridges at ~6 weeks (Byskov, 1986). During fetal life, male germ cells continue to proliferate up to about week 22 of gestation (Hilscher, 1991). Within the fetal ovary, following oogonial replication by mitosis, nests of syncytial germ cells form and are linked by cytoplasmic bridges. The germ cells subsequently enter meiosis only to arrest at diplotene of the first meiotic division (Hilscher, 1991). At this time point, the germ cells become surrounded by somatic cells, thus forming primordial follicles. This process, allowing communication between oocyte and somatic cell, is believed to be crucial for the survival of primordial follicles which may be required to remain in that arrested state for up to 50 years (Gosden, 1995).

Studies in rodents have highlighted the importance of the c-kit proto-oncogene receptor and its ligand, the kit ligand (stem cell factor), in migration of germ cells from the yolk sac to the developing gonad and in their subsequent survival and development (Manova *et al.*, 1990; Godin *et al.*, 1991; Pesce *et al.*, 1993). For example, analysis in mice of the effects of mutations of the White Spotting and Steel loci (encoding c-kit and the kit ligand respectively) has allowed the demonstration of the importance of this ligand–receptor pair in multiple stem cell lineages including melanogenesis and haematopoiesis as well as gametogenesis (Besmer, 1991; Ashman, 1999). c-kit is predominantly expressed by germ cells in the rodent testis although it has also been suggested to be expressed by Leydig cells (Manova *et al.*, 1990), whereas kit ligand is expressed by a wider range of cell types. The presence of a functional c-kit receptor has been implicated in spermatogonial proliferation, survival and adhesion to Sertoli cells (Loveland and Schlatt, 1997). Vincent *et al.* have demonstrated expression of c-kit by pachytene spermatocytes and proposed that the kit/kit ligand interaction is essential for meiosis (Vincent *et al.*, 1998).

mRNA encoding c-kit and kit ligand have been detected in fetal mouse ovaries between embryonic days 8 and 14.5, consistent with a role in germ cell migration and proliferation (Driancourt *et al.*, 2000). In their review, Driancourt and

colleagues used ovaries from mice in which one copy of the kit gene was replaced by a lac-z reporter construct (Bernex *et al.*, 1996) to demonstrate that *c-kit* mRNA is not present in oogonia in fetal ovaries on day 15.5, but is transcribed at high levels in oocytes in primordial and growing follicles. These results are in agreement with the other findings (Manova *et al.*, 1990) using in-situ hybridization studies. Functional effects of kit ligand/c-kit in the ovary may persist into adult life, for example in the regulation of persistence of meiotic arrest (Horie *et al.*, 1991; Ismail *et al.*, 1997) and activation of primordial follicle growth (Yoshida *et al.*, 1997; Parrott and Skinner, 1999).

Studies identifying the sites of expression of c-kit in the human fetus have been very limited compared with those in rodents and there are inconsistencies between the results so far reported. Horie and co-workers used specific immunohistochemistry to detect c-kit protein on frozen sections from a number of human tissues, and in their paper immunopositive staining of single sections from a human fetal testis (18 weeks) and human fetal ovary (20 weeks) was shown (Horie *et al.*, 1993). However, other investigators have suggested that c-kit is not detectable in the fetal testis after 15 weeks gestation (Rajpert de-Meyts *et al.*, 1996). It is notable that in one study (Rajpert de-Meyts *et al.*, 1996), c-kit remained detectable in intersex testes until later in gestation, and other studies from the same group have demonstrated that c-kit is a marker of carcinoma *in situ* (CIS), a pre-malignant lesion thought to be associated with persistence of fetal-type germ cells in the adult testis (Rajpert de-Meyts and Skakkebaek, 1994). Consistent with the suggestion that signalling via c-kit is important in normal male germ cell development and function, alterations in c-kit/kit ligand expression have also been demonstrated in some patients with defective spermatogenesis (Mauduit *et al.*, 1999), with reduced expression associated with increased germ cell apoptosis.

As the second trimester is the major time for the regulation of germ cell numbers in the female (Baker, 1963) and is a period of continuing testicular development (Wartenberg, 1989), we have examined the expression and localization of c-kit in the human gonad between 13 and 20 weeks of development. Our studies have demonstrated that *c-kit* mRNA and protein are expressed specifically in germ cells of both sexes during this critical period.

## Materials and methods

### Tissues

Human fetal gonads were obtained following medical termination of pregnancy. Women gave consent according to national guidelines (Polkinghorne, 1989) and the study was approved by the Lothian Paediatrics/Reproductive Medicine Research Ethics Sub-Committee. Termination of pregnancy was induced by treatment with mifepristone (200 mg orally) followed by prostaglandin E<sub>1</sub> analogue (Gemeprost; Beacon Pharmaceuticals, Tunbridge Wells, UK) 1 mg 3 hourly per vaginam. None of the terminations were for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Gestational age was determined by ultrasound examination prior to termination and confirmed by subsequent direct measurement of foot length. A total of

20 specimens was used for this study, divided equally between male and female.

Ovaries and testes were dissected free, and either fixed for immunohistochemical analysis or snap-frozen and stored at  $-70^{\circ}\text{C}$ . Fixation was carried out in Bouin's fluid for 5 h, followed by transfer to 70% ethanol prior to processing into paraffin using standard methods.

### Isolation of RNA and synthesis of cDNA from whole tissues

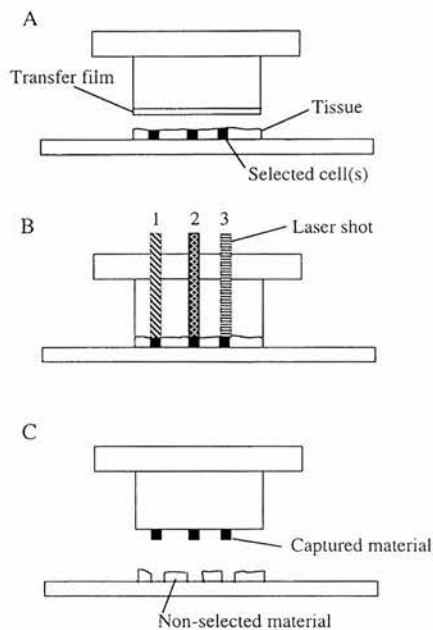
Total RNA was extracted from snap-frozen samples of fetal ovary (13–21 weeks,  $n = 10$ ) and testis (14–19 weeks,  $n = 6$ ) using the RNeasy mini kit (Qiagen, Crawley, UK). RNA was treated with DNase (Gibco, Paisley, UK) and reverse transcription performed using a first strand cDNA synthesis kit (Roche Diagnostics, Lewes, UK). Briefly, 1  $\mu\text{g}$  total RNA was incubated with oligo (dT)<sub>18</sub> primer for 10 min at  $65^{\circ}\text{C}$  and then placed on ice. A reaction mix comprising buffer, 1 mmol/l each deoxynucleotide triphosphate (dNTP), ribonuclease inhibitor and 50 IU reverse transcriptase, was added to each tube in a total volume of 50  $\mu\text{l}$  and the tubes were then incubated at  $40^{\circ}\text{C}$  for 2 h.

### Isolation of RNA by laser capture microscopy

Sections (5  $\mu\text{m}$ ) were cut from paraffin wax-embedded 19 week human fetal testis samples and mounted on plain, uncoated, glass slides. Sections were dewaxed in xylene, rehydrated then subjected to immunostaining. To visualize the cells within the seminiferous cords, an anti-Müllerian hormone (AMH) polyclonal antibody was used as detailed below except that the protocol was modified for short, typically 10 min, incubation times at each step to reduce the chance of RNA degradation, and RNase inhibitor (200 IU/ml, Promega) was included in all the immunohistochemical reagents. After colour development with diaminobenzidine (DAB), the sections were dehydrated through graded alcohols and finally xylene. Sections were stored in a vacuum desiccator for at least 30 min prior to capture. Care was taken throughout to avoid RNase contamination of sections and all aqueous solutions were prepared with DEPC-treated water.

Individual cell fragments were recovered from the stained sections by microdissection using the PixCell II LCM system (Arcturus Engineering Inc., Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, each section was overlaid with a thermoplastic membrane mounted on optically transparent caps and cell fragments were captured by focal melting of the membrane due to laser activation (Figure 1). The parameters of the laser shot used in this study were: spot size 7.5  $\mu\text{m}$  in diameter, power 45 mW and duration time 0.5 ms. The same parameters and number of laser shots (~600) were used for each cell type to normalize the amount of cellular material isolated.

