

Molecular mechanisms by which estrogen causes ovarian epithelial cell dysplasia

Nhung Hong Vuong

November 6, 2017

Department of Cellular and Molecular Medicine

University of Ottawa

Ottawa, Ontario, Canada

This thesis is submitted as a partial fulfilment of the
Ph.D. program in Cellular and Molecular Medicine.

© Nhung Hong Vuong, Ottawa, Canada, 2018

Contributions

Manuscripts submitted:

- **N.H. Vuong**, D. Cook, L. Forrest, L. Carter, P. Robineau-Charette, J. Kofsky, K. Hodgkinson, and B.C. Vanderhyden. Single-cell RNA-sequencing reveals transcription dynamics of estrogen-induced dysplasia in the ovarian surface epithelium. *Nature Communications*. Submitted.
- **N.H. Vuong**, O. Salah Salah, and B.C. Vanderhyden. 17 β -Estradiol sensitizes ovarian surface epithelium to transformation by suppressing *Disabled-2* expression. *Scientific Reports*. Submitted.
- K. Hodgkinson, **N.H. Vuong**, L. Forrest, B. Djordjevich, and B.C. Vanderhyden. *GREB1* is a novel estrogen receptor-regulated tumour promoter that is frequently expressed in ovarian cancer. *Oncogene*. Submitted.

Contributions by students under supervision of Nhung Vuong:

- Omar Salah Salah performed immunohistochemical (IHC) staining shown in Figure 7, Figure 10, Figure 11, Figure 16, and Figure 17, and Appendix E; and IHC quantification for Figure 16.
- Joshua Kofsky performed Image J quantification of SA- β Gal staining for Figure 22 and Figure 37, anchorage independent growth assays for Figure 23, and validation of KRAS^{G12D} over-expression in Figure 21.

Contributions by other lab members:

- Colleen Crane performed IHC staining for Figure 2.
- Kendra Hodgkinson performed IHC staining for Figure 39D.
- Lauren Carter performed IHC staining for Figure 34 and Appendix E.
- Pascale Robineau-Charette performed IHC staining for Figure 34 and Appendix E.
- David Cook performed bioinformatics for FiguresFigure 25, Figure 26, Figure 27, Figure 28, Figure 29, Figure 35, Figure 36, and Figure 39A.
- Laura Forrest performed IHC staining for Figure 39C and Appendix E.
- Olga Collins isolated OSE from mouse ovaries and human ascites cells from patient ascites fluid.
- Ken Garson infected *Dab2*-floxed OSE with KRAS^{G12D} retrovirus.
- Galaxia Rodriguez helped with interpreting the single cell sequencing data in the context of immunology.

Contributions by collaborators:

- Ken Korach provided our lab with *Esr1* conditional knockout mice.
- Elizabeth Smith from Xiang-Xi Xu's lab provided our lab with primary isolations of *Dab2*-floxed OSE.

Contributions by core facilities:

- OHRI StemCore Laboratories helped with hybridizing, staining, and scanning the Affymetrix GeneChip miRNA 4.0 Array. They also contributed to single-cell RNA sequencing experiments by allowing us to use their Fluidigm C1 system, assisted us assessing cDNA library quality, and ran the sequencing.
- Ottawa Cell Biology and Image Acquisition core facility gave us access to the Zeiss LSM 800 confocal microscope system and Imaris Image Analysis Software.

Funding:

- Canadian Institutes of Health Research (CIHR) grant
- Ontario Graduate Scholarship
- Queen Elizabeth II Graduate Scholarships in Science and Technology
- CIHR Reproduction, Early Development, and the Impact on Health Training Program

Thesis Advisory Committee Members:

- Jonathan Lee
- Jonathan Copeland
- Christine Pratt

Abstract

The initiating events of ovarian cancer remain unknown, but an established risk factor is use of estrogen therapy by post-menopausal women where there is a positive correlation between duration of use and risk for disease. Mouse models of ovarian cancer have shown that exposure to exogenous 17 β -estradiol (E2) accelerates tumour onset so this study aims to investigate the E2 signalling mechanisms responsible for sensitizing ovarian epithelial cells to transformation. By developing model systems that are responsive to E2 manipulation, we showed that E2 induces the formation of epithelial dysplasias both *in vitro* and *in vivo*. microRNA microarray was used to discover that E2 up-regulates microRNA-378 via the ESR1 pathway, resulting in the down-regulation of a tumour suppressor gene called *Disabled-2 (Dab2)*. E2 suppression of *Dab2* was found to result in increased proliferation, loss of contact inhibition, epithelial dysplasia, and increased sensitivity to transformation. This mechanism was also found to be active in mouse fallopian tube epithelium and human ovarian cancer cells. Single-cell RNA sequencing and trajectory analysis was subsequently used to explore additional signalling mechanisms that might contribute to the emergence of dysplastic lesions induced by E2. Multiple molecular signalling pathways dysregulated by E2 were identified and this revealed several possible biomarkers to be investigated for early detection of ovarian cancer. In the context of a current lack of strategies for ovarian cancer prevention or early detection, this work represents a significant advance in our understanding of how E2 promotes ovarian cancer initiation.

Table of Contents

Contributions.....	ii
Abstract.....	iv
List of Tables	viii
List of Figures.....	ix
List of Abbreviations	xi
Acknowledgements	xiv
Chapter 1: Introduction	1
1.1: Ovarian Cancer Overview	1
1.1.1. Ovarian cancer statistics	1
1.1.2. Histological subtypes of ovarian cancer	1
1.1.3. Risk Factors for ovarian cancer.....	3
1.1.4. Estrogen replacement therapy is a risk factor for ovarian cancer	3
1.2: Ovarian Cancer Initiation	4
1.2.1. Cells of origin for ovarian cancer	4
1.2.2. Ovarian Surface Epithelium	5
1.2.3. Fallopian Tube Epithelium.....	5
1.3: Estrogen effects on OSE and FTE	6
1.3.1. 17 β -estradiol.....	6
1.3.2. Mechanisms of E2 action	7
1.3.3. E2 effects on OSE.....	8
1.3.4. E2 effects on FTE	8
1.4: Effects of exogenous E2 in mouse models	10
1.4.1. E2 accelerates tumour onset in a mouse model of ovarian cancer	10
1.4.2. Prolonged E2 exposure causes pre-neoplastic OSE lesions	10
1.4.3. E2's influence on cell morphology is context dependent.....	11
1.5: Disabled-2, a regulator of OSE structure and function.....	12
1.5.1. Epithelial cell polarity	12
1.5.2. <i>Disabled-2 (Dab2)</i>	13
1.5.3. DAB2 in ovarian cancer.....	14
1.5.4. <i>Dab2</i> knockout mice	14
1.5.5. E2 regulation of polarity and <i>Dab2</i>	14
1.6: Rationale, hypothesis, and objectives.....	17
1.6.1. Objectives.....	17
Chapter 2: Materials and Methods	19
2.1: Mouse Experiments	19
2.1.1. Mice strains and care	19
2.1.2. Hormone pellet implants.....	19
2.2: Cell Culture Experiments.....	19
2.2.1. Isolation and maintenance of primary human ascites cultures	19
2.2.2. Isolation and maintenance of mouse OSE and MASE primary cultures.....	20
2.2.3. Culture media for <i>in vitro</i> assays	21
2.2.4. Assessment of cell proliferation	21
2.2.5. Knockout of <i>Esr1</i> and <i>Dab2</i> in OSE <i>in vitro</i> using adenovirus.....	21
2.2.6. Blocking ESR1 activity.....	22

2.2.7. microRNA (miRNA) mimic assays	22
2.2.8. Over-expression of KRAS ^{G12D} in <i>Dab2</i> -floxed OSE.....	23
2.2.9. Senescence-associated- β gal staining and analysis.....	23
2.2.10. Low attachment growth assay	24
2.2.11. Colony formation assay	24
2.3: Molecular Techniques	24
2.3.1. Immunohistochemical staining of tissue	24
2.3.2. Western blot analysis.....	25
2.3.3. Quantitative polymerase chain reaction (qPCR) analysis	26
2.3.4. Immunofluorescence (IF) staining.....	27
2.3.5. Microscopy.....	28
2.3.6. Immunofluorescence image analysis.....	28
2.3.7. Chromatin Immunoprecipitation (ChIP).....	29
2.3.8. miRNA microarray	30
2.4: Single-cell sequencing and analysis	30
2.4.1. Single-cell cDNA library preparation and sequencing.....	30
2.4.2. scRNA-Seq data processing	31
2.4.3. scRNA-Seq data filtering.....	32
2.4.4. Normalization and imputation of expression values.....	32
2.4.5. Clustering and differential expression between clusters.....	33
2.4.6. Functional enrichment analysis of scRNA-Seq data.....	34
2.4.7. Identification of marker genes of each cluster	34
2.4.8. Pseudotime analysis of ovarian epithelial cell populations	35
2.5: Data availability	35
2.5.1 miRNA microarray	35
2.5.2. Single-cell sequencing data and code	35
Chapter 3: Results.....	36
3.1. In vitro model in which E2 induced OSE dysplasia can be studied.....	36
3.1.1. OSE cells <i>in vitro</i> can establish a polarized epithelial monolayer.....	36
3.1.2. OSE cells <i>in vitro</i> are capable of undergoing EMT	36
3.1.3. E2 induced OSE dysplasia is reproducible <i>in vitro</i>	39
3.2. E2 suppression of <i>Dab2</i>.....	43
3.2.1. OSE dysplasia is associated with a reduction in <i>Dab2</i> expression and function	43
3.2.2. <i>Dab2</i> suppression by E2 is via the ESR1 pathway.....	46
3.2.3. Investigation of <i>Dab2</i> 's putative estrogen response element.....	50
3.2.4. E2 suppresses <i>Dab2</i> by up-regulation of miR-378.....	52
3.2.5. <i>Dab2</i> suppression by E2 is observed in mouse FTE.....	55
3.3. Loss of <i>Dab2</i> causes OSE dysplasia and sensitizes cells to transformation.	58
3.3.1. Loss of <i>Dab2</i> is sufficient to cause OSE hyperplasia.....	58
3.3.2. Loss of <i>Dab2</i> is sufficient to cause disorganization of OSE monolayer	58
3.3.3. Loss of <i>Dab2</i> sensitizes OSE to transformation.....	62
3.4. Transcription dynamics of estrogen-induced dysplasia in the OSE	62
3.4.1. E2 induced dysplasias demonstrate aberrant expression in polarity proteins	62
3.4.2. Single-cell profiling of control and E2 treated OSE cells	66
3.4.3. Heterogeneity in OSE cell response to E2	68
3.4.4. Distinct cell populations <i>in vitro</i>	74
3.4.5. Pseudotime analysis reveals phenotypic divergence	86
3.4.6. Trajectory models foci formation	91
3.4.7. <i>Greb1</i> is uniquely up-regulated in E2 induced stratified OSE	94

Chapter 4: Discussion	96
4.1: <i>In vitro</i> model of E2 induced OSE dysplasia.....	97
4.2: E2 promotes ovarian cancer by suppressing <i>Dab2</i>	97
4.2.1. E2 suppression of <i>Dab2</i> is mediated by the ESR1 pathway.....	97
4.2.2. E2 responsive miRNAs contribute to <i>Dab2</i> suppression and OSE dysplasia.....	98
4.2.3. miR-378's relevance in ovarian cancer.....	99
4.2.4. E2 induced OSE dysplasia is downstream of <i>Dab2</i> suppression.....	100
4.2.5. <i>Dab2</i> mediates contact inhibition via proper localization of CDH1.....	101
4.2.6. DAB2 is a tumour suppressor.....	102
4.2.7. Physiological relevance of <i>Dab2</i> suppression by E2 in healthy OSE	103
4.2.8. Physiological relevance of DAB2 in normal FTE biology and dysplastic lesions....	104
4.2.9. E2 may also sensitize FTE to transformation.....	104
4.2.10. E2 regulation of DAB2 is conserved in humans.....	105
4.3: Other mechanisms of E2 induced OSE dysplasia	106
4.3.1. E2 promotes proliferation and cell survival pathways.....	106
4.3.2. E2 prioritizes sustained proliferation over maintaining epithelial architecture.....	108
4.3.3. Strategies for early detection and prevention of ovarian cancer	109
4.3.4. Investigation of <i>Greb1</i> 's role in ovarian cancer initiation	110
Conclusions.....	110
References.....	113
Appendices.....	123
Appendix A: <i>In vitro</i> OSE cell adenovirus infection optimization and validation.....	124
Appendix B: miRNA mimic details and optimization experiment	126
Appendix C: KRAS ^{G12D} over-expression vector.....	127
Appendix D: List and details of antibodies used for immunohistochemistry staining.....	128
Appendix E: Immunohistochemistry staining antibody controls.....	129
Appendix F: Validation of Amido Black as western blot loading control.....	131
Appendix G: DAB2 antibody validation for western blot analysis.....	132
Appendix H: List and probing parameters for antibodies used in western blot analysis....	133
Appendix I: qPCR endogenous control validation for E2 assays	134
Appendix J: Probe validation for qPCR analysis.....	135
Appendix K: qPCR probes, primers, miRNA assay sequences	137
Appendix L: List and details of antibodies used for immunofluorescent staining.....	138
Appendix M: Immunofluorescent staining “no primary” controls.....	139
Appendix N: Primer sequences used for chromatin immunoprecipitation of ESR1	141
Appendix O: Single cell capture quality control	142
Appendix P: Control vs. E2 microRNA array results.....	144
Appendix Q: Differential expression results between Clusters 1 and 2 (k=2).....	145
Appendix R: Differential expression results between Clusters 2 and 3 (k=3).....	148
Appendix S: Pseudotime branch-dependent gene expression results.....	149

List of Tables

Table 1: Top 5 KEGG pathways associated with E2 responsive miRNAs in MASE	53
Table 2: Top 5 terms associated with genes more highly expressed in Clusters 1 & 2....	73
Table 3: Top 5 terms associated with genes more highly expressed in Clusters 2 & 3....	76
Table 4: Area under receiver operator characteristic curves	77
Table 5: Top 5 terms associated with each cluster of branch-dependent genes	90

List of Figures

Figure 1: Histological Subtypes of Ovarian Cancer.....	2
Figure 2: Suppression of DAB2 in OSE cells & ovarian tumours <i>in vivo</i> by E2	16
Figure 3: Hypothesis schematic.....	18
Figure 4: OSE cells in culture form adherens junctions upon cell-cell contact	37
Figure 5: OSE cells in culture demonstrate proper localization of polarity proteins	38
Figure 6: OSE cells in culture are responsive to TGF β induced EMT.....	40
Figure 7: Prolonged E2 stimulation alters OSE cell morphology	41
Figure 8: OSE cells become stratified with prolonged E2 exposure.....	42
Figure 9: Prolonged E2 exposure causes an increase in hyperplastic OSE.....	44
Figure 10: Prolonged E2 exposure suppresses <i>Dab2</i> in OSE.....	45
Figure 11: E2 induced stratified cells had loss or mislocalization of CDH1	47
Figure 12: DAB2 suppression occurs before morphological changes & is mediated via the ESR1 pathway.....	48
Figure 13: Validation of <i>Esr1</i> knockout by AdCre infection in <i>Esr1</i> -floxed OSE	49
Figure 14: The putative ERE proximal to the TSS of <i>Dab2</i> does not bind ESR1.....	51
Figure 15: E2 suppresses <i>Dab2</i> via up-regulation of miR-378	54
Figure 16: Prolonged E2 exposure suppressed DAB2 & increased dysplasia in mouse FTE.56	
Figure 17: E2 induced FTE disorganization affects both PAX8 positive & negative cells	57
Figure 18: Validation of <i>Dab2</i> knockout by AdCre infection in <i>Dab2</i> -floxed OSE.....	59
Figure 19: Loss of <i>Dab2</i> is sufficient to increase OSE proliferation	60
Figure 20: Loss of <i>Dab2</i> is sufficient to cause disorganization of OSE monolayer	61
Figure 21: Validation of KRAS ^{G12D} over-expression in <i>Dab2</i> -floxed OSE.....	63
Figure 22: Loss of <i>Dab2</i> prevents oncogene induced senescence.....	64
Figure 23: Loss of <i>Dab2</i> sensitizes OSE cells to transformation	65
Figure 24: E2 exposure causes deregulation of polarity related proteins in OSE	67
Figure 25: Single-cell profiling of control & dysplastic E2 treated OSE cells	69
Figure 26: Unsupervised clustering reveals distinct heterogeneity in OSE	70

Figure 27: Assessing E2 responsiveness	72
Figure 28: Unsupervised clustering can segregate control & E2 treated OSE	75
Figure 29: PCA cluster markers to be validated by IF	79
Figure 30: Morphologies observable in control or E2 treated cultures highlighted by IF actin staining	80
Figure 31: Validation of cluster 1 marker LTBP2 by IF	81
Figure 32: Validation of cluster 2 marker PTGIS by IF	83
Figure 33: Validation of cluster 3 marker IGFBP5 by IF	84
Figure 34: Cluster marker validation <i>in vivo</i> by IHC	85
Figure 35: Columnar & stratified OSE cells represent different stages of dysplasia	87
Figure 36: Expression dynamics of divergent control & E2 treated phenotypes	89
Figure 37: E2 promotes survival	92
Figure 38: E2 promotes proliferation	93
Figure 39: Prolonged E2 exposure leads to up-regulation of GREB1 in stratified OSE cells	95
Figure 40: Molecular signaling mechanisms of estrogen-induced dysplasia in OSE	107

List of Abbreviations

°C	degrees Celsius
3'UTR	3-prime untranslated region
ACK	ammonium-chloride-potassium
ACTB	beta actin
AdCre	adenovirus expressing Cre recombinase
AdGFP	adenovirus expressing GFP
AKT	v-akt murine thymoma viral oncogene homolog 1 (aka Akt1)
ANOVA	analysis of variance
aPKC	atypical PKC
aPKC _ι	atypical PKC iota
AUC	area under the receiver operator characteristic curve
bp	base pair(s)
BRCA1	breast cancer type 1 susceptibility protein
BRCA2	breast cancer type 2 susceptibility protein
cAMP	cyclic adenosine monophosphate
CDH1	cadherin-1 (aka E-cadherin)
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus
COL1A2	collagen type I alpha 2
Cre	Cre recombinase
CSS	charcoal-stripped serum
CT	cycling threshold
CTNNB1	catenin beta-1 (aka Beta-catenin)
DAB	diaminobenzidine
Dab2	disabled-2
DAPI	4',6-diamidino-2-phenylindole
DMBA	7,12-dimethylbenz[a]anthracene
DMEM-F12	Dulbecco's modified eagle medium: nutrient mixture f-12
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
E2	17 beta-estradiol
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptor(s)
ERE	estrogen response element(s)
ERK	extracellular signal-regulated kinase family (aka Mapk1)
ESR1	estrogen receptor 1 (aka alpha)
ESR2	estrogen receptor 2 (aka beta)
EtOH	ethanol
EZR	ezrin
FBS	fetal bovine serum
FTE	fallopian tube epithelium

G	gravity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GP1B	glycoprotein 1 (aka Gp130)
GREB1	growth regulation by estrogen in breast cancer 1
HIF1A	hypoxia inducible factor 1 alpha subunit
HRP	horseradish peroxidase
IF	immunofluorescence
IFC	integrated fluidic circuit
IGFBP4	insulin like growth factor binding protein 4
IGFBP5	insulin like growth factor binding protein 5
IgG	immunoglobulin G
IHC	immunohistochemistry
IL6	interleukin 6
IP	immunoprecipitation
JAK2	janus kinase 2
JNK	c-jun n-terminal kinase (aka Mapk8)
Ki67	marker of proliferation ki-67 (aka Mki67)
KO	knockout
KRAS	v-Ki-Ras2 kirsten rat sarcoma viral oncogene homolog
KRAS ^{G12D}	mutant KRAS where glycine is substituted with aspartic acid at position 12
KRT19	keratin 19 (aka Ck19)
KRT7	keratin 7
LH	luteinizing hormone
logFC	logarithmic fold change
LoxP	locus of X-over P1
LTBP2	latent-transforming growth factor beta-binding protein 2
M	molar
MAPK	mitogen-activated protein kinase(s)
MAS	mouse ascites-derived ovarian cancer cell line
MASC	MAS line derived from a control mouse
MASE	MAS line derived from an e2 treated mouse
MEK	mitogen-activated protein kinase kinase (aka Map2k) family
MEM	minimum essential media
mg	milligram(s)
min	minute(s)
miRNA	micro RNA
mL	milliliter(s)
mM	millimolar
M-PER	mammalian protein extraction reagent
MPP	methyl-piperidino-pyrazole
mRNA	messenger RNA
MYC	V-myc avian myelocytomatosis viral oncogene homolog
NA	numerical aperture
NCBI	National Center for Biotechnology Information

ng	nanogram(s)
nM	nanomolar(s)
OSE	ovarian surface epithelium
PAX8	paired box 8
PBS	phosphate-buffered saline
PC1	principal component 1
PC2	principal component 2
PC3	principal component 3
PCA	principal component analysis
PCR	polymerase chain reaction
PFU	plaque forming unit
PI3K	phosphoinositide-3-kinase(s)
pmols	picomoles
PPIA	peptidylprolyl isomerase a
PTEN	phosphatase and tensin homolog
PTGIS	prostaglandin i2 synthase
PVDF	polyvinylidene difluoride
QPCR	quantitative reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute 1640 medium
RQ	relative quantity
RT-PCR	reverse transcription-polymerase chain reaction
S.E.M.	standard error of the mean
SCID	severe combined immunodeficiency
sec	second(s)
siRNA	short interfering RNA
SNAI1	snail family zinc finger 1
SV40	Simian vacuolating virus 40
TAg	sv40 large t-antigen
tgCAG-TAg	transgenic mouse model where inducible TAg is driven by the chicken ACTB promoter
TGFβ	transforming growth factor beta
TMA	tissue microarray
TNF	tumor necrosis factor
TSS	transcription start site
v/v	volume/volume
w/v	weight/volume
WT	wild type
ZO1	zonula occludens-1 (aka tight junction protein 1; Tjp1)
αMEM	alpha minimum essential medium
μg	microgram(s)
μL	microliter
μM	micromolar
μm	micrometer

Acknowledgements

First and foremost, I'd like to acknowledge my stellar supervisor Dr. Barbara Vanderhyden for taking me on as a graduate student. It was a huge leap of faith given that my only qualification at the start was "enthusiasm". Barb is truly a master scholar and a saint as she not only helped me focus my energy into becoming a productive, high caliber scientist; but also had the patience to teach me how to think critically, write concisely, and speak clearly. Thank you for all the time you've invested in my academic success and making sure I get through life in general. You did a good job on this one boss.

In the lab, a lot of credit goes to Ken, Olga, Liz, and Josee for developing my skills at the bench side and always making time to help me with lab work, new ideas, and dealing with existential crises such as running out of DNase or coping with new health and safety rules. Thank you for keeping the lab running smoothly all these years, making sure I only ended up in the emergency once, and booting me in the right direction during my less clever moments. As for my outstanding students Omar, Brendan, and Josh: thank you for all the hard work you boys put into your projects and your display of resilience as I slowly figure out how to be a mentor. Hopefully you've all come out of my wing mostly unscathed and will move on to making awesome scientific discoveries – thanks to your positive and negative controls.

Honourable mention goes to all the members of Biohazard: our brilliantly fluorescent yellow lab frisbee team that is never short on fun. Thank you all for being there to (in)validate my feelings, celebrate my successes, and laughing away my failures. My liver has aged substantially since our first game, but my spirit is more than ever alive. It is never a dull moment with this band of aspiring adults; and for better or for worse, I hope we never change. It goes without saying that my better half David deserves an honorary degree as he was the engine that kept this project going. A full day of negative data never seemed so bad because he always made sure I had delicious food at home, and clean socks to start again tomorrow. Thank you for keeping our life together as I pursue my passion for science. I know it's not easy sharing me with lab work, nor living with a professional student.

Finally, thank you to my family for supporting my education all these years. It's been a long while and perhaps nobody believes me anymore, but guess what folks?! I think I may be done school.

Chapter 1: Introduction

1.1: Ovarian Cancer Overview

1.1.1. Ovarian cancer statistics

A woman's lifetime risk of developing ovarian cancer is 1 in 75, and her chance of dying due to the disease is 1 in 100 (Reid et al. 2017). It has the highest mortality rate of all cancers in the female reproductive system with a five-year survival of only 45% (Canadian Cancer Society 2016). Women who develop the disease tend to remain asymptomatic until later stages of metastasis, but if ovarian cancer is detected early, the five-year survival rate increases to 92% (Ellison 2015; American Cancer Society 2016). This highlights the need to understand the initiating events of ovarian cancer so that better strategies for early detection and disease prevention can be developed.

1.1.2. Histological subtypes of ovarian cancer

Ovarian cancer is not one disease but it is made of multiple subtypes that have unique sites of origin, clinical behaviour, and molecular aberrations (**Figure 1**). On the surface, ovarian cancer is divided into three histological types based on the cell type from which it arises: epithelial, stromal, or germ cell (Reid et al. 2017). The most predominant form is epithelial ovarian cancers which make up 90% of all cases and can be further broken down into five subtypes based on histology and biomarker expression (**Figure 1b**): high-grade serous, low-grade serous, endometrioid, clear cell, and mucinous (Jayson et al. 2014). Given the variety of forms in which ovarian cancer presents itself, it is a common goal in ovarian cancer research to elucidate the multiple cells of origin

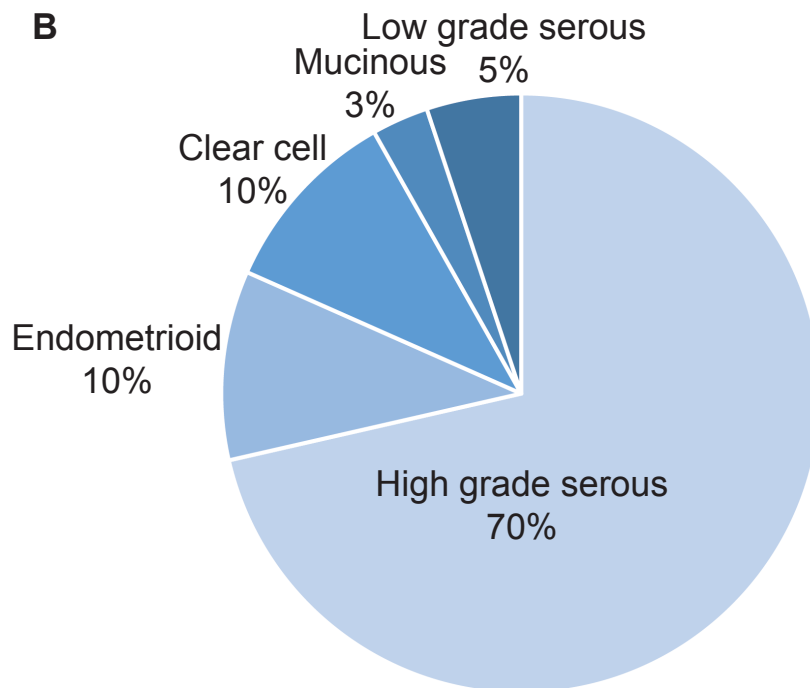
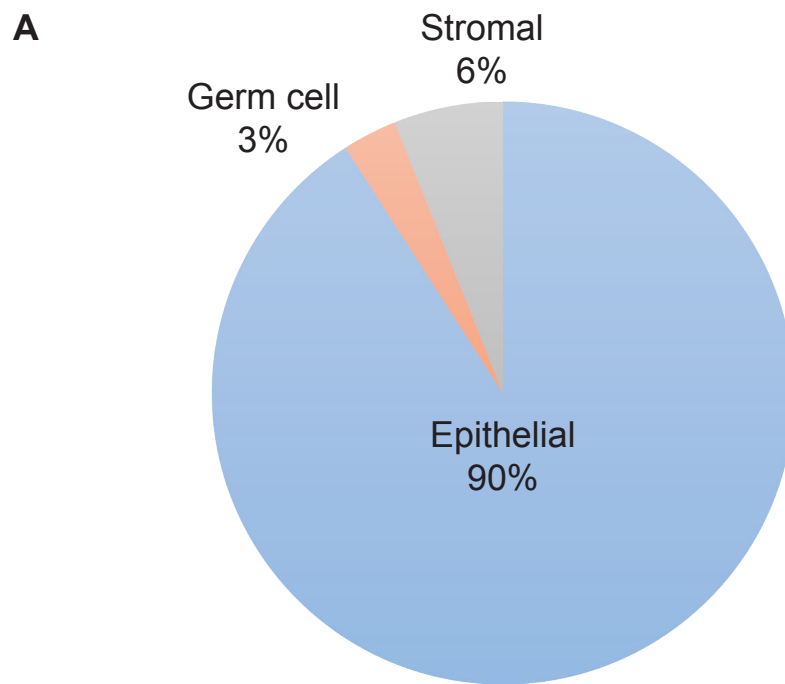


Figure 1: Histological Subtypes of Ovarian Cancer. (A) Cell types of ovarian cancer. **(B)** Subtypes of epithelial ovarian cancer based on biomarker expression. **(A-B)** Statistical information acquired from Jayson *et al.* (2014) and Reid *et al.* (2017).

and risk factors that contribute to each histological subtype.

1.1.3. Risk Factors for ovarian cancer

Our current understanding of ovarian cancer initiation revolves around factors that increase or decrease risk, but the molecular signalling events leading to transformation remain unclear. Ovarian cancer in women under the age of 40 is rare and the few cases observed are mainly germ cell tumours (Webb & Jordan 2016). The incidence for epithelial ovarian cancer starts to rise after 40 and peaks around 60-70 years of age. At least 10% of ovarian cancers are due to hereditary factors such as BRCA1/BRCA2 mutations and Lynch syndrome (mutations in mismatch repair genes) (Jayson et al. 2014). The majority of ovarian cancers, however, are not associated with any known mutations and a woman's risk for developing the disease is dependent on their cumulative exposure to environmental and lifestyle factors. In addition to genetics, reproductive history, endometriosis, obesity, and estrogen replacement therapy are associated with increased ovarian cancer risk (Webb & Jordan 2016). Factors that are considered protective against ovarian cancer include: parity and oral contraceptive use; and to a lesser extent: lactation, regular physical activity, and avoidance of cigarettes (Reid et al. 2017).

1.1.4. Estrogen replacement therapy is a risk factor for ovarian cancer

Meta-analysis of 52 epidemiological studies investigating menopausal hormone use and ovarian cancer risk found that 55% of women who developed ovarian cancer had also used estrogen replacement therapy. The increased relative risk for ovarian cancer

was significant for high-grade serous and endometrioid but not for other ovarian cancer subtypes. This risk increases with duration of use and declines when the estrogen therapy is stopped, although the risk isn't completely mitigated (Collaborative Group on Epidemiological Studies of Ovarian Cancer 2015). In patients with malignant ovarian cancer, there is a significant positive correlation between plasma estrogen levels, stage of disease, and tumour volume. This correlation is further supported when patients are treated with chemotherapy, where a decrease in tumour volume is also associated with a marked decrease in plasma estrogen levels (Mählck et al. 1988).

Together, epidemiological studies have shown a positive correlation between use of estrogen therapy and the development of ovarian cancer, while Mählck *et al.* (1988) suggests that continued E2 stimulation is important for tumour progression as the tumour appears to promote estrogen secretion.

1.2: Ovarian Cancer Initiation

1.2.1. Cells of origin for ovarian cancer

Many studies have shown, by investigating tumour histology, molecular profiles, and mouse models of ovarian cancer, that inclusion cysts derived from the ovarian surface epithelium (OSE) and the fimbrial fallopian tube epithelium (FTE) can be cells of origin for high- and low-grade serous ovarian cancers (Auersperg 2013). Recent advances in high through-put techniques have allowed proteomics and genome-wide association studies to further support that both cell types are capable of giving rise to serous ovarian cancers (Coscia et al. 2016; Coetzee et al. 2015). New data are suggesting that endometrioid and clear cell tumours can arise from endometrial cells that implant on the peritoneal wall and ovary due to retrograde menstruation (Reid et al. 2017; Taniguchi

2017).

