Endometrial stem/progenitor cells in endometrial regeneration, carcinogenesis and aging

Thesis Submitted

In Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

By

SHAFIQ MUKHTAR SYED

B.V.Sc (Vet); M.V.Sc. (Biotechnology)

THE UNIVERSITY OF
NEWCASTLE
AUSTRALIA

TO

Faculty of Health,

School of Biomedical Sciences and Pharmacy

The University of Newcastle, Australia

May 2018

TABLE OF CONTENTS

DECLARATIONS (PART A)

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision.

The thesis contains scholarly work of which I am a co-author. For each such work a written statement, endorsed by my supervisor, attesting to my contribution to the joint work has been included.

*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 in the text. 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.*

***** Unless an Embargo has been approved for a determined period.***

Candidate Signature: Date: 24/ 5/ 2018

Shafiq Syed dd/mm/yyyy

DECLARATIONS (PART B)

I hereby certify that to the best of my knowledge the work for this thesis entitled "Endometrial stem/progenitor cells in endometrial regeneration, carcinogenesis and aging" 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 and 3, and about 80-90% of the scholarly work described in chapter 4 has been carried out by the Research Higher Degree candidate Shafiq Syed. Outlined below are the items that the candidate has contributed towards the fulfillment of the work described in this thesis:

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

Supervisor Signature: ..... Date: 24/ 5/ 2018

Pradeep S. Tanwar dd/mm/yyyy

ACKNOWLEDGMENTS

Firstly, I would like to express my sincere gratitude to my principal supervisor Dr. Pradeep S. Tanwar for providing me with an opportunity to work on one of the most exciting projects in his lab. His unrelenting support, guidance and patience always kept me motivated during my PhD study. He always stood by my side through some of the tougher times during this journey and made sure that I have access to all the required resources. His unparalleled support and patience helped me nurture my own ideas. I could not have imagined having a better mentor. Besides my principal supervisor, I would like to thank my co-supervisor, Dr. Janet Bristow for her thoughtful advice, suggestions and encouragement.

I would also like to acknowledge my fellow lab mates for their help, support and co-operation.

Many thanks to the members of Department of Medical Biochemistry for their assistance throughout the journey.

My sincere thanks also go to the ASU team at HMRI particularly Rebecca Bosevski, Nick Bosevski, Carol Allport and Nicole Morris who were exceptionally helpful to me. They really took great care of my animals and always made sure that everything I needed at the Bioresources facility was readily accessible to me without any hiccups.

Last but not the least, I would like to thank my parents and brothers for their unconditional love and support throughout this journey. Special thanks to my wife for her exceptional understanding and forgiving me for not spending my time with her the way she deserved.

LIST OF ABBREVIATIONS

3D	3-dimensional
αSMA	Alpha smooth muscle actin
ABCG2	ATP-binding cassette sub-family G membrane 2
ABCB5	ATP-binding cassette B5
AFM	Atomic force microscopy
Amhr2	Anti-Müllerian hormone receptor 2
APC	Anaphase promoting complex
ASCs	Adult stem cells
AUC	Area under the ROC curve
BAD	Bcl2-associated death promoter
Bis-AA	Bis-acrylamide
BMDSCs	Bone marrow-derived stem cells
BrdU	Bromodeoxyuridine
CAFs	Cancer associated fibroblasts
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CDKN1B	Cyclin-dependent kinase inhibitor 1B
CIC	Cancer initiating cells
CK8	Cytokeratin 8
CIdU	Chloro-deoxyuridine
CM	Conditioned media
COC	Cumulus oocyte complex
DAPI	4', 6-diamidino-2-phenylindole
E2	Estradiol
EC	Endometrial cancer
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIC	Endometrial intraepithelial carcinoma
eMSC	Endometrial mesenchymal stem
ER	Estrogen receptor
ERK	Extracellular-signal-regulated kinase
ERKO	Estrogen receptor knockout
EYFP	Enhanced yellow fluorescent protein
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor

FIGO	International Federation of Gynecology and Obstetrics
FOXO	Forkhead box O
GE	Glandular epithelium
GFP	Green fluorescence protein
GSK3	Glycogen synthase kinase 3
H2B-GFP	H2bj protein and green fluorescent protein fusion protein complex
Hand2	Heart- and neural crest derivatives-expressed protein
H & E	Hematoxylin and Eosin
HBSS	Hanks balanced salt solution
HGF	Hepatocyte growth factor
HGFP	H2bj protein and green fluorescent protein fusion protein complex
HLA	Human leukocyte antigen
IGF	Insulin-like growth factor
IdU	Iodo-deoxyuridine
IK	Ishikawa
IPF	Idiopathic pulmonary fibrosis
ITO	Indium tin oxide
K8	Keratin 8
LE	Luminal epithelium
LEF	Lymphoid enhancer binding factor
LGR5	Leucine Rich Repeat Containing G Protein-Coupled Receptor 5
Lkb1	Liver kinase B1
LMD	Laser capture micro-dissection
LOX	Lysyl oxidase
LRCs	Label retaining cells
MALDI-MSI	Matrix assisted laser desorption/ionization mass spectrometry imaging
MAPK	Mitogen-activated protein kinase
MD	Müllerian duct
MDM2	Mouse double minute 2 homolog
MET	Mesenchymal-epithelial transition
MMP	Matrix metalloproteinases
MSI	Microsatellite instability
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NES	Normal endometrial stroma
Ng2	Neural/glial antigen 2
PAA	Polyacrylamide
PDGFRα	Platelet-derived growth factor alpha
PFA	Paraformaldehyde

pFAK	Phospho-focal adhesion kinase
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PND	Post-natal day
Poly-HEMA	Poly (2-hydroxyethyl methacrylate)
PTEN	Phosphatase and tensin homolog
PR	Progesterone receptor
RFP	Red fluorescence protein
RIPA	Radioimmunoprecipitation assay
ROC	Receiver operating characteristic
RTK	Receptor tyrosine kinase
Sca-1	Stem cell antigen-1
SFM	Serum-free media
SHG	Second harmonic generation
SOX-2	SRY(Sex determining region Y)-box 2
SP	Side population
SSEA-1	Stage-specific embryonic antigen-1
STAT3	Signal transducer and activator of transcription 3
SUSD2	Sushi domain containing 2
TA	Transit amplifying
TAM	Tamoxifen
TCF	T-cell factor
TCGA	The Cancer Genome Atlas
TGF β	Transforming growth factor β
TIE	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TMA	Tumor microarray
TP53	Tumor suppressor p53
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Abstract

Uterus is one of the most regenerative organs of human body, and, is regulated by ovarian sex hormones. Throughout the reproductive life it undergoes >400 cycles of degeneration and regeneration during which endometrium, the inner lining of uterus, regrows completely to a thickness of 4-10mm every month. Such a remarkable regeneration potential can only be explained by the existence of adult stem cells in uterus. Adult stem cells have the potential to contribute to the pool of cancer initiating cells (CICs). Thus, identifying these stem cells and understanding their regulation will provide newer insights into endometrial physiology and disease. However, owing to the lack of uterine stem cell specific markers, there is no definitive evidence for the existence of uterine stem cells. Nearly 85% of endometrial cancers (EC) are estrogen-driven. Once reproductive life is over, and menopause sets in, there is a marked reduction in the circulating levels of ovarian hormones due to decline in the follicular reserves. Interestingly, the risk of EC development increases with age, and is 10 times higher in women aged 50 and above. Therefore, it remains unclear how aging causes EC development despite having diminished ovarian follicular reserves and therefore, the reduced levels of estrogen. Identifying uterine stem cells within their resident niches and the key regulatory pathways involved, and establishing uterine cell lineage hierarchy is crucial to investigate how dysregulation in these pathways lead to the development of EC. It might also provide newer insights into the pathogenesis of endometriosis, particularly in cases where endometrial tissue grows ectopically outside the peritoneal cavity.

