Understanding the role of the mTOR signalling pathway in the ovary during folliculogenesis, PCOS and hyperandrogenism

Thesis Submitted

In Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

By

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TO

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I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, 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. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

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I hereby certify that to the best of my knowledge the work for this thesis entitled "" has been carried out under my supervision, in the School of Biomedical Sciences and Pharmacy at The University of Newcastle, Australia, and that all of the scholarly work described in chapters 2, 3 and 4 has been carried out by the Research Higher Degree candidate Lisa Sercombe. Outlined below are the items that the candidate has contributed towards the fulfilment of the work described in this thesis:

- Contributed to the conception and design of the studies
- Conducted and designed most of the experiments
- Critically analysed and interpreted the results
- Prepared and organised the figures
- Contributed in drafting and conceptualising the thesis chapters
- Contributed in formatting initial and revised versions of the thesis chapters.

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Pradeep S. Tanwar dd/mm/yyyy

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List of abbreviations

α-SMA	Smooth muscle alpha
17-OHP	17-hydroxyprogesterone
3B-HSD2	3-beta-hydroxysteroid dehydrogenase type II
A4	Androstenedione
AARS	Alanine--trna ligase, cytoplasmic
Acn	Acetonitrile
ACOT1	Acyl-coenzyme A thioesterase 1
ADPRH	[Protein ADP-ribosylarginine] hydrolase
AKR1D1	Aldo-keto reductase family 1 member D1
THOC4	THO complex subunit 4
AMPK	AMP-activated protein kinase
APP	Amyloid precursor protein
AR	Androgen receptor
PMCA1, ATP2B1	Plasma membrane calcium-transporting atpase 1
BSA	Bovine serum albumin
UHRF1	E3 ubiquitin-protein ligase UHRF1
CAPG	Macrophage-capping protein
CBR3	Carbonyl reductase [NADPH] 3
CDC37	Hsp90 co-chaperone Cdc37
CK2	Casein kinase 2
CLIC4	Chloride intracellular channel protein 4
CO3A1	Collagen alpha-1(III) chain
COPG	Coatomer subunit gamma-1
CPT2	Carnitine O-palmitoyltransferase 2, mitochondrial
CSE1L	Exportin-2/ chromosome segregation like-1 protein
CST	Cell signalling technology
CYP11A1	Side-chain cleavage enzyme
CYP17α1	17α-hydroxylase
DAB	Diaminobenzidine
Dbt, MARS	Methionine--tRNA ligase, cytoplasmicLipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial
Deptor	DEP-domain-containing mtor-interacting protein

DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
dpn	Day post-natal
DSHB	Developmental studies hybridoma bank
EGF	Epidermal growth factor
EIF2	Eukaryotic initiation factor 2
EIF3G	Eukaryotic translation initiation factor 3 subunit G
EIF3J	Eukaryotic translation initiation factor 3 subunit J-A
EIF3K	Eukaryotic translation initiation factor 3 subunit k
EIF3M	Eukaryotic translation initiation factor 3 subunit M
EIF4	Eukaryotic initiation factor 4
EIF4E	Eukaryotic translation initiation factor 4E
EIF5	Eukaryotic translation initiation factor 5
FBS	Fetal bovine serum
FDR	False discovery rate
FSH	Follicle stimulating hormone
GAMT	Guanidinoacetate N-methyltransferase
GAP	Gtpase-activating proteins
GlaGLA	Alpha-galactosidase A
Gnai2G-alpha i2	Guanine nucleotide-binding protein G(i) subunit alpha-2
GnRH	Gonadotropin releasing hormone
GSTT1	Glutathione S-transferase theta-1
H3K4	Histone H3 lysine 4
HCF-1, Hcf-1	Host cell factor 1
Hsp90	Heat shock protein 90
IF	Immunofluorescence
IGF-I	Insulin-like growth factor-I
IHC	Immunohistochemistry
ILKAP	Integrin-linked kinase-associated serine/threonine phosphatase 2C
IPA	Ingenuity pathway analysis
IR	Insulin receptor
ISOC2A	Isochorismatase domain-containing protein 2A