1.2.2. Ovarian Surface Epithelium

The OSE is normally a thin epithelial monolayer that covers the ovary but has been documented to show heterogeneous morphologies where a mix of mesenchymal, squamous, cuboidal, columnar, and stratified phenotypes can be observed on the same ovary (Okamura & Katabuchi 2003). Unlike other reproductive cell types that derive from coelomic epithelial differentiation during fetal development, OSE cells have retained uncommitted pluripotent characteristics that allow them to change their morphology in response to environmental factors such as ovulation. This allows the OSE to remain quiescent as squamous or cuboidal cells on a non-ovulated ovary, but also to quickly respond to ovulation by rapid proliferation and assuming a more migratory mesenchymal morphology for wound repair (Auersperg 2001).

1.2.3. Fallopian Tube Epithelium

The FTE is a pseudostratified epithelium where every FTE cell is in contact with the basement membrane, but the folded nature of the fallopian tube gives FTE cells a superficial appearance of being stratified (Stewart & Behringer 2012). The FTE is divided into ciliated and secretory cell populations and the fallopian tube itself is comprised of 3 sections that are histologically recognizable by the ratio of ciliated to secretory cells, extent of longitudinal folds, and thickness of smooth muscle that surrounds the stromal layer (Stewart & Behringer 2012). Closest to the ovary is the infundibulum which has the highest ratio of ciliated to secretory FTE cells, the greatest

amount of epithelial folding, and is thinly surrounded by smooth muscle. Furthest from the ovary and adjacent to the uterus is the isthmus which is predominantly made of secretory FTE cells, has the least amount of folding, and has the thickest lining of smooth muscle. The ampulla exists in between the infundibulum and isthmus, and has median characteristics of both ends.

At present, the FTE is thought to be the site of origin for 70% of high-grade serous ovarian cancers (Kramer 2013), suggesting that the remaining 30% stems from OSE. Current strategies for culturing FTE cells, however, requires them to be immortalized by perturbing the p53 and pRb tumour suppressor pathways (Karst & Drapkin 2012), making it a less desirable system for determining how healthy epithelial cells progress towards cancer. Given that primary mouse OSE cells are more amenable to growing in culture, we used OSE cells as an *in vitro* model system to initially elucidate the molecular mechanisms of estrogen signaling, then extended our observations to FTE *in vivo* to determine if the findings are relevant in both cell types.

1.3: Estrogen effects on OSE and FTE

1.3.1. 17 β -estradiol

There are three naturally occurring estrogens in women: 17 β -estradiol, estrone, and estriol. In this study, estrogen refers to 17 β -estradiol (E2) because it is the major secreted estrogen in premenopausal women, it has the most potent effects on the estrogen receptors, and it is the form that is used or mimicked in estrogen replacement therapy (Barrett et al. 2009). The ovary is the main source of E2 and it is the primary steroid hormone that regulates female reproduction and secondary sex characteristics (Vrtačnik

et al. 2014).

1.3.2. Mechanisms of E2 action

E2 mediates its effects through two nuclear receptors, ESR1 and ESR2, and a membrane associated G-protein coupled estrogen receptor (GPER1). In the presence of E2, ESR1 will homodimerize to function as a transcription factor by binding to estrogen response elements (ERE) in proximity to the promoter region of genes. Upon recruitment of the transcriptional machinery, ESR1 degradation by the ubiquitin–proteasome pathway is required for efficient transcription of ESR1 target genes (Zhou & Slingerland 2014). ESR2 is generally thought to be an antagonist of ESR1 where it can heterodimerize with ESR1 to block ESR1 transcriptional activity. Various tissue types express differing ratios of ESR1:ESR2 as an added layer of control for regulating cell responsiveness to circulating E2 (Britt & Findlay 2002). In ovarian tumours, the ratio of ESR1:ESR2 is increased where ESR1 expression is elevated and ESR2 is diminished, suggesting that neoplastic transformation by E2 is driven by the ESR1 pathway (Couse & Korach 1999). GPER1 is the latest estrogen receptor to be discovered. E2 binding to GPER1 induces rapid cellular events such as cAMP production, calcium mobilization, and activation of MAPK and PI3K/Akt pathways (Prossnitz & Barton 2014). The literature on GPER1's role in ovarian cancer is currently limited and conflicting because GPER1 has been suggested to be an ovarian cancer tumour suppressor (Ignatov et al. 2013), but activation of its down-stream targets, MAPK and PI3K/Akt, are typically associated with pathways that are active in cancer (Kanehisa et al. 2017). Although GPER1 has been observed in ovarian tumours, its presence in OSE cells has not been established.

1.3.3. E2 effects on OSE

OSE cells express both *Esr1* and *Esr2* (Gulliver & Hurst 2012) and *in vivo*, OSE are exposed to 400-fold higher concentrations of E2 than found in serum (Plaino et al. 2000). In a mouse study, Gotfredson and Murdoch (2007) showed that OSE cells are bathed in follicular fluid at ovulation. At 2 days after ovulation, the cells become more stratified, but eventually settle back into a simple squamous and cuboidal morphology 4-8 days post-ovulation. Mice that have been exposed to high exogenous E2 have increased areas of stratification and columnar cells before and after ovulation relative to control, untreated mice (Gotfredson & Murdoch 2007; Laviolette et al. 2010). In the rabbit, E2 stimulates OSE growth and can lead to the formation of papillary structures that are reminiscent of serous ovarian cancer (Bai & Nicosia 2000). When human OSE cells were isolated and grown in collagen gel cultures, addition of E2 caused OSE cells to form luminal structures that were keratin positive and appeared to be polarized (Okamura & Katabuchi 2003). This suggests that E2 promotes epithelial differentiation of human OSE, a notion that is supported in mice where immature mice injected with E2 showed a rapid and significant increase in *Cdh1* (epithelial marker) levels in OSE (MacCalman et al. 1994). ESR1 is currently thought to be the predominant mediator of E2 transcriptional effects in the OSE (Auersperg 2001), but this may simply be due to the lack of attention given to investigating the role of ESR2 and GPER1 in OSE.

1.3.4. E2 effects on FTE

Although both secretory and ciliated cells express *ESR1* (Li et al. 2017), the molecular signalling pathways regulated by E2 in these cells are not well established.

Three dimensional culture systems for mouse oviducts and baboon fallopian tubes (to closely mimic human fallopian tubes) show that FTE cells do not proliferate in response to E2 or ovulation (King et al. 2011). E2 however, is thought to play a role in regulating the structure and function of FTE cells where it promotes the differentiation of ciliated cells and increases secretory cell activity (Okada et al. 2004; Chen et al. 2013). Studies observing porcine oviduct epithelial cultures in estrous conditions over 2.5 days have demonstrated that when E2 levels are high, FTE cells have increased height and show loss of cellular polarity relative to diestrous conditions where E2 levels are low (Chen et al. 2013).

The function of E2 signalling in key aspects of female fertility (such as follicle development and establishment of the uterine lining) has been well established, but the role of E2 in OSE and FTE cell function is less understood, perhaps because of their apparently quiescent nature relative to other cell types of the female reproductive tract. It has become clear, however, that epithelial ovarian cancer stems from OSE or FTE and understanding how different risk factors contribute to their transformation will be critical in understanding ovarian cancer initiation. In the context of E2 signalling, the literature so far has demonstrated that E2 stimulation can result in aberrant OSE and FTE morphology (Bai & Nicosia 2000; Gotfredson & Murdoch 2007; Laviolette et al. 2010; Chen et al. 2013) and ESR1 appears to be the dominant receptor in ovarian cancer cells (Couse & Korach 1999).

1.4: Effects of exogenous E2 in mouse models

1.4.1. E2 accelerates tumour onset in a mouse model of ovarian cancer

A transgenic mouse model of ovarian cancer called tgCAG-TAg was developed by our lab to study exogenous factors that may affect disease onset and progression (Laviolette et al. 2010). Cre-loxP technology was used in the design of the mouse model, where adenoviral vectors expressing Cre recombinase (AdCre) are injected under the ovarian bursal membrane. This results in infection of OSE cells and deletion of a loxP-stop sequence and the expression of SV40 large T antigen (TAg), an oncoprotein. Generally, there is a latency of 80-90 days after AdCre injection before tumours are evident. Control mice that were induced to develop tumours had a median survival of 113 days after AdCre injection. Mice that were exposed to E2 via a subcutaneous slow-release 60-day pellet (to model hormone replacement therapy), however, had a median survival of only 50 days after AdCre injection. Since E2 treated mice reached their end-point 30-40 days before microscopic tumours were observed in control mice, this indicated that E2 can accelerate tumour onset.

1.4.2. Prolonged E2 exposure causes pre-neoplastic OSE lesions

To begin investigating how E2 induced early onset of tumours in the tgCAG-TAg mice, ovaries from mice injected with adenovirus expressing green fluorescent protein (AdGFP; controls for AdCre injection) and inserted with E2 pellets were evaluated to see if E2 had any effect on OSE independent of TAg expression. After 60 days of exogenous E2 exposure, these mice developed pre-neoplastic lesions on the OSE. These pre-neoplastic lesions were comprised of two distinct morphological changes in the OSE with

a significant increase in the incidence of both columnar epithelial cells and stratified hyperplastic cells observable on the same ovary. One or both of these kinds of OSE dysplasias are thought to be pre-neoplastic lesions, because when tgCAG-TAg mice are injected with AdCre to activate TAg, patches of TAg negative OSE remain as a squamous monolayer of epithelial cells, whereas TAg positive cells assume a dysplastic phenotype, similar to mice treated with E2 alone, days before they invade the ovary (Laviolette et al. 2010).

1.4.3. E2's influence on cell morphology is context dependent

In normal epithelial cells, E2 signalling is known to promote epithelial differentiation and inhibit epithelial to mesenchymal transition (EMT) (Lee 2006). In various ovarian cancer cell lines, however, E2 has been reported to induce EMT (Park et al. 2008). This suggests that E2's ability to influence morphology is context dependent where it can shift from being an inhibitor to an activator of EMT during tumour progression. Exactly when and how this shift occurs is not well understood. In our lab, tgCAG-TAg mice treated with E2 pellets developed tumours that showed stronger epithelial differentiation relative to placebo. Cancer ascites cell lines derived from tgCAG-TAg mice treated with placebo (MASC) or E2 pellets (MASE) showed that when they are injected in SCID mice, MASE tumours better maintain a differentiated epithelial histology. Unlike many cancers that tend to dedifferentiate into a more immature state, transformed OSE cells tend to acquire Mullerian epithelial phenotypes such as the differentiated epithelium of the fallopian tube (Auersperg 2002). The basis for epithelial differentiation in OSE transformation is still under investigation, but the current literature

presents two hypothesis to explain the selective advantage: (1) CDH1, an inducer of epithelial differentiation, can mediate adhesion to prevent induction of apoptosis through anoikis (Kantak & Kramer 1998); (2) Mullerian differentiation causes OSE cells to acquire receptors that will respond to hormones and growth factors that promote carcinogenesis. Despite the potential advantages of epithelial features, transformed OSE cells must still overcome the tumour suppressive mechanisms of cell polarity to progress to ovarian carcinoma (Lee & Vasioukhin 2008). Microarray analysis of MASE tumours showed that even though tumours derived from E2 treated mice demonstrate epithelial differentiation, these tumours have significant increases in expression of genes involved in both differentiation and migration relative to placebo (Laviolette et al. 2014), suggesting that E2 derived tumours are capable of acquiring mechanisms that promote a mesenchymal phenotype.

1.5: *Disabled-2*, a regulator of OSE structure and function

1.5.1. Epithelial cell polarity

Cell polarity is important for maintaining proper structure, function, and regulation of epithelial tissues. Epithelial cells are polarized in such a way where their apical side is exposed and their basal side is attached to a basement membrane. The apical and basal domains are separated by tight and adherens junctions consisting of proteins such as ZO1, CDH1, and CTNNB1. ZO1 and CDH1 will anchor cells together and CDH1 links to the cytoskeleton through CTNNB1 (Yano et al. 2017). Membrane cytoskeleton proteins such as EZR are located at the apical side of OSE cells and interact with membrane proteins to help stabilize the cytoskeleton–plasma membrane linkage

(Maniti et al. 2012). Protein complexes such as the PAR3-PAR6-atypical PKC (aPKC) localize to the apical membrane domain to maintain the apical identity by activating EZR (Wald et al. 2008). These proteins are just a few of the many complex interactions required for cell polarity. Maintenance of cell polarity is important for epithelial cells because it provides positional cues for surface localization. Loss of cell polarity is considered a hallmark of epithelial cancer because epithelial cells undergoing transformation require dysregulation of pathways mediating polarity to become hyperplastic (Lee & Vasioukhin 2008).

1.5.2. Disabled-2 (*Dab2*)

Like all epithelial cells, OSE cell proliferation and morphology are tightly regulated by the asymmetrical distribution of polarity proteins that provide positional cues for surface localization and growth inhibition (Auersperg 2001). An important regulator of OSE cell polarity is a tumour suppressor called disabled-2 (DAB2). DAB2 is an adaptor protein that is critical for the polarized distribution of cell surface proteins such as CDH1 (Yang et al. 2007), and it regulates cellular response to growth control factors such as TGF β (Tao et al. 2014). *Dab2* was originally named *DOC2* (differentially expressed in ovarian carcinoma 2) at the time of its discovery because it was consistently present in normal OSE but absent in all ovarian cancer cell lines (Mok et al. 1994). Since its discovery, *DAB2* has been confirmed to be expressed in normal human OSE but its expression is lost in 85% of ovarian carcinomas (Fazili et al. 1999; Zhang et al. 2014).

1.5.3. DAB2 in ovarian cancer

In contrast to the normal OSE monolayer that is DAB2 positive and mostly Ki67 negative, cells in ovarian tissue where the OSE layer is dysplastic or transformed are DAB2 negative and Ki67 positive (Fazili et al. 1999; Yang et al. 2006). IHC staining of DAB2 in prophylactic oophorectomy tissues consistently shows a transition of DAB2 positive to DAB2 negative cells existing on the same ovary as the OSE layer transitions from a morphologically normal monolayer to morphologically dysplastic regions. These dysplastic regions are associated with pre-neoplastic features such as multicell-layered (papillary) epithelia, invaginations, and inclusion cysts. In regions displaying adenomas and microscopic adenocarcinomas, DAB2 continues to be undetectable (Roland et al. 2003).

1.5.4. Dab2 knockout mice

Dab2 homozygous knockout mice are embryonic lethal due to extensive disorganization of embryonic cells (Yang et al. 2002). *Dab2* heterozygous mice are viable and only adult female mice display aberrant cellular morphology, which consists of uterine hyperplasia and OSE dysplasia (fallopian tube morphology was not reported in this study) (Yang et al. 2006). In that study, OSE dysplasia was not consistently observed in *Dab2* heterozygous mice until they were 6 months old and by this point, the mice were also sterile.

1.5.5. E2 regulation of polarity and *Dab2*

In normal epithelial cells, E2 regulates polarity by increasing the expression of

CDH1, a protein commonly associated with the epithelial phenotype (Lee 2006). E2 indirectly achieves this by inhibiting SNAIL1 (a transcription factor that mediates EMT in part by inhibiting the transcription of *Cdh1*). CDH1 expression is critical for maintaining polarity because it establishes the adherens junction necessary for monolayer formation and DAB2 has been demonstrated to ensure proper localization of CDH1 (Yang et al. 2007). The influence of E2 on *Dab2* expression has only been investigated in mammary epithelial cells of virgin mice that normally have undetectable levels of DAB2 (Tao et al. 2014). In these cells, Tao *et al.* demonstrated that E2 alone did not result in any appreciable increase in DAB2. Beyond this, no study has investigated the possible regulation of *Dab2* by E2.

Thus, at the start of this thesis research, it was unknown how E2 causes OSE dysplasias and whether the actions of E2 are mediated by suppression of *Dab2*. Preliminary DAB2 IHC staining experiments using ovaries from tgCAG-TAg mice injected with AdGFP showed that placebo mice normally have an OSE layer that is highly positive for DAB2. In areas of hyperplasia in E2 treated mice, DAB2 expression was lost (**Figure 2A**). Tumours derived from tgCAG-TAg mice injected with AdCre showed a trend for lower *Dab2* expression in E2 treated mice than placebo pellet mice (p-value=0.1; **Figure 2B**). Tumours derived from SCID mice injected intraperitoneally with MASE and treated with an E2 or placebo pellet showed that E2 significantly decreased *Dab2* expression (**Figure 2C**). A genome-wide screen has identified an ERE proximal to the transcriptional start site of *Dab2* in mice and humans (Bourdeau et al. 2004), suggesting that *Dab2* may be a direct target of E2 action. These observations formed the foundation for this study.

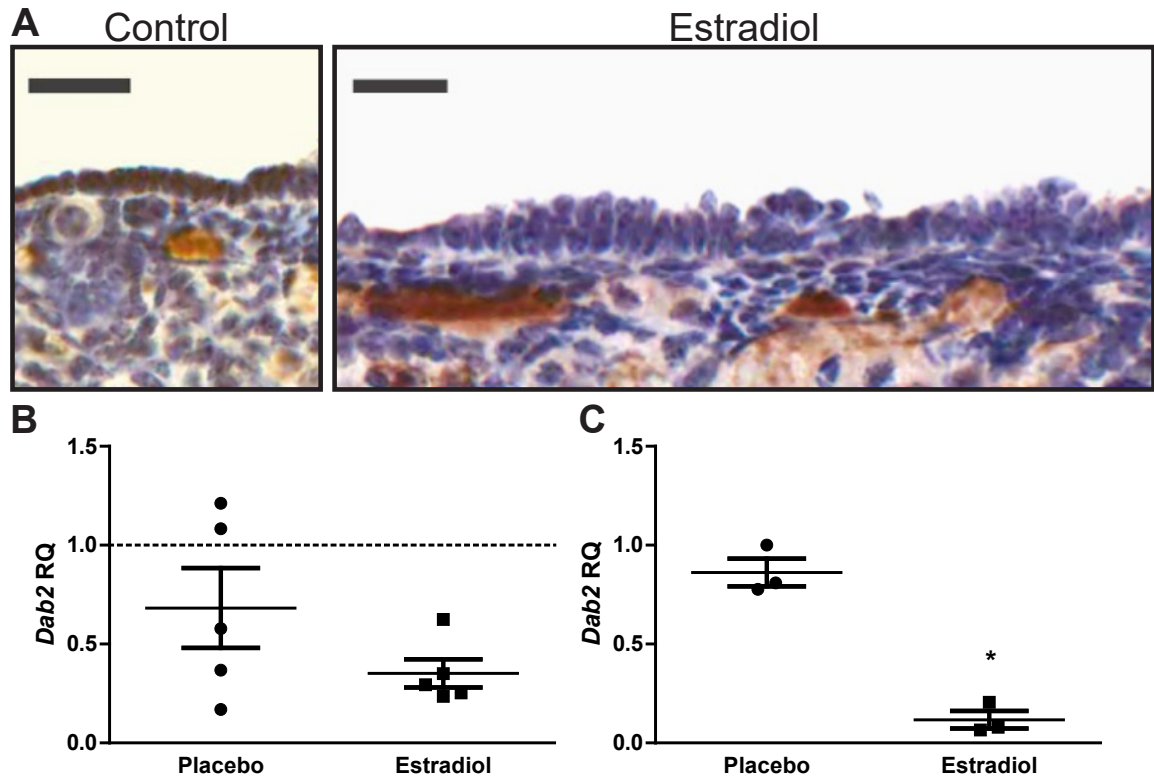


Figure 2: Suppression of DAB2 in OSE cells & ovarian tumours *in vivo* by E2. (A) Ovaries from tgCAG-TAg/AdGFP mice retrieved 60 days after insertion of placebo or E2 pellet and stained for DAB2. Scale bar=25 m. (B) Tumour tissue derived from tgCAG-TAg/AdCre mice treated with an E2 or placebo pellet. Mice were euthanized and tumours were collected on average 50 days after AdCre injection and subcutaneous insertion of pellet. Dotted line denotes wild-type ovary reference sample that was normalized to 1. n=5; p-value=0.16; unpaired t-test. (C) Tumours derived from SCID mice injected intraperitoneally with MASE cells and treated with E2 or placebo pellet. Tissue collected on average 58 days after injection of cells and subcutaneous insertion of pellet. n=3; *: p<0.05 compared to control; unpaired t-test.

1.6: Rationale, hypothesis, and objectives

The mechanism by which E2 causes OSE dysplasia is unknown but given the preliminary *in vivo* data (**Figure 2**), the phenotypic similarities between ovaries of *Dab2* heterozygous mice and E2 treated mice, and the loss of DAB2 in human ovarian tissue progressing towards transformation, **we hypothesized that E2 down-regulates *Dab2* and prolonged exposure leads to epithelial disorganization and increased proliferation resulting in OSE dysplasia that renders them more susceptible to transformation (Figure 3).** After the initial focus on *Dab2*, we used an unbiased approach to explore other signalling pathways dysregulated by E2 to provide a more global explanation for how E2 may increase OSE sensitivity to transformation. As most studies linking E2 and ovarian cancer have been correlative or descriptive, the overall aim of this study was to provide a more mechanistic explanation for the earlier onset of ovarian cancer caused by E2 exposure.

1.6.1. Objectives

- (1) To develop an *in vitro* model in which E2 induced OSE dysplasia can be studied.
- (2) To determine if E2 down-regulates *Dab2* and the mechanism by which it occurs.
- (3) To determine if loss of *Dab2* is sufficient to cause OSE dysplasia and sensitize OSE to transformation.
- (4) To explore additional molecular signalling pathways dysregulated by E2 that could lead to OSE dysplasia and susceptibility to transformation.

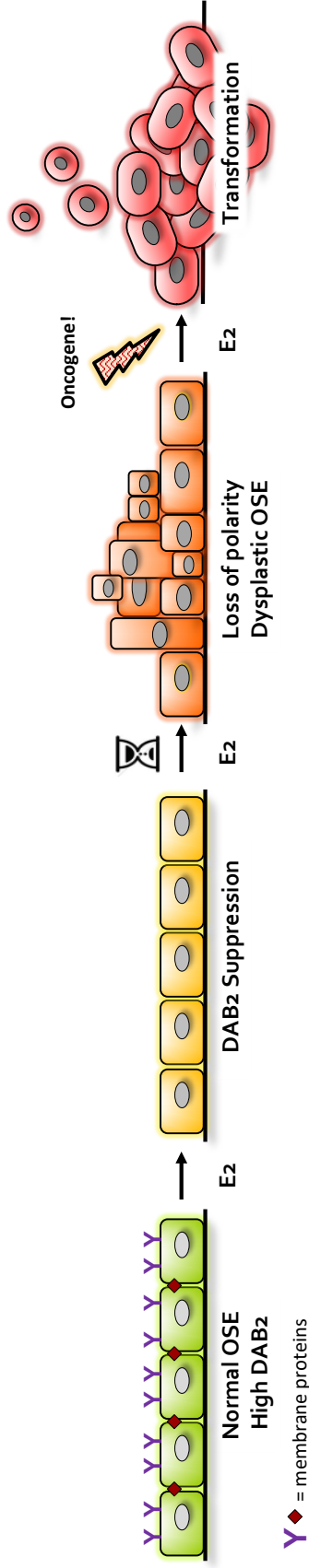


Figure 3: Hypothesis schematic. E2 down-regulates DAB2 and prolonged exposure leads to epithelial disorganization and increased proliferation resulting in OSE dysplasia that renders them more susceptible to transformation.

Chapter 2: Materials and Methods

2.1: Mouse Experiments

2.1.1. Mice strains and care

Experiments involving mice were all performed according to the Canadian Council on Animal Care Guidelines for the Care and Use of Animals on a protocol approved by the University of Ottawa Animal Care Committee. FVB/N mice were used for experiments and they were housed in the University of Ottawa animal care facility.

2.1.2. Hormone pellet implants

Mice were anaesthetized then exogenous E2 was delivered via subcutaneous implantation of 60-day time released E2 pellets (0.25 mg/pellet) or placebo control pellets (Innovative Research of America, SE-121). Mice (placebo pellet, n=5; E2 pellet, n=5) were euthanized using CO₂ 60 days after pellet implant and their reproductive tracts were fixed in formalin for 24 hours then processed and embedded in paraffin by the University of Ottawa pathology lab.

2.2: Cell Culture Experiments

2.2.1. Isolation and maintenance of primary human ascites cultures

Ascites fluid was collected from patients with ovarian cancer who consented to donate these samples to the Ottawa Ovarian Cancer Tissue Bank for research purposes. The fluid was spun at 400 G for 10 mins and the cell pellet collected. ACK lysis buffer (Quality Biological Inc.) was used to lyse red blood cells when evident. Cells were

washed with phosphate-buffered saline (PBS) and re-suspended in RPMI-1640 medium (Hyclone), then plated in a tissue culture flask pre-incubated with 2.5 mL autologous ascites fluid. Once established, human ascites cells were maintained at 37°C in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco). Human ascites cells used for experimentation were between passages 5-10.

2.2.2. Isolation and maintenance of mouse OSE and MASE primary cultures

OSE cells were isolated from mouse ovaries as previously described (Gamwell et al. 2012). *Esr1*-floxed OSE were acquired from ovaries of *Esr1* conditional knockout mice, provided by Ken Korach (Hewitt et al. 2010) and *Dab2*-floxed OSE were isolated by Elizabeth Smith from ovaries of *Dab2* conditional knockout mice (Moore et al. 2013). In both *Esr1* and *Dab2* conditional knockout models, infection with AdCre will result in loxP site deletion by Cre recombinase (within exon 3 for *Esr1* and exons 3-4 for *Dab2*) that results in a stop sequence that truncates the mRNA sequence. MASE ovarian cancer cells were isolated from ascites fluid of E2 treated tgCAG-TAg mice (Laviolette et al. 2010) in a similar fashion to human ascites isolation. All mouse cells were maintained at 37°C in OSE medium that consists of α MEM medium (Corning) with 5% FBS, 2 ng/mL epidermal growth factor (Sigma), and 0.01 mg/mL insulin-transferrin-sodium selenite supplement (Roche). Based on previous experience in the lab, estrogen responsiveness of cells in culture is dependent on baseline *Esr1* expression levels (Laviolette et al. 2014). For all *in vitro* E2 experiments in this study, *Esr1* expression levels in cell isolations were measured by qPCR and only isolations that maintained at least 50% of baseline *Esr1* mRNA expression relative to whole ovary were used.

2.2.3. Culture media for *in vitro* assays

Cells were seeded into tissue culture plates such that cells would be 80-90% confluent by the end of the experiment. Cells were allowed to attach overnight in OSE media. The next day, cells were washed with PBS and the media were changed to steroid-free media consisting of 5% charcoal stripped FBS in phenol red free DMEM-F12 (Sigma), 2 ng/mL epidermal growth factor, and 0.01 mg/mL insulin-transferrin-sodium selenite supplement. Cells were allowed to acclimatize to steroid-free media for 48 hours before E2 treatment. Unless otherwise stated, 100 nM E2 was used to treat cells all *in vitro* assays. This dose was previously determined by our lab to give the most consistent E2 response by cells in culture and was deemed within physiological range for OSE (Plaino et al. 2000). An equivalent volume of 100% EtOH (vehicle) was added to control plates for a final concentration of 0.0002% EtOH. Media were not changed for short term experiments (1-48 hours), but were refreshed every 3-4 days for long term experiments (2-15 days).

2.2.4. Assessment of cell proliferation

OSE cells were seeded and treated with E2 as described above and then counted after 5, 10, and 15 days. Cells were counted by a Vi-CELL Cell Counter (Beckman Coulter) that also measured cell viability using the trypan blue dye exclusion method.

2.2.5. Knockout of *Esr1* and *Dab2* in OSE *in vitro* using adenovirus

Conditional knockout OSE cells were suspended in serum-free α MEM media and infected with AdGFP or AdCre at 100 pfu/cell. Infected OSE cells were then seeded into

tissue culture plates and incubated at 37°C for 1 hour before FBS (10%) was added to the media. Cells continued to incubate at 37°C for 6 hours, then the fluid was changed to fresh 5% OSE medium. The next day, AdGFP infected OSE cells were observed with an EVOS FL microscope to confirm successful adenovirus infection of cells. Media were replaced and the cells then left to recover from adenovirus infection for 1 week before use in experiments. See **Appendix A** for *in vitro* OSE cell adenovirus infection validation and optimization experiments.

2.2.6. Blocking ESR1 activity

MASE cells were seeded and switched to steroid-free media as described above. One hour prior to addition of 10 nM E2, MASE cells were pre-incubated with 1 μ M methyl-piperidino-pyrazole (MPP) (Sigma), an ESR1 specific antagonist, or an equivalent volume of DMSO (vehicle). Cells were lysed for protein isolation 48 hours after E2 treatment.

2.2.7. microRNA (miRNA) mimic assays

MASE cells were seeded and allowed to attach overnight, washed with PBS the next day, and then changed to steroid-free media to minimize any E2 suppression of *Dab2*. The Lipofectamine RNAiMax reagent was used according to the manufacturer's instructions to deliver 5, 10, or 20 pmols of miR-378 mirVana Mimic (ThermoFisher Scientific). A mirVana miRNA Mimic Negative Control #1 was used as a scrambled control and ddH₂O was used for mock transfections. Following transfection, cells were incubated for 2 days at 37°C before they were lysed for protein collection. See **Appendix**

B for additional details on miRNA mimics and assay optimization experiment.

2.2.8. Over-expression of KRAS^{G12D} in *Dab2*-floxed OSE

Dab2-floxed OSE were infected with a Moloney based retroviral expression vector encoding KRAS^{G12D} and hygromycin resistance. KRAS^{G12D} expressing cells were selected using 2 µg/mL hygromycin B (Sigma). See **Appendix C** for map of KRAS^{G12D} over-expression vector.

2.2.9. Senescence-associated-βgal staining and analysis

To assess senescence-associated (SA)-βgal staining in *Dab2*-floxed OSE cells over-expressing HYG/KRAS^{G12D}, 200 000 cells were seeded onto glass cover-slips and incubated for 3 days. To assess SA-βgal staining in normal OSE exposed to E2 over 15 days, cells were seeded onto glass coverslips and treated with E2 as described above. Cells were then fixed with 4% paraformaldehyde (w/v, in PBS) for 10 mins, then incubated with SA-βGal staining solution (Itahana et al. 2013) overnight at 37°C. Coverslips were mounted onto slides using ProLong Diamond antifade mountant (ThermoFisher Scientific) and allowed to cure for 24 hours. Slides were visualized using an EVOS 507 XL Core imaging system under phase contrast and bright field to best capture morphology and SA-βGal stain, respectively. Quantification of SA-βgal was performed using Fiji version 1.0. SA-βgal-positive (SA-βgal+) cell pixels were identified using automated colour deconvolution (Methyl Green DAB vector, colour 1) on bright field images. SA-βgal- pixels were quantified by subtracting bright field pixels from the corresponding phase contrast pixels using Image Calculator. Percent SA-βgal+ cells were

determined using ratio of SA- β gal⁺ cell pixels/total cell pixels.

2.2.10. Low attachment growth assay

250 000 *Dab2*-floxed OSE cells over-expressing HYG or KRAS^{G12D} were seeded into 24-well low attachment plates (Corning) containing OSE medium. Cells were incubated at 37°C for 3 days, then trypsinized and viable cells were counted using a Vi-CELL Cell Counter. The mouse ovarian cancer cell line, MASE, was used as a positive control for the assay.