Total RNA was extracted from micro-dissected samples with the Micro RNA Isolation Kit (Stratagene, La Jolla, CA, USA). After incubation with 200  $\mu\text{l}$  of denaturing buffer and 1.6  $\mu\text{l}$  of  $\beta$ -mercaptoethanol at room temperature for 10 min, the sample was extracted with 20  $\mu\text{l}$  of 2 mol/l sodium acetate, 220  $\mu\text{l}$  phenol and 60  $\mu\text{l}$  chloroform:isoamyl alcohol (24:1). The aqueous phase was mixed with 1  $\mu\text{l}$  of 10 mg/ml carrier glycogen and then precipitated with 200  $\mu\text{l}$  of isopropanol. After a 70% ethanol wash followed by drying in air, the pellet was resuspended in 10  $\mu\text{l}$  of RNase free H<sub>2</sub>O. The extracted RNA was reverse transcribed using 10 pmol random hexamer primers and 200 IU of Superscript II (Gibco BRL) reverse transcriptase according to the manufacturers instructions. An aliquot of cDNA was then amplified using a modified degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) protocol (Kasai *et al.*, 2000) using the primer UN1, 5'-CCGAC-



**Figure 1.** Laser capture microdissection. (A) The transfer polymer attached to a cap is brought into contact with the fixed, stained tissue section. (B) Areas of transfer polymer in contact with the selected cell fragments are activated using the laser pulse, the diameter of which can be varied. (C) The cap is removed with the selected cells attached, non-selected material is left behind. Captured material can now be used in a number of downstream applications; in this study mRNA was extracted and gene expression examined.

TCGAGNNNNNATGTGG-3' in a total volume of 25  $\mu$ l. 5  $\mu$ l of amplified cDNA was then used in subsequent PCR reactions using primer sets for specific sequences.

#### Amplification of specific cDNA by PCR

##### *c-kit*

PCR was performed by incubating either 1  $\mu$ l (whole tissue extracts) or 5  $\mu$ l (samples from LCM) cDNA with Taq DNA polymerase (AGS Gold; Hybaid, Ashford, UK) in buffer containing 0.2 mmol/l of each dNTP and forward and reverse oligonucleotide primers. Two control tubes were run in parallel, one in which water replaced the RNA and a second omitting reverse transcriptase to ensure that there was no genomic DNA contamination. PCR amplification conditions consisted of an initial denaturation step at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 45 s; a final extension period at 72°C for 5 min completed the amplification. Three sets of primers specific for human *c-kit* (Vandebark *et al.*, 1992) were used, one for cDNA from whole tissue extracts (set 1), the other two following laser capture of samples [sets 2 and 3 (nested)]. All three pairs of primers were designed to span an intron to ensure that genomic DNA was not amplified. Set 1, 5'-AAGGACTTGAGGTTTATTCCT-3' (sense) and 5'-CTGACGTTTCATAATTGAAGTC-3' (antisense), amplified a product of 345 bp; set 2, 5'-GTGGTTAAAGGAAACGCTCG-3' and 5'-CATACATTTTCAGCAGGTGCG-3', amplified a product of 232 bp; set 3, 5'-AAGTGGATGGCACCTGAAAG-3' and 5'-GAACCTTA-GAATCGACCGGCA-3', amplified a product of 138 bp from within the product of primer set 2. Primers for the constitutively expressed gene *GAPDH* were used to confirm the integrity of the RNA and efficacy of the PCR reaction. The identity of all PCR products were confirmed by direct sequencing using an Applied Biosystems 373A automated sequencer.

#### Anti-Müllerian hormone (AMH)

The following primers were used to identify samples containing Sertoli cell mRNA recovered by LCM (all primers were based on the sequence of human AMH; accession no. NP000470): set 1, 5'-TGCAACA-CCGGTGACAGGCAG-3' and 5'-GCAGCCCAGCCCTCGTCA-CAG-3', amplified cDNA 238 bp; set 2 (nested), 5'-GCTGCCTTGCCCTCTAC-3' and 5'-GAACCTCAGCGAGGGTG-TT3', amplify a product of 117 bp from within the product of primer set 1.

#### Immunohistochemistry

Sections (5  $\mu$ m) were mounted on TESPA (Sigma, Poole, Dorset)-coated slides, dewaxed and rehydrated. Endogenous peroxidase activity was inhibited by incubation in 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min. After a wash in water, slides were transferred into Tris-buffered saline (TBS; 0.05 mol/l Tris, 0.85% NaCl, pH 7.6) for 5 min and blocked for 30 min in normal rabbit serum (NRS; Diagnostics Scotland, Carlisle, UK) diluted 1:4 in TBS containing 5% bovine serum albumin (NRS/TBS/BSA). Sections were then blocked with avidin (0.01 mol/l; 15 min) and biotin (0.001 mol/l; 15 min; both from Vector, Peterborough, UK) with washes in TBS in between. The primary antibody (anti-c-kit goat polyclonal; cat. No. M14 Santa Cruz) was applied at a dilution of 1 in 300 in NRS/TBS/BSA at 4°C overnight. Sections were washed and incubated for 30 min with biotinylated rabbit anti-goat antibody (Dako, Cambridge) diluted 1:500 in NRS/TBS/BSA. Following washes in TBS, sections were incubated with avidin-biotin-horseradish peroxidase linked complex (Dako) according to the manufacturer's instructions. Bound antibody was visualized using 3,3'-diaminobenzidine tetrahydrochloride (Dako). A second anti-c-kit primary antibody (rabbit polyclonal, dilution 1:30; Dako) was also used in some experiments. Testis sections were stained with AMH primary antibody (rabbit polyclonal, gift of Dr R.Rey, Buenos Aires, Argentina) used at a dilution of 1:500 following antigen retrieval using citrate buffer (0.01 mol/l, pH 6.0, pressure cooked for 2.5 min). Swine anti-rabbit second antibody (Dako) was used in both cases. Primary antibodies were omitted as negative controls.

Sections were counterstained with haematoxylin, dehydrated, mounted and visualized by light microscopy. Images were captured using an Olympus Provis microscope (Olympus Optical Co., London) equipped with a Kodak DCS330 camera (Eastman Kodak), stored on a Macintosh PowerPC computer and assembled using Photoshop 5 (Adobe, Mountain View, CA, USA). A total of six ovaries and six testes were examined using immunohistochemistry.

#### Immunoblotting

Fetal ovaries ( $n = 2$ ) and testes ( $n = 4$ ) were homogenized in denaturing buffer [58 mmol/l Tris pH 6.8, 1% sodium dodecyl sulphate (SDS), 1% glycerol; all from Sigma]. Samples (10  $\mu$ g protein) were diluted with an equal volume of reducing loading buffer (187 mmol/l Tris pH 6.8, 2% SDS, 2%  $\beta$ -mercaptoethanol, 1% sucrose, 0.01% bromophenol blue) and boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 7.5% acrylamide gel in parallel with prestained protein molecular weight markers (Biorad, CA, USA) and blotted onto PDF membranes (Amersham Pharmacia, Buckinghamshire, UK) overnight using wet blot apparatus (Biorad). Thereafter, membranes were blocked for 2 h at room temperature in 0.02 mol/l TBS (pH 7.6) containing 3% w/v BSA (Sigma). Membranes were washed in TBS with 0.1% Tween-20 (TBST) and then incubated for 2 h with the primary antibody (anti-c-kit goat polyclonal, 1:750, Santa Cruz) in TBST with 1% BSA. Primary antibody was omitted as a negative control. Bound antibody was detected using a rabbit anti-goat HRP linked secondary



antibody (1:6000, Dako) and the enhanced chemiluminescence visualization system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

## Results

### Expression of *c-kit* mRNA

By RT-PCR, a single cDNA (345 bp) was amplified from RNA extracted from both fetal ovaries and testes at all gestational ages examined (13–21 weeks) (Figure 2A). Although the PCR was not quantitative, the amount of mRNA detected appeared higher in samples from testis.

Analysis of cell populations recovered from fixed sections of a week 19 testis by laser capture microscopy showed that at this age expression of *c-kit* mRNA was confined to the germ cell population (gonocytes) and was not expressed in either Sertoli cells or within the interstitium (Figure 2B–D).