Itpr2I,P3R2	Inositol 1,4,5-trisphosphate receptor type 2
KLHL11	Kelch-like protein 11
LAMB2	Laminin subunit beta-2
LAMP2	Lysosome-associated membrane glycoprotein 2
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LFQ	Label-free quantification
LH	Luteinising hormone
LKB1, STK11	Liver Kinase B1/ serine/threonine kinase 11
MBNL2	Muscleblind-like protein 2
MEM	Minimum essential media
MLL	Mixed-lineage leukaemia
mLST8, GBL	Mammalian lethal with Sec13 protein 8, G protein beta subunit-like
mTOR	Mammalian target of rapamycin
mTORC1/2	Mammalian target of rapamycin complex 1/2
NEAA	Non-essential amino acids
NLRP14	NACHT, LRR and PYD domains-containing protein 14
NLRP5	NACHT, LRR and PYD domains-containing protein 5
OPLAH	5-oxoprolinase
$P450_{arom}$	Cytochrome P450 aromatase
P4EBP1	Phospho-4e binding protein 1
p70S6K	Ribosomal protein S6 kinase beta-1
PADI6	Protein-arginine deiminase type-6
PAFAH1B2	Platelet-activating factor acetylhydrolase IB subunit beta
PEPCK2	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial
PCNA	Proliferating cell nuclear antigen
PCOS	Polycystic ovarian syndrome
PDK1	Phosphoinositide dependent protein kinase-1
PDLIM4	PDZ and LIM domain protein 4
PFA	Paraformaldehyde
PHPT1	14 kDa phosphohistidine phosphatase
PI3K	Phosphoinositide 3-kinase
PIN4	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 4
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate

PKD	Polycystic kidney disease
Pkm	Pyruvate kinase PKM
PPLBL2	Putative phospholipase B-like 2
PLD	Polycystic liver disease
PLD	Phospholipase D
PLD3	Phospholipase D3
PMSG	Pregnant mare serum gonadotrophin
POF	Premature ovarian failure
PP2A	Protein phosphatase 2
PPP1CB	Serine/threonine-protein phosphatase PP1-beta catalytic subunit
PPP1CC, PP1ɣ	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit
pS6, Rps6RPS6	Phospho-ribosomal protein S6
Psmg1DSCR2	Proteasome assembly chaperone 1
PTEN	Phosphatase and tensin homolog
PtpaPPP2R4	Serine/threonine-protein phosphatase 2A activator
Rap1bRAP1B	Ras-related protein Rap-1b
Rap2bRAP2B	Ras-related protein Rap-2b
Raptor	Regulatory associated protein of mtor
RHEB	Ras homolog enriched in brain
Rictor	Rapamycin insensitive companion of mtor
RNH1	Ribonuclease inhibitor
RPL11	60S ribosomal protein L11
RPL31	60S ribosomal protein L31
Rpl7	60S ribosomal protein L7
RPL7A	R60S ribosomal protein L7a
RPS10	40S ribosomal protein S10
RPS15	40S ribosomal protein S15
RPS23	40S ribosomal protein S23
RPS29	40S ribosomal protein S29
Rps6ka3, RSK2	Ribosomal protein S6 kinase alpha-3
Rps8	40S ribosomal protein S8
S6K1/2	S6 kinase 1/2
Sh3gl1, ENDOA2	Endophilin-A2

SHBG	Sex hormone binding globulin
SNPs	Single nucleotide polymorphisms
SNTB2	Beta-2-syntrophin
SPE	Solid phase extraction
SVS4	Seminal vesicle secretory protein 4
SVS5	Seminal vesicle secretory protein 5
T	Testosterone
TBC17	Tre2-Bub2-Cdc16 (TBC) 1 domain family, member 7
TFA	Trifluoroacetic
THOP1	Thimet oligopeptidase
TLE6	Transducin-like enhancer protein 6
TMED4	Transmembrane emp24 domain-containing protein 4
TSC1/2	Tuberous sclerosis complex 1/2
1UAP1L1	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1
URI	Unconventional prefoldin RPB5 interactor
Vinexin	Sorbs3
XRN2	5'-3' exoribonuclease 2