2.2.11. Colony formation assay

Dab2-floxed OSE cells over-expressing HYG or KRAS were seeded into soft agar as previously described (McCloskey et al. 2014). Briefly, a base layer consisting of a 1:1 mix of OSE medium and agarose was used to coat the bottom of a 6-well dish. Once the base layer solidified, a top layer consisting of a 1:1:1 mix of 2.5×10^4 cells from single-cell suspension, OSE medium, and agarose was added on top. Cells suspended in OSE medium and agarose were incubated at 37°C for 7 days then visualized using the EVOS XL imaging system. Colonies were manually counted, in a blinded fashion, using the Image J cell counter tool.

2.3: Molecular Techniques

2.3.1. Immunohistochemical staining of tissue

All IHC staining of mouse tissues was performed using 3-5 mice per treatment group. Human ovarian cancer samples (4 low-grade serous, 16 high-grade serous, and 20

endometrioid) stained were part of a tissue microarray acquired from the Cooperative Human Tissue Network (University of Virginia, USA) or The Ottawa Hospital (Canada). IHC was performed using a previously described protocol (McCloskey et al. 2014). Briefly, paraffin sections were deparaffinized in xylene and rehydrated in a graded series of alcohols. Antigen retrieval was conducted in citrate acid based antigen unmasking solution (Vector Laboratories) then slides were incubated in 3% hydrogen peroxide to block endogenous peroxidase activity. The Avidin/Biotin blocking kit (Vector Laboratories) was used for blocking and tissues were incubated with primary antibody diluted in antibody diluent (Dako) overnight at 4°C in a humidified chamber. Sections were incubated with Envision secondary reagents (Dako) and developed with 0.2% diaminobenzidine and 0.001% hydrogen peroxide solution. Slides were counterstained with haematoxylin, dehydrated in a series of alcohols and xylenes, and then mounted onto coverslips with Permount (Fisher Scientific). Images were acquired using ScanScope CS2 (Leica Biosystems, Concord, Canada). See **Appendix D** for details on antibodies used for IHC and their staining parameters. IHC quantifications were performed using the Aperio software. DAB2 staining of FTE was quantified using the Aperio positive pixel count algorithm (version 10.1) where DAB2 positive pixels were normalized to total pixel count. FTE dysplastic regions were quantified using the inclusion and exclusion pen tools where stratified regions (μm^2) were normalized to total epithelium (μm^2).

2.3.2. Western blot analysis

Cells were lysed using M-PER mammalian protein extraction reagent

(ThermoFisher Scientific) containing 1x protease inhibitor cocktail (Sigma). Protein samples were separated using NuPAGENovex 4-12% Bis-Tris protein gels (ThermoFisher Scientific) and electro transferred onto a PVDF membrane (Emd Millipore). Blots were incubated in blocking buffer (Tris-buffered saline, 0.1% Tween 20, 5% non-fat powdered milk) for 1 hour at room temperature before probing with primary antibody (diluted in blocking buffer) overnight at 4°C. The next day, blots were probed with goat anti-rabbit IgG (H + L)-HRP conjugate secondary antibody (Bio-Rad) diluted 1:5000 in blocking buffer for 1 hour at room temperature, then visualized using ECL Substrate (Bio-Rad) in an chemiluminescence Alpha Imager system (Protein Simple). Amido Black stain (40% methanol, 10% acetic acid, 0.1% (w/v) Amido Black) was used as the western blot loading control. Image J software was used for densitometric analysis of protein bands and total lane density of Amido Black was used as loading normalizer. Amido Black loading control (**Appendix F**) and DAB2 antibody (**Appendix G**) have been optimized for quantitative western blot analysis using the conditions described above. See **Appendix H** for list of antibodies used for western blot analysis and their probing parameters.

2.3.3. Quantitative polymerase chain reaction (qPCR) analysis

For mRNA analysis, mRNA was isolated from cells using an Illustra RNA extraction kit (GE Healthcare Life Sciences) and reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad). The resulting cDNA was mixed with PrimeTime probes (Integrated DNA Technologies) and iTaq Universal Probes Super mix (Bio-Rad), and then run using a 7500Fast system (Applied Biosystems). For miRNA analysis, miRNA

was isolated from cells using a miRNeasy miRNA isolation kit (Qiagen). TaqMan MicroRNA assays for miR-378 and snoRNA202 (Applied Biosystems) in combination with the TaqMan MicroRNA Reverse Transcription Kit and TaqMan Fast Universal PCR Master Mix were used to generate cDNA and run qPCR using the 7500Fast system. See **Appendix I** for validation of *Tbp* and snoRNA202 as qPCR endogenous loading controls. See **Appendix J** for a summary of qPCR probe validation results and details on how calibration curves were acquired. See **Appendix K** for nucleotide sequences of probes and primers used for qPCR.

2.3.4. Immunofluorescence (IF) staining

Cells were seeded onto glass coverslips and treated with E2 for up to 15 days as described above. Cells were washed twice with PBS then fixed with 4% paraformaldehyde (w/v, in PBS) for 15 mins, permeabilized with 0.2% Triton X-100 (v/v, in PBS) for 10 mins, and then blocked with 5% normal goat serum (v/v, in PBS) (Sigma) for 1 hour at room temperature. Cells were probed with primary antibody diluted in blocking buffer overnight at 4°C. The next day, cells were probed with AlexaFluor488 goat anti-mouse/rabbit secondary antibody (ThermoFisher Scientific) diluted in blocking buffer for 1 hour at room temperature in the dark. Cells were mounted onto slides using ProLong Gold antifade mountant with DAPI and maintained in the dark at room temperature for 24 hours to allow mounting media to cure. See **Appendix L** for details on antibodies used for IF and their staining parameters. See **Appendix M** for IF “no-primary” controls. All IF experiments have at least two independent experimental replicates and “n” refers to fields of view.

2.3.5. Microscopy

Coloured bright field and phase contrast images of cells were acquired using an EVOS XL Core imaging system (ThermoFisher Scientific). The EVOS FL Cell Imaging System (ThermoFisher Scientific) was used to validate AdGFP infection in OSE cells. Wide-field fluorescence images were acquired using a Zeiss Axioskop 2 microscope using EC Plan Neofluar 10x/0.30NA Ph1, 20x/0.50NA Ph2, 40x/0.75NA Ph2 objectives. Confocal images were acquired using a Zeiss LSM510 META AxioVert200 system using a Plan-Neofluar 40x/1.3NA DIC EC oil objective or a Zeiss LSM800 AxioObserver Z1 system using Plan-Apochromat 40x/1.3NA oil objective.

2.3.6. Immunofluorescence image analysis

Wide-field fluorescence images were processed using AxioVision v4.8 software. Quantification of Ki67 staining was done in Image J using the cell counter plugin. Processing and analysis of confocal images were performed in the Zen 2 Blue software (Carl Zeiss) or using Imaris v8 (Bitplane). For IF images that were analysed in Imaris, images are shown as z-stack maximum intensity projections of DAPI (blue) or actin (orange) channels. Proteins of interest (green) are represented as solid surface objects created based on the dynamic range of all control and E2 treated images acquired within the biological replicate. The surface object intensity threshold was set based on the maximal intensity value of the dynamic range. Percent Ki67+ cells was determined using the ratio of Ki67+nuclei/total nuclei (DAPI) (n=6). Cell membrane co-staining was not ideal due to permeabilization required for IF antibodies so actin was used as an alternative means to estimate cell height (n=6). Cell height was determined by actin

surface rendering in Imaris. Due to mounting of the coverslip, cell height was generally limited to $< \sim 20 \mu\text{m}$.

2.3.7. Chromatin Immunoprecipitation (ChIP)

MASE cells were treated with 100 nM E2 for 45 mins, and then cross-linked with 1% formaldehyde for 30 mins at room temperature. Cross-linking was quenched by adding 125 mM glycine for 10 mins at room temperature. Cells were sonicated with Diagenode's Bioruptor for 30 sec on high and 60 sec off for 80 cycles at 4°C to produce 150-200 bp fragments. To bind antibody to Dynabeads magnetic beads, 5 μg of ESR1 antibody (Santa Cruz, sc-542) or normal mouse IgG (control for non-specific interactions) (Millipore) was incubated with 20 μl of magnetic beads overnight at 4°C the day prior to immunoprecipitation (IP). 250 μg of DNA was used per IP and DNA was pre-cleared using 1 $\mu\text{l/mL}$ salmon sperm (Sigma), 10 $\mu\text{l/mL}$ ovalbumin (Sigma), and 10 $\mu\text{l/mL}$ Dynabeads magnetic beads for 1 hour at 4°C. 10% of pre-cleared chromatin was saved as "Input" and IP of DNA with antibody-bound beads occurred overnight at 4°C. The next day, the beads bound by immune-complexes were collected using a Magna GrIP Rack (Millipore) and washed twice with low-salt, high-salt, and LiCl washes. The IP materials were eluted from the beads by incubating the samples with elution buffer at 65°C for 10 mins at 1400 rpm in an Eppendorf Thermomixer. Reverse cross linking and protease treatment was performed on each sample including input. The IP genomic DNA fragments were isolated via phenol:chloroform:isoamyl alcohol extraction, precipitated with ethanol, then pelleted. The air-dried DNA pellet was resuspended in nuclease-free water. HotStarTaq DNA Polymerase Kit (Qiagen) in combination with primers spanning

the putative *Dab2* ERE and *Greb1* ERE1 and ERE2 were used to amplify DNA.

Amplified PCR products were ran on a 4% agarose gel containing RedSafe Nucleic Acid Staining Solution (Intron Biotechnology) and bands were visualized with an EpiChem II Darkroom transilluminator (UVP Laboratory). See **Appendix N** for primers used for ChIP.

2.3.8. miRNA microarray

miRNA acquired from three biological replicates of MASE cells treated with E2 for 24 hours were prepared for an Affymetrix GeneChip miRNA 4.0 Array with the FlashTag Biotin HSR RNA labeling kit (Affymetrix). Affymetrix Transcriptome Analysis Console Software was used to analyze the resulting array data and microRNA.org (<http://www.microrna.org>) was used for target prediction analysis. KEGG pathway analysis on significantly up-regulated miRNAs was performed using DIANA-miRPath v3.0 software and the DIANA-microT-CDS algorithm (Vlachos et al. 2015).

2.4: Single-cell sequencing and analysis

2.4.1. Single-cell cDNA library preparation and sequencing

Cells were processed according to the Fluidigm C1 Single-Cell mRNA Seq HT IFC v2 protocol, using a medium (10-17 μ m) integrated fluidic circuit (IFC). Briefly, while the IFC was being primed in the Fluidigm C1, each sample was trypsinized, strained through a 20 μ m filter, and diluted to 300 cells/ μ L. Each sample was loaded into separate sample inlets on the IFC, along with a LIVE/DEAD viability stain (ThermoFisher Scientific, L-3224). After the “Cell Load and Stain” C1 program was run,

each capture site was visually inspected using an EVOS FL Cell Imaging System to score the number and viability of cells in each capture site (**Appendix O**). The samples were then run through the Lysis, Reverse Transcription, Preamplification, and Harvest programs on the C1, and the 20 cDNA libraries corresponding to each column of capture sites were removed from the IFC to further prepare each library for sequencing. Each library was quantified using the Qubit dsDNA HS assay and diluted to 1.5 ng/μL before 1.25 μL of each sample with 2.5 μL of Tagment DNA Buffer and 1.25 μL of Amplification Tagment Mix from the Nextera XT kit was incubated at 55°C for 10 mins. The reaction was neutralized with 1.5 μL of NT buffer and PCR amplified with Nextera indices and PCR parameters recommended in the Fluidigm protocol. Libraries were cleaned-up with AMPure XP beads and quantified again with the Qubit dsDNA HS assay. Tagmentation was assessed using the Advanced Analytical Fragment Analyzer and sample molarity was calculated using the average fragment size. Samples were pooled at equal molarity, spiked with 20% PhiX, and sequenced together in a single high-output 150 cycle NextSeq500 run with read 1 set to 26 bp and read 2 set to 75 bp.

2.4.2. scRNA-Seq data processing

Fastq files corresponding to the 20 cDNA libraries were downloaded directly from Illumina Basespace and were demultiplexed into 800 fastq files, each representing one of the 800 capture sites on the Fluidigm C1 IFC, using Fluidigm's standalone API script, which segregates reads based on the protocol's "row" barcode present in the 26bps of read 1. Transcripts were quantified from the resulting 75bp read 2 fastq files using Kallisto (Bray et al. 2016), and gene-level read counts were imported into R using the

tximport package (Soneson et al. 2016). The scater package (McCarthy et al. 2017) was used for quality control, normalization, and most exploratory visualization of the data.

2.4.3. scRNA-Seq data filtering

Capture sites that were not annotated to include only one live cell were first filtered from the data. The average number of reads-per-cell in the remaining data was approximately 120 000. Cells with less than 20 000 reads were removed, as they may represent individual capture sites with inefficient lysis or RT, and may affect downstream analysis. Additionally, cells with over 250 000 reads-per-cell were removed, as they may represent captures sites containing more than one cell that were not detected during the annotation process. After filtering, the distribution of sequencing depth was normally distributed. Because the number of detected genes in each cell can drive a large amount of variation, cells above or below three median absolute deviations of the median number genes detected were filtered to remove outliers and reduce this effect. Cells with a high proportion of mitochondrial-associated reads (higher than three median absolute deviations of the median) were also removed as they often represent poor-quality cells. Lastly, genes were only retained in downstream analysis if they had at least one count in a minimum of 10 cells. The final expression matrix included the expression of 14 299 genes across 636 cells.

2.4.4. Normalization and imputation of expression values

Cells of the filtered expression matrix were clustered based on their log2-transformed counts using the quickCluster function of the R package scan (Lun,

McCarthy, et al. 2016), with the minimum cluster size set to 30 cells. Scale factors for each cell were then calculated by deconvoluting pool-based size factors as described previously (Lun, Bach, et al. 2016), using the implementation in the `computeSumFactors` function of the `scrn` package. Each cell's expression profile was then scaled by the resultant cell-specific size factor. To restore structure in the data that may be lost as the result of drop-out, characteristic of scRNA-Seq, we performed data imputation on log-transformed normalized counts using the MAGIC algorithm (van Dijk et al. 2017) with the following parameters: `n_pca_components=20`, `t=4`, `ka=3`, `k=9`, `epsilon=1`. These values were used for downstream clustering and pseudotime analysis.

2.4.5. Clustering and differential expression between clusters

Log-transformed, imputed, and normalized counts (hereon referred to simply as expression values) were used to define clusters of related cells. k-means-based consensus clustering was performed using SC3 (Kiselev et al. 2017) to segregate the cells into 2 or 3 clusters. The optimal number of clusters was determined by finding the value of k that maximized average silhouette width. k=3 was optimal, with an average silhouette width of 0.96, and provided biologically relevant clustering. We also noted that k=2 segregated the two large groupings of cells along the first principal component, so we also explored this clustering pattern in downstream analysis. Comparisons were made between clusters 2 and 3 in the k=3 groupings, and between clusters 1 and 2 in the k=2 groupings. Differential expression analysis was performed by fitting a generalized linear model to the expression values of each gene and filtering for genes with a q-value < 0.05 and a log2 fold change > 0.5.

2.4.6. Functional enrichment analysis of scRNA-Seq data

The DAVID Gene Functional Classification Tool (Huang, Sherman & Lempicki 2009; Huang, Sherman & Lempicki 2009b) was used to explore KEGG pathways and biological process GO terms significantly associated with differentially expressed genes between clusters in PCA and clusters of genes with similar expression dynamics throughout pseudotime (EASE p-value<0.05). In both PCA and trajectory analysis, the top 5 terms ranked by EASE p-value were determined by equally subjecting each cluster to filtering parameters. Terms were removed if there was intra-cluster term conflict, (i.e.: “positive regulation of apoptotic process” and “negative regulation of apoptotic process” appearing in the same cluster), if term is not unique to one cluster (to remove inter-cluster term conflict), if the biological process had 10 or more child terms (to eliminate broad and uninterpretable processes such as “translation” or “protein phosphorylation”), or if they could be reasonably agreed upon as not applicable to the model system (i.e.: liver regeneration, Alzheimer's disease, etc.). KEGG pathways were given priority because this category provides the most information for biological interpretation of data.

2.4.7. Identification of marker genes of each cluster

Marker genes for each cluster were identified using SC3 (Kiselev et al. 2017), which constructs a binary classifier for each gene based on the mean expression of the gene in each cluster. The area under the receiver operator characteristic curve (AUC) was used to determine the accuracy of the gene as a marker for the cluster. The AUC values were ranked, and top-ranking genes for each cluster were chosen for validation by IF based on commercial availability of antibody and quality of stain (assessed by

preliminary antibody optimization experiments).

2.4.8. Pseudotime analysis of ovarian epithelial cell populations

A pseudotime trajectory of all cells was constructed using the reversed graph embedding DDRTree method implemented in monocle (Qiu, Mao, et al. 2017). The root branch was defined as the branch comprising cells from each condition. A generalized linear model was fit to the expression values with an interaction term to model branch-specific expression dynamics as a function of pseudotime. Because the imputation and normalization eliminate a large amount of variation, many genes have a q-value of less than 0.05. Instead of filtering on q-value, the interaction coefficients for each gene were collected and ranked.

2.5: Data availability

2.5.1 miRNA microarray

Data and details for miRNA microarray of MASE cells treated with E2 for 24 hours (n=3) can be found on Geoprofiles:

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE98391>.

2.5.2. Single-cell sequencing data and code

All data, code, and analysis notebooks are available at:

<https://github.com/dpcook/scRNASeq-Estrogen>

Chapter 3: Results

3.1. *In vitro* model in which E2 induced OSE dysplasia can be studied

3.1.1. OSE cells *in vitro* can establish a polarized epithelial monolayer

OSE cells are a morphologically transient cell type that can switch between mesenchymal and epithelial morphology in response to environmental factors (Auersperg 2001). To determine if OSE cells isolated off the ovary can maintain their phenotypic plasticity when seeded as adherent monolayers in culture, we first observed their ability to properly form cell-cell contact upon reaching confluence (**Figure 4-5**). When OSE cells are sub-confluent, individual OSE cells assume a mesenchymal phenotype. With increasing confluence, neighbouring OSE cells up-regulated adherens junction proteins CDH1 and CTNNB1, and tight junction protein ZO1 that all localize to the lateral membrane. The establishment of these cell-cell contacts cause OSE to aggregate into a monolayer of cobble-stone shaped cells and assume a more epithelial phenotype. The proportion of cells expressing these junctional proteins increases proportionally to confluency. Closer examination of these epithelial monolayers by confocal microscopy demonstrate that CDH1, CTNNB1, and ZO1 were properly localized as they were often observed to be concentrated at lateral membranes adjacent to other cells; however, these junctional proteins were notably absent from areas of membrane that lacked contact with neighbouring cells.

3.1.2. OSE cells *in vitro* are capable of undergoing EMT

Given that the mechanism of E2 induced OSE dysplasia is unknown and EMT is

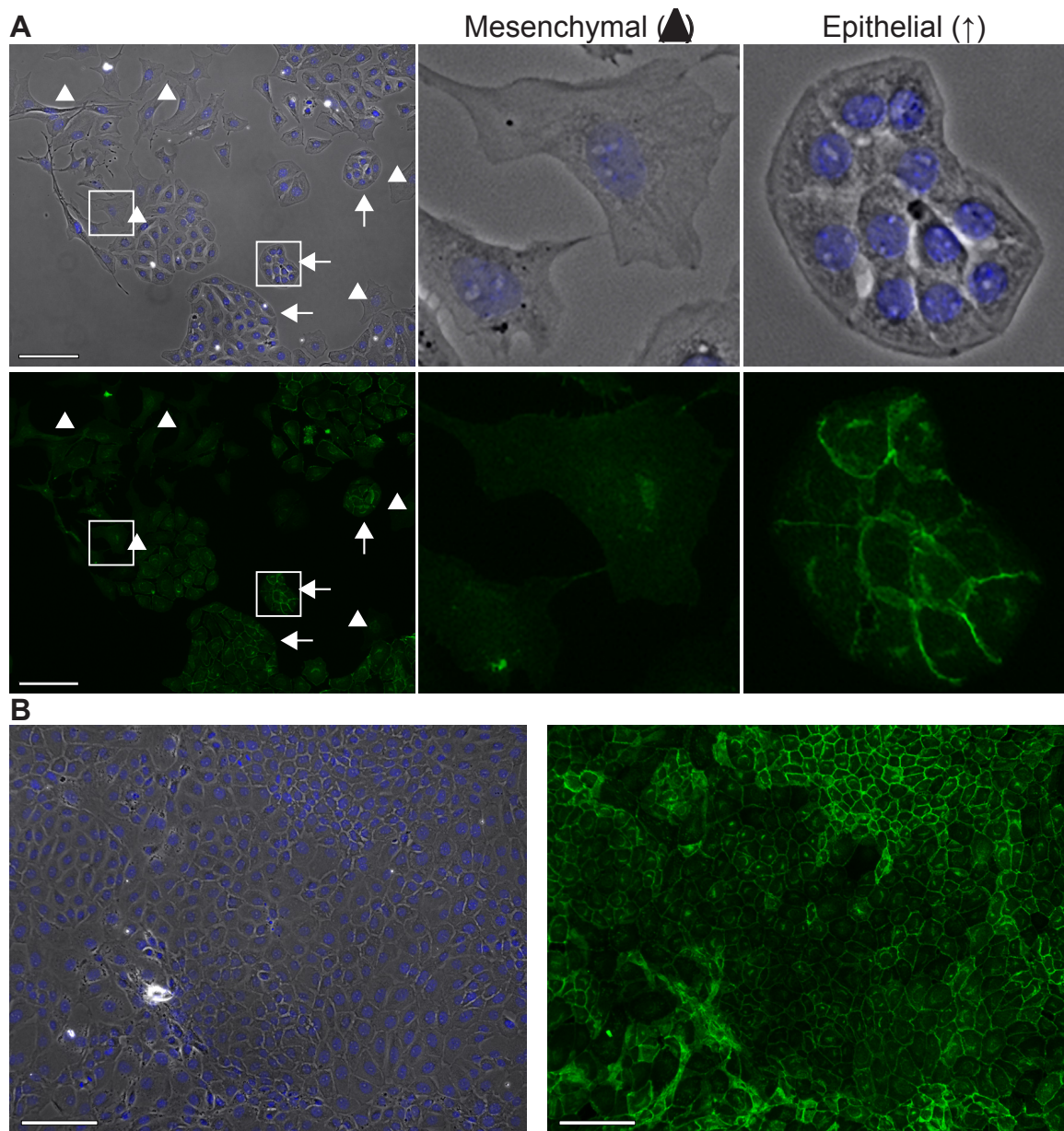


Figure 4: OSE cells in culture form adherens junctions upon cell-cell contact. OSE cells were seeded at same time then stained for CDH1 by IF while OSE were sub-confluent (**A**) or when OSE become confluent (**B**). (**A-B**) n=9. Phase contrast and DAPI (blue) images are merged. CDH1 staining (green) for the same field of view is also shown. Scale bar=100 μ m. Images were acquired using a Zeiss Axioskop 2 microscope. In (**A**), arrowheads indicate mesenchymal cells and arrows indicate epithelial cells. Boxed areas are shown at higher power in the panels to the right to show representative mesenchymal and epithelial cell.

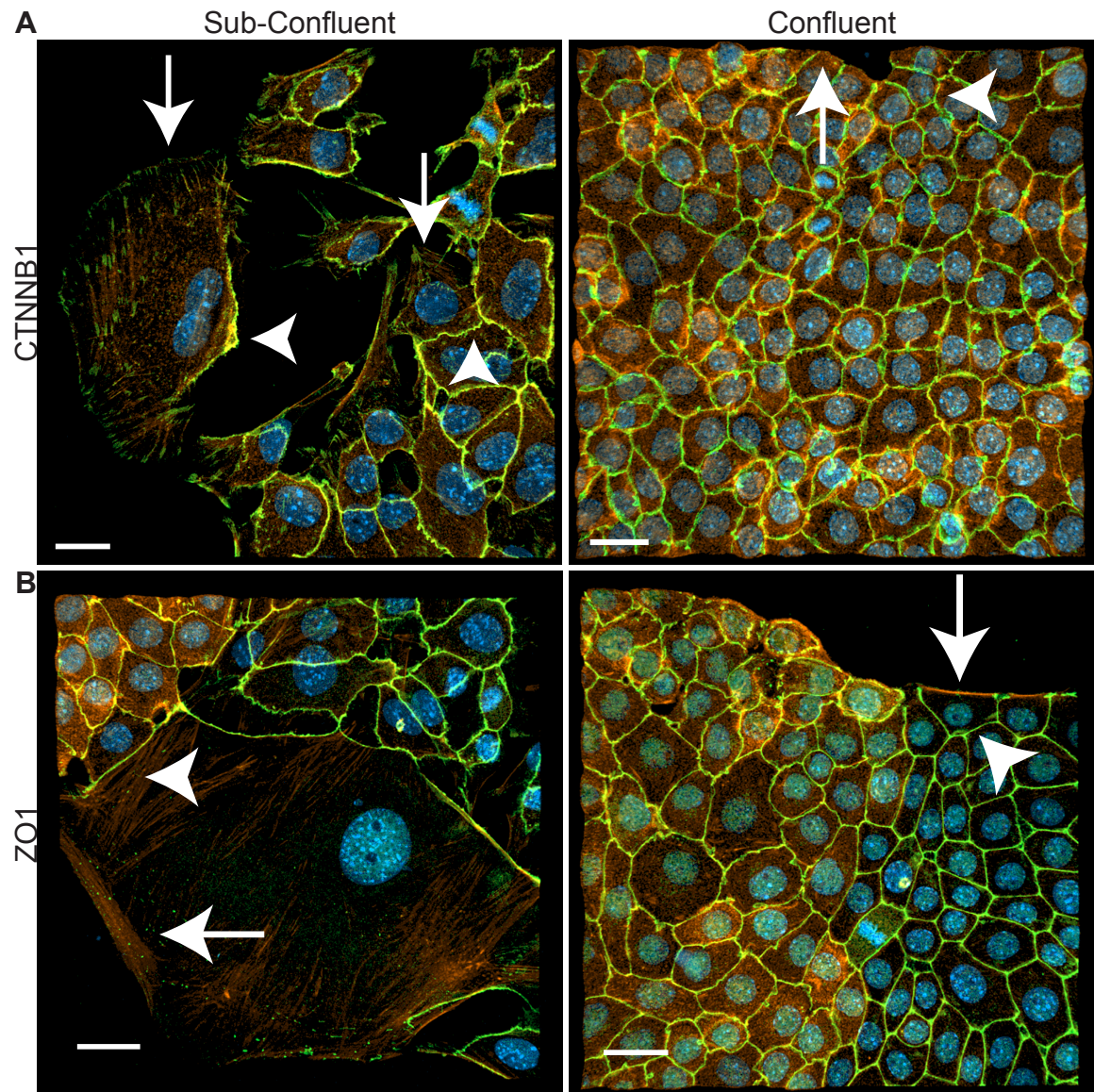


Figure 5: OSE cells in culture demonstrate proper localization of polarity proteins. OSE cells stained for CTNNB1 (n=6) (A) and ZO1 (n=6) (B) by IF. Arrows point to cell membranes that are not adjacent to other cells and are low in CTNNB1 or ZO1. Arrowheads point to cell membranes that are adjacent to a neighbouring cell and have high CTNNB1 or ZO1 expression. Green = CTNNB1 or ZO1; blue = DAPI; orange = Actin; yellow = overlapping pixels from the CTNNB1/ZO1 and Actin channels. Images were acquired using a Zeiss LSM800 AxioObserver Z1 system and analyzed using Zen 2 Blue. Scale bars=20 μ m.

an important step for oncogenesis in ovarian cancer, it was important to confirm that our *in vitro* model system can also reflect the OSE cell's ability to undergo EMT so to not rule out the possibility that OSE dysplasia is a result of E2 driven EMT. TGF β is a cytokine that is produced by the ovary and is the prototypic inducer of EMT in many biological systems including OSE cells (Zhu et al. 2010). OSE cells in culture treated with TGF β underwent morphological changes characteristic of EMT where epithelial cells became flattened and spindle shaped (**Figure 6**). These results in combination with above results (**Figure 4-5**) confirmed that OSE cells seeded in standard adherent monolayer conditions are capable of displaying the full range of OSE plasticity observed *in vivo* and is therefore an acceptable model system to investigate the molecular mechanisms normally involved in maintaining the OSE monolayer, and how these mechanisms become dysregulated as OSE cells transition into pre-neoplastic lesions as a result of prolonged E2 exposure.

3.1.3. E2 induced OSE dysplasia is reproducible *in vitro*

Mice that received exogenous E2 via subcutaneous insertion of an E2 pellet had increased areas of hyperplastic and columnar OSE relative to mice with placebo pellets (**Figure 7**), as this lab has reported previously (Laviolette et al. 2010). This dysplastic phenotype was reproduced in culture by maintaining OSE cells in culture medium containing 100 nM E2 for 15 days (**Figure 8**). Quantification of proliferation and Ki67 staining (a marker of proliferation) verified that OSE cells displayed both increased

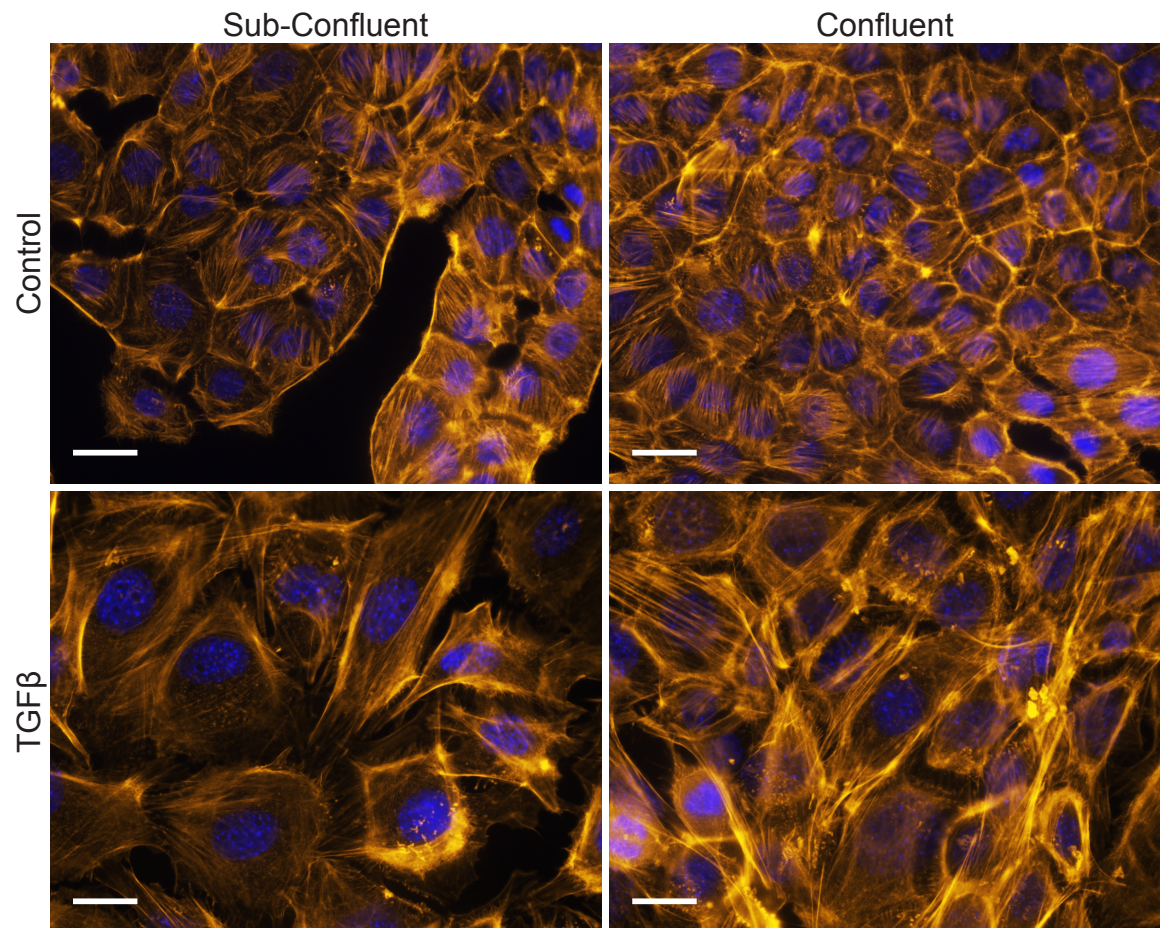


Figure 6: OSE cells in culture are responsive to TGFβ induced EMT. OSE cells treated with 10 ng/mL TGFβ for 4 days and then stained for Actin by IF. n=6/treatment. Orange = Actin; Blue = DAPI. Scale bars=20 μm. Images acquired using a Zeiss Axioskop 2 microscope.