In this thesis, we have identified uterine stem cells within their resident niches and also define their specific markers. We used a suite of transgenic mouse models to label genetically the uterine epithelial and mesenchymal lineages, and then traced the fate of these labelled cells to identify adult stem cells. We showed that endometrial epithelium self-renews during development, growth and regeneration throughout life. This self-renewal process is fueled by a subset of glandular epithelial cells which are Wnt-responsive and express Axin2. These Axin2+ glandular epithelial cells act as long-lived bi-potent epithelial stem cells that give rise to the both glandular and luminal epithelium. In response to tamoxifen administration, an estrogen agonist and a known inducer of EC, the progenies of these $Axin2^{+}$ cells comprise the majority of the resulting epithelial hyperplastic lesions, the precursor of EC. Thus $Axin2^{+}$ cells are likely the cells of origin for the EC. Furthermore, we show that there is no flux of cells between epithelial and mesenchymal lineages. In fact, all the uterine mesenchymal cells, including stromal fibroblasts and myometrium arise from embryonic Ng2-expressing pericytes, which act as uterine mesenchymal stem cells. These results show that Wnt signaling is responsible for the maintenance of endometrial epithelial stem cells, and, is crucial for endometrial homeostasis and cancer development. We further show that it is the hyperactive Wnt signaling in aged endometrium that is responsible for the development of endometrial hyperplasia, despite the reduction in estrogen levels. This upregulation in Wnt signaling is in turn mediated by age-related changes in endometrial stroma, which results in its altered extracellular matrix (ECM) dynamics

and increased stiffness. The transduction of altered ECM compliance by epithelial cells to result in hyperactive Wnt signaling is mediated through integrin-focal adhesion kinase (FAK) signaling.

In conclusion, we have defined uterine cell lineage hierarchy and identified epithelial and mesenchymal stem cell specific markers. Furthermore, we identified the role of Wnt signaling in endometrial cancer development in aged women. By studying age-related endometrial stromal changes, we also defined a prognostic stromal cell signature for EC patients

Thesis overview

I. Introduction

Uterus is an important organ from at least two perspectives. From the perspective of stem cell biology, it is the only organ where there is massive physical shedding of its inner lining including epithelium, stroma and the associated vasculature, following which 4-10 mm of new tissue regrows within 4-10 days every 28 days in humans. This massive degeneration and, more importantly, the scar less regeneration process, that too at such a massive scale, is akin to the regeneration potential of invertebrate life forms. Yet, unfortunately so, not even a single study, over the past decade, has been able to provide a clear understanding of the cellular hierarchy of uterine cell lineages, and/or the existence of the stem cells. From the perspective of reproductive biology, its massive regenerative potential is the key to the sustenance of mammalian life, and most of the uterine disorders, including infertility, endometriosis, adenomyosis and EC, are almost always the result of aberrant endometrial regeneration. Most of the literature on uterine regeneration is based on mere in vitro/transplantation assays and/or histological marker analyses. Due to the lack of uterine stem cell specific markers and therefore the suitable mouse models, genetic lineage tracing, which is the robust technique to trace the fate of any labelled cell under *in vivo* conditions, is not possible. We decided to label genetically all the uterine epithelial and mesenchymal lineages to assess the flux of labelled cells between these compartment, enabling us to map the putative stem cells to particular lineage compartments, and subsequently use more specific promoters to identify the stem cells within their resident niches. Therefore, we generated a suite of transgenic mouse models to perform the lineage tracing experiments to identify uterine stem cells. We hypothesized that once identified, it will be possible to study the key regulatory pathways involved and their dysregulation in EC development, particularly in aged women.

II. Aims

The studies presented in this thesis aim to:

1. Understand the mechanisms underlying endometrial homeostasis and regeneration.
2. Identify and characterize endometrial epithelial stem cells, their role in epithelial regeneration and the pathways regulating these stem cells.
3. Role of Wnt signaling in age-associated EC development.