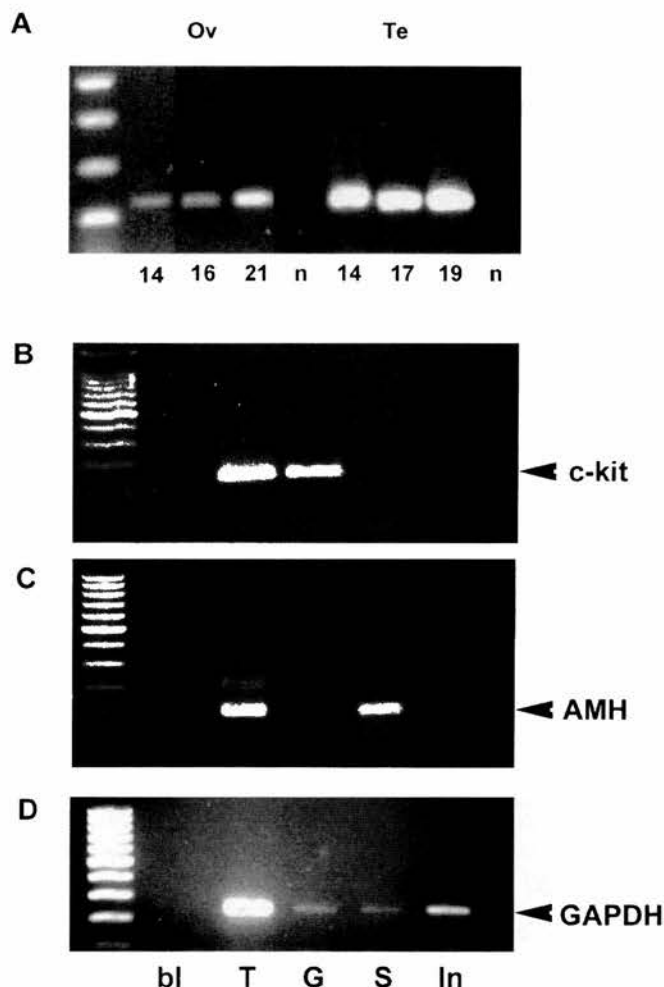
### Immunohistochemistry

*c-kit* protein was detected by immunohistochemistry in all specimens examined, across the gestational range 14–21 weeks (ovary) and 13–19 weeks (testis) (Figure 3, representative sections from a total of six ovaries and six testes). Both anti-*c-kit* antibodies tested gave similar staining patterns. In the ovary, positive staining was seen in oogonia and oocytes (Figure 3A–C), with the great majority of germ cells being stained. The surface epithelium, the ovarian stroma and the pregranulosa cells of primordial follicles were all immunonegative. This was particularly apparent in the 21 week gestation specimen in which there were a large number of primordial follicles which were not present at earlier gestations (Figure 3C). It was notable that although the *c-kit* protein appeared to be concentrated at the cell membrane of the oogonia at earlier gestational ages (Figure 3A and B), at 21 weeks the protein was clearly spread throughout the cytoplasm of the oocytes (Figure 3C).

Within the testis, *c-kit* protein was localized to gonocytes within the testicular cords (Figure 3D–F) consistent with the cell specific expression of *c-kit* mRNA. Although most gonocytes were immunopositive at all ages examined (13–19 weeks), some immunonegative cells were also present (e.g. 17 weeks, Figure 3F). As with the ovary, immunohistochemical staining was concentrated at the germ cell membrane (Figure 3F). *c-kit* immunoreactivity was not detected in the peritubular cells, interstitial cells (Figure 3F) or the surface epithelium (not shown). Sertoli cells were identified by immunostaining for AMH (Figure 3G). No staining was seen on sections of ovary (Figure 3H) or testis (Figure 3I) in which the primary antibody was not included.

### Immunoblotting

The presence of *c-kit* protein in both fetal ovary and testis was confirmed by immunoblotting. A prominent 145 kDa band corresponding to the size of the transmembrane receptor protein was detected in samples from gonadal tissues of both sexes (Figure 4). There was no immunoreactivity in the absence of

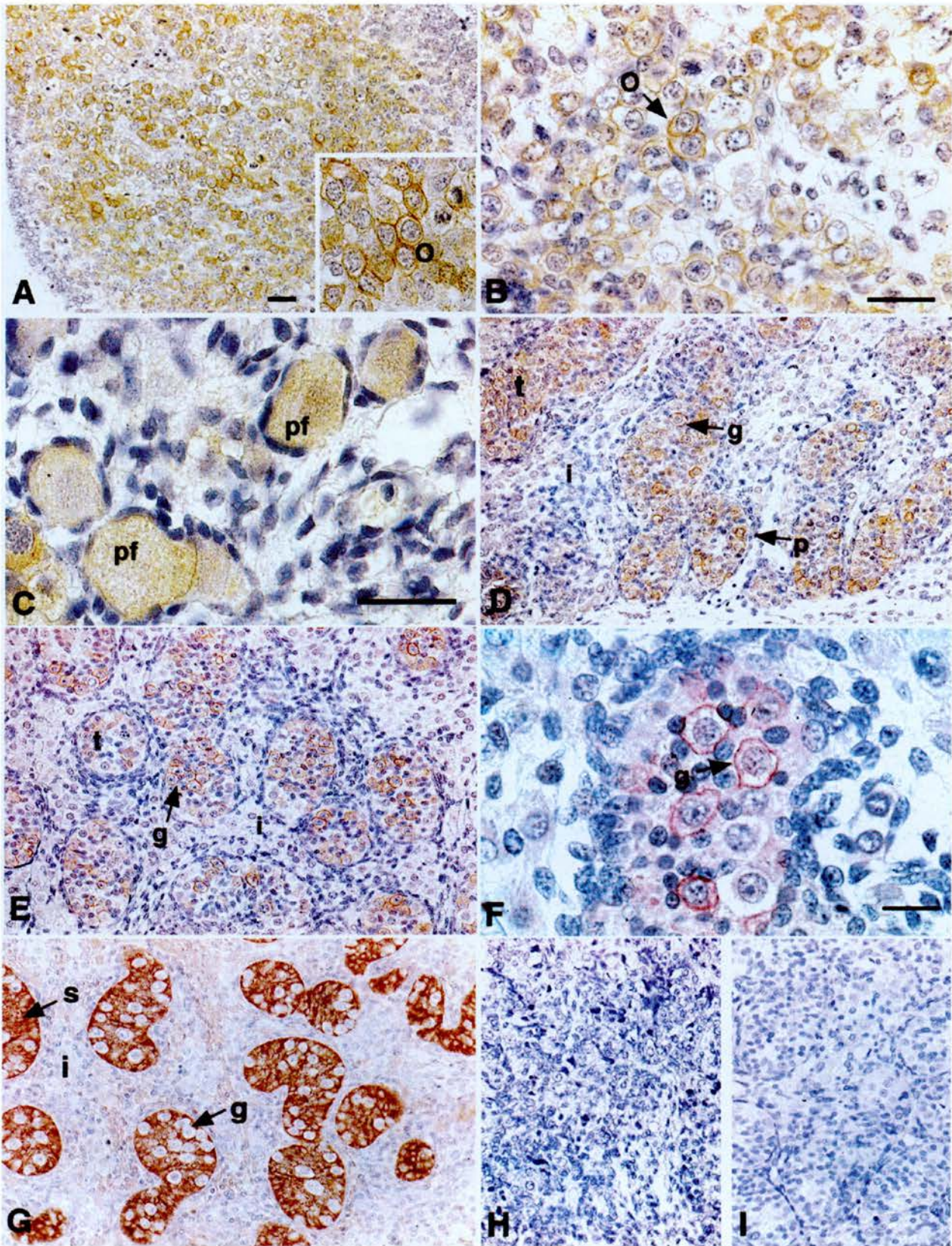


**Figure 2.** Expression of *c-kit* mRNA in human fetal ovary and testis. (A) Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of samples extracted from whole ovaries obtained at 14, 16 and 21 weeks, and testis at 14, 17 and 19 weeks as indicated. A product of 345 bp was detected in each sample. Lanes labelled 'n' contained samples in which reverse transcriptase was not included. Molecular weight marker: PCR marker (Promega). (B, C, D) Results of RT-PCR analysis of cell fragments recovered from a 19 week fetal testis by laser capture as described in Materials and methods. Tissues were immunostained for AMH to identify the positions of Sertoli cells and gonocytes within the seminiferous cords. The mRNA for *c-kit* (panel B) was detected in gonocytes (G) and whole testis (T) but not in Sertoli cells (S) or the interstitium (In). *AMH* (panel C) was only detected in S or T but not in G or In. *GAPDH* (panel D) was detected in all samples. Lane bl denotes the blank in which reverse transcriptase was not included. Molecular weight marker: 100 bp ladder.

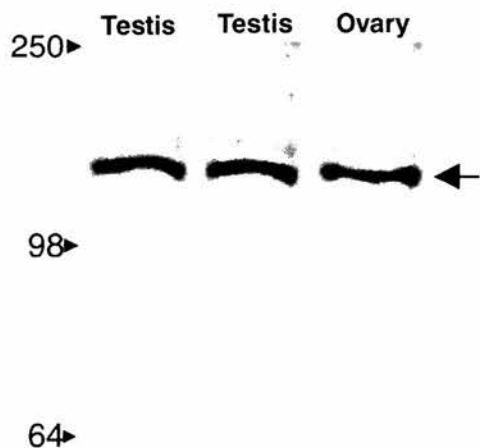
the primary antibody. The experiment was repeated three times with similar results.