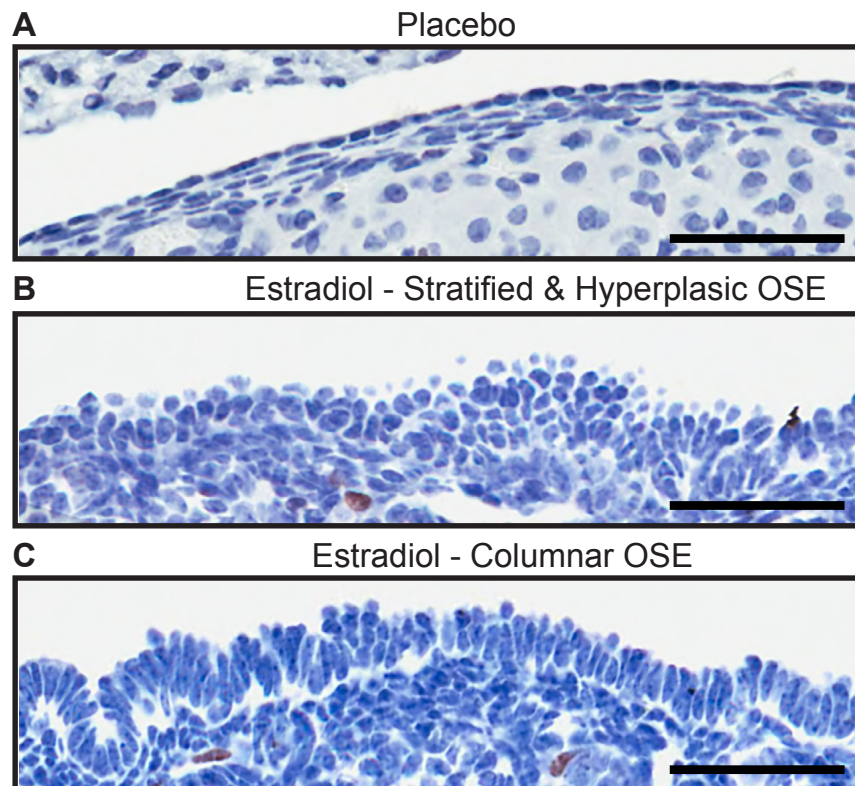


Figure 7: Prolonged E2 stimulation alters OSE cell morphology. Representative images of OSE layer from FVB/n mice 60 days after implantation of placebo (A) and E2 (B-C) pellet. n=5/treatment group. (B) Area of stratified and hyperplasic OSE. (C) Area of columnnar OSE. Scale bars=50 μ m.

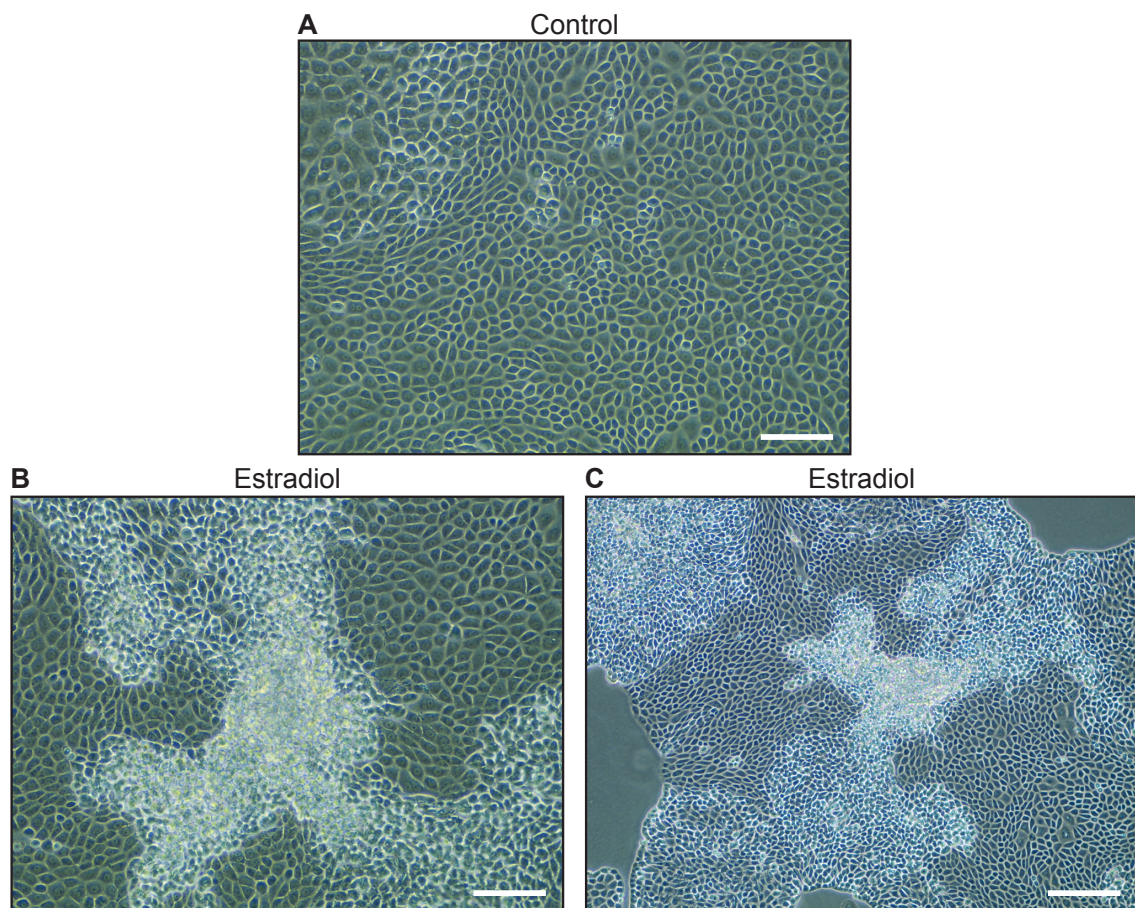


Figure 8: OSE cells become stratified with prolonged E2 exposure. Phase contrast images of OSE cells on day 15 of E2 exposure acquired using EVOS XL Core imaging system. **(A)** Confluent area in control plate; scale bar=100 μm . **(B)** Confluent area of E2 treated cells; scale bar=100 μm . **(C)** Sub-confluent area of E2 treated cells; scale bar=200 μm .

number and proliferation of cells in response to E2 stimulation (**Figure 9**). Phase contrast images showed that E2 treated OSE cells formed foci of stratified cells on top of an underlying OSE cell monolayer after prolonged E2 exposure, whereas control OSE cells remained as an organized monolayer (**Figure 8A-B**). Foci of stratified cells were observable even in areas of sub-confluence in E2 treated dishes (**Figure 8C**), demonstrating that E2 treated cells were not becoming stratified due to over-confluence, but more likely because the mechanisms conferring proper positional cues for formation of an organized monolayer were being deregulated with prolonged E2 stimulation. Small areas of stratified cells begin to be observable 10 days after E2 exposure, and beyond 15 days the proportion of stratified cells increased but no additional phenotypic changes were observed.

3.2. E2 suppression of *Dab2*

3.2.1. OSE dysplasia is associated with a reduction in *Dab2* expression and function

OSE cells exposed to continuous E2 showed a decrease in *Dab2* expression in both animal and cell culture studies. IHC of ovaries from wild-type mice with E2 pellets showed loss of DAB2 associated with areas of OSE dysplasia (**Figure 10A**). OSE cells exposed to E2 for 15 days in culture also showed a reduction of *Dab2* at the mRNA and protein levels (**Figure 10B-C**). IF staining revealed that DAB2 is globally reduced in all E2 treated cells but OSE cells aggregated in foci of stratified cells have the greatest decrease in DAB2 compared to the surrounding monolayered cells, often having no detectable expression (**Figure 10D**).

Previous work using mouse endoderm cells has demonstrated that DAB2 can

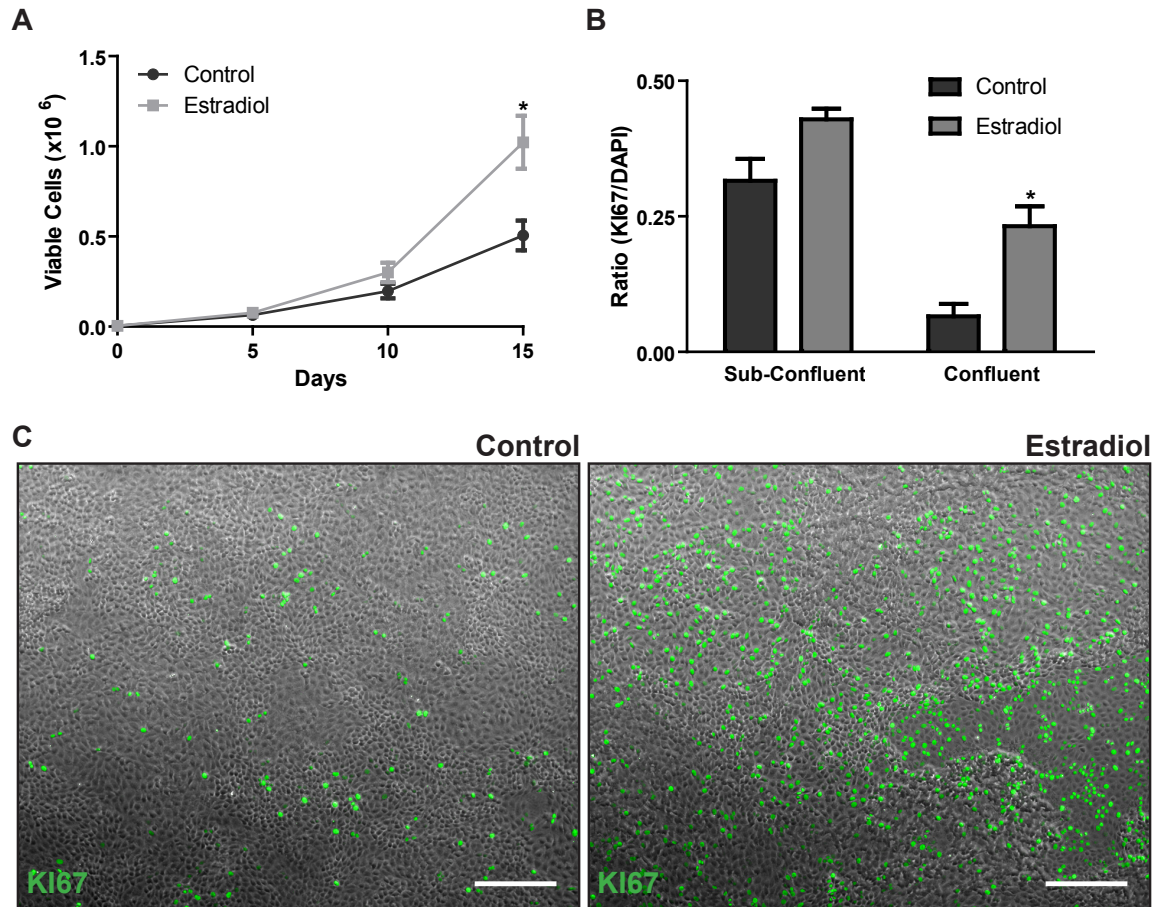


Figure 9: Prolonged E2 exposure causes an increase in hyperplastic OSE. Primary cultures of OSE cells exposed to E2 for 15 days. **(A)** Growth curve counting viable cells. **(B)** Proportion of Ki67 positive cells relative to DAPI in sub-confluent and confluent areas of the culture plate. 3-6 fields of view/group. **(A-B)** $n=3$; $*=p<0.05$; paired two-way ANOVA with Bonferroni post-test. **(C)** Merged image of phase contrast and IF staining of Ki67 (green) in area of confluence acquired using Zeiss Axioskop 2 microscope; scale bars=200 μm .

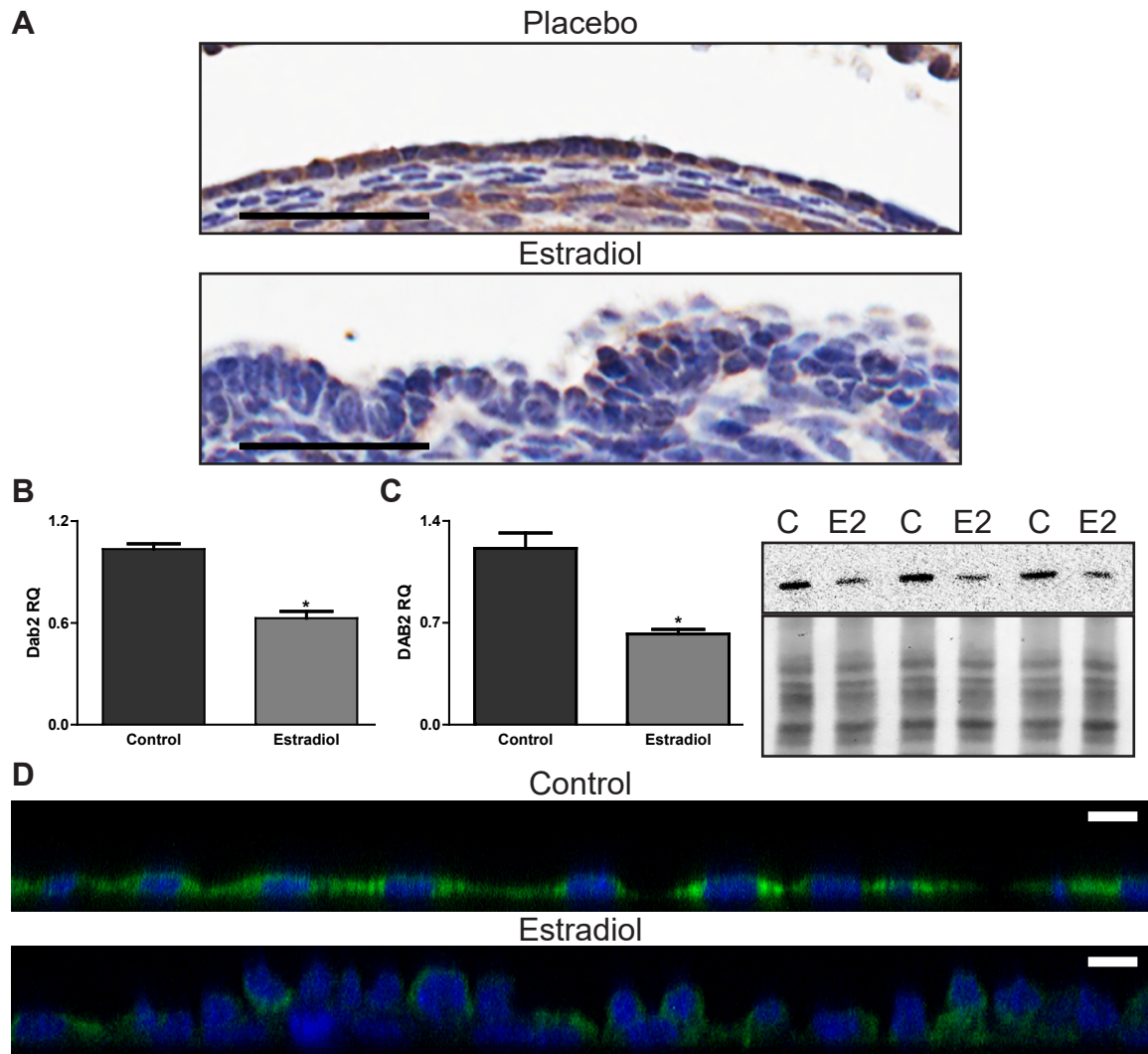


Figure 10: Prolonged E2 exposure suppresses *Dab2* in OSE. (A) Mouse ovaries stained for DAB2 60 days after insertion of placebo or E2 pellet. All IHC sections were stained for DAB2 in parallel on the same day with identical conditions (n=3/treatment); scale bars=50 μ m. See **Appendix E** for DAB2 IHC staining controls. (B-D) Expression of *Dab2* in OSE cells after 15 days of E2 treatment in culture. (B) *Dab2* mRNA levels measured by qPCR. RQ=Relative Quantity. (C) DAB2 protein levels measured by western blot analysis. Image of blot shows results from 3 biological replicates. n=3; *=p<0.05, paired t-test. (D) Z-stack of cultured OSE cells stained for DAB2 by IF. Images were acquired using a Zeiss LSM510 META AxioVert200 system and analyzed using Zen 2 Blue software. Blue=DAPI; Green=DAB2; scale bars=10 μ m; n=6-8.

mediate the proper localization of CDH1 (Yang et al. 2007). When mouse ovaries and OSE cells were stained for CDH1, it was clear that prolonged E2 exposure consistently resulted in two distinct OSE phenotypes: (1) areas of stratified cells with loss or mislocalization of CDH1; and (2) areas of columnar OSE with increased CDH1 expression at the lateral membranes (**Figure 11**).

3.2.2. *Dab2* suppression by E2 is via the ESR1 pathway

To determine if *Dab2* suppression is directly due to E2 repression or is simply a result of dysplasia, OSE cultures were treated with E2 and suppression of DAB2 protein was observed by 48 hours (**Figure 12**). At this time, no cell dysplasia nor changes in morphology were evident. Therefore, *Dab2* suppression occurs before the E2 induced dysplasia in OSE cells.

Esr2 transcripts were not detectable by qPCR analysis in cultured OSE cells, suggesting that *Dab2* suppression is primarily via the ESR1 pathway. To test this hypothesis, we infected primary cultures of *Esr1*-floxed OSE cells with AdCre to knockout *Esr1* (**Figure 13**). *Esr1*-WT OSE readily down-regulated DAB2 following 48 hours of E2 exposure but *Esr1*-KO OSE showed no change between control or E2 treatment (**Figure 12B**). To determine if *Dab2* suppression via the ESR1 pathway might be a more generalized mechanism, we used a different assay and cell type. MASE (mouse ovarian cancer) cells were pre-incubated with an ESR1 specific antagonist, MPP, for 1 hour before treatment with E2 for 48 hours. Like knockout of *Esr1* in OSE cells, inhibition of ESR1 in MASE cells with MPP prevented DAB2 suppression by E2 (**Figure 12C**). Interestingly, both knockout of *Esr1* in OSE cells and inhibition of ESR1

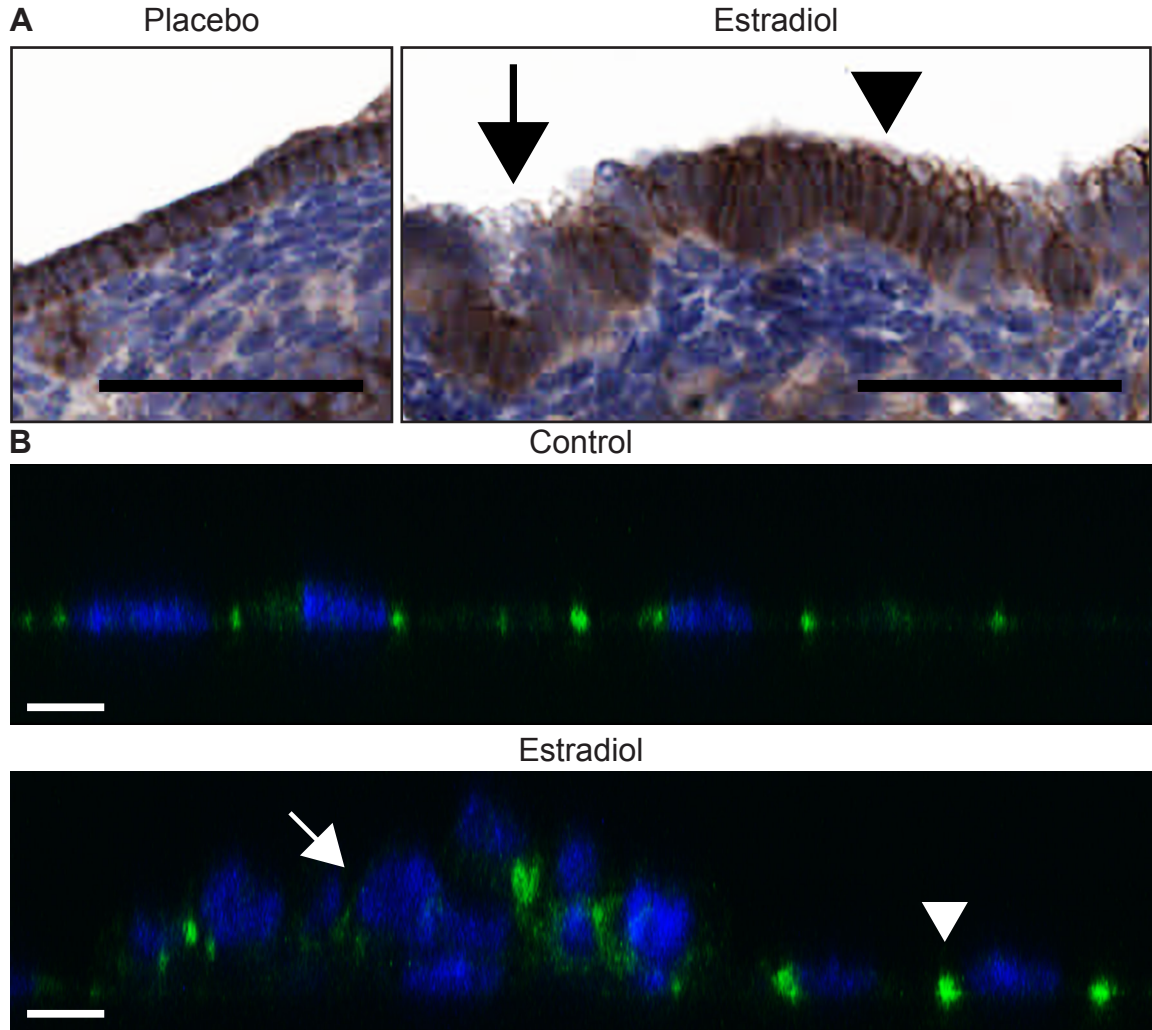


Figure 11: E2 induced stratified cells had loss or mislocalization of CDH1. (A) Mouse ovaries 60 days after insertion of placebo or E2 pellet and stained for CDH1; n=3/treatment; scale bar=50 μ m. See **Appendix E** for CDH1 IHC staining controls. **(B)** Z-stack of cultured OSE cells stained for CDH1 by IF after 15 days of E2 treatment, n=6/treatment. Images were acquired using a Zeiss LSM510 META AxioVert200 system and analyzed using Zen 2 Blue software. Blue=DAPI; Green=CDH1; scale bars=5 μ m. Arrows indicate loss of expression or mislocalization of CDH1 in areas of stratified cells. Arrowheads indicate columnar OSE cells with increased CDH1 at lateral membrane.

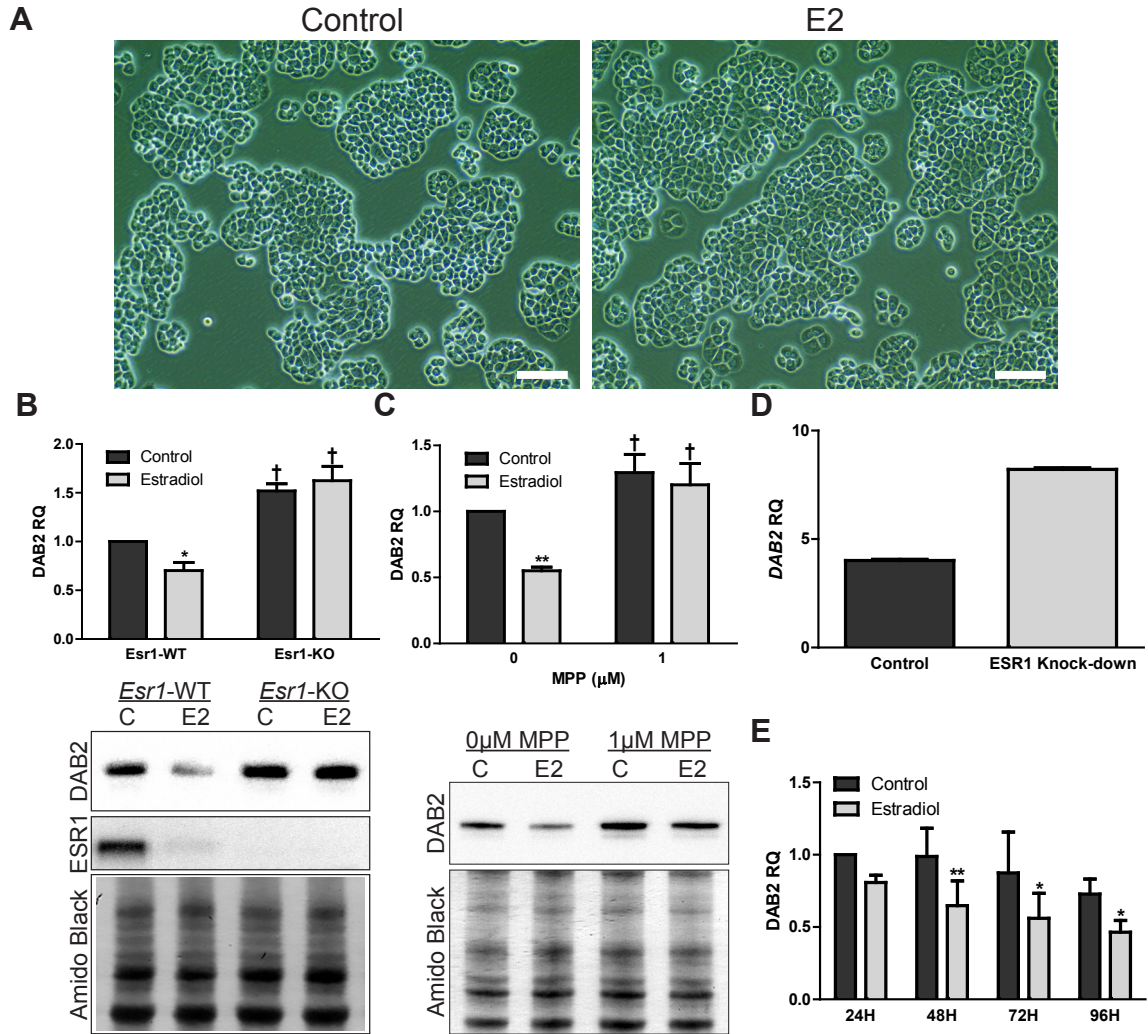


Figure 12: DAB2 suppression occurs before morphological changes & is mediated via the ESR1 pathway. (A) Phase contrast images comparing control vs. E2 treated OSE cells at 48 hours; images were acquired using an EVOS XL Core microscope; scale bars=100 μm. (B) *Esr1*-floxed OSE infected with AdGFP (*Esr1*-WT) or AdCre (*Esr1*-KO) to knockout *Esr1* were then treated with E2 for 48 hours. Western blot analysis, n=4; each replicate is from independent adenovirus infections. * =p-value<0.05, E2 significantly different relative to control; † =p-value<0.05, *Esr1*-KO significantly different relative to *Esr1*-WT. (C) MASE treated with MPP and 10 nM E2 for 48 hours. Western blot analysis, n=3. **=p-value<0.01, E2 significantly different relative to control; † = 1 μM MPP significantly different relative to 0 μM MPP. (D) *DAB2* expression from microarray comparing MCF7 breast cancer cells (control) vs. *ESR1* silenced MCF7 cells. Data was acquired from Geoprofiles (Saleh et al. 2011). (E) Primary culture of human ovarian cancer ascites cells with or without E2 for up to 96 hours. *DAB2* expression is significantly reduced due to E2 treatment but is not changed over time. Western blot analysis, n=3; **=p-value <0.01, *=p-value <0.05 Control vs. E2. (B-E) RQ=Relative Quantity. (B, C, E) Two-way ANOVA with Bonferroni post-test.

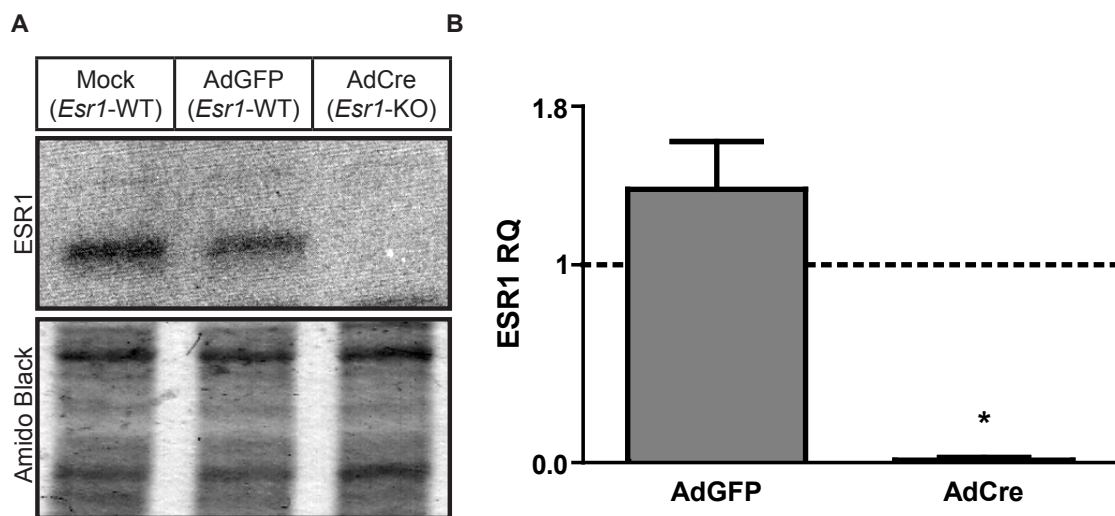


Figure 13: Validation of *Esr1* knockout by AdCre infection in *Esr1*-floxed OSE. Successful knockout of *Esr1* using primary cultures of OSE isolated from *Esr1* conditional knockout mice (*Esr1*-floxed OSE) (Hewitt et al. 2010). **(A-B)** *Esr1*-floxed OSE were probed for ESR1 by western blot 1 week following adenovirus infection. **(A)** Representative image of validation western blot. **(B)** Quantification of ESR1 expression by densitometry analysis of western blots. n=4; *=p-value<0.05; two-tailed paired t-test. Dotted line denotes Mock infected cells that were normalized to 1.

by MPP in MASE resulted in a significant increase in DAB2 expression. This phenomenon was also independently observed in ESR1 positive human breast cancer cells where microarray data comparing control and *ESR1* siRNA transfected MCF7 cells showed that knockdown of *ESR1* increased *DAB2* expression (**Figure 12D**). Lastly, to further investigate if E2 regulation of *DAB2* is translatable to humans, ovarian cancer ascites cells were isolated and expanded in culture. Consistent with the results in mouse cells, DAB2 protein levels were maximally suppressed 48 hours after E2 exposure (**Figure 12E**).

