# Investigation into the Expression and Localisation of c-kit and the Regulation of Kit Ligand Gene Expression in the Adult Human Ovary

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**B.Sc (Biomedical Science), B.HSc. (Hons)**

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The most beautiful thing we can experience is the mysterious.
It is the source of all true art and science.

*Albert Einstein*

Question everything. Learn something. Answer nothing.

*Euripides*

I love fools' experiments. I am always making them.

*Charles Darwin*

This thesis is dedicated to my mum and dad.

Thank you for everything.

# ABSTRACT

Folliculogenesis is a complex process that is central to the ovary's primary function, the production of healthy oocytes. One of the essential ligand/receptor pairs that mediates folliculogenesis is kit ligand (KITL), a granulosa-derived cytokine growth factor, and its receptor, c-kit. Since their discovery two decades ago, the KITL/c-kit system has been extensively studied in animal models, in particular the mouse, in which it has been demonstrated to be crucial for normal folliculogenesis and fertility. To date, little investigation into KITL and c-kit has been performed in the adult human ovary. Previously, this laboratory showed abnormally increased KITL protein levels in human polycystic ovaries (PCO) compared to non-PCO, suggesting that KITL may contribute to several PCO phenotypes according to the range of actions KITL has been shown to have in animal folliculogenesis. Thus, this thesis aimed to characterise KITL and c-kit expression and localisation in the adult human ovary, including polycystic ovaries, and examined regulation of KITL gene expression by endocrine and intraovarian factors.

To perform these studies, human ovarian tissues were obtained. These included granulosa cell subtypes cumulus and mural granulosa cells from women undergoing assisted reproductive technology treatment at infertility clinics, fresh ovarian cortex from the Royal Adelaide Hospital and archival paraffin-embedded human ovarian tissue from the Institute of Medical and Veterinary Sciences. The human granulosa tumour cell line, KGN, was also used as a model.

KITL and c-kit isoforms were demonstrated to be present in the human ovary throughout follicle development. KITL-2 was shown to be expressed primarily by granulosa cells representing preantral follicles, while KITL-1 was the predominant isoform expressed in preovulatory granulosa cells, suggesting that KITL-2 may play a greater role during early follicle development which then diminishes in preovulatory follicles with increased KITL-1 levels. Both c-kit mRNA isoforms were found to be present in human ovarian cortex. Examination of c-kit localisation throughout follicle development by immunohistochemistry revealed that all follicular cell types in preantral and antral follicles expressed c-kit protein. This may suggest that KITL has an unknown autocrine function in granulosa cells unique to the human ovary, as animals models have previously demonstrated c-kit staining to be confined to the theca layer and the oocyte. c-kit staining patterns were found to be different in PCO compared to non-PCO preantral and antral follicles, suggesting a potential involvement for c-kit in PCO pathology. Collectively these results suggest, as demonstrated in various animal models, that the KITL/c-kit system is present in the human ovary and may have some involvement in the mediation of human folliculogenesis.

Regulation of KITL gene expression was examined using KGN and cumulus cells. Based on previous studies, the candidate regulatory factors that were examined included androgen receptor (AR), endocrine factor follicle-stimulating hormone (FSH), theca-derived factor keratinocyte growth factor (KGF) and oocyte-secreted factors bone morphogenetic factor-15 (BMP-15) and growth differentiation factor-9 (GDF-9). Of these candidate factors, GDF-9 was found to directly decrease KITL gene expression in KGN

cells and cumulus cells via ALK 4/5/7 receptors. There was also some evidence for a slight reversal of the GDF-9 effect on KITL expression by the addition of the potent androgen 5α-dihydrotestosterone (DHT). The results of these studies indicated KITL gene expression is regulated by GDF-9 in human granulosa cells and are consistent with observations of negative regulation of KITL expression in mouse granulosa cells.