## Discussion

The results presented demonstrate unequivocally that the expression of *c-kit* mRNA and protein occurs in the germ cells of both ovary and testis in the human fetus during the second trimester.



**Figure 3.** Immunohistochemical localization of c-kit (A–F) and anti-Müllerian hormone (AMH) (G) proteins to fixed tissue sections from human fetal ovary and testis. (A) 14 week ovary; inset shows oocyte staining for c-kit, (B) 17 week ovary, (C) 21 week ovary, (D) 14 week testis, (E) 16 week testis, (F) 17 week testis, (G) 17 week testis, AMH. (H and I) Representative sections of fetal ovary and testis respectively omitting anti-c-kit antibody. Staining for c-kit and AMH is brown. G = gonocyte; i = interstitium; o = oocyte; p = peritubular cells; pf = primordial follicle; s = Sertoli cells; t = tubules. Scale bar in A represents 200  $\mu$ m and applies to A, D, E, G, H and I; scale bar on B is 50  $\mu$ m and also applies to A inset; scale bars on C and F are both 25  $\mu$ m.



**Figure 4.** Western blot of *c-kit* in human fetal ovary and testis. Total protein samples (10  $\mu$ g) from ovary (14 weeks gestation) and testes (15 and 17 weeks gestation) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to nylon membrane and incubated with anti-*c-kit* antibodies. Positions of molecular weight markers are indicated; the arrow marks the position of the single protein band which migrated with an apparent molecular size of ~145 kDa. No immunoreactive bands were detected in the absence of primary antibody (not shown).

In the rodent ovary, expression of the *c-kit* gene has been detected very early in development and is high in proliferating germ cells (Manova and Bachvarova, 1991); however, expression stops when oogonia enter meiosis (Manova *et al.*, 1990). In the human ovary, entry into meiosis occurs over a wide timescale, being detectable as early as 11 weeks gestation (Gondos *et al.*, 1986) although it is believed to be maximal at ~20 weeks (Baker, 1963). Thus over the range of gestations examined in the present study, many oogonia will continue to proliferate by mitosis while an increasing proportion enter meiosis. The absence of a closely defined time at which mitosis ceases and meiosis is initiated throughout the ovary is likely to account for the detection of *c-kit* mRNA in whole tissue samples at all gestations examined up to 21 weeks, at which time many primordial follicles were present. The detection of *c-kit* protein in all specimens examined up to and including 21 weeks gestation is consistent with a previous report in sheep where *c-kit* protein, but not mRNA, was detected in oocytes undergoing meiosis (Tisdall *et al.*, 1999). It is possible that the apparent change in intracellular localization of *c-kit* protein from the membrane to the cytoplasm seen in human primordial follicles may be related to this underlying change in gene expression. *c-kit* protein has been previously localized to the oocytes of a 20 week gestation fetus (Horie *et al.*, 1993), but in that study, which was undertaken on frozen sections, it was unclear whether *c-kit* was localized to the cytoplasm or cellular membrane.

The range of gestational ages examined in this study covers the period of definitive histogenesis of the ovary, when the finite population of oocytes which will survive within primordial follicles and thus determine reproductive potential is regulated. *c-kit* and its ligand, stem cell factor or kit ligand, may have an important role in these processes. Mutations in

the genes for these factors result in loss of primordial germ cells (Besmer *et al.*, 1993), and effects of kit ligand/*c-kit* signalling on germ cell survival and protection from apoptosis have been demonstrated *in vivo* and *in vitro* (Godin *et al.*, 1991; Pesce *et al.*, 1993; Yee *et al.*, 1994). It has been suggested that the pro-survival effects of *c-kit* in the ovary may be mediated by increased expression of the anti-apoptotic factor, Bcl-2 (Tilly, 1996). Such studies have also suggested a role for this pathway in regulation of the onset of primordial follicle formation and growth of primary follicles (Yoshida *et al.*, 1997). Kit ligand/*c-kit* may also have a role in later folliculogenesis: both promotion of follicle development and maintenance of arrest of meiosis have been suggested (Horie *et al.*, 1991; Ismail *et al.*, 1997; Parrott and Skinner, 1999). Kit ligand appears to have effects on surrounding stromal cells in addition to the oocyte (Parrott and Skinner, 2000). *c-kit* is also present in the adult human ovary, in both oocytes and granulosa cells (Tanikawa *et al.*, 1998) and has been suggested to have an autocrine role in the ovarian surface epithelium (Parrott *et al.*, 2000). *c-kit* expression appeared to be confined to germ cells within the ovary in the present study: no consistent staining of stromal cells was observed.

The technique of laser capture microscopy (LCM) was developed at the National Institutes of Health in the USA to allow sampling of individual, or groups of cells from complex tissues in such a way that mRNA and/or proteins could be extracted from them and analysed (Emmert-Buck *et al.*, 1996). The method has recently been applied to the recovery of seminiferous tubules from frozen sections of mouse testis (Suárez-Quian *et al.*, 2000). In the present study, the LCM microscope was used with the laser set to the smallest size available (7.5  $\mu$ m) to allow for sampling of single gonocytes. Recently the size of male germ cells in the human fetal testis (7–10 weeks) has been reported as being 9  $\mu$ m in diameter (Bendsen *et al.*, 2001). To enable us to locate individual gonocytes, fixed tissue sections were used and these were stained using a modified immunohistochemical technique. The use of fixed sections meant that only short fragments of cDNA could be identified by RT-PCR (Goldsworthy *et al.*, 1999) and we employed a nested PCR strategy to increase signal intensity and specificity. We believe that this is the first time LCM methodology has been used to sample individual cell types from the human fetal testis and it has allowed us to show that *c-kit* mRNA is expressed in fetal gonocytes at 19 weeks gestation.

In a previous study on human fetal testes, *c-kit* protein was not detected in the testis beyond 15 weeks gestation using immunohistochemistry (Rajpert de-Meyts *et al.*, 1996). In another study, presence of *c-kit* in the human fetal testis was reported in cells described as spermatogonia in a fetus of 18 weeks gestation (Horie *et al.*, 1991). The results we have obtained using specific immunohistochemistry and Western analysis all demonstrate that *c-kit* protein is expressed in the fetal germ cells up to and including 19 weeks of gestation. Gonocytes are believed to be the cell of origin of gonadoblastomas (Jorgensen *et al.*, 1997), and it has been suggested that prolonged expression of *c-kit* in germ cells in individuals with intersex conditions may be a component of abnormal germ

cell development in such individuals who are at increased risk of testicular neoplasia (Rajpert de-Meyts *et al.*, 1996). Following laser capture of cells from a 19 week fetus, we failed to detect expression of *c-kit* mRNA in interstitial cells. Although this result is based on a single stage of development, it was in agreement with a lack of immunostaining in the interstitium at all ages examined and would not therefore be consistent with findings in the mouse (Manova *et al.*, 1990). Studies of mice in which mutations in *c-kit* or its ligand have been well documented and result in a failure in migration of germ cells into the genital ridge (Besmer *et al.*, 1993); however, the use of a blocking antibody against *c-kit* has led to the suggestion that *c-kit* is important in proliferation of differentiated spermatogonia (Yoshinga *et al.*, 1991) and protection from apoptosis (Packer *et al.*, 1995). A role in spermatogonial differentiation rather than proliferation has also been suggested on the basis of experiments involving transplantation of germ cells into testes of *Steel* mice (Ohata *et al.*, 2000) and other studies have shown a role for the *kit*-*kit* ligand in meiosis (Vincent *et al.*, 1998). Taken together, these data reinforce the importance of *c-kit* and its ligand in multiple cell lineages both during development and in adulthood.

In conclusion, this study demonstrates conclusively that *c-kit* mRNA and protein are expressed in oogonia during the transition from rapid proliferation by mitosis to the formation of primordial follicles, and in gonocytes of the developing testis during the second trimester. *c-kit* has been demonstrated to be crucial for germ cell migration, survival and proliferation in the mouse: the present results suggest that *c-kit* is likely to be of similar importance in the human.