3.2.3. Investigation of *Dab2*'s putative estrogen response element

Since *Dab2* regulation by the ESR1 pathway was shown to be conserved between OSE and MASE cells, MASE cells were used to further investigate the mechanism by which E2 down-regulates *Dab2* in the following two sections due to technical limitations of primary OSE cultures in terms of cell number yield and tolerance to transfection. In a time-course experiment, E2 suppressed the abundance of *Dab2* transcripts to less than 50% within 3 hours of E2 exposure (**Figure 14A**). Given the rapid suppression of *Dab2* mRNA, this suggested that the down-regulation of *Dab2* could be mediated by the direct binding of ESR1 to a putative ERE identified in proximity to the transcription start site (TSS) of *Dab2* that is conserved in both mouse and humans (Bourdeau et al. 2004). Using ChIP, we found that ESR1 does not bind to this putative ERE upon E2 treatment (**Figure 14B**). To confirm that these results were not due to technical error, pull-down of RNA POL II with the mouse *Gapdh* promoter (a constitutively expressed gene)

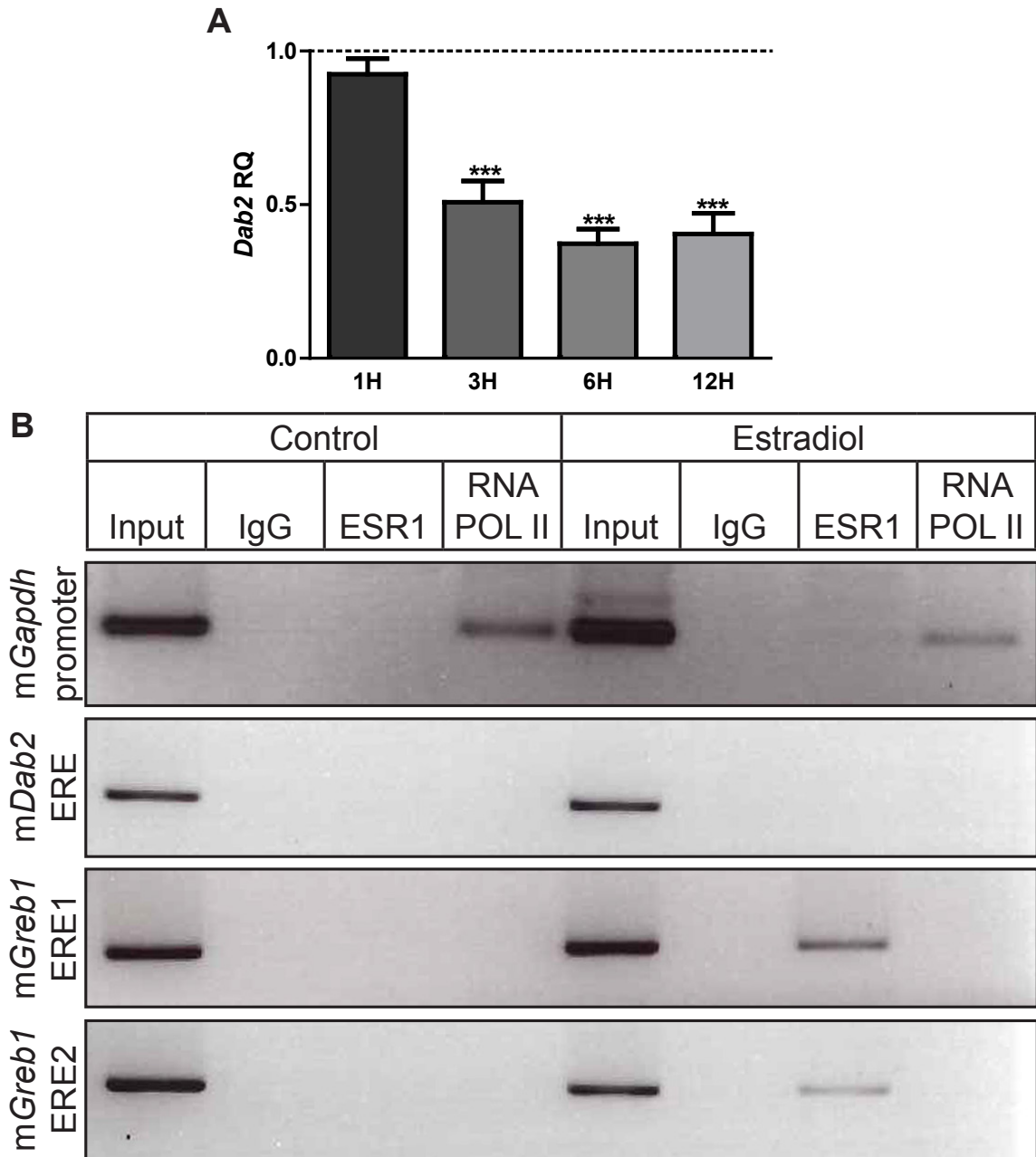


Figure 14: The putative ERE proximal to the TSS of *Dab2* does not bind ESR1. (A) E2 treatment time-course with MASE cells for 1-12 hours. qPCR analysis, n=3; ***= $p < 0.001$; one-way ANOVA. Control (untreated) is normalized to 1 and is represented by dashed line. (B) ChIP of control and E2 treated MASE cells. Cross-linked cell lysates were pulled-down with IgG, ESR1, and RNA POL II antibodies. DNA eluted from input and pull-down samples were subsequently probed by primers spanning the mouse *Gapdh* promoter, *Dab2* ERE, and two *Greb1* EREs.

confirmed that the ChIP assay was successful for both treatment groups, and primers spanning two mouse *Greb1* EREs (a known ESR1 target in MASE cells) were used as a positive control for the ESR1 antibody. IP with normal mouse IgG confirmed that the primers used do not enrich non-specific interactions. These results lead us to the conclusion that the putative *Dab2* ERE is not a functional one in ovarian epithelial cells.

3.2.4. E2 suppresses *Dab2* by up-regulation of miR-378

In light of the ChIP results, we investigated the possibility that *Dab2* is suppressed by miRNAs that are up-regulated by E2. Both mouse and human studies have demonstrated that E2 can regulate miRNA expression (Ferraro et al. 2012; Klinge 2009; Klinge 2015), and that miRNAs can target the *Dab2* transcript (Du et al. 2014; Chao et al. 2012). miRNA microarray analysis comparing control vs. E2 treated MASE cells revealed that E2 regulates the expression of 110 miRNAs (**Appendix P**). KEGG pathway analysis of these E2 responsive miRNAs reveal that they are predominantly involved in regulating fatty acid metabolism, Hippo signalling, and adherens junctions (**Table 1**).

Of the miRNAs that had the highest increase in response to E2 (> 2-fold change), miR-378 was the only miRNA that had a seeding sequence capable of targeting the 3-prime untranslated region (3'UTR) of both human and mouse *Dab2* transcripts (**Figure 15A**). Using qPCR analysis to validate the miRNA microarray results, we confirmed that miR-378 is up-regulated by E2 (**Figure 15B**) and that this up-regulation is via the ESR1 pathway since E2 induction of miR-378 is not observed when MASE cells were pre-treated with MPP, an ESR1 antagonist (**Figure 15C**). An E2 time-course experiment shows that E2 induction of miR-378 occurs within the same time frame as *Dab2*

Table 1: Top 5 KEGG pathways associated with E2 responsive miRNAs in MASE.

KEGG Pathway	p-value	# genes	# miRNAs
Fatty acid biosynthesis	5.75E-10	7	14
Fatty acid metabolism	8.22E-10	24	36
Hippo signaling pathway	1.31E-09	90	58
Biosynthesis of unsaturated fatty acids	1.77E-08	15	28
Adherens junction	2.13E-07	49	47

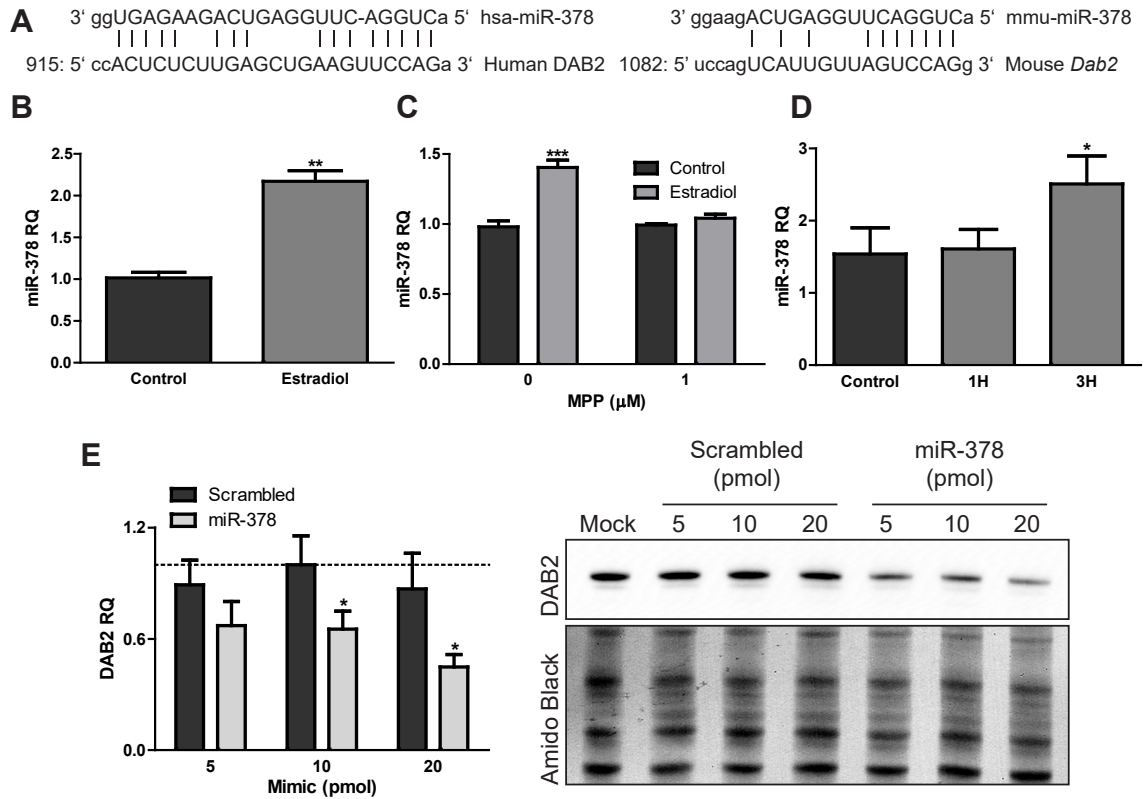


Figure 15: E2 suppresses *Dab2* via up-regulation of miR-378. (A) Alignment of miR-378 relative to *Dab2* transcript in mouse and human. (B) MASE cells were treated with E2 for 24 hours and differential expression of miR-378 was measured by qPCR analysis to validate miRNA microarray results. n=3; ** =p-value<0.01; unpaired t-test. (C) MASE cells were treated with 10 nM E2 with/without MPP for 24 hours. qPCR analysis, n=3; ***= p<0.001; two-way ANOVA with Bonferroni post-test. (D) MASE cells were treated with E2 for 1-3 hours. n=3; * = p<0.05; one-way ANOVA. (E) MASE cells were transfected with water (Mock) or 5, 10, or 20 pmols of miR-378 mimic or mirVana miRNA Mimic Negative Control #1 (scrambled control). Western blot analysis, n=3. * = p-value<0.05 miR-378 significantly different relative to scrambled; two-way ANOVA. Mock is normalized to 1 and is represented by dashed line. Representative blot is shown. (A-E) RQ=Relative Quantity.

transcript suppression (**Figure 15D**) and using miRNA mimic assays, miR-378 was confirmed to be capable of suppressing DAB2 expression (**Figure 15E**).

3.2.5. *Dab2* suppression by E2 is observed in mouse FTE

The FTE is an alternative site of origin for epithelial ovarian cancer and with DAB2 loss observed in 85% of all ovarian cancers (Fazili et al. 1999; Zhang et al. 2014), this suggests that prolonged E2 exposure and *Dab2* suppression may be relevant factors contributing to sensitizing the FTE to transformation as well. The FTE is a pseudostratified epithelium where the undulating nature of the tissue gives the epithelial cells a superficial appearance of being stratified, but in actuality, every cell is in contact with the basement membrane (Stewart & Behringer 2012). Consistent with this, oviducts isolated from placebo mice showed a pseudostratified arrangement of FTE cells that line the underlying stroma as a single layer of epithelial cells (**Figure 16-17**). DAB2 showed a more intense apical staining in cells lining the fimbria and became more evenly distributed in the cytoplasm in FTE cells lining the ampulla and isthmus (**Figure 16A-B**). When mice were exposed to E2 for 60 days, there were no changes in proliferation observed by Ki67 staining compared to placebo mice (**Figure 16C**), but all segments of the FTE showed a significant decrease in DAB2 (**Figure 16D**) and increased regions of dysplasia (**Figure 16E**) where we observed a significantly higher proportion of stratified FTE cells in the infundibulum and thickening of the FTE layer in the ampulla and isthmus. Due to the folding nature of the oviduct, it is possible for areas of stratified FTE cells to be observed as an artifact of histological sectioning but blinded analysis of our tissue sections using the Aperio software confirms that E2 treated mice display a

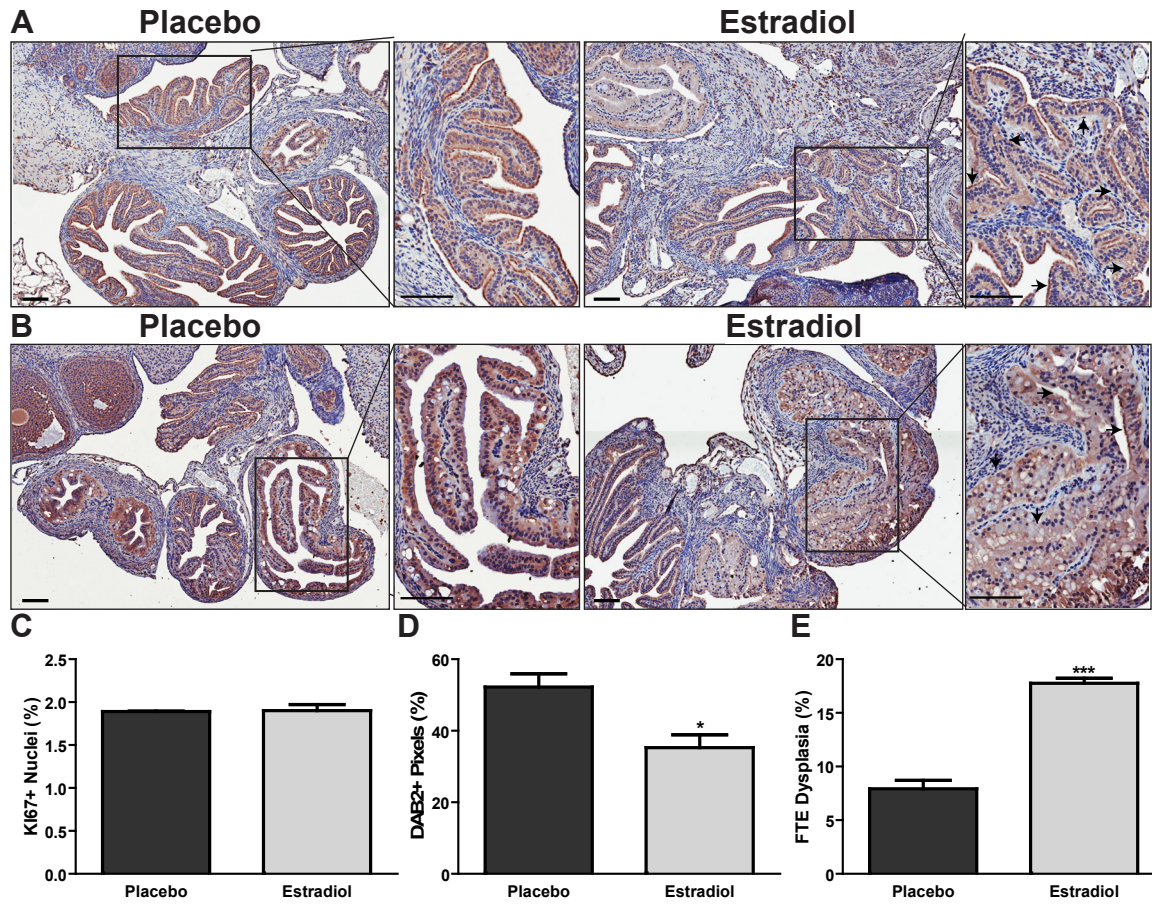


Figure 16: Prolonged E2 exposure suppressed DAB2 & increased dysplasia in mouse FTE. (A-B) Mouse oviducts stained for DAB2 60 days after insertion of placebo or E2 pellet; scale bars=100 μ m. See **Appendix E** for DAB2 IHC staining controls. Comparison of DAB2 staining in fimbria (A) and ampulla (B) between placebo and E2 treated mice. Arrows point to areas of dysplasia. (C) Quantification of DAB2 expression in all segments of mouse FTE by pixel count. %=DAB2+ pixels/total pixels. n=3; *=p-value <0.05, unpaired t-test. (D) Quantification of area of dysplastic FTE/total FTE area in all segments of mouse FTE. n=3; ***=p-value <0.001, unpaired t-test. (E) Quantification of Ki67+ nuclei over total nuclei. n=3.

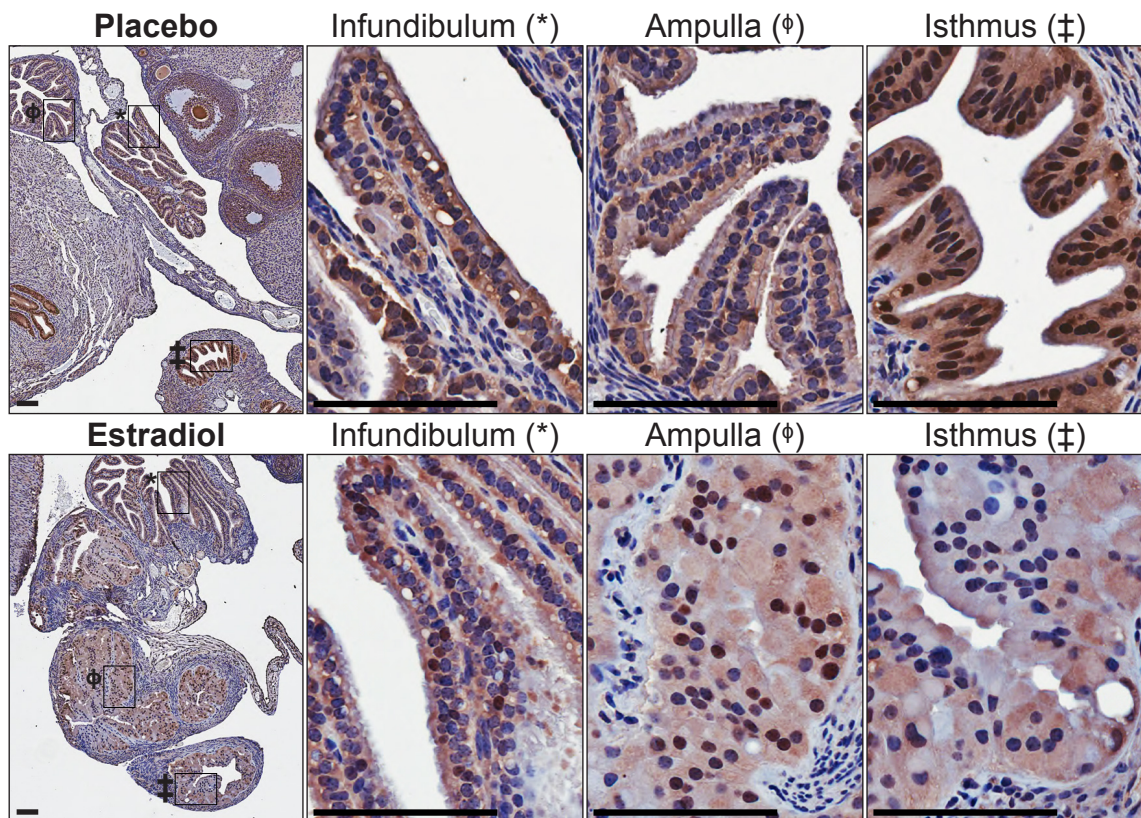


Figure 17: E2 induced FTE disorganization affects both PAX8 positive & negative cells. Oviducts stained for PAX8 from mice 60 days after implantation of placebo or E2 pellet; scale bars=100 μm. See **Appendix E** for PAX8 IHC staining controls.

significantly higher proportion of stratified FTE relative to placebo mice. Staining for PAX8 (a marker of secretory cells) showed that both PAX8 positive and negative cells became dysplastic in response to E2 (**Figure 17**). Like OSE cells, these results demonstrate that prolonged E2 exposure can lead to FTE dysplasia that is associated with DAB2 suppression.

3.3. Loss of *Dab2* causes OSE dysplasia and sensitizes cells to transformation

3.3.1. Loss of *Dab2* is sufficient to cause OSE hyperplasia

To determine if the E2 induced OSE hyperplasia is a direct result of *Dab2* suppression, primary cultures of *Dab2*-floxed OSE were infected with AdCre to knockout the *Dab2* gene (**Figure 18**). Results from proliferation experiments comparing *Dab2*-WT vs. *Dab2*-KO OSE cells demonstrated that even when they are cultured in steroid-free media, deletion of *Dab2* is sufficient to increase OSE proliferation (**Figure 19A**). Ki67 staining showed that *Dab2*-WT and *Dab2*-KO OSE have similar proportions of proliferating cells when the cells are sub-confluent. However, when the cells become confluent, less than 20% of *Dab2*-WT cells are Ki67+ whereas 40% of *Dab2*-KO OSE cells remain Ki67+ despite obvious over-crowding of cells (**Figure 19B-C**).

3.3.2. Loss of *Dab2* is sufficient to cause disorganization of OSE monolayer

Like other OSE cell isolations, *Dab2*-WT OSE cells formed a cobblestone-patterned monolayer upon reaching confluence, whereas knockout of *Dab2* resulted in disorganization of the OSE monolayer (**Figure 19C**). Knocking out *Dab2* does not change CDH1 protein expression levels (**Figure 20A**), but when stained for CDH1 by IF, *Dab2*-WT cells show membrane localization of CDH1, whereas membrane localization is

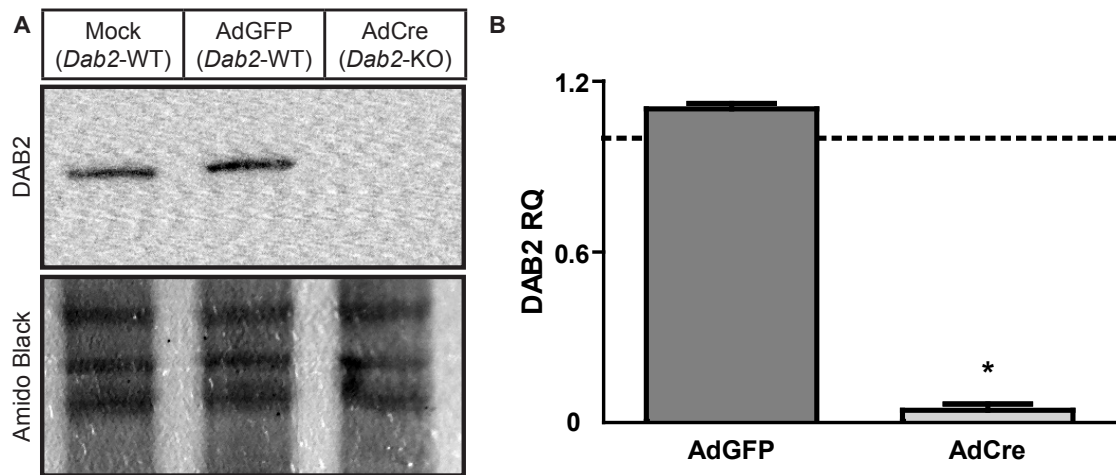


Figure 18: Validation of *Dab2* knockout by AdCre infection in *Dab2*-floxed OSE. Successful knockout of *Dab2* using primary cultures of OSE isolated from *Dab2* conditional knockout mice (*Dab2*-floxed OSE) (Moore et al. 2013). **(A-B)** *Dab2*-floxed OSE were probed for DAB2 by western blot 1 week following adenovirus infection. Knockout of DAB2 only occurs in presence of AdCre and adenovirus infection (AdGFP) alone does not suppress DAB2 relative to Mock. **(A)** Representative image of western blot probing for DAB2. **(B)** Quantification of DAB2 protein levels in using densitometry analysis of western blots. n=3; *=p-value<0.05; two-tailed paired t-test. Dotted line denotes Mock infected cells that were normalized to 1.

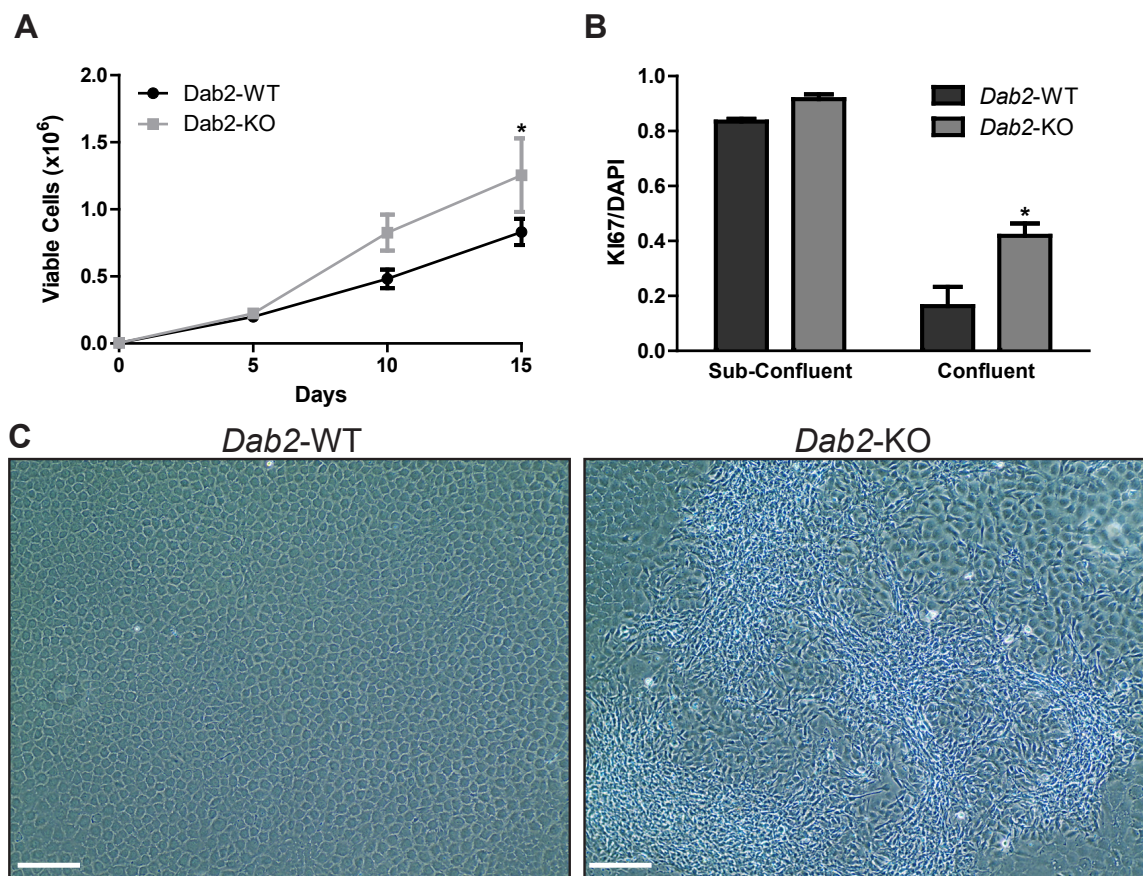


Figure 19: Loss of *Dab2* is sufficient to increase OSE proliferation. (A) Proliferation of *Dab2*-WT and *Dab2*-KO OSE cells for up to 15 days in hormone-free media. n=3; each replicate is from independent adenovirus infections. *=p-value<0.05 control vs. E2; two-way ANOVA. (B) Proportion of Ki67+ cells relative to DAPI in *Dab2*-floxed OSE. n=3; *=p-value<0.05 *Dab2*-WT vs. *Dab2*-KO; two-way ANOVA. (C) Phase contrast images of *Dab2*-floxed OSE on day 15; images were acquired using an EVOS XL Core microscope; scale bar=100 μ m.

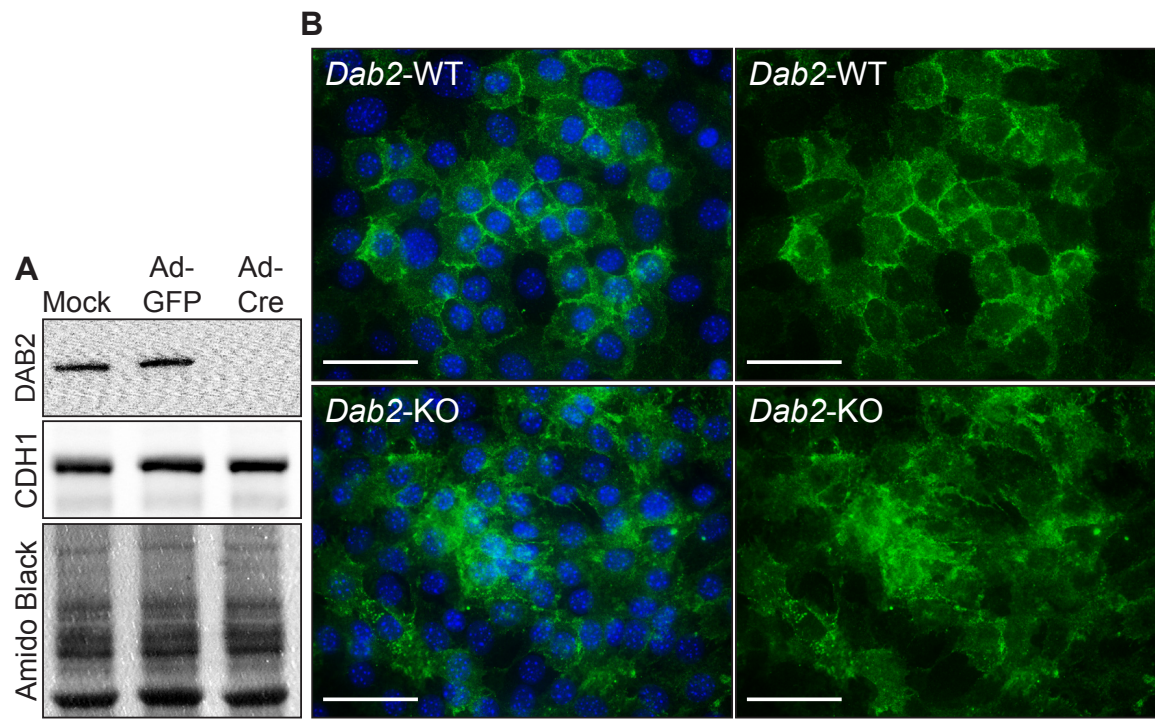


Figure 20: Loss of *Dab2* is sufficient to cause disorganization of OSE monolayer. (A) Western blot probing for DAB2 and CDH1 in mock, AdGFP (*Dab2*-WT), and AdCre (*Dab2*-KO) infected *Dab2*-floxed OSE cells, n=3. (B) IF image of *Dab2*-floxed OSE stained for DAPI (blue) and CDH1 (green) using a Zeiss Axioskop 2 microscope; n=7-8; scale bar=50 μ m.

less frequently observed in *Dab2*-KO cells (**Figure 20B**).