Abstract

Polycystic ovary syndrome (PCOS) is a complex and heterogeneous reproductive and endocrine disorder. Symptoms of PCOS include hyperandrogenism, polycystic ovaries and infrequent or absent menstruation. PCOS is also the leading cause of anovulation-associated infertility in women. Reduced fertility in PCOS patients arises due to follicular dysfunction, which culminates in the continuous arrest of mid antral follicles followed by polycystic remodelling. The cause of PCOS is ill-defined because the disease pathophysiology is poorly understood. Previous research has alluded to the dysfunction of the mammalian target of rapamycin (mTOR) signalling pathway in PCOS. However, no study thus far has comprehensively investigated the relationship between the mTOR signalling pathway and PCOS. Hyperandrogenism is thought to be a key pathogenic factor in PCOS, as women with clinical androgen excess and animal models exhibit many of the ovarian features of PCOS. Yet, the precise mechanisms through which hyperandrogenism causes reproductive dysfunction in PCOS is unclear. Animal models of PCOS are limited because they indirectly induce PCOS via systemic hyperandrogenism, which requires several months to produce the desired phenotype. Culturing ovaries *in vitro* can overcome these limitations of PCOS animal models. *In vitro* methods have been widely used in research to establish ovarian development and biology, toxicology, and also ovarian disorders. Thus, *in vitro* models are a promising methodology to characterise the molecular mechanisms related to hyperandrogenism, the mTOR signalling pathway and PCOS.

The capacity of ovary culture models to recapitulate *in situ* counterparts is ill-defined, particularly the molecular mechanisms. Therefore, the first point of investigation of this thesis was a comparative proteomic analysis of *in vitro* cultured ovaries. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomic approach was used to compare the protein profile of ovaries cultured *in vitro* to those *in situ*. The results showed that overall, ovaries cultured *in vitro* are similar to that *in situ*, as they share the majority of proteins and most of which are comparably expressed. In addition, we demonstrated that mTOR pathways were conserved within ovaries cultured *in vitro.* Accordingly, *in vitro* cultured ovaries were able to signal through mTOR pathway(s) similar to if they remained *in vivo*. Taken together, organ culture models are an appropriate methodology to investigate the ovarian mechanisms of PCOS. Chapter two of this thesis characterised the expression of mTOR pathway markers within PCOS cysts. This analysis revealed that mTOR pathway signalling within the granulosa cells lining PCOS cysts was differentially deregulated. Based upon this differential mTOR pathway activity as well as histological differences, two cyst types were characterised within PCOS ovaries, multilayered and flattened. In the same chapter (chapter

3), the cause of aberrant mTOR signalling in PCOS was investigated using an *in vitro* culture model of moderate ovarian hyperandrogenism. This revealed that moderate hyperandrogenism induces abnormal mTOR signalling similar to that of multilayered PCOS cysts, suggesting a relationship between androgen excess, cystogenesis and mTOR signalling activity in PCOS ovaries. The final chapter (chapter 4), utilised an *in vitro* model to examine how androgen excess progressively impairs ovarian function. A label-free quantification (LFQ) LC-MS/MS proteomic approach was used to characterise the protein profile of moderate and severe ovarian hyperandrogenism. Ingenuity pathway analysis showed that of moderate and severe hyperandrogenism deregulates mTOR signalling comparable to the multilayered and flattened cysts of PCOS ovaries, respectively. These results again suggest that hyperandrogenism deregulated mTOR signalling in PCOS.

This thesis, for the first time, identified that mTOR signalling is deregulated in PCOS ovaries. Furthermore, hyperandrogenism in PCOS progressively promotes cystogenesis in a dual manner, (1) first attenuating pS6K signalling triggering follicular arrest and granulosa apoptosis, (2), later by increasing mTOR signalling to preserve cysts and the functions of their granulosa cell layer. Together, this finding showcases a mechanism through which androgen excess increasingly deregulates the mTOR signalling in PCOS ovaries and thereby promotes cystogenesis. Current treatment methods for PCOS are limited because they only pacify symptoms. The novelty of this thesis is that we identified a novel therapeutic target, which is based upon molecular mechanisms of PCOS rather than symptoms.