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# Matrix metalloproteinases and tissue inhibitors of metalloproteinases in human fetal testis and ovary

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Matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) are major regulators of tissue remodelling of the extracellular matrix (ECM) and may also be involved in the control of growth factor availability. We have investigated their production and localization in the developing human gonad during mid-gestation using zymographic techniques and immunohistochemistry. The secretion of MMP-2, MMP-9 and all four TIMP was demonstrated from both testis and ovary, with the predominant gelatinase produced by both being MMP-2. In the testis, MMP-1, MMP-2, MMP-9 and all TIMP family members were localized to the interstitium and to varying degrees within the tubules. MMP-9 and TIMP-4 were abundant in both Sertoli cells and gonocytes and MMP-1 and TIMP-1 were localized in particular to Sertoli cells. In the ovary, all TIMP and MMP-1, MMP-2 and MMP-9 were localized to the oogonium/oocyte cytoplasm with varying intensities and MMP-1, TIMP-2 and TIMP-3 were also detected in the ovarian stroma. This study demonstrates that MMP-1, MMP-2, MMP-9 and all TIMP family members are secreted by the developing ovary and testis and are localized to specific cell and tissue sites. MMP and TIMP are likely to play a role in ECM remodelling during gonadal development and also in the cell and matrix interactions that control a range of cellular functions.

*Key words:* fetus/matrix metalloproteinase/ovary/testis/TIMP

## Introduction

The development of the human fetal gonad is a complex process involving dramatic structural changes, the control mechanisms of which remain unclear. In both sexes, primordial germ cells migrate to the nephrogonadoblastic ridge where they replicate by mitosis. Invasion of mesonephric cells results in the formation of testicular cords containing Sertoli cells and gonocytes in the testis, while in the ovary the ovarian stroma divides clusters of oogonia which subsequently form primordial follicles (Byskov, 1986; Motta *et al.*, 1997). By that stage, oogonia have entered meiosis and arrest at the diplotene stage. In both fetal ovary and testis, the extracellular matrix (ECM) provides the scaffold to which cells attach and also, by binding to specific cell surface receptors, modulates their function (Vu and Werb, 1991). Remodelling of the ECM may play an integral role in fetal gonadal development, including cell migration, organization, differentiation and function.

Matrix metalloproteinases (MMP) are a family of enzymes essential for proteolytic degradation of the ECM. A family of four specific tissue inhibitors of MMP (TIMP) have also been identified and the extent of ECM remodelling depends on the ratio of MMP to TIMP (Salamonsen, 1996). Regulation of ECM remodelling by MMP and TIMP is vital to provide an

environment that supports initiation of growth, migration and differentiation by a range of mechanisms (Behrendtsen and Werb, 1997; Giannelli *et al.*, 1997; Nagase and Woessner, 1999; Li *et al.*, 2000). These proteins can act from within the matrix and also at the cell surface, where, for example, MMP-2 and MMP-9 are known to bind to heparan sulphate proteoglycans. They are thus positioned for interaction with cell surface adhesion molecules or receptors and for regulating the turnover of these molecules (Yu and Woessner, 2000).

MMP and TIMP also regulate proliferation of a variety of cell types (Edwards *et al.*, 1996a) and are involved in the regulation of cytokines and their receptors both directly and indirectly via effects on the ECM. Growth factors bound to ECM are biologically inactive and must be liberated and in some cases activated before binding to receptors (Vu and Werb, 2000); for example, MMP-9 proteolytically activates latent transforming growth factor- $\beta$  (TGF- $\beta$ ) (Yu and Stamenkovic, 2000). MMP may also control bioavailability by cleaving binding proteins, thus MMP-1 can degrade insulin-like growth factor binding protein (IGFBP) into fragments with low affinity for insulin growth factor (IGF), thus increasing the bioavailability of IGF (Rajah *et al.*, 1995). Regulation of proteolytic degradation of the ECM may therefore provide an

important mechanism for controlling growth factor availability and activity, thus influencing tissue differentiation during organ development. Secretion of MMP and TIMP is under the control of a wide range of cytokines and growth factors (Nagase and Woessner, 1999), such as platelet-derived growth factor (PDGF) (Johnson and Knox, 1999) and TGF- $\beta$  (Edwards *et al.*, 1996b). Sex hormones such as progesterone are also significant in regulation of ECM remodelling via inhibition of MMP-1, MMP-3 and MMP-7 synthesis and stimulation of TIMP-1 and TIMP-2 production (Imada *et al.*, 1994; Marbaix *et al.*, 1995). Signalling pathways also lead to expression of particular MMP genes as in the case of MMP-1 which is mediated by the MAP kinase pathway (Reunanen *et al.*, 1998).

The MMP are produced by a variety of ovarian cell types including mature oocytes, granulosa cells and luteal cells in the rat (Bagavandoss, 1998), and in the bovine ovary, MMP-9 and TIMP-1 have been associated with follicular growth (Kaiura *et al.*, 2000; McCaffery *et al.*, 2000). TIMP have also been identified in the gonadal tissue of various species including the adult human (Curry *et al.*, 1990). The objective of this study was to establish the secretion and localization of a range of MMP and all TIMP family members to determine their possible role in the development of the fetal testis and ovary.

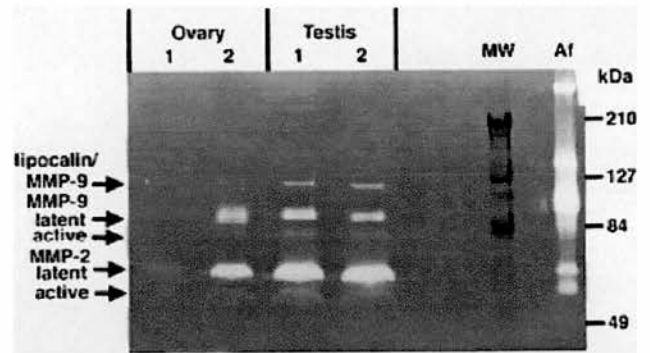
## Materials and methods

### Collection of tissue samples

Gonadal tissue was collected from 21 fetuses after termination of pregnancy induced by priming with mifepristone (200 mg, orally) followed 48 h later by prostaglandin E<sub>1</sub> analogue (Gemprost, Upjohn) 1 mg 3 hourly p.v. All fetuses appeared morphologically normal. The gestational age was assessed by the date of the last menstrual period and by ultrasound scanning during pregnancy, and confirmed by foot length measurement post mortem. The gonads were removed and either placed in a sterile Petri dish containing minimal essential medium  $\alpha$  (MEM $\alpha$ ; Gibco, Paisley, UK) prior to culture or were immediately fixed in Bouin's fluid for histological analysis. Tissues were collected under the approval of the Lothian Research Ethics Committee in accordance with the Guidelines of the British Government (Polkinghorne, 1989). Informed consent was obtained from each of the patients undergoing termination of pregnancy.

### Explant culture

Gonads from two fetuses of each sex (ovaries at 12 and 14 weeks gestation, and testes at 17 weeks gestation) were dissected free of adherent tissues using sterile technique, bisected longitudinally and then cut into slices ~0.5 mm thick. Three tissue fragments were cultured on blocks of 2% agarose gel in 12-well plates (Transwell, Costar, High Wycombe, UK) in 0.4 ml of medium, sufficient to form a meniscus at the level of the tissue. The medium comprised MEM $\alpha$  containing 3 mg/ml bovine serum albumin, antibiotics (100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin sulphate, 0.125  $\mu$ g/ml amphotericin), insulin, transferrin and selenium (5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin and 5  $\mu$ g/ml sodium selenite), 2 mmol/l glutamine and 2 mmol/l pyruvate (all chemicals supplied by Sigma, Poole, Dorset). The cultures were maintained at 37°C in 5% CO<sub>2</sub> in air in a humidified incubator. After 48 h the media were collected in a sterile container and frozen at -20°C prior to analysis by zymography. Histological analysis of cultured tissue confirmed that morphology was maintained and the tissue was viable.



**Figure 1.** Gelatin zymography gel showing gelatinase activity (visualized as lighter bands) in conditioned medium from explant cultures of fetal ovary and testis. Ovary 1, 14 weeks; ovary 2, 12 weeks; testis 1 and 2, 17 weeks. The predominant gelatinase activity is due to MMP-2 (latent form; 72 kDa). Molecular weight (MW) markers are as indicated (kDa). A sample of human term amniotic fluid (Af) was used as a positive control.

### Detection of gelatinase activities by zymography

Activities of MMP-2 and MMP-9 were determined using gelatinase zymography, as described previously in detail by this laboratory (Riley *et al.*, 1999a). Briefly, samples of conditioned medium were lyophilized, reconstituted in 0.1% SDS and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% gels; Minigel apparatus; BioRad, Hemel Hempstead, Herts, UK) containing gelatin (1 mg/ml) using non-reducing conditions. The presence of SDS both activates the latent forms of MMP and dissociates them from their inhibitors, so all forms are detected. Gels were washed [twice, 2.5% (v/v) Triton X-100] and incubated in digestion buffer [200 mmol/l NaCl, 50 mmol/l Tris, 5 mmol/l CaCl<sub>2</sub>, 1  $\mu$ mol/l ZnCl<sub>2</sub>, 0.02% (v/v) Brij-35, pH 7.6] for 18 h at 37°C. Gels were stained (0.5% Coomassie Blue R250 in 30% methanol/10% glacial acetic acid in H<sub>2</sub>O) for 3 h at 23°C, then destained (staining solution omitting Coomassie Blue), revealing localized regions where the substrate has been degraded. Human amniotic fluid collected at term during labour was used as a positive control which clearly demonstrates the latent forms of MMP-2 (72 kDa), MMP-9 (92 kDa), a lipocalin-proMMP-9 complex (120 kDa) and dimeric MMP-9 (~210 kDa).