3.3.3. Loss of *Dab2* sensitizes OSE to transformation

Oncogene induced senescence is a phenomenon observed in culture where untransformed cells will undergo senescence in response to increased oncogenic activity in an attempt to prevent the cell's progression into cancer (Courtois-Cox et al. 2008). To determine if loss of *Dab2* would allow OSE to escape senescence and thereby sensitize them to transformation, *Dab2*-floxed OSE were infected with a KRAS^{G12D} over-expression vector (**Figure 21**) and assessed for senescence by measuring SA- β gal activity. Consistent with the known ability of KRAS^{G12D} to cause oncogene induced senescence, *Dab2*-WT OSE expressing KRAS^{G12D} showed a significant increase in SA- β gal activity while the percentage of senescent cells did not increase in *Dab2*-KO OSE (**Figure 22**). To determine the tumour suppressive capacity of *Dab2*, *Dab2*-floxed OSE over-expressing KRAS^{G12D} were assessed for transformation *in vitro* using two independent assays: low attachment growth and colony formation in soft agar. Loss of *Dab2* allowed KRAS^{G12D} over-expressing OSE to survive under both anchorage-independent growth conditions (**Figure 23**) while loss of *Dab2* or up-regulation of KRAS^{G12D} alone did not mediate these changes.

3.4. Transcription dynamics of estrogen-induced dysplasia in the OSE

3.4.1. E2 induced dysplasias demonstrate aberrant expression in polarity proteins

In addition to *Dab2*, we wanted to explore alternative polarity related and/or growth control pathways dysregulated by E2 that also contribute to OSE dysplasia. Given

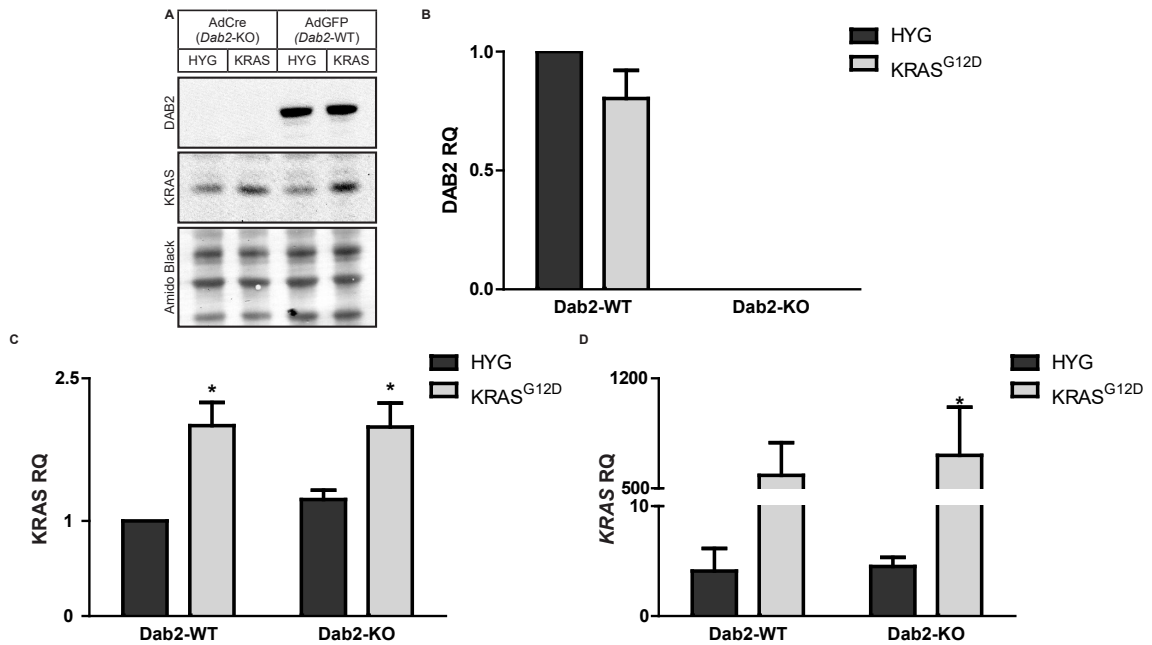


Figure 21: Validation of $KRAS^{G12D}$ over-expression in *Dab2*-floxed OSE. Successful over-expression of $KRAS^{G12D}$ in *Dab2*-floxed OSE. *Dab2*-floxed OSE infected with HYG or $KRAS^{G12D}$ over-expression vector validated by western blot (A-C) and qPCR analysis (D); * = $KRAS^{G12D}$ significantly different relative to HYG (p-value<0.05), two-way ANOVA with Bonferroni post-test. (A) Representative image western blot image. (B) Quantification of DAB2 relative quantity (RQ) by western analysis (n=3). (C) Quantification of $KRAS^{G12D}$ by western blot analysis (n=4). (D) Quantification of $KRAS^{G12D}$ mRNA levels by qPCR analysis (n=4).

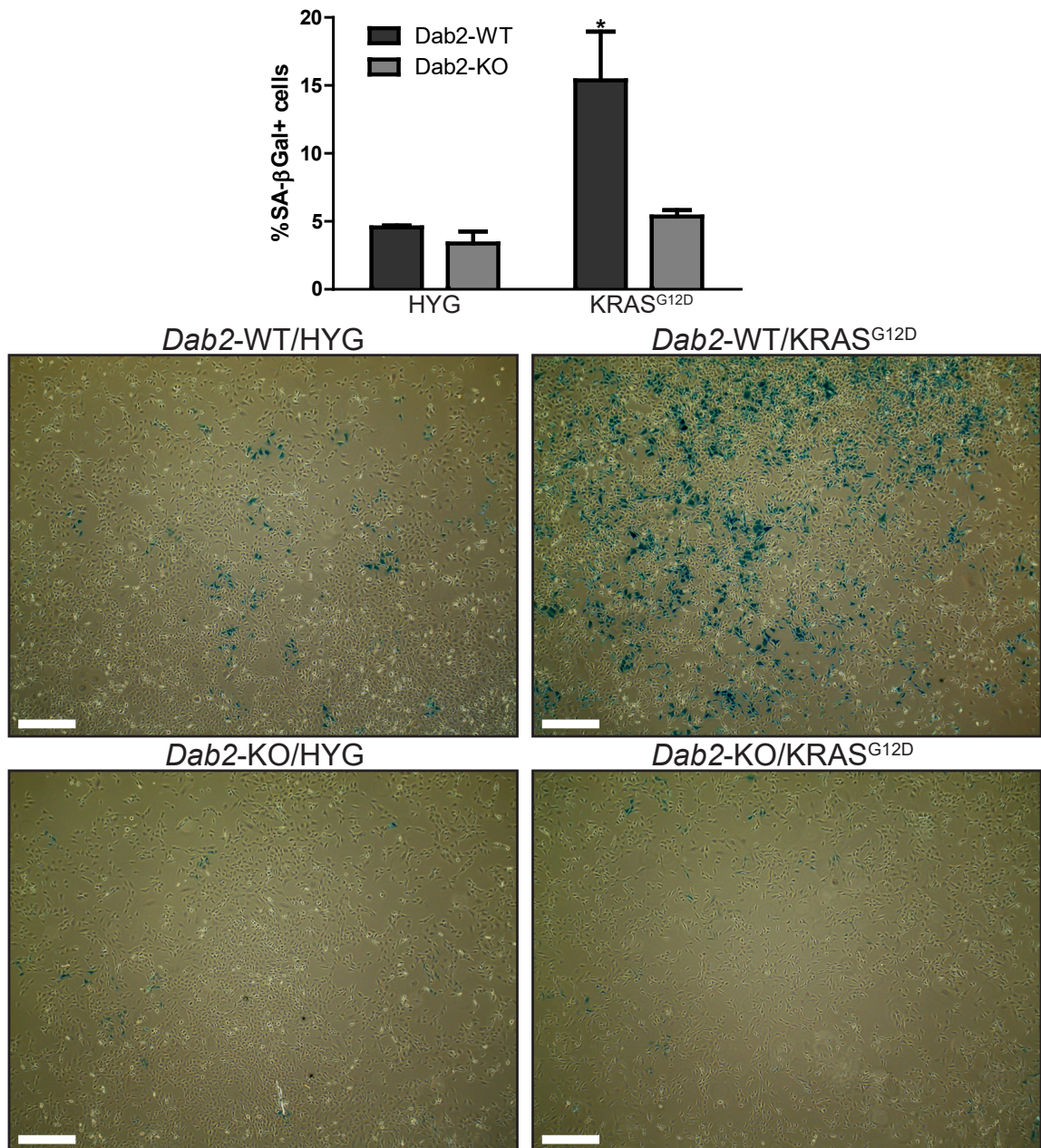


Figure 22: Loss of *Dab2* prevents oncogene induced senescence. *Dab2*-floxed OSE infected with KRAS^{G12D} or empty vector (HYG) and stained with X-gal. Senescence-associated (SA)-βgal+ cells were identified using Image J Feulgen light green colour 2 colour deconvolution. %=SA-βgal+ pixels/total cell pixels; 3 fields of view/group (n=3; *=p-value <0.05 *Dab2*-WT vs. *Dab2*-KO; two-way ANOVA). Representative images acquired using EVOS XL Core imaging system are shown. Scale bars=500 μm.

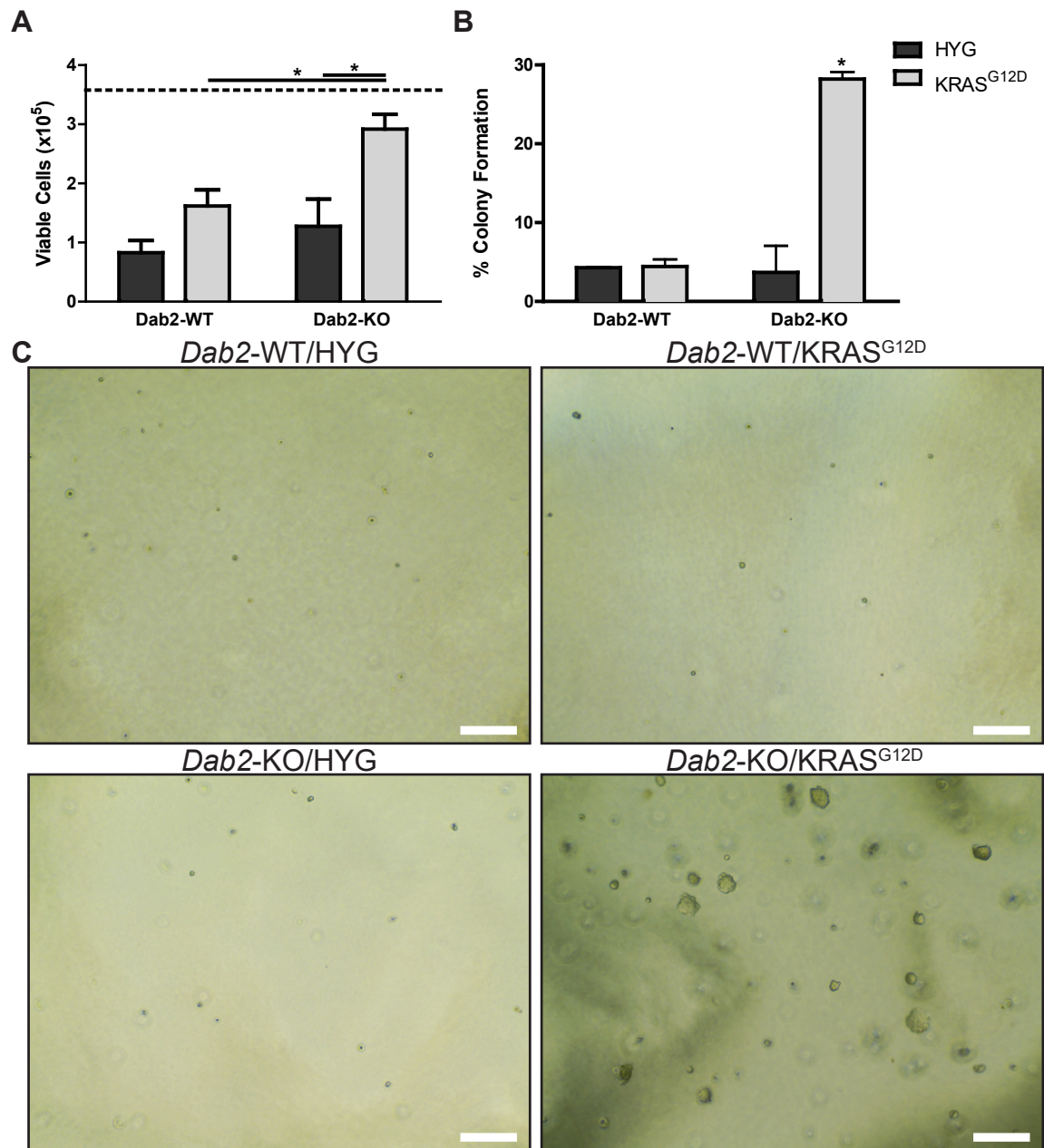


Figure 23: Loss of *Dab2* sensitizes OSE cells to transformation. *Dab2*-floxed OSE cells infected with KRAS^{G12D} or HYG in low attachment growth assay (A) and colony formation assay (B-C). (A) Cell viability measured after 3 days in low-attachment plates. n=3; *=p-value <0.05; two-way ANOVA with Bonferroni multiple comparisons post-test. Dotted line denotes mean of MASE, a mouse ovarian cancer cell line that was used as positive control. (B) % colonies=#colonies/(colonies+single cells). n=3; 2 fields of view per biological replicate; *=p-value <0.05; two-way ANOVA with Bonferroni multiple comparisons post-test. (C) Representative images from colony formation assay acquired using EVOS XL Core imaging system are shown. Scale bar=500 μ m.

the drastic phenotypic changes observed in E2 treated OSE cells, we expected to see equally drastic changes in polarity proteins responsible for maintaining OSE morphology. As a starting point, we first investigated polarity proteins documented to be differentially expressed in ovarian cancer compared to normal OSE (Wang et al. 2013; Bhagat et al. 2013; Zhang et al. 2006; Song et al. 2005; Yoshida et al. 2009). Western blot analysis showed, however, that prolonged E2 exposure only caused modest aberrant changes in SNAI1, CK19, CDH1, and CTNNB1 relative to controls while no changes were observed in NF2, PRKCI, EZR (**Figure 24**).

Since IF staining of OSE cell cultures and IHC staining of mouse ovaries showed uneven DAB2 suppression amongst E2 treated cells (**Figure 10**) and CDH1 staining revealed that areas of columnar OSE have increased CDH1 expression while adjacent areas of stratified OSE have decreased CDH1 (**Figure 11**), it became clear that the OSE cell population on the same ovary or in the same dish are asynchronous and/or heterogeneous in their response to E2 and this would confound conventional methods used to measure transcript and protein expression changes. To better tease apart this mixed population and to understand how columnar and stratified cells arise, single-cell RNA-Sequencing (scRNA-Seq) was applied to our *in vitro* model of E2 induced OSE dysplasia.

3.4.2. Single-cell profiling of control and E2 treated OSE cells

E2 treated OSE cultured over 15 days were confirmed to have substantial dysplasia, relative to control cells, with disparate morphological changes present in the

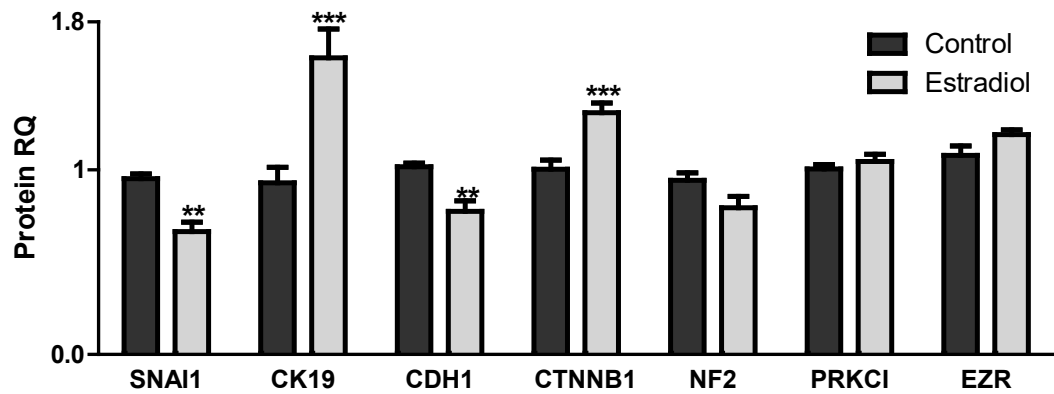


Figure 24: E2 exposure causes deregulation of polarity related proteins in OSE. Western blot analysis of SNAI1, CK19, CDH1, CTNNB1, NF2, PRKCI, and EZR from protein lysate collected from cultured OSE cells 15 days after E2 exposure. n=3; *=p<0.05, **=p-value<0.01, ***=p-value<0.001; control vs. E2 two-way ANOVA with Bonferroni multiple comparisons post-test.

same dish (**Figure 25A**). Cells were then individually captured and barcoded using the Fluidigm IFC system, and then had their pooled barcoded cDNA sequenced. After processing and filtering out poor quality libraries (**Figure 25B, Appendix O**), the dataset consisted of 329 control and 260 E2 treated cells, with a median of approximately 120 000 counts/cell and 4000 genes/cell (**Figure 25C**).

Principal component analysis (PCA) revealed two distinct clusters of cells along the first principal component (PC1) that did not segregate based on E2 treatment (**Figure 25D**). PC2 and PC3, however, did correlate with the treatment. No known technical variable or typical biological source of variation (e.g. cell cycle) correlated with these principal components, suggesting they represent biological variation of interest.

3.4.3. Heterogeneity in OSE cell response to E2

To determine the gene expression differences that drive structure in the PCA, we used SC3 (Kiselev et al. 2017)—a consensus-based k-means clustering algorithm—to cluster the data. Given the two distinct populations along PC1, we first set $k=2$ to define two clusters, splitting the cells along the first principal component (**Figure 26**). We then used monocle (Qiu, Hill, et al. 2017) to identify 1,132 differentially expressed genes between clusters (**Appendix Q**).

Since the cells of the rightmost cluster did not spread along PC2 according to E2 exposure, while the cells of the left cluster did, we predicted that these two clusters captured differences in the cells' ability to respond to E2. None of the additional principal components captured E2 associated differences in the cells of the right cluster, and increasing the k parameter did not cleanly segregate these cells by treatment. The estrogen receptors *Esr1* and *Esr2* were detected in 23.4% and 4.9% of cells, respectively,

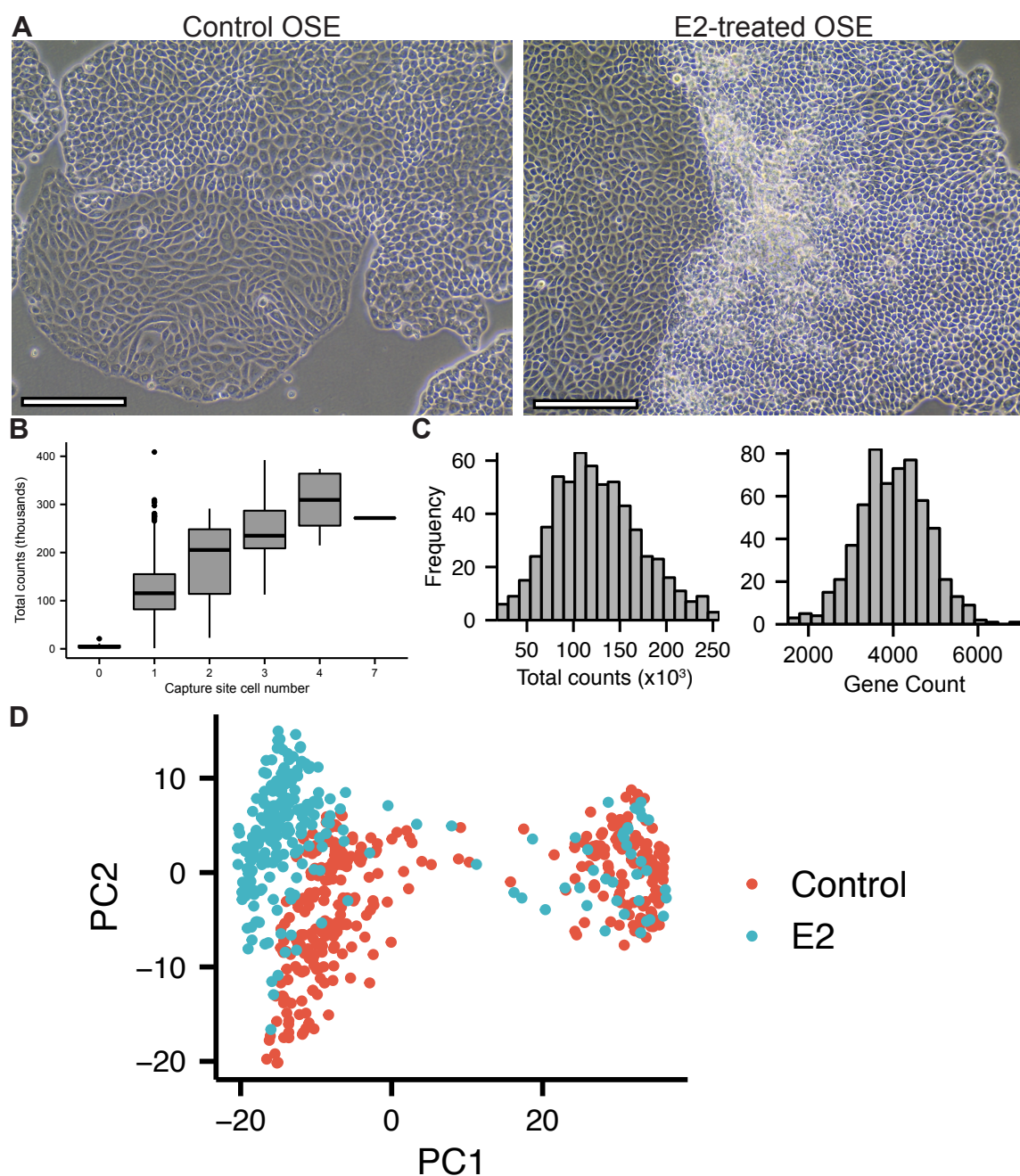


Figure 25: Single-cell profiling of control & dysplastic E2 treated OSE cells. (A) Phase contrast images of control and E2 treated OSE cells acquired using an EVOS XL Core microscope; scale bar=200 μm . (B) Boxplots of each library's transcript counts relative to the number of cells that were observed in the corresponding Fluidigm C1 capture sites. (C) Histogram of the number of transcript counts per cell following quality control filtering. (D) PCA of scRNA-Seq data from OSE cells. Data points are coloured by treatment.

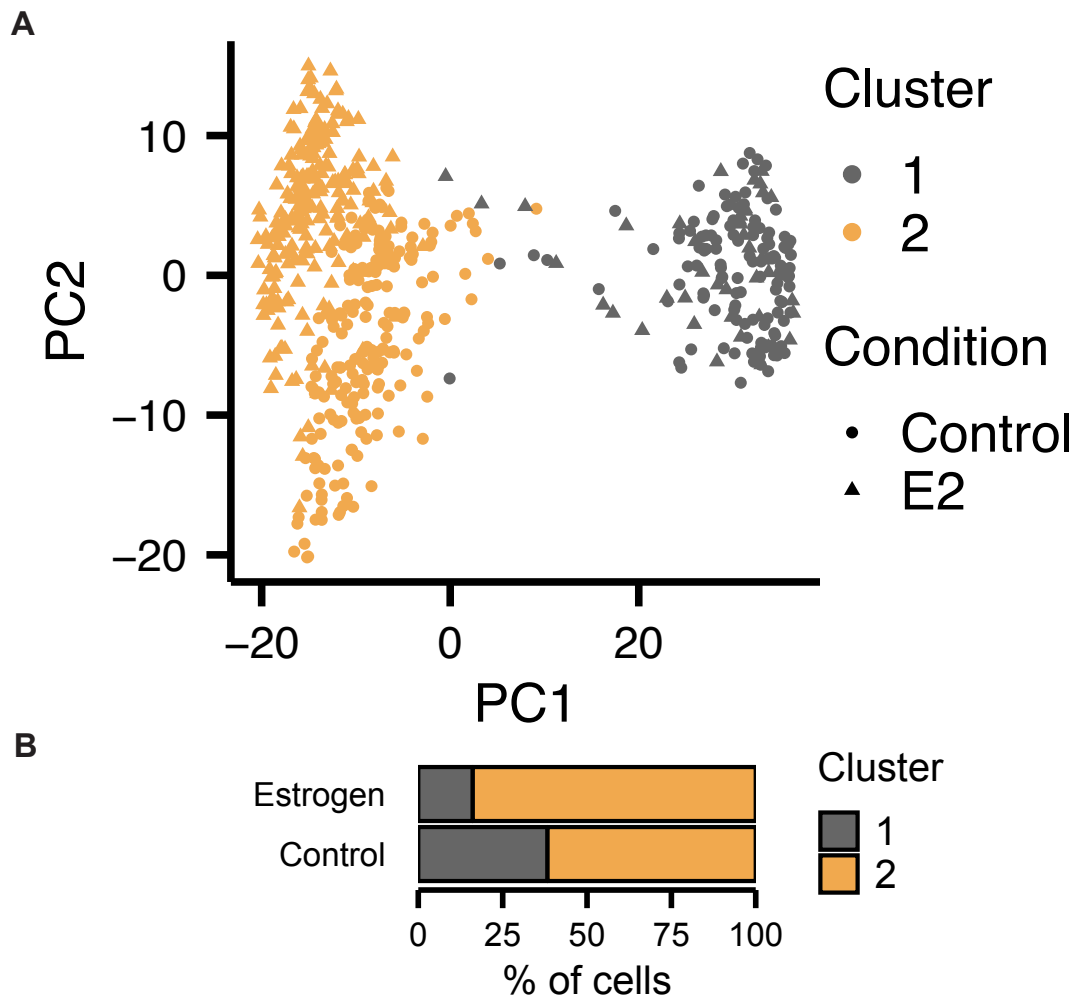


Figure 26: Unsupervised clustering reveals distinct heterogeneity in OSE. (A) PCA of OSE cells coloured by cluster for $k=2$ clustering. **(B)** Stacked barplot displaying the percent of cells from each condition within each cluster.

and *Gper1* was not detected in any cell (**Figure 27**). *Esr1* is the predominant mediator of the transcriptional effects of E2 signalling in the OSE (Auersperg 2001) and differential expression analysis deemed *Esr1* to be expressed significantly higher in the left cluster along PC1 (cluster 2; $q=0.02$). This suggests that the right cluster along PC1 (cluster 1) may represent a population of cells that are E2 unresponsive. *Esr1* expression may not be a reliable measure for estrogen responsiveness, however, because upon E2 binding to ESR1, processes for both the upregulation and degradation of *Esr1* mRNA and protein are required for proper execution of ESR1 transcription-mediated effects (Zhou & Slingerland 2014; Ing 2016), thereby making it a transient target for an otherwise very active pathway. For this reason, *Igfbp4* was used as marker for E2 responsiveness because previous studies have shown that *Igfbp4* contains a *bona fide* ERE, conserved between mice and humans, that is constitutively bound to ESR1 in a ligand independent manner (Bourdeau et al. 2004). Cell lines confirmed to be devoid of ESR1 do not show binding of ESR1 at the *Igfbp4* ERE but cells confirmed to be ESR1 positive have enriched ESR1 binding at this locus even in the absence of E2. Consistent with these findings, *Igfbp4* expression distinguishes the two clusters with up to 32-fold higher expression in the E2 responsive cluster (**Figure 27**).

Functional enrichment analysis of differentially expressed genes between the right and left clusters was performed to explore the biological differences between these two clusters. The E2 responsive cluster seems more metabolically active than the unresponsive cluster, as demonstrated by the top ontology terms for this cluster (**Table 2**). The E2 unresponsive cluster was characterized by regulation of Actin cytoskeleton and, surprisingly, pathways in cancer. However, the majority of genes in this term are

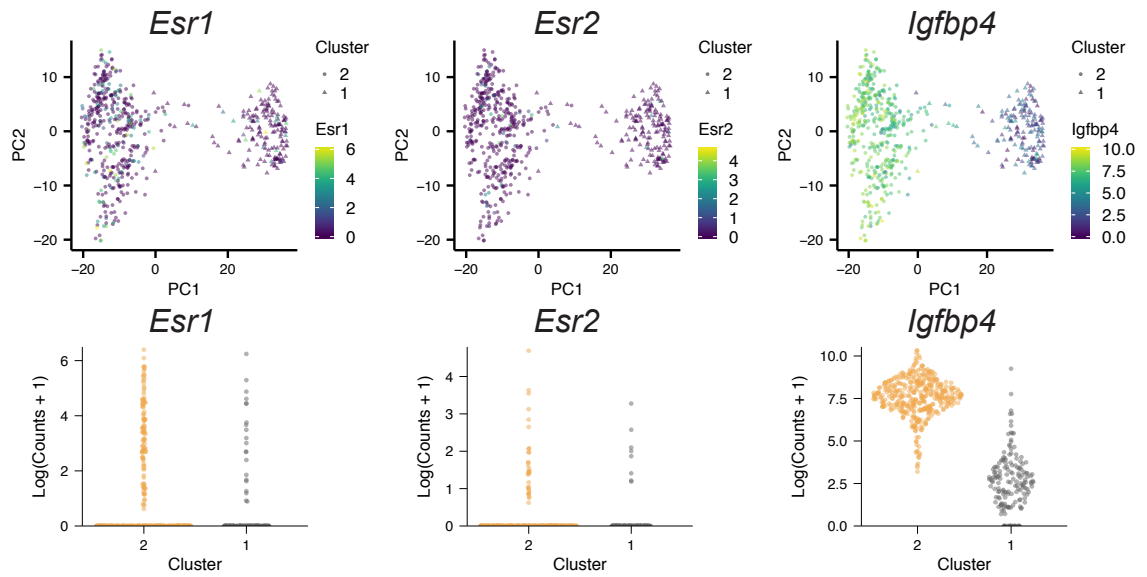


Figure 27: Assessing E2 responsiveness. Top row: PCA of OSE cells, coloured by the logged counts of *Esr1* (left), *Esr2* (middle), and *Igfbp4* (right). Bottom row: Plot showing the distribution of expression levels (logged counts) within each cluster.

Table 2: Top 5 terms associated with genes more highly expressed in Clusters 1 & 2 (k=2); p-value<0.05. All terms are KEGG pathways.