Evidence shown in this thesis suggests that the ratio of KITL isoforms in granulosa cells changes throughout human folliculogenesis. Follicular target cells for KITL signalling were found to include granulosa cells, theca cells and the oocyte, suggesting that the KITL/c-kit system may have potential roles throughout human folliculogenesis as demonstrated in animal models. Furthermore, this thesis has demonstrated that GDF-9 directly regulates KITL gene expression in human granulosa cells. From these results, this thesis proposes an *in vivo* model in which abnormally low levels of GDF-9, shown by a previous study to be characteristic of PCOS oocytes, results in increased KITL levels and this effect may be further exacerbated by the reversal of GDF-9 inhibition by excess androgen. This thesis has provided a greater understanding of the molecular mechanisms involved in human folliculogenesis which may be of use in future therapeutic strategies and diagnosis in assisted reproductive technology, and provide a basis for understanding human ovarian function and ovarian disease.

# DECLARATION

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

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Astrud R R Tuck

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# PUBLICATIONS ARISING FROM THIS THESIS

**Manuscripts in Preparation for Submission to Scientific Journals**

Tuck AR, Robker, RL, Norman RJ, Tilley WD, Hickey TE. Expression and localisation of kit ligand and c-kit in the adult human ovary. To be submitted to Fertility and Sterility.

Tuck AR, Robker, RL, Norman RJ, Tilley WD, Hickey TE. Regulation of kit ligand gene expression by endocrine and ovarian factors in human granulosa cells. To be submitted to Molecular Reproduction and Development.

**Abstracts Published in the Proceedings of Scientific Meetings**

Tuck AR, Tilley WD, Hickey TE. Expression of kit ligand is increased in polycystic ovaries. Australian Society for Medical Research Annual Scientific Meeting of the SA Division, Adelaide, SA, 2008.

Tuck AR, Tilley WD, Hickey TE. The role of kit ligand in the pathology of polycystic ovary syndrome. University of Adelaide Faculty of Health Sciences Postgraduate Expo, Adelaide, SA, 2008.

Hickey TE, Tuck AR, Dodd T, Norman RJ, Tilley WD. Increased kit ligand in polycystic ovaries. Annual Meeting of the Endocrine Society, San Francisco, CA, USA, 2008.

Hickey TE, Tuck AR, Dodd T, Norman RJ, Tilley WD. Increased expression of an androgen receptor regulated gene, kit ligand, in polycystic ovaries. Annual Meeting of the Society for Reproductive Biology, Melbourne, VIC, 2008.

Tuck AR,Yang X, Tilley WD, Hickey TE. Kit ligand expression and regulation in human polycystic ovaries. Australian Society for Medical Research Annual Scientific Meeting of the SA Division, Adelaide, SA, 2009.

Tuck AR, Robker RL, Tilley WD, Hickey TE. Kit ligand expression and regulation in human ovarian granulosa cells. Annual Meeting of the Endocrine Society of Australia, Adelaide, SA, 2009.

Tuck AR, Robker RL, Tilley WD, Hickey TE. Kit ligand expression and regulation in human ovarian granulosa cells. University of Adelaide Faculty of Health Sciences Postgraduate Expo, Adelaide, SA, 2009.

Tuck AR, Tilley WD, Hickey TE. Increased kit ligand expression in human polycystic ovaries. 14$^{th}$ World Congress of Gynaecological Endocrinology, International Society of Gynaecological Endocrinology, Florence, Italy, 2010.

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Tuck AR, Robker RL, Norman RJ, Tilley W,D Hickey TE. Characterisation of c-kit expression and localisation in human ovaries. Research Centre for Reproductive Health and Centre for Stem Cell Research, Research Day, Adelaide, SA, 2011.