### Detection of TIMP by reverse zymography

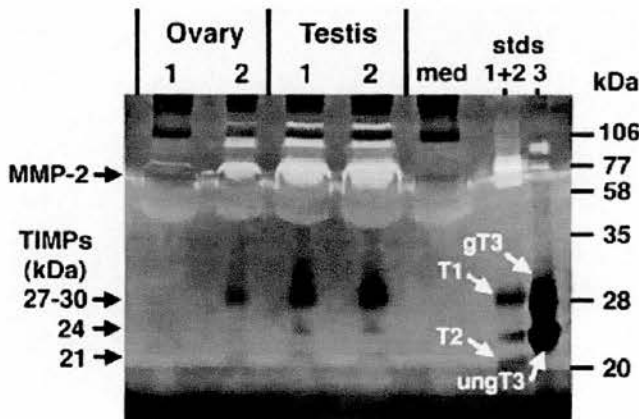
The activities of TIMP were detected by reverse zymography as described previously using a commercially available kit (University Technologies Inc., Calgary, Canada) with some minor adaptations (Riley *et al.*, 1999b). Culture medium samples were lyophilized, reconstituted in 0.1% SDS and separated by PAGE (12% gels) containing gelatin (1 mg/ml) and a preparation of MMP-2 (conditioned medium from BHK-21 cells which constitutively express MMP-2; University Technologies Inc.) using a minigel apparatus. Gels were washed [wash buffer: 50 mmol/l Tris, 5 mmol/l CaCl<sub>2</sub>, 2.5% (v/v) Triton X-100; for 2.5 h at 23°C] and incubated in digestion buffer (wash buffer excluding Triton X-100) at 37°C for 17 h. Gels were stained (0.5% Coomassie Blue R250 in 30% methanol/10% glacial acetic acid) and destained (staining buffer omitting the Coomassie Blue). The TIMP inhibitory activity appeared as dark bands against a lighter background. TIMP were identified and characterized by comparison with molecular weight markers (BioRad), control standard solutions containing mouse TIMP-1, TIMP-2 and the glycosylated and unglycosylated forms of TIMP-3 (University Technologies Inc.), and also human amniotic fluid, which contains all TIMP isoforms

**Table I.** Primary antibodies for matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) used for immunostaining of human fetal ovary and testis

Antibody	Species raised	Optimal dilution ( $\mu\text{g/ml}$ )	Source
MMP-1	Mouse	2	Chemicon International Inc., Harrow, UK
MMP-2	Mouse	5	Calbiochem, Nottingham, UK
MMP-9	Mouse	10	Insight Biotechnology, Wembley, Middlesex, UK
TIMP-1	Rabbit	2	Sigma, Poole, Dorset, UK
TIMP-2	Rabbit	5	Triple Point Biologics, Forest Grove, OR, USA
TIMP-3	Rabbit	5	Sigma, Poole, Dorset, UK
TIMP-4	Rabbit	5	Chemicon International Inc., Harrow, UK

**Table II.** Semi-quantitative analysis of cellular and spatial localization of matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) by immunohistochemistry in the fetal testis between 13 and 19 weeks gestation

	Peritubular cells	Gonocytes	Sertoli cells	Interstitial	Surface epithelium	Vascular endothelium
MMP-1	+/-	+	++	+++	+	+
MMP-2	-	+	+/-	++	+/-	+
MMP-9	-	++	++	++	+	+
TIMP-1	+/-	+	++	++	+/-	+
TIMP-2	-	+/-	+/-	++	-	+
TIMP-3	+/-	-	-	+++	-	+
TIMP-4	-	++	++ ++ -	+	-	+

**Figure 2.** Reverse zymography gel demonstrating the secretion of tissue inhibitors of metalloproteinases (TIMP) (visualized by darker bands) into culture medium from explant cultures of fetal ovary and testis. Ovary 1, 14 weeks; ovary 2, 12 weeks; testis 1 and 2, 17 weeks. Three predominant bands of TIMP activity are observed at 27–30, 24 and 21 kDa. The standards (stds) of TIMP-1 and TIMP-2 (T1: TIMP-1 as a broad band at 27–30 kDa; T2: TIMP-2 at 21 kDa) and TIMP-3 (gT3: glycosylated TIMP-3 at 28–30 kDa; ungT3: unglycosylated TIMP-3 form at 24 kDa) are indicated by arrows. Molecular weight markers are as indicated (kDa). A control of unconditioned medium showing no activity is shown (med).

(Riley *et al.*, 1999b). Analysis of samples by PAGE with gelatin substrate omitted demonstrated no significant detectable underlying protein staining at the molecular weights at which TIMP were observed, demonstrating the specificity of the reverse zymography for detection of TIMP activity.

**Table III.** Semi-quantitative analysis of cellular and spatial localization of matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) by immunohistochemistry in the fetal ovary between 13 and 21 weeks gestation

	Oocyte cytoplasm	Ovarian stroma	Surface epithelium	Vascular endothelium
MMP-1	++	+/-	+/-	+
MMP-2	++	-	+/-	+
MMP-9	++	-	+/-	+
TIMP-1	+++	-	+/-	+
TIMP-2	+/-	+/-	-	+
TIMP-3	+/-	+/-	+/-	+
TIMP-4	++	-	-	+

#### Localization of MMP-1, MMP-2, and MMP-9 and TIMP by immunohistochemistry

Immunoreactive MMP-1, MMP-2 and MMP-9, and TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were localized in tissues using immunohistochemistry and antibodies characterized and described in detail previously (Riley *et al.*, 1999b). In brief, tissue sections (5  $\mu\text{m}$ ) were mounted on silane-coated slides. Sections were washed with histoclear (National Diagnostics, Atlanta, GA, USA) to remove the wax, rehydrated and endogenous peroxidase activity inhibited by incubation in  $\text{H}_2\text{O}_2$  (3% in  $\text{H}_2\text{O}$ ; 20 min). Sections were washed and a blocking step applied (5% normal goat or horse serum, appropriate to the primary antibody and detection system used; 30 min). The sections were blocked with avidin and then biotin (Vector Labs, Burlingame, CA, USA; 15 min each block) to inhibit any endogenous avidin-biotin interactions with the antibodies and then incubated overnight with the primary antibody at 4°C in a humidified atmosphere. Optimal antibody concentrations were established in a series of preliminary experiments and information on all primary antibodies used is detailed