Cluster	Ontology Term	Genes
E2 Unresponsive (Cluster 1)	Ubiquitin mediated proteolysis	ANAPC1, UBE2D2A, XIAP, VHL, UBA2, UBA3, ANAPC4, PIAS2, CUL4B, PARK2, HERC2, UBE2H, CUL1, UBE2R2
	Regulation of actin cytoskeleton	ACTB, FGF8, HRAS, GNG12, NCKAP1, ARPC1A, KRAS, PAK3, GSN, ITGAV, ARAF, RAC1, BRK1, WASL, F2R, APC
	Endocytosis	EPN3, HRAS, CHMP3, KIF5B, CHMP4B, CAPZA2, TGFB2, MET, ARF5, CLTC, RAB7, ARPC1A, AP2A2, RAB31, FAM21, GBF1, WASL, IQSEC1, F2R
	Pathways in cancer	BMP4, FGF8, HRAS, CTBP1, XIAP, VHL, GNAI1, MET, ARNT2, TGFB2, GNG12, PTEN, MMP2, COL4A6, LAMA1, SMO, KRAS, AGTR1A, ITGAV, ARAF, RAC1, PIAS2, F2R, APC
	Biosynthesis of unsaturated fatty acids	SCD1, SCD2, FADS1, HSD17B12, HACD4
E2 Responsive (Cluster 2)	Oxidative phosphorylation	ATP5D, NDUFB3, NDUFB4, COX7A2, UQCRC1, NDUFA6, NDUFB9, CYC1, NDUFA7, ATP5G2, NDUFA10, COX5B, COX6C, NDUFS7, SDHB, NDUFS5, UQCR10, UQCRH, SDHC, SDHD, ATP5O, COX6B2, ATP5H, COX17, ATP5J
	Metabolic pathways	ATP5D, SAT1, UQCRC1, CYP2S1, CYC1, PGD, PTGS1, ACOT1, OGDH, COX5B, PDHB, FDFT1, NDUFS7, ST3GAL1, PKM, CRYL1, UQCR10, NDUFS5, GOT1, PTGIS, TKFC, ST3GAL4, CPOX, ST3GAL6, ATP5O, COX17, ATP5H, ATP5J, ACAA2, PFKL, BST1, CBR3, NDUFA10, COX6C, ACADVL, PGM2, PLCE1, DHRS3, PANK3, PRDX6, UQCRH, ABAT, MDH1, NDUFB3, NDUFB4, ALDOC, NDUFB9, HMGCS1, ATP5G2, PLPP2, ALDH1A2, AKR1A1, PLA2G12A, CKMT1, DAD1, MAN1A, PNPO, IDH1, PCYT1A, FH1, COX6B2, B4GALT4, ENO1, MAT2A, NDUFA6, NDUFA7, APRT, CSGALNACT1, SDHB, RDH10, SDHC, SDHD, PSAT1, PGK1, SCP2, GUK1, SMPD3, SMPD2, NNMT
	Carbon metabolism	ME2, PFKL, ALDOC, PGD, ESD, OGDH, PDHB, PKM, SDHB, GOT1, TKFC, SDHC, SDHD, IDH1, FH1, PSAT1, PGK1, ENO1, MDH1
	Citrate cycle (TCA cycle)	SDHB, SDHC, SDHD, IDH1, FH1, OGDH, PDHB, MDH1
	Mineral absorption	HMOX2, SLC30A1, MT2, ATP1A3, CYBRD1, MT1, TRF

involved in evading apoptosis [*Xiap* (Kanehisa et al. 2017), *Bmp4* (Nilsson & Skinner 2003)]. Although some pro-proliferative signals were present (*Kras*, *Hras*, and *Araf*), there was also significant up-regulation of *Pten*, a tumour suppressor that is sufficient to inhibit the proliferative signals of KRAS in OSE (Zhang et al. 2016). Cells in this cluster had a significant 2.87 log fold decrease in *Krt19*, an epithelial morphology marker in OSE cells, while having high expression of *Actin*, *Mmp2*, and *Rac1*—key genes for cell motility, filopodia, and lamellipodia formation. Thus, cells in the E2 unresponsive cluster are predicted to be mesenchymal in morphology and more quiescent than E2 responsive cells.

The initial choice of the $k=2$ parameter was based on the observed PCA structure; however, we found that $k=3$ produced optimal clusters, maximizing the cells' average silhouette width. Interestingly, this segregated the E2 responsive cluster by condition and retained the unresponsive cluster (**Figure 28**). A total of 189 genes were differentially expressed between the two condition-specific clusters (**Appendix R**). Control cells of the responsive cluster had higher expression of genes involved in wound healing, aging, response to hypoxia, negative regulation of *Notch* signalling, and NOD-like receptor signalling. E2 treated cells had an up-regulation in genes involved in metabolic, *PI3K-Akt*, *VEGF*, and *Rap1* signaling pathways (**Table 3**).

3.4.4. Distinct cell populations *in vitro*

To confirm that the clustering patterns identified in the scRNA-Seq data represented distinct populations of cells *in vitro*, we defined marker genes for each cluster. Genes in each cluster were ranked based on their AUC values (**Table 4**) then top-ranking genes *Ltbp2* (unresponsive cluster), *Ptgis* (control-specific cluster), and *Igfbp5*

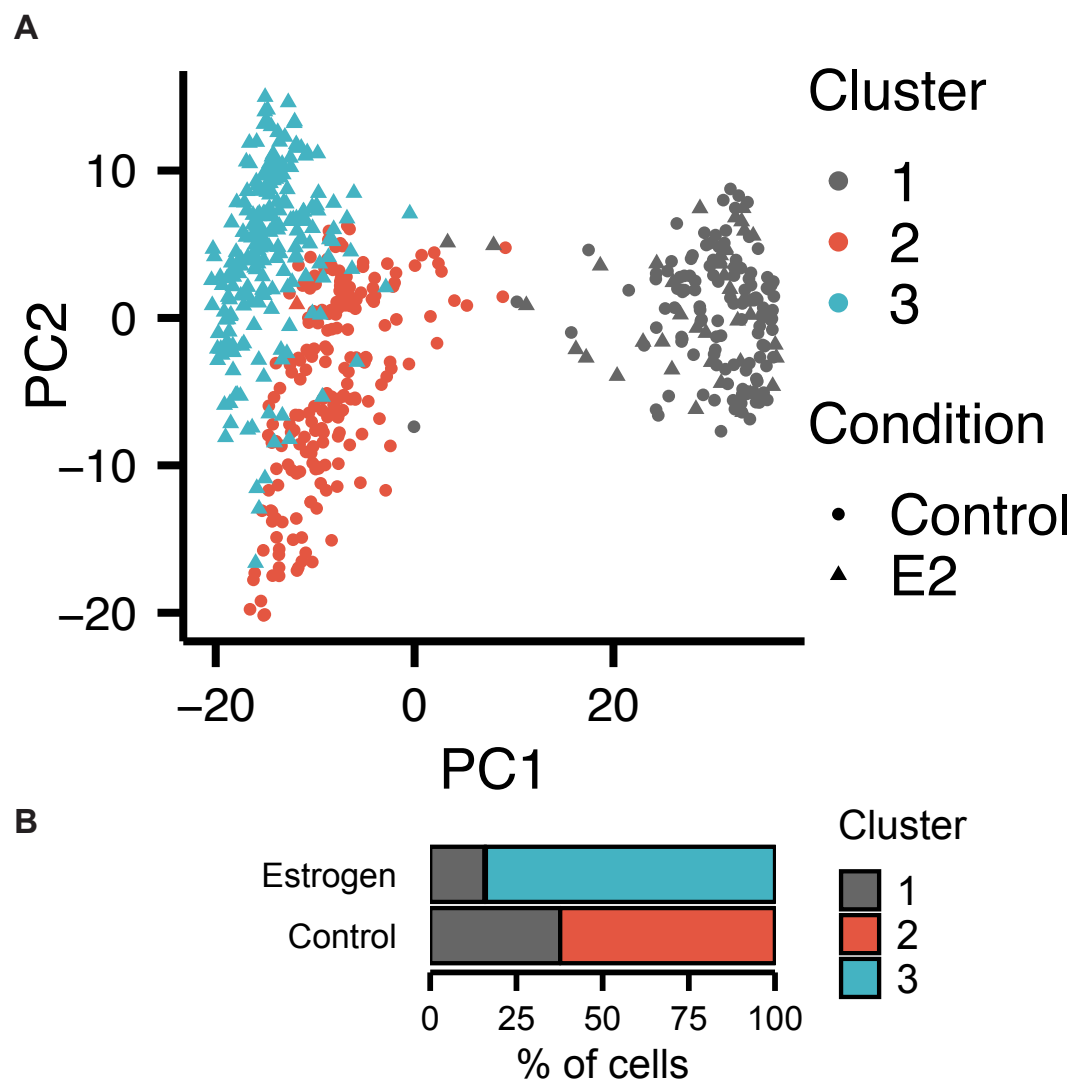


Figure 28: Unsupervised clustering can segregate control & E2 treated OSE. (A) PCA of OSE cells coloured by cluster for $k=3$ clustering. **(B)** Stacked barplot displaying the percent of cells from each condition within each cluster.

Table 3: Top 5 terms associated with genes more highly expressed in Clusters 2 & 3 (k=3); p-value<0.05. GO = gene ontology; KEGG = KEGG pathway.

Cluster	Ontology Term	Genes
Control (Cluster 2)	GO: Positive regulation of wound healing	CCL2, KANK1, MYLK
	GO: Aging	CCL2, CTGF, CRYAB, TIMP2, TIMP1
	GO: Negative regulation of Notch signaling pathway	PEAR1, NFKBIA, MMP14
	GO: Response to hypoxia	CCL2, PTGIS, CRYAB, CASP1, MMP14
	KEGG: NOD-like receptor signaling pathway	CCL2, NFKBIA, CASP1
E2 (Cluster 3)	KEGG: Oxidative phosphorylation	SDHB, ND4L, NDUFA7, ATP6V1B2, PPA2
	KEGG: PI3K-Akt signaling pathway	MAP2K1, FGF9, IL4RA, COL6A2, COL6A1, JAK1, KITL
	KEGG: Rap1 signaling pathway	CDC42, MAP2K1, FGF9, KITL, PRKD3
	KEGG: VEGF signaling pathway	CDC42, MAP2K1, MAPKAPK2
	KEGG: Metabolic pathways	FUT8, NDUFA7, ATP6V1B2, SDHB, ALDH1A2, CRYL1, ND4L, RDH10, PANK3, ST3GAL6, GCSH, IDH1, PCCA

Table 4: Area under receiver operator characteristic curves. Top 10 significant (q-value<0.05) genes for cluster1-3 by PCA, ranked by AUROC value.

Cluster	Gene	AUROC
1 (E2 Unresponsive)	Col1a2	0.991
	C1s2	0.946
	Tm4sf1	0.847
	Tmsb10	0.843
	Msln	0.842
	Cyp1b1	0.828
	Lrrfip1	0.812
	Ltbp2	0.793
	Mmp2	0.792
	Gpc3	0.774
2 (Control)	Ptgis	0.83
	Thy1	0.79
	Angptl7	0.786
	Igfbp6	0.783
	Prph	0.763
	Hspb1	0.76
	Tspan8	0.759
	Casp1	0.749
	Zfp579	0.737
	Loxl1	0.736
3 (E2)	Igfbp5	0.897
	Ctsh	0.877
	Cd55	0.837
	Tacc1	0.834
	Krt19	0.786
	Pank3	0.779
	Idh1	0.755
	Mapkapk2	0.752
	Emilin2	0.747
	Il4ra	0.744

(E2 specific cluster) were chosen for validation by IF (**Figure 29**). Although *Ltbp2* was not the top hit for the E2 unresponsive cluster (cluster 1), it was the first on the list to have a commercially available antibody that could be used for IF.

Both control and E2 treated samples contained cells that had classical mesenchymal and epithelial morphologies present in the same dish. Mesenchymal OSE cells were recognizable as very large flat cells with ruffled membranes that appeared migratory while epithelial cells tended to be tightly associated with neighbouring cells in a cobblestone arrangement. Closer examination of the epithelial population showed that they can be further separated into squamous, cuboidal, columnar, and stratified epithelial cells based on the arrangement and estimated height of the actin cytoskeleton (**Figure 30**). Squamous epithelial cells are the flattest but differ from mesenchymal cells because they are cobblestone shaped and display epithelial markers such as ZO1 (**Figure 5**). Cuboidal epithelial cells are taller than squamous cells and have actin projections that extend towards the apical membrane. Columnar and stratified cells have the tallest axial actin projections and they are distinguishable from each other based on DAPI (nuclear) stain where cells in a stratified region have nuclei that stack on top of each other. Staining of all 3 marker proteins demonstrated that their expression is correlated with morphology – not treatment, however treatment significantly influenced the proportion of cells of each morphology within the population.

LTBP2 (Latent-transforming growth factor beta-binding protein 2) is a secreted protein that was detected at high levels in the cytoplasm and extracellular matrix surrounding mesenchymal cells in both control and E2 treated dishes. In contrast, LTBP2 expression was relatively low in the majority of epithelial OSE (**Figure 31**). PTGIS

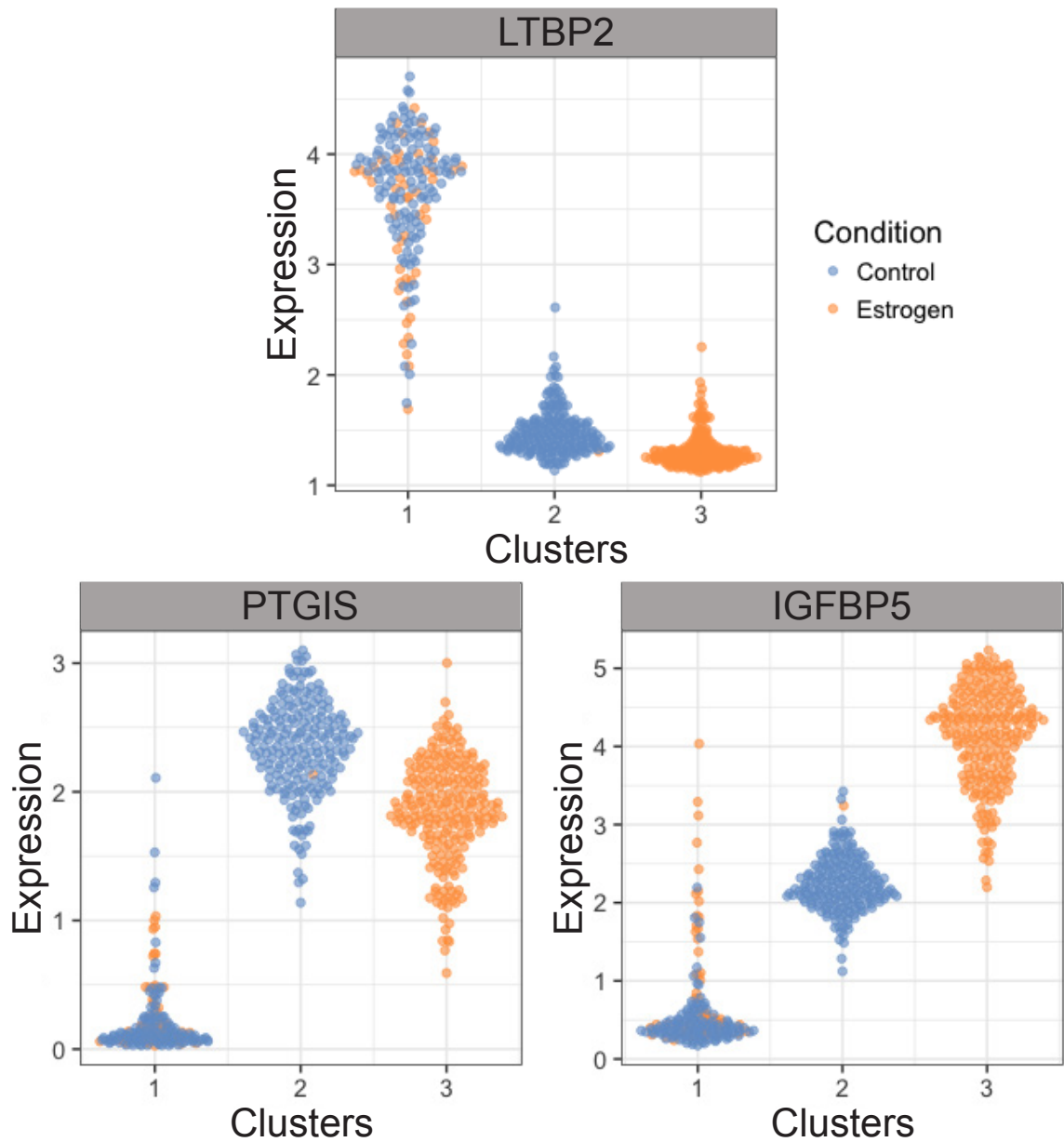


Figure 29: PCA cluster markers to be validated by IF. Expression of cluster markers *Ltbp2* (Cluster 1), *Ptgis* (Cluster 2), and *Igfbp5* (Cluster 3) for control and E2 treated cells across each cluster.

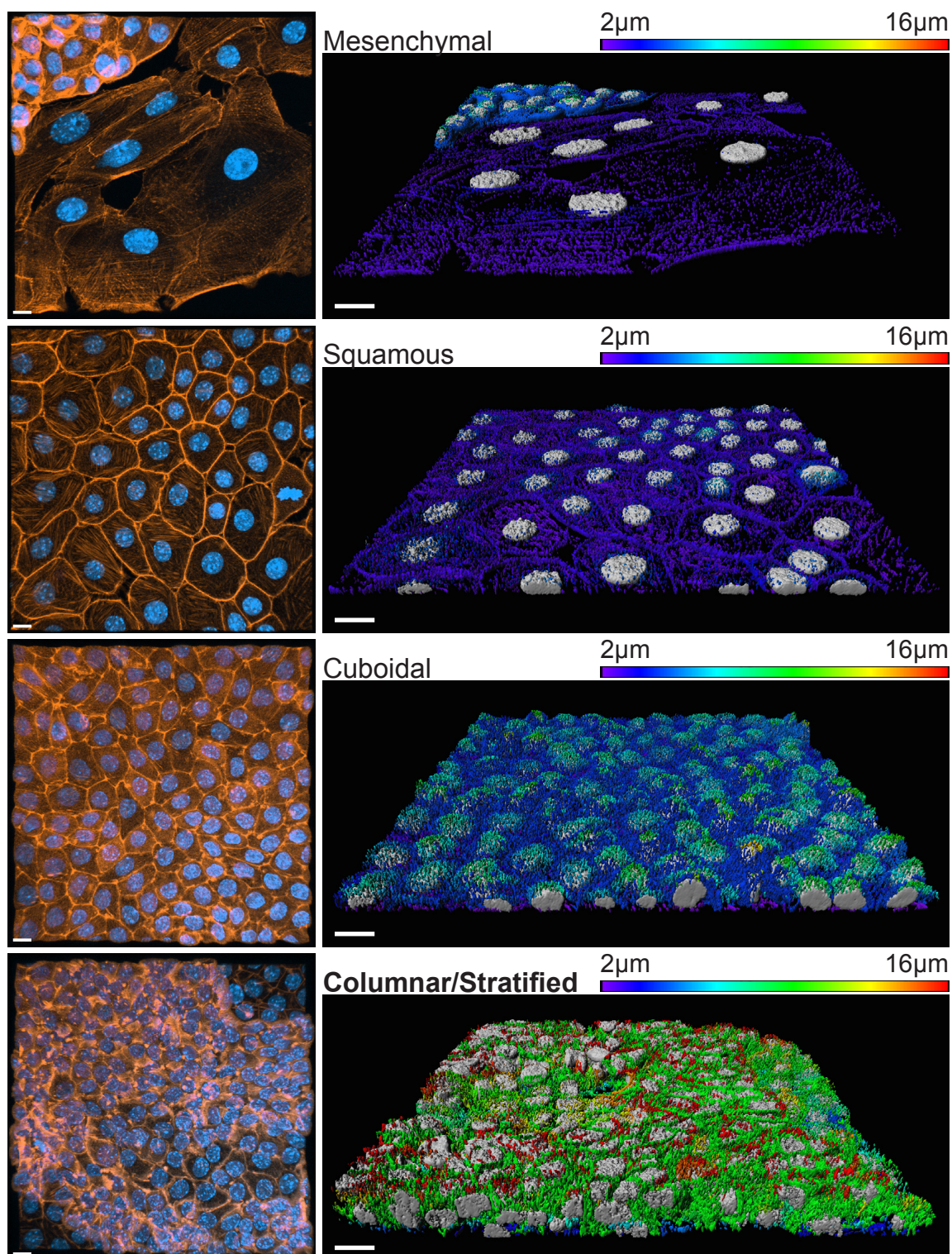


Figure 30: Morphologies observable in control or E2 treated cultures highlighted by IF actin staining. Images were acquired using a Zeiss LSM800 AxioObserver Z1 system and analyzed using Imaris. **Left:** Z projections of orange=Actin, blue=DAPI. Scale bars=10 μm . **Right:** Surface rendering of actin that was pseudo-coloured to reflect Z-position; white=DAPI. Scale bars=15 μm .

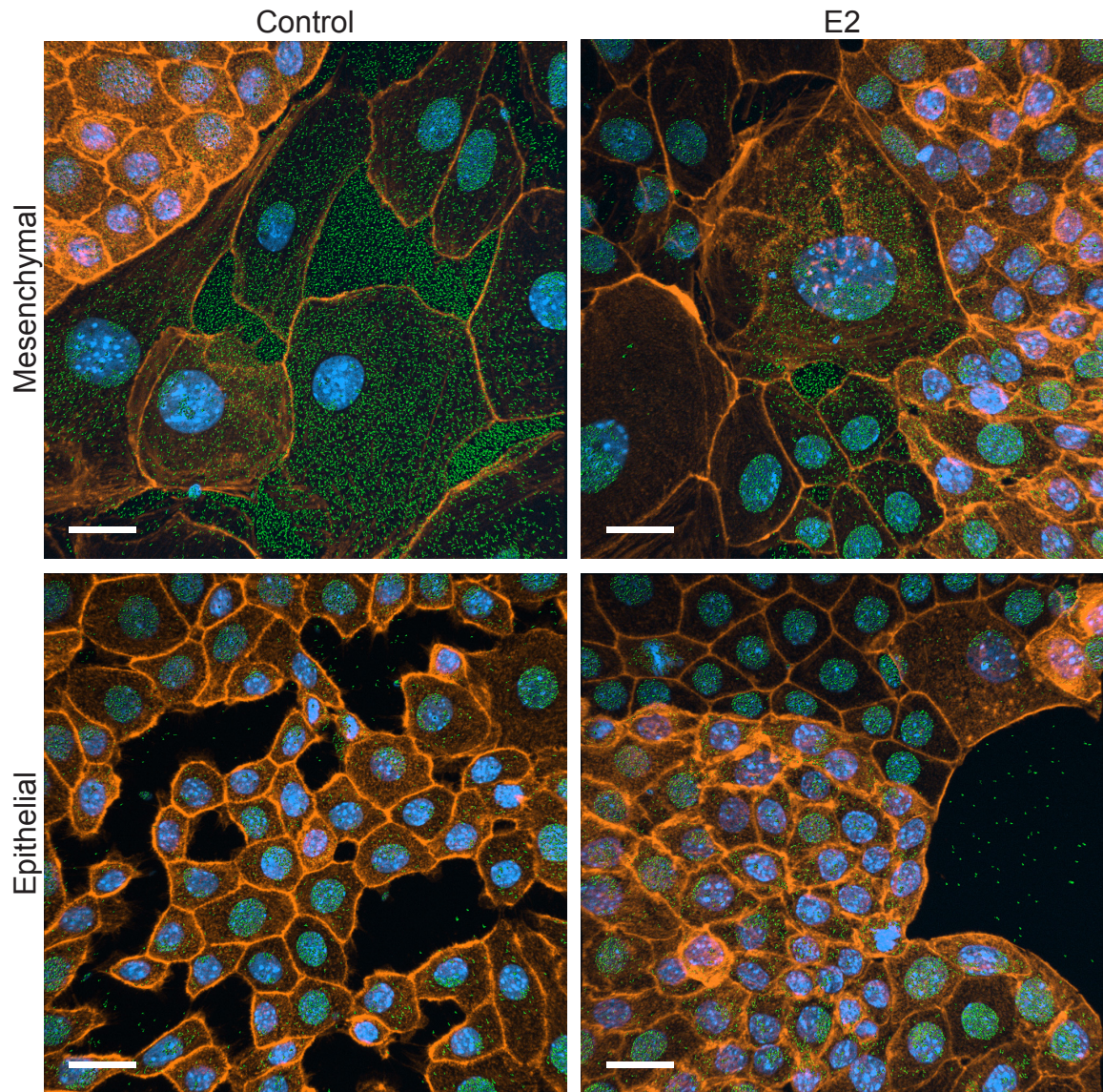


Figure 31: Validation of cluster 1 marker LTBP2 by IF. Control and E2 treated OSE cells stained for LTBP2 by IF (n=9-10). Images were acquired using a Zeiss LSM800 AxioObserver Z1 system and analyzed using Imaris; Green=LTBP2, orange=Actin, blue=DAPI. Scale bars=20 μ m.

(Prostaglandin I2 synthase) is an endoplasmic reticulum membrane protein that contributes to the “cellular response to hypoxia” ontology term in control cells (**Table 3**). It was modestly expressed in all cells, with a typically perinuclear localization but was notably abundant throughout the cytoplasm of squamous epithelial cells which made up a large proportion of the control population and only a small proportion of E2 treated population (**Figure 32**). IGFBP5 (Insulin-like growth factor binding protein 5) was absent in squamous OSE, but was detected in the cytoplasm of cuboidal, columnar, and stratified OSE – morphologies typically found in E2 treated cultures and less so in control cultures (**Figure 33**).

Although several morphologies were described, these validation results support the PCA clustering where both control and E2 treated populations have a subpopulation of mesenchymal OSE cells that uniquely secretes LTBP2, and each population/cluster is distinguishable from the others based on their respective over-representation of PTGIS and IGFBP5 positive cells. The results also revealed that these cluster markers are associated with OSE cell morphologies that appear to be driven by treatment, where control populations have a high frequency of PTGIS-high, IGFBP5-low squamous epithelial cells and E2 treated populations have a high prevalence of PTGIS-low, IGFBP5-high columnar and stratified OSE cells.

To determine if these subpopulations are found *in vivo*, ovaries from mice treated with exogenous E2 were evaluated by IHC with these same markers (**Figure 34**). E2 treated mice had significant areas of columnar and stratified OSE, as previously reported (Laviolette et al. 2010), and these cells had faint PTGIS expression, but strong punctate apical staining of IGFBP5. Unlike control cells *in vitro*, placebo mice remain subject to

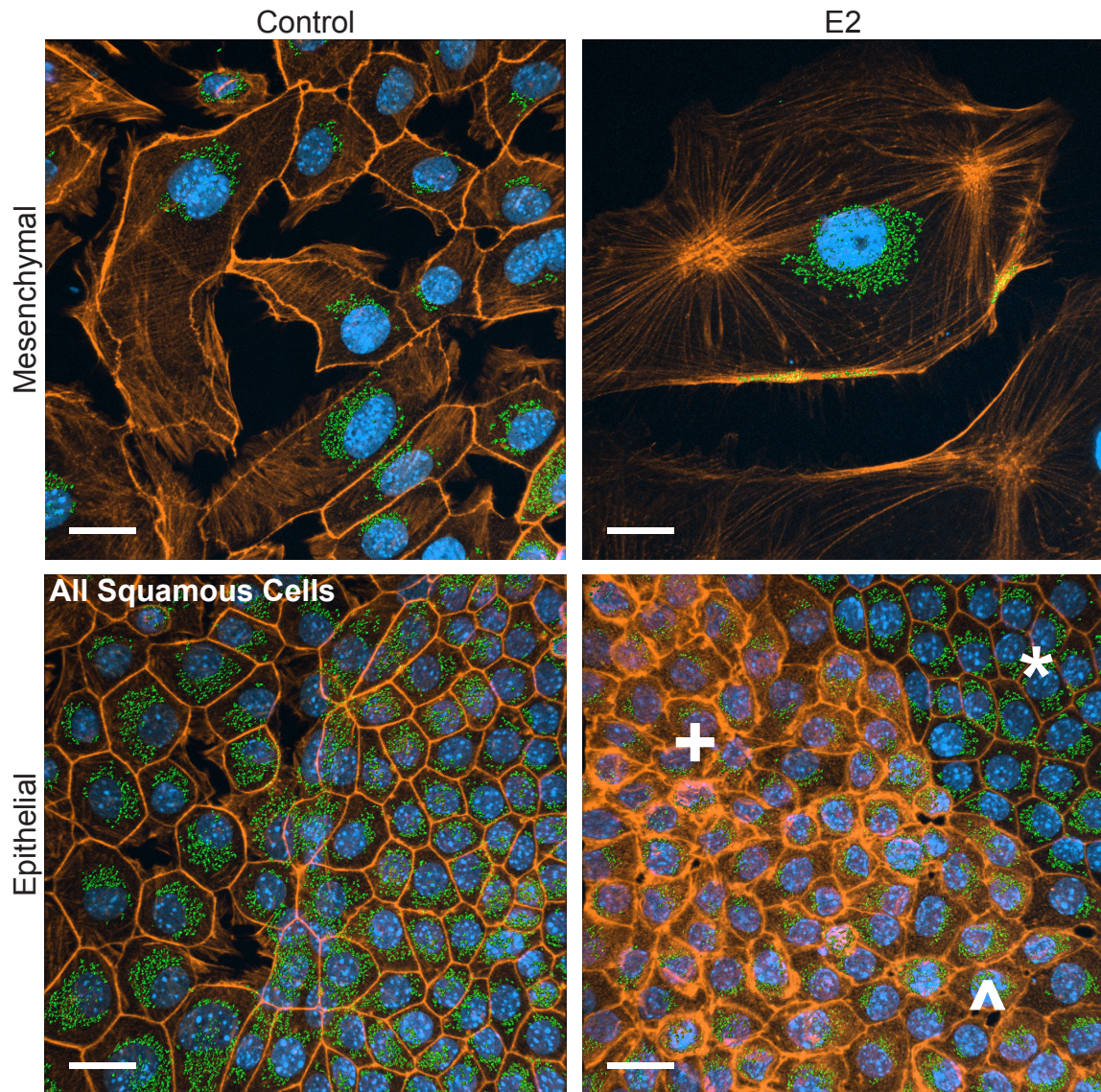


Figure 32: Validation of cluster 2 marker PTGIS by IF. Control and E2 treated OSE cells stained for PTGIS (n=13) by IF. Images were acquired using a Zeiss LSM800 AxioObserver Z1 system and analyzed using Imaris; Green=PTGIS, orange=Actin, blue=DAPI. Scale bars=20 μ m. Epithelial OSE cells from E2 cultures has squamous (*), cuboidal (^), and columnar cells (+) in field of view.