# ABBREVIATIONS

| | |
|---|---|
| 3,4-DCI | 3,4-dichloroisocoumarin |
| A | antrum |
| ALK | anaplastic lymphoma kinase |
| ANOVA | analysis of variance |
| AR | androgen receptor |
| ARE | androgen response element |
| ART | assisted reproductive technology |
| bFGF | basal fibroblast growth factor |
| BL | basal lamina |
| BMI | body mass index |
| BMP | bone morphogenetic factor |
| BMPR | bone morphogenetic factor receptor |
| bp | base pair |
| BSA | bovine serum albumin |
| cAMP | cyclic adenosine monophosphate |
| CA | California |
| CAlb | corpus albicans |
| CC | cumulus cells |
| cDNA | complementary DNA |

| | |
|---|---|
| CL | corpus luteum |
| $CO_2$ | carbon dioxide |
| COC | cumulus-oocyte-complex |
| COOH | carboxyl group |
| DAB | 3,3'-Diaminobenzidine |
| DBD | DNA binding domain |
| DCC | dextran coated charcoal |
| DCC-FBS | dextran coated charcoal-fetal bovine serum |
| DFP | diisopropylfluorophosphate |
| DHT | 5α-dihydrotestosterone |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DNase 1 | deoxyribonuclease 1 |
| EDTA | ethylenediamine tetra-acetic acid |
| EGF | epidermal growth factor |
| ERK | extracellular signal-regulated kinase |
| EtOH | ethanol |
| FAI | free androgen index |
| FBS | fetal bovine serum |
| FSH | follicle stimulating hormone |
| FSHR | follicle stimulating hormone receptor |
| g | gram |
| GC | granulosa cells |

| | |
|---|---|
| GDF | growth differentiation factor |
| GnRH | gonadotrophin releasing hormone |
| h | hour |
| hCG | human chorionic gonadotrophin |
| HSP | heat shock protein |
| IGF-1 | insulin-like growth factor-1 |
| IgG | immunoglobulin |
| IU | international units |
| IVF | *in vitro* fertilisation |
| IVM | *in vitro* maturation |
| kb | kilo base |
| kD | kilo Dalton |
| KGF | keratinocyte growth factor |
| KITL | kit ligand |
| L | litre |
| LBD | ligand binding domain |
| LH | luteinising hormone |
| M | molar |
| mA | milliampere |
| MAP | mitogen activated protein |
| MAPK | mitogen activated protein kinase |
| mg | milligram |
| MGC | mural granulosa cells |

| | |
|---|---|
| min | minute |
| mL | millilitre |
| mM | millimolar |
| mRNA | messenger RNA |
| NaCL | sodium chloride |
| ng | nanogram |
| $NH_2$ | amino group |
| nmol | nanomolar |
| NTD | amino-terminal domain |
| O | oocyte |
| OHF | hydroxyflutamide |
| PBS | phosphate-buffered saline |
| PCO | polycystic ovaries |
| PCOS | polycystic ovarian syndrome |
| PCR | polymerase chain reaction |
| PMA | phorbol 12-myristate 13-acetate |
| POF | premature ovarian failure |
| PTX3 | pentraxin 3 |
| qPCR | quantitative polymerase chain reaction |
| RIPA | radioimmunoprecipitation assay buffer |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |

| | |
|---|---|
| RT | reverse transcriptase |
| S | stroma |
| SA | South Australia |
| SCF | stem cell factor |
| SD | standard deviation |
| sec | second |
| SEM | standard error of the mean |
| SHBG | steroid hormone binding globulin |
| SMAD | mothers against decapentaplegic protein |
| StAR | steroidogenic acute regulatory protein |
| T | theca layer |
| TβR | transforming growth factor β receptor |
| TBS | tris buffered saline |
| TBST | tris buffered saline-tween 20 |
| TGF | transforming growth factor |
| UK | United Kingdom |
| USA | United States of America |
| V | volt |

## Other:

| | |
|---|---|
| °C | degrees Celsius |
| µg | microgram |
| µl | microlitre |

| | |
|---|---|
| μm | micron |
| μM | micromolar |