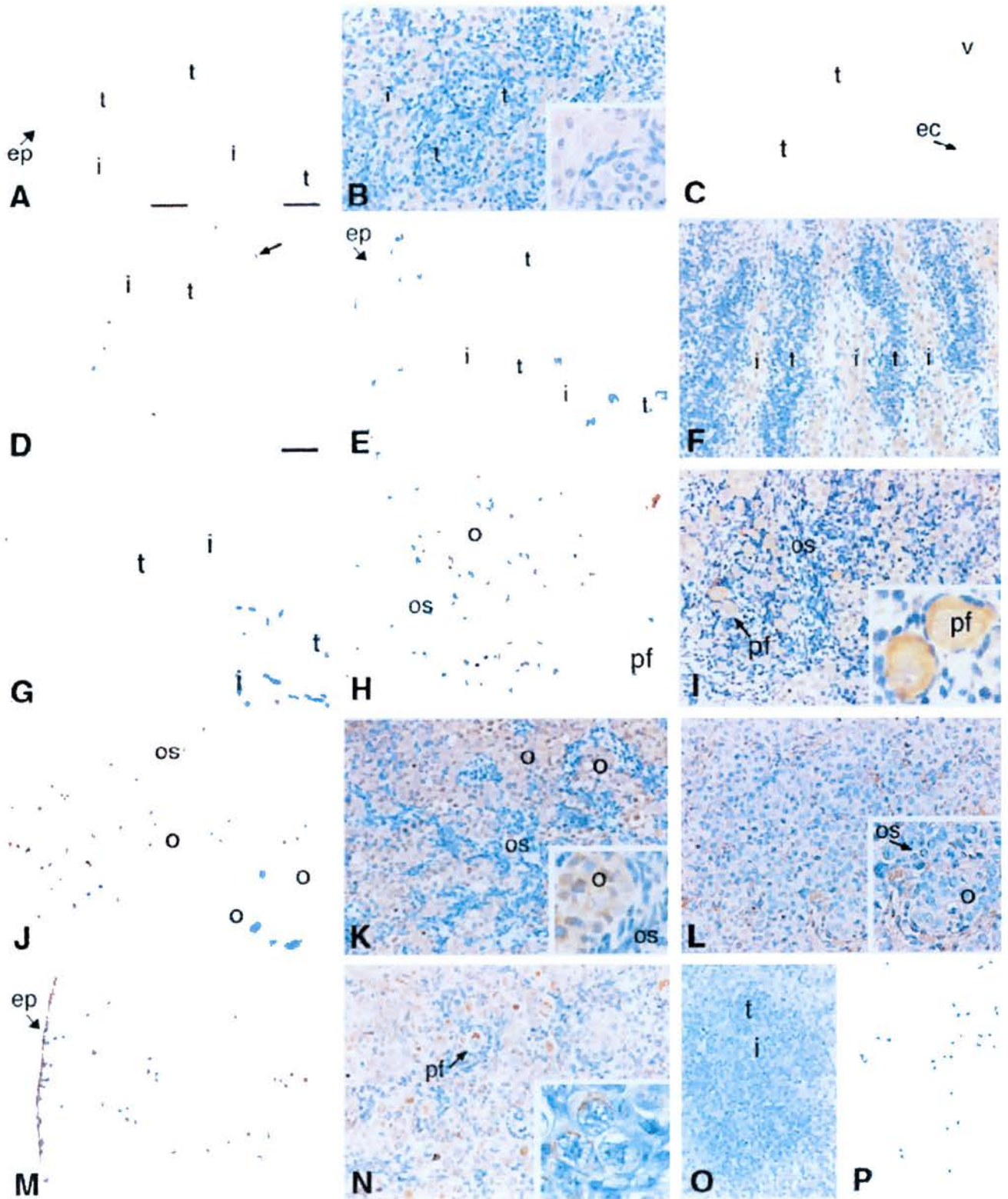


Figure 3. For legend see facing page.

in Table I. Primary antibody was detected using a goat anti-rabbit or horse anti-mouse biotinylated second antibody as appropriate, and an avidin-peroxidase complex according to the manufacturer's instructions (Vector Labs). The primary antibody was omitted for negative controls (see representative section Figure 3). Term human fetal membranes were used as a positive control (Riley *et al.*, 1999b). Sections were counterstained with haematoxylin, dehydrated, mounted and visualized by light microscopy.

#### Analysis of immunohistochemistry

Immunostaining of tissue sections was assessed semi-quantitatively for both the ovary and testis using + and - symbols as a measure of the intensity and amount of staining in particular cell types (Tables II and III) + indicates pale staining in this cell type, ++ indicates marked staining, and +++ signifies intense immunostaining. These scores also reflect that the majority of cells of that particular type have stained positively. A score of +/- means that some but not most of these cells have stained, while a score of - means that there is no positive staining in any cell of this type.

## Results

### Secretion of MMP and TIMP by the fetal testis

In culture medium conditioned by the fetal testis for 48 h, high levels of gelatinase activity were detected at 120, 92, 86, 72 and 66 kDa. These molecular weights correspond to the MMP-9-lipocalin complex, the latent form of MMP-9, active MMP-9, MMP-2-activated protein in the latent form and the active form of MMP-2 respectively (Figure 1). The predominant gelatinase activity detected was the latent form of MMP-2.

The examination of TIMP activity in the testis using reverse zymography showed predominant gelatinase inhibitory activity at 27–30 kDa, which corresponds to the molecular weights of TIMP-1, glycosylated TIMP-3, and TIMP-4 (Figure 2). Bands of activity were also present at 24 kDa (corresponding to unglycosylated TIMP-3) and 21 kDa (corresponding to TIMP-2). These bands aligned with standards to TIMP-1, TIMP-2 and glycosylated and unglycosylated TIMP-3. Reverse zymography is unable to distinguish precisely between the TIMP isoforms of 27–30 kDa molecular weight. The presence of these TIMP of similar molecular weights, including TIMP-4, was therefore confirmed by immunohistochemistry.

### Localization of MMP and TIMP in the fetal testis

Sections of human testis from 10 fetuses between 13 and 19 weeks gestation were used for analysis. Table II shows the spatial and cellular localization of MMP and TIMP immunoreactivity

and relative intensity of staining, and Figure 3A–G and O shows representative photomicrographs. MMP-1 was found in abundance in the cytoplasm of the interstitial cells and to a lesser extent in the surface epithelium (Figure 3A). MMP-1 was also distributed among some of the peritubular cells and within the testicular cords, particularly in Sertoli cells. MMP-2 was predominantly localized to the interstitium of the testis and also found within the cytoplasm of some of the tubular cells, being more prevalent in gonocytes than Sertoli cells (Figure 3B). It was also present in the surface epithelium but absent in the peritubular cells. MMP-9 was present chiefly within the testicular cords and was also present in some of the interstitial cells and surface epithelium (Figure 3C). Like MMP-2, MMP-9 was not observed in the peritubular cells.

TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were also immunolocalized within the fetal testis. TIMP-1 was localized to the cytoplasm of the interstitial cells and also within the testicular cords, staining Sertoli cells in particular (Figure 3D). TIMP-2 was predominantly localized to the interstitium, there being little within the cords and no immunoreactivity in the peritubular cells or surface epithelium (Figure 3E). Staining for TIMP-3 was intense within the interstitium and was also present although to a lesser degree in some of the peritubular cells (Figure 3F). There was no TIMP-3 immunostaining within the cords and surface epithelium. TIMP-4 was present mainly in the interstitial cells but there was also strong positive staining within the Sertoli cell and gonocyte cytoplasm (Figure 3G). It was absent from both the peritubular cells and the surface epithelium. Immunostaining was also observed in the vascular endothelium for MMP-1, MMP-2, MMP-9 and TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Table II). No major changes were seen in immunostaining for any MMP or TIMP examined over the gestational range examined. Sections incubated without primary antibody showed no non-specific staining (Figure 3O).

### Secretion of MMP and TIMP by the fetal ovary

Analysis using zymography of culture medium conditioned by the fetal ovary for 48 h demonstrated, in the two samples examined, that the predominant gelatinase activity was identified at 72 kDa molecular weight, corresponding to the latent form of MMP-2 (Figure 1). The active form of MMP-2, which has a molecular weight of 66 kDa, was also detected but at a lower level. Gelatinase activity was also detectable at 92 kDa, corresponding to latent MMP-9, and a less intense band of activity was observed at 120 kDa, corresponding to the MMP-

**Figure 3.** Localization of matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) in human fetal gonadal tissue. (A) MMP-1 in fetal testis. Arrow denotes surface epithelium (ep). (B) MMP-2 in fetal testis (C) MMP-9 in fetal testis. Arrow indicates endothelial cells (ec). (D) TIMP-1 in fetal testis. Arrow indicates peritubular cells. (E) TIMP-2 in fetal testis. Arrow denotes the surface epithelium. (F) TIMP-3 in fetal testis. (G) TIMP-4 in fetal testis. (H) MMP-1 in fetal ovary. Inset demonstrates primordial follicles (pf) stained with MMP-1 (I) MMP-2 in fetal ovary. Inset shows primordial follicles with staining for MMP-2 in oocyte cytoplasm. (J) MMP-9 in fetal ovary. Inset shows oocytes stained with MMP-9. (K) TIMP-1 in fetal ovary. (L) TIMP-2 in fetal ovary. Inset shows oocytes and rete ovarii staining with TIMP-2. (M) TIMP-3 in fetal ovary. Arrow denotes surface epithelium. (N) TIMP-4 in fetal ovary. Arrow denotes primordial follicle. Inset shows oocyte staining for TIMP-4. (O) Representative section of fetal testis omitting primary antibody. (P) Representative section of fetal ovary omitting primary antibody. I = interstitium; t = tubules; os = ovarian stroma; o = oocyte. Scale bar represents 100  $\mu$ m for panels A, B, C and E; 50  $\mu$ m for panels D and F–P; and 12  $\mu$ m for insets in panels A–C, E, G–L and N.

9–lipocalin complex. A faint band of active MMP-9 (86 kDa) was also detected. In one sample, MMP-2 (latent and active) and MMP-9 were identified in much smaller amounts.