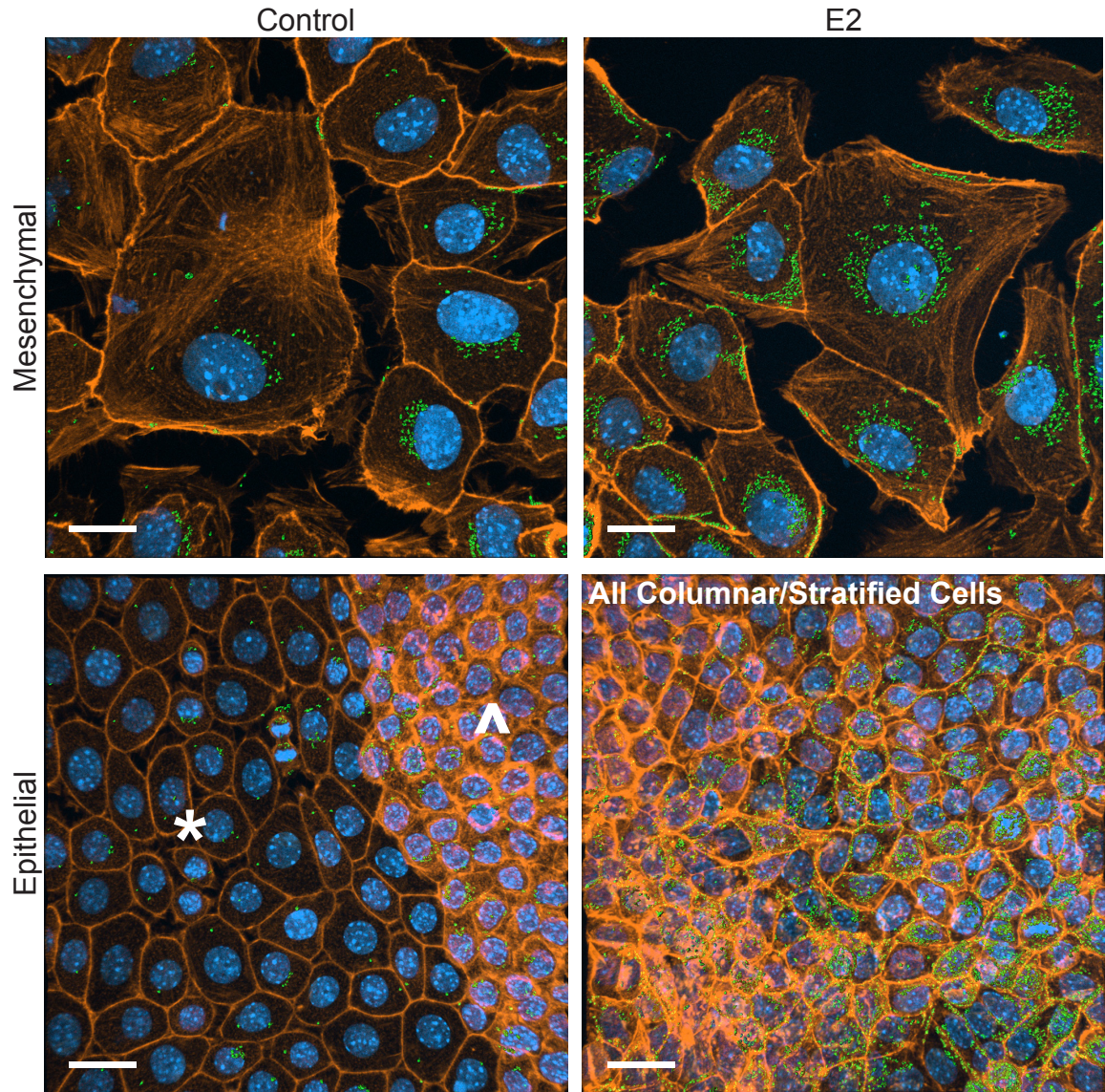


Figure 33: Validation of cluster 3 marker IGFBP5 by IF. Control and E2 treated OSE cells stained for IGFBP5 (n=9-10) by IF. Images were acquired using a Zeiss LSM800 AxioObserver Z1 system and analyzed using Imaris; Green=IGFBP5, orange=Actin, blue=DAPI. Scale bars= 20 μ m. Epithelial OSE cells from control cultures has squamous (*) and cuboidal (^) cells in field of view.

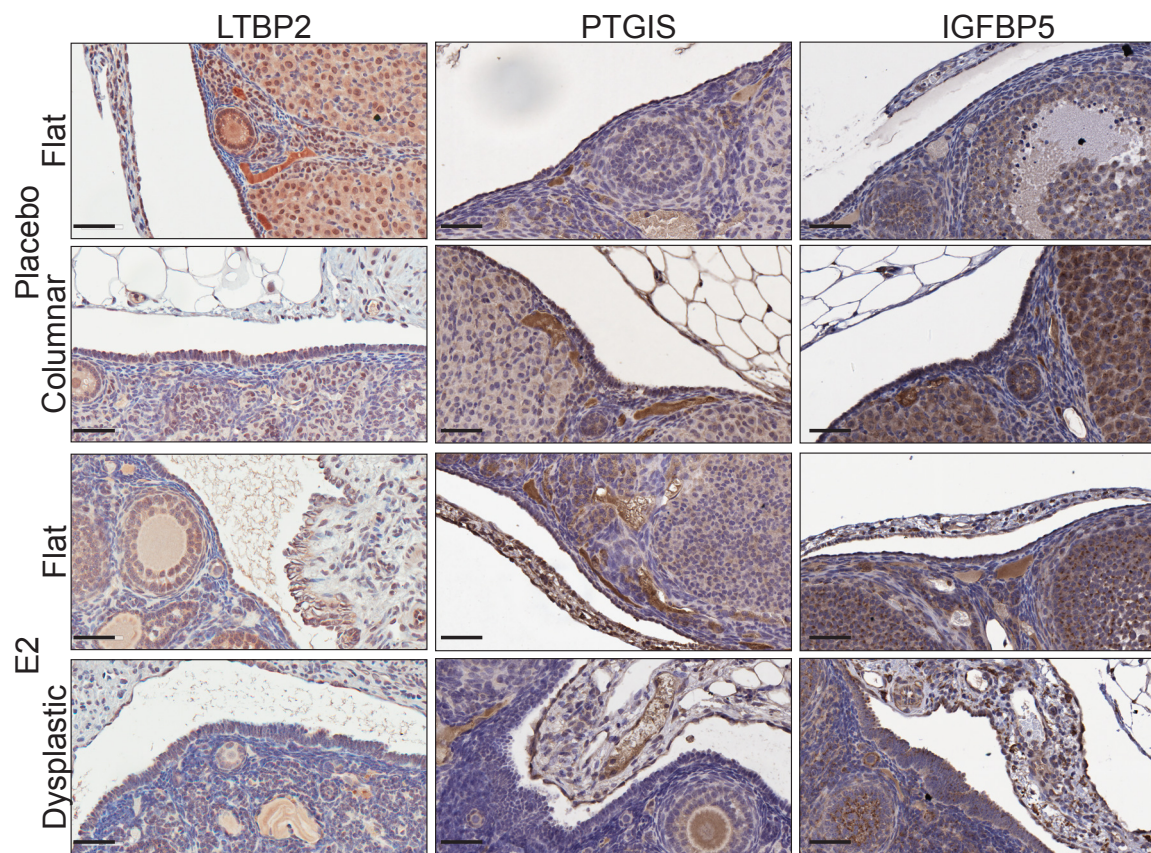


Figure 34: Cluster marker validation *in vivo* by IHC. IHC staining of ovaries from placebo and E2 treated mice for LTBP2 (placebo n=3; E2 n=5), PTGIS (placebo n=5; E2 n=5), and IGFBP5 (placebo n=5; E2 n=5). Scale bars=50 μ m. See **Appendix E** for LTBP2, PTGIS, and IGFBP5 IHC staining controls.

endogenous estrus cycle activity and some areas of IGFBP5 positive and PTGIS negative columnar OSE were observable, but the majority of OSE were PTGIS positive and IGFBP5 negative squamous to cuboidal cells. LTBP2 staining was inversely proportional to cell height where it was most intense in squamous OSE cells and absent in columnar or stratified cells. Staining for LTBP2 by IHC did not clearly highlight mesenchymal cells *in vivo* as it did *in vitro* but this may be a limitation of the technique as mesenchymal OSE cells are difficult to observe by IHC due to their orientation and flat nature. Aside from this, IHC staining of placebo and E2 treated mouse ovaries with PTGIS and IGFBP5 yielded results consistent with the *in vitro* IF data where marker staining was associated with cell morphology, which was in turn influenced by treatment.

3.4.5. Pseudotime analysis reveals phenotypic divergence

Following E2 exposure, we observed a significant increase in areas of columnar and stratified OSE cells that were observable in both sub-confluent and confluent monolayers. The smearing of E2 cells along the principal components (**Figure 28**), rather than discrete clustering, supports a model where the formation of these dysplastic regions is an asynchronous process. Therefore, we hypothesized that sampling cells for scRNA-Seq at a single time point would capture cells at various stages of foci formation, allowing us to use pseudotime analysis to reconstruct the trajectory of transcriptional changes that occur throughout the process.

We used monocle (Qiu, Hill, et al. 2017) to construct a transcriptional trajectory of all the cells, naive to the condition that the cells came from. This algorithm pseudotemporally ordered the cells by ranking their distance along the trajectory from the cell of the E2 unresponsive branch farthest away from the branch point (**Figure 35**). The

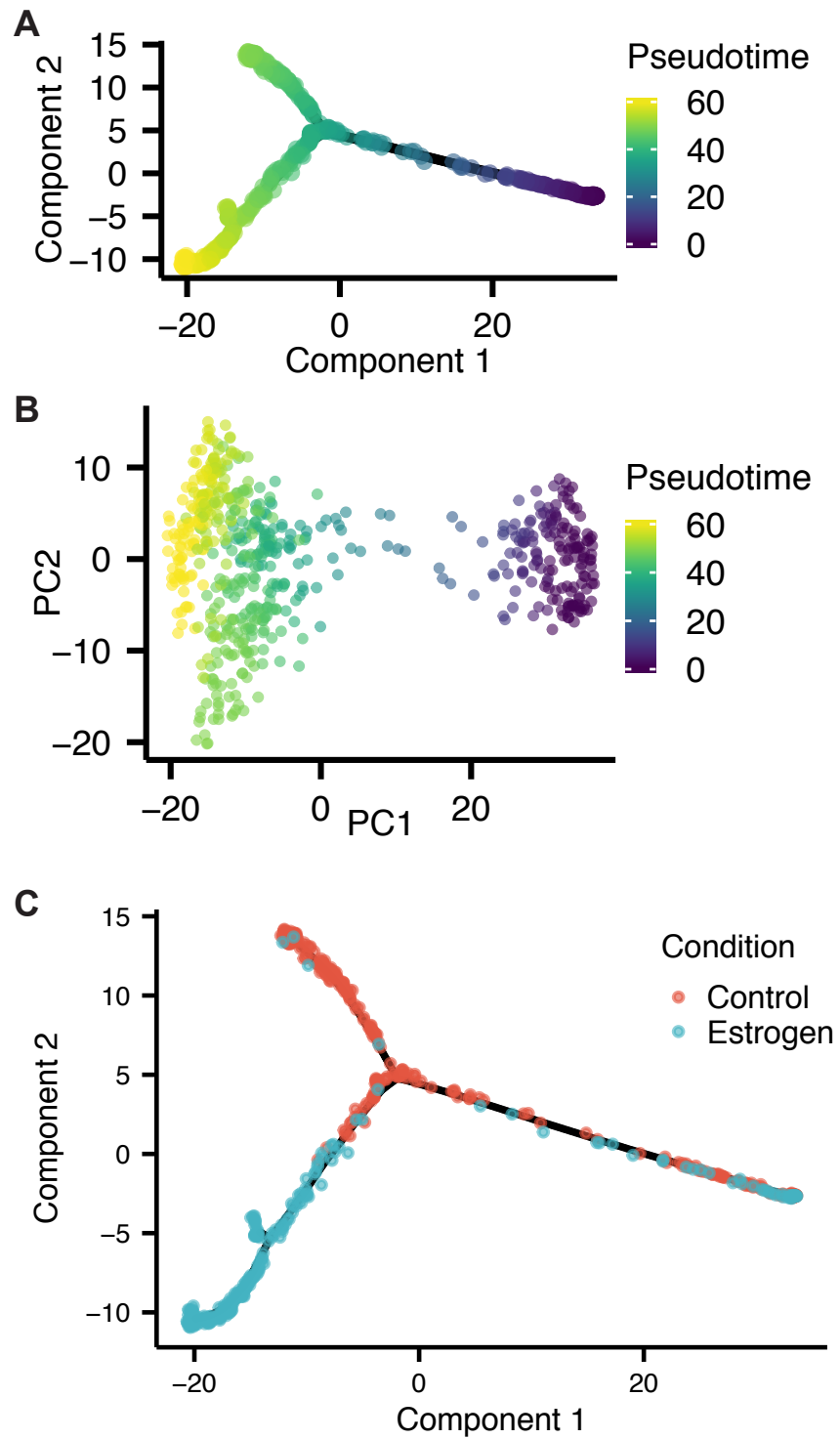


Figure 35: Columnar & stratified OSE cells represent different stages of dysplasia. (A) Pseudotemporal trajectory of control and E2 treated OSE cells constructed by Monocle coloured by pseudotime value. (B) PCA coloured by pseudotime value. (C) Pseudotemporal trajectory coloured by treatment.

resulting trajectory was characterized by a branch common to cells from both conditions (representing the E2 unresponsive cluster) that diverges from a branch point into two condition-specific branches. This can be interpreted as a linear trajectory for each condition, where depending on whether the cells had been exposed to E2, the trajectory diverges into a different state space. If the columnar and stratified states were “lineages” arising from a common precursor cell, we would expect a branched pattern in the E2 specific trajectory, but if they arose from two distinct cell populations, we would expect two disconnected trajectories in the E2 treated cells. Given that the trajectory has a good sampling density throughout, and that each condition’s trajectory seems linear, this data supports the hypothesis that the columnar and stratified phenotypes represent different stages of OSE cell dysplasia. Additionally, the trajectory connects the E2 unresponsive population with the responsive population, suggesting that cells can transition between these two states.

After assigning a pseudotime value to cells, we identified 693 genes with branch-dependent gene expression dynamics (see **Appendix S** for list of genes that make up cluster 1-5 from pseudotime branch-dependent gene expression results). Clustering of these genes based on their expression dynamics identified groups with common expression patterns (**Figure 36**). We found that OSE cells cultured long-term in a steroid-free environment continually acquire characteristics of cell stress as they diverge from the branch point. There is induction of genes associated with cell aging, inflammatory pathways, positive regulation of cell death, and other immunogenic signals for apoptosis and clearance (Clusters 1-3; **Table 5**). E2 treated cells show either no change or a decrease in these genes, but show increased expression of genes involved in metabolic

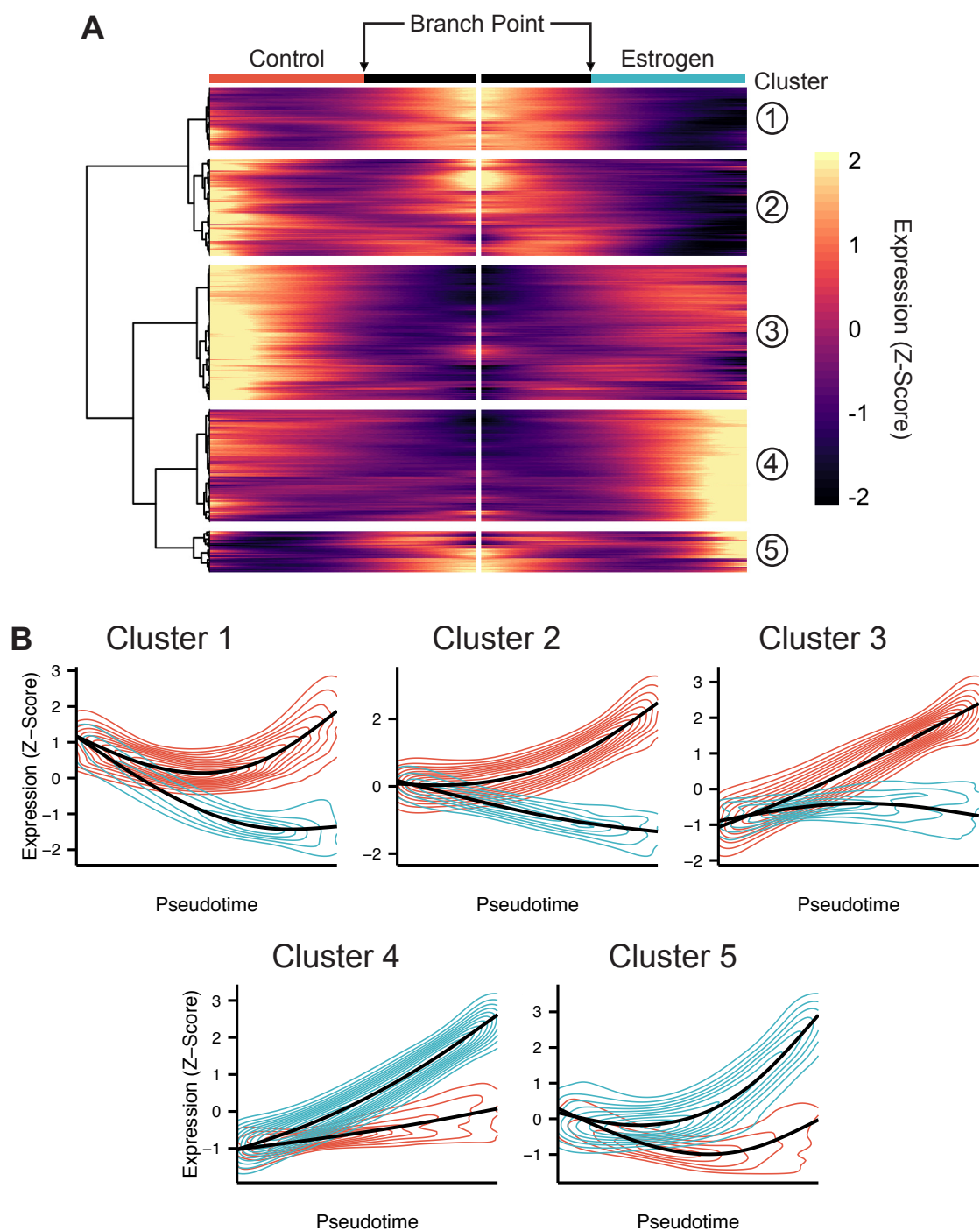


Figure 36: Expression dynamics of divergent control & E2 treated phenotypes. (A) Clustered heatmap of genes with branch-dependent gene expression as a function of pseudotime. The center of the heatmap represents the root cell (pseudotime=0). Cells are ordered by increasing pseudotime value, diverging outwards from the heatmap's center, corresponding to the two condition-specific trajectories. **(B)** Expression dynamics of genes from each cluster. The black line represents the average scaled (Z-score) expression values, which is surrounded by the two-dimensional kernel density estimate. Values are separated by branch (control-dominated branch: red; estrogen: blue).

Table 5: Top 5 terms associated with each cluster of branch-dependent genes, as shown in **Figure 36**.

Cluster	Ontology Term	Genes
1	GO: Complement activation	C1RA, C1S1, C1S2
	GO: Ribosomal small subunit assembly	RPS27, RPS17, RPS27RT
	GO: Tumor necrosis factor-mediated signaling pathway	TNFRSF1A, LTBR, KRT18
	GO: Cell-cell adhesion	LIMA1, LDHA, TLN1, LRRFIP1, TMEM2
	GO: Positive regulation of cell death	EGR1, BMP4, CTGF
2	KEGG: TNF signaling pathway	ICAM1, ATF4, CCL2, CEBPB, NFKBIA, FAS
	GO: Regulation of cell shape	EPB41L2, ICAM1, CCL2, CSNK1G2, PLXNB2, ITGA7, SLC9A3R1
	GO: Positive regulation of protein targeting to membrane	STOM, CCL2, MYO1C
	GO: Cellular response to interleukin-1	ICAM1, CCL2, CEBPB, SAA3
	GO: Aging	ATP2B1, CCL2, CRYAB, SHC1, TIMP1
3	KEGG: Mineral absorption	ATP1B1, MT2, MT1, TRF
	GO: Positive regulation of proteasomal ubiquitin-dependent protein catabolic process	DAB2, CLU, PSEN2, SIRT2, TRIB1
	GO: Positive regulation of I-kappaB kinase/NF-kappaB signaling	S100A4, ZDHHC17, TGM2, FKBP1A, EEF1D, CASP1, ECM1
	GO: Positive regulation of nuclear-transcribed mRNA poly(A) tail shortening	ZFP36, BTG2, PABPC1
	GO: Cellular response to interferon-beta	TMEM173, IFI47, TGTP2, GBP2
4	KEGG: Pathways in cancer	WNT5A, DVL3, ADCY2, MAP2K1, FGF9, ROCK2, TGFB3, KITL, CDC42, FOS, HIF1A, SOS2, JAK1
	KEGG: Proteoglycans in cancer	WNT5A, CDC42, NANOG, HIF1A, MAP2K1, ROCK2, MAPK14, SOS2
	KEGG: Prolactin signaling pathway	FOS, SOCS2, MAP2K1, MAPK14, SOS2
	KEGG: PI3K-Akt signaling pathway	MAP2K1, FGF9, SOS2, IL4RA, COL6A2, COL6A1, JAK1, KITL, G6PC3, SYK
	KEGG: Gap junction	ADCY2, MAP2K1, SOS2, GUCY1A3, GJA1
5	KEGG: Glyoxylate and dicarboxylate metabolism	GCSH, ACAT1, MDH2
	KEGG: Fatty acid metabolism	ACADM, SCD2, ACAT1
	KEGG: PPAR signaling pathway	ACADM, SCD2, FABP5
	KEGG: Insulin resistance	PPP1CA, OGT, INSR
	GO: Circadian regulation of gene expression	PPP1CA, OGT, BHLHE40

activity, pathways in cancer, and proliferation via PI3K-Akt signaling (Clusters 4-5; **Table 5**). Additionally, E2 treated cells progressively lose expression of genes associated with regulating cell shape and cell-cell adhesion, which likely contributes to the development of dysplasia (Clusters 1-2; **Table 5**).

3.4.6. Trajectory models foci formation

While the characteristics of the transcriptional trajectory are consistent with the observed biology of the culture system, we sought to validate that the structure of the trajectory relates to foci formation in the E2 treated cells and cell stress in the control cells. To do this, SA- β Gal activity was used as a marker for stressed cells (Courtois-Cox et al. 2008) and phase contrast images of the same field of view was overlaid to track cell morphology (**Figure 37**). Ki67 staining by IF was used as a marker for proliferating cells (Juríková et al. 2016) and actin staining was once again used to track cell morphology and approximate cell height (**Figure 38**). Mesenchymal and relatively flat (~ 8.8 - $9.6 \mu\text{m}$) E2 unresponsive cells likely occupying the common branch, were SA- β Gal-negative and sometimes Ki67-positive in both control and E2 treated cultures. Also common between control and E2 treated cultures were areas of sub-confluent OSE where cells in small epithelial rafts or cells on the periphery of larger rafts were cuboidal in morphology, ~ 13 - $14 \mu\text{m}$ in height, SA- β Gal-negative, and $\sim 50\%$ Ki67-positive. These areas of proliferative and cuboidal OSE cells, seen in both control and E2 treated cultures, are likely cells that exist shortly before phenotypic divergence. As the rafts become larger and more confluent, the divergence predicted by trajectory analysis becomes apparent. Confluent control cells remain similar in height to sub-confluent cells ($12.6 \mu\text{m}$) and remain SA- β Gal-negative, but become less Ki67-positive. The final phenotype observed in control

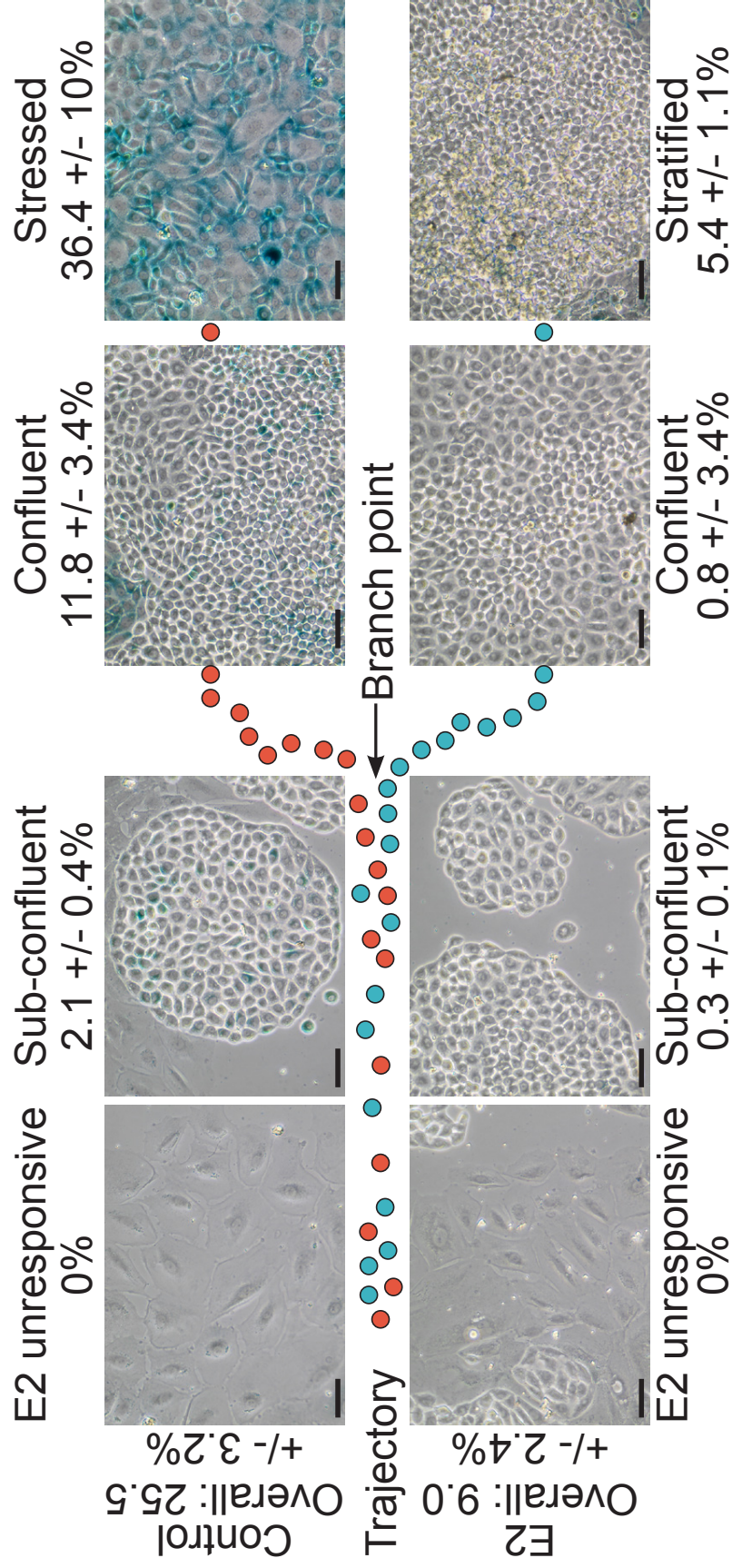


Figure 37: E2 promotes survival. Control and E2 treated OSE cells stained for SA-βgal. Overall %SA-βgal, n=7 using 4x objective; %SA-βgal/morphology, n=6-7 using 40x objective. Mean +/- S.E.M. Scale bars= 50 μm. Representative images shown is a result of phase contrast overlaid with bright field.

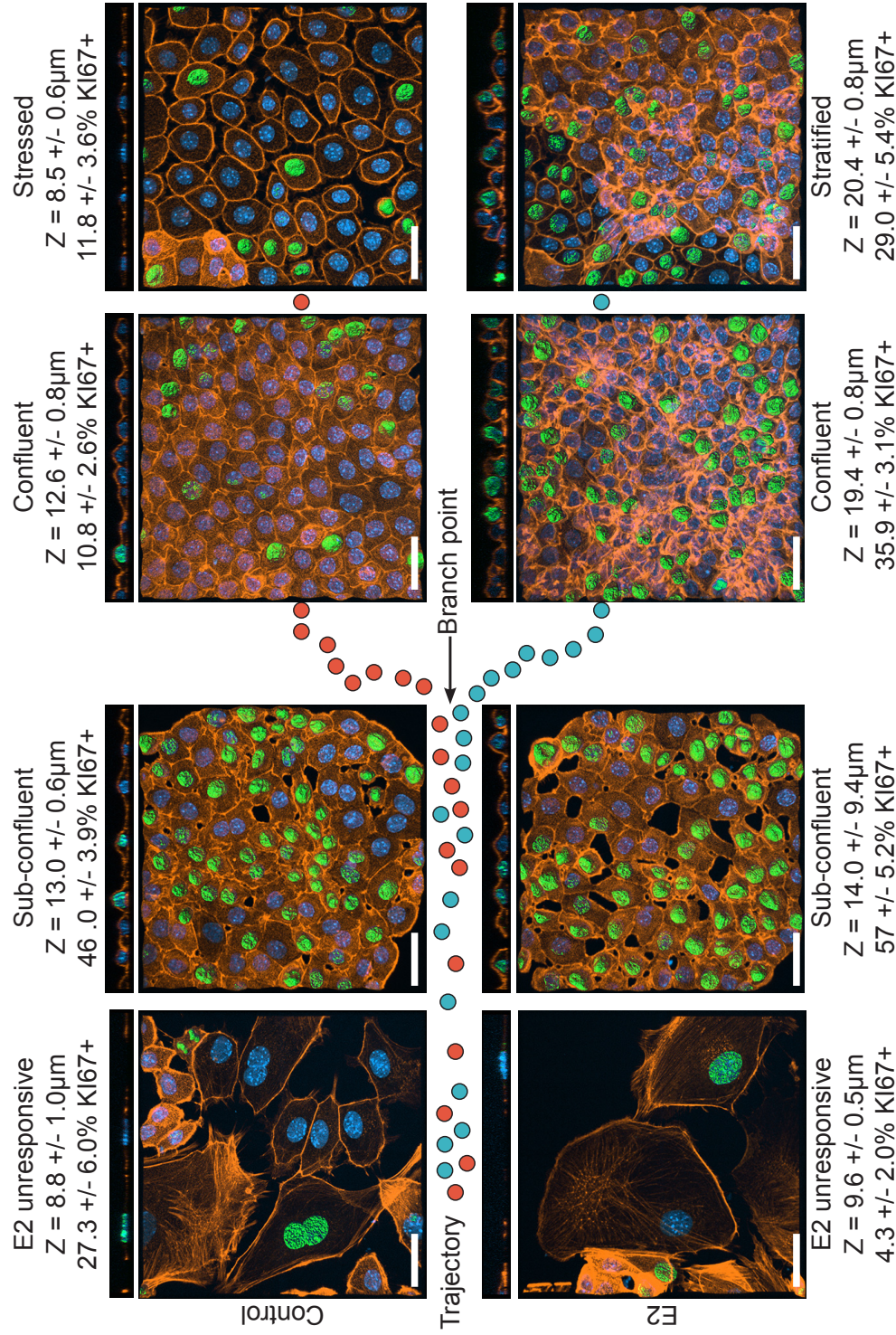


Figure 38: E2 promotes proliferation. Control and E2 treated OSE cells stained for Ki67 by IF. Green=Ki67, orange=Actin, blue=DAPI. %Ki67+=Ki67+nuclei/DAPI; n=6. Z=cell height; n=5-6. Mean±/S.E.M. Scale bars=30 μm. Representative images display z-stack maximal intensity projections of Actin and DAPI channels merged with Ki67 surface rendering. Axial view of field is also shown. Images were acquired using a Zeiss LSM800 AxioObserver Z1 system and analyzed using Imaris.

cultures, and presumably the last stage in the control-dominant branch of the trajectory, was SA- β Gal-positive cells that remain mostly Ki67-negative and have become enlarged and flattened in morphology (8.5 μ m), characteristic of senescent cells (Terzi et al. 2016). In contrast, E2 treated cells remain proliferative at confluence and either progressively assume a columnar morphology or become stratified (19.4-20.4 μ m). No substantial difference in height was observable between columnar and stratified cells due to mounting of the coverslip. Consistent with the few E2 treated OSE cells existing in the control branch of the trajectory, some areas of SA- β Gal-positive cells are seen in E2 treated cultures, but most cells are SA- β Gal-negative.

3.4.7. *Greb1* is uniquely up-regulated in E2 induced stratified OSE

Of all the genes with branch-dependent gene expression dynamics, *Greb1* stood out as a gene in need of further analysis because *GREB1* is a known E2 responsive gene that is over-expressed in human ovarian cancer relative to healthy human OSE, has never been reported to be induced by E2 in untransformed cells, and is known to promote cancer progression (Laviolette et al. 2014; Hodgkinson & Vanderhyden 2014). In the trajectory, *Greb1* belonged in cluster 4 and was only detected in 37% of E2 treated cells that existed beyond the branch point (**Figure 39A**). IF staining of OSE cultures demonstrated that all control cells are GREB1-negative and GREB1 is only present in E2 induced stratified OSE (**Figure 39B**). Since GREB1 positive cells exist at the end of pseudotime in our trajectory and maps back to stratified OSE in culture, the combined data suggests that the modelled trajectory represents the process of E2 induced foci