III. Organization of the thesis

The “Thesis overview” provides an introduction to the thesis and summarizes the chapters presented in the thesis. The preface to each chapter briefly explains the results found and the rationale behind each study. Each preface is followed by the study in detail.

Chapter 1, entitled "**Endometrial stem/progenitor cells in endometrial regeneration, carcinogenesis and aging**" provides a review of the existing literature. It serves as a background to this thesis and provides information on the existence of the putative endometrial stem cells, and their role in endometrial regeneration and age-associated EC development.

Chapter 2, entitled "**Mechanisms of endometrial regeneration**" addresses the first aim of the thesis, that is, to understand the mechanisms underlying endometrial homeostasis and regeneration. Here we used various mouse models to show that endometrial epithelium is highly plastic, and self-renews during development, growth and regeneration. We also show that this self-renewal is fueled by a subset of epithelial cells residing in endometrial glands and luminal epithelial crypts. Importantly, there is no contribution of stromal-epithelial transition, bone marrow derived stem cells (BMDSCs) or native mesenchymal stem cells towards the epithelial self-renewal neither during homeostasis nor during regeneration. It is important to note that due to the lack of robust *in vivo* lineage tracing methods, over the past decade, a number of *in vitro* and transplantation assays-based studies have implicated the contribution of these processes towards endometrial regeneration. Furthermore, we identified embryonic Ng2-expressing pericytes as mesenchymal stem cells that give rise to all the uterine mesenchymal cell lineages including the stromal fibroblasts and myometrium.

Chapter 3, entitled "**Role of Wnt signaling in endometrial regeneration**" addresses the second aim of the thesis. After establishing that endometrial epithelium displays heterogeneity in the proliferation potential, and that a subset of epithelial cells residing in glands or luminal crypts fuel epithelial self-renewal, we focused on endometrial glands to identify the epithelial stem cell. We were able to show that a sub-population of glandular epithelial cells that are Wnt responsive and express Axin2 are the long-lived bipotent epithelial stem cells of the endometrium. These $Axin2^+$ cells self-renew and give rise to glandular epithelial cells, as well as the luminal epithelial cells over long term. Importantly, a singly labeled $Axin2^+$ cell responds to tamoxifen administration, an estrogen agonist and a known inducer of EC, and results in epithelial hyperplasia, a precursor for EC. Thus, these Wnt-responsive $Axin2^+$ cells are likely the cells of origin for EC. In addition, these cells act as ovariectomy-resistant endometrial stem cells and are analogous to castration-resistant prostate stem cells. Thus, Wnt signaling is necessary for the maintenance of endometrial epithelial stem cells and plays a critical role in endometrial regeneration.

Chapter 4, entitled "**Aging, Wnt, and endometrial cancer**" addresses the final aim of the thesis, that is, the role of Wnt-signaling in age-associated EC development. Here we show that age-related changes in endometrial stroma result in aberrant composition and deposition of ECM resulting in increased stromal stiffness. These changes in turn result in the upregulation of Wnt signaling pathway mediated through integrin-focal adhesion kinase (FAK) signaling, which ultimately results in endometrial hyperplasia, a precursor for EC. Therefore, we propose hyperactive Wnt signaling, resulting in possible dysregulation of endometrial epithelial stem cells,

as a mechanism underlying the EC development in aged women. By studying the stromal changes in aged endometrium and EC tissues, we further defined a stromal signature that can predict the long-term survival of EC patients.

Finally, the thesis discussion summarizes the findings of chapters 2, 3 and 4. Briefly, the studies presented in this thesis have functionally identified and characterized for the first time endometrial epithelial and mesenchymal stem cells and defined the hierarchy of uterine cell lineages. Wnt signaling is important for the maintenance of epithelial stem cells. With age, the changes in the compliance of endometrial stroma due to altered ECM composition results in the hyperactive Wnt signaling in endometrial epithelium leading to the development of endometrial hyperplasia, a precursor for EC.