A broad spectrum of TIMP activity was detected in culture medium conditioned by the fetal ovary for 48 h by reverse zymography (Figure 2). Three bands of gelatinase inhibitory activity were present at 27–30 kDa (corresponding to the molecular weight of TIMP-1, glycosylated TIMP-3 and TIMP-4), at 24 kDa (corresponding to unglycosylated TIMP-3) and 21 kDa (corresponding to TIMP-2). As with zymography, one of the samples showed very low levels of TIMP activity.

#### *Localization of MMP and TIMP in the fetal ovary*

Sections of fetal ovaries from 11 fetuses between 13 and 21 weeks gestation were used for analysis. Table III and Figure 3H–N and P describe and demonstrate the spatial and cellular localization of MMP and TIMP and relative intensity of immunostaining found. As with the fetal testis, no major or systematic variation in immunostaining was seen for any MMP or TIMP examined with increasing gestational age.

MMP-1 was abundantly present in the cytoplasm of the oocytes throughout the ovarian cortex and was also localized to some of the cells of the ovarian stroma and the surface epithelium, although this immunoreactivity was less intense (Figure 3H). MMP-2 was predominantly found in the oocyte cytoplasm and was also present in some of the cells of the surface epithelium but was absent from the ovarian stroma (Figure 3I). A similar staining pattern in the oocyte cytoplasm and surface epithelium was found with MMP-9 (Figure 3J).

All four members of the TIMP family were also localized within the fetal ovary. TIMP-1 was found chiefly in the cytoplasm of the oocytes but was also present in the surface epithelium (Figure 3K). TIMP-2 was weakly associated with the oocyte cytoplasm but was more localized to the ovarian stroma and was absent from the surface epithelium (Figure 3L). TIMP-3 was widely distributed, immunostaining some of the oocyte cytoplasm, ovarian stroma and cells of the surface epithelium (Figure 3M). Immunostaining for the TIMP-4 antibody was restricted to the oocyte cytoplasm (Figure 3N). MMP-1, MMP-2, MMP-9 and all four TIMP were also localized to the vascular endothelium (Table III). No immunostaining was observed in the negative control (Figure 3P).

#### **Discussion**

This study demonstrates the presence of MMP-1, MMP-2, MMP-9 and all four TIMP family members in the human fetal ovary and testis during mid-gestation. MMP-2 appears as the predominant gelatinase MMP secreted by the gonads, being most abundant in its latent form. All of the TIMP family were secreted by both the testis and ovary. These data therefore indicate the likely involvement of MMP and TIMP during this time of gonadal development. In the ovary, this is a period of intense oogonal proliferation, and the time for entry of an increasing number of oogonia into meiosis (Baker and Neal, 1974; Gondos *et al.*, 1986). Towards the end of this period, there is migration of granulosa cell precursors from the mesonephros-derived ovarian stroma into the clusters of

oocytes with subsequent formation of primordial follicles (Byskov, 1986; Motta *et al.*, 1997; McNatty *et al.*, 2000). While such marked structural changes are not occurring in the testis, the tubules having formed earlier in development, there is continuing proliferation of the various cell types, and marked steroidogenic activity in the fetal Leydig cells (Majdic *et al.*, 1998).

Using zymographic techniques, we are unable to comment on the absolute amounts of MMP and TIMP present as they are not quantitative, but they do allow relative intensities of activity to be established. However, one of the ovarian samples analysed (at 14 weeks gestation) appeared to be producing much lower levels of MMP and TIMP than the other samples tested. This may be due to a change in secretion levels over gestation or to a delay in receiving the tissue after termination. Immunolocalization does not specifically identify the cellular site of production of these TIMP and MMP as they are secreted and may bind to the ECM or directly to the cell; however, it is likely that the MMP and TIMP have been directly secreted at these sites to mediate specific functions (Vu and Werb, 2000). The drugs used to induce termination included mifepristone and a prostaglandin E<sub>1</sub> analogue. Mifepristone is a potent anti-progestin and anti-glucocorticoid, which also has anti-oestrogenic effects (Teutsch and Philibert, 1994). Although concentrations of mifepristone reaching the chorionic villi are low compared with those in serum and decidua (Wang *et al.*, 1994), it may have some effect on the fetal gonadal tissue, yet the nature and extent of any such effects are currently unknown. Exogenous prostaglandin E<sub>1</sub> is also used to induce uterine contractions and promote cervical dilatation at termination. Prostaglandins are involved in many aspects of normal ovarian function and it is uncertain whether these concentrations used in the termination procedure might cross the placenta to the fetus and then have an effect on the fetal gonadal tissue (Greystoke *et al.*, 2000).

Both MMP and TIMP are likely to be involved in the tissue remodelling that accompanies the rapid growth, differentiation and structural changes of the fetal gonads in the second trimester. Interactions between MMP and TIMP are probably important in controlling both remodelling of fibrillar collagen (by MMP-1), an important structural matrix component, and also of collagen IV (by MMP-2 and MMP-9), a major component of basement membranes. Thus, cells can be permitted to grow, differentiate and undergo mitosis. Cell migration may also be allowed (Giannelli *et al.*, 1997), as seen for instance in the formation of primordial follicles in the ovary that occurs at this time in development and the movement of gonocytes from a central location within the testicular tubule to lying adjacent to the basement membrane. MMP and TIMP influence many cellular functions (Salamonsen, 1996; Vu and Werb, 2000) and may play other roles within the gonads. The interaction between a cell and its surrounding matrix, for instance via integrins and focal adherins, is a vital regulator of cell function (Brooks *et al.*, 1996; Giancotti, 1997; Steffensen *et al.*, 1998) and studies on roles of MMP at the cell surface have shown that they can stimulate cell proliferation through interaction with cytokines (Edwards *et al.*, 1996a). MMP-1, MMP-2 and MMP-9 bind to heparan sulphate proteoglycans

on the cell surface (Fisher *et al.*, 1994; Yu and Woessner, 2000), possibly preventing diffusion of the MMP and conferring a high degree of local control for tissue remodelling, cell–matrix interactions and local modulation of cytokine shedding or degradation. The binding of MMP-2 and MMP-9 to heparan sulphate proteoglycan therefore may have an effect on processes involving excessive tissue breakdown, such as angiogenesis. As we have observed vascular staining in both testis and ovary for MMP-1, MMP-2 and MMP-9 and all four TIMP, it is likely that these proteins are involved in angiogenesis within the gonadal tissue (Yu and Woessner, 2000). MMP-1 may also have another role in angiogenesis as it enhances smooth muscle cell migration within the vessel wall by degrading collagen to gelatin, leaving this available for the action of gelatinases such as MMP-2 (Pilcher *et al.*, 1997).

MMP and TIMP may also regulate cell cycle progression or death (Boudreau *et al.*, 1996). TIMP-3 induces apoptosis of colon carcinoma cells (Smith *et al.*, 1997), and in mammary cells inhibition of MMP activity rescues cells from apoptosis (Schedin *et al.*, 2000). The number of germ cells within the ovary reaches a peak at ~20 weeks gestation (Baker and Neal, 1974) with a parallel increase in the number of atretic cells. The rapid increase and subsequent loss of germ cell numbers at this time is likely to have a major impact on the complement of primordial follicles. The regulatory mechanisms involved are fundamental to the determination of reproductive lifespan but are poorly understood. The presence of MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-4 in the oocytes suggests that they may potentially regulate survival signals and therefore possibly affect cell proliferation. MMP also regulate growth factor activity by cleaving the proteins that bind them. Both MMP-1 and MMP-2 can degrade IGFBP allowing IGF to become active (Rajah *et al.*, 1995; Vu and Werb, 2000). IGF, its receptor and binding proteins are expressed in the human ovary and have been implicated in follicular development (Zhou and Bondy, 1993a,b). In addition, TIMP also stimulate proliferation directly in other cell systems (Hayakawa *et al.*, 1994) and MMP activity and its control by TIMP regulates activation of cytokines such as tumour necrosis factor  $\alpha$  (McGeehan *et al.*, 1994) at the cell–matrix interface. The TIMP-1–procathepsin L complex has been previously suggested to be a potent activator of steroidogenesis in the rat testis and is secreted by Sertoli cells (Boujrad *et al.*, 1995). The present data suggest that TIMP-1 is mainly localized to the Sertoli cells within the testicular cords, thus it may play a similar steroidogenic role in the human fetal testis.

In conclusion, this study demonstrates that MMP and TIMP are secreted by the human fetal gonad during mid-gestation and are localized with discrete cellular and spatial distributions within the fetal testicular and ovarian tissue. These results suggest that MMP and TIMP are involved in ECM remodelling at this time. They may also play a role in paracrine regulation of functions including germ cell proliferation by regulating growth factor availability and action and possibly also by regulating steroidogenesis in the testis. This study provides a basis from which to work towards further assessment of the functions of MMP and TIMP within the human fetal gonad during this period of structural change and development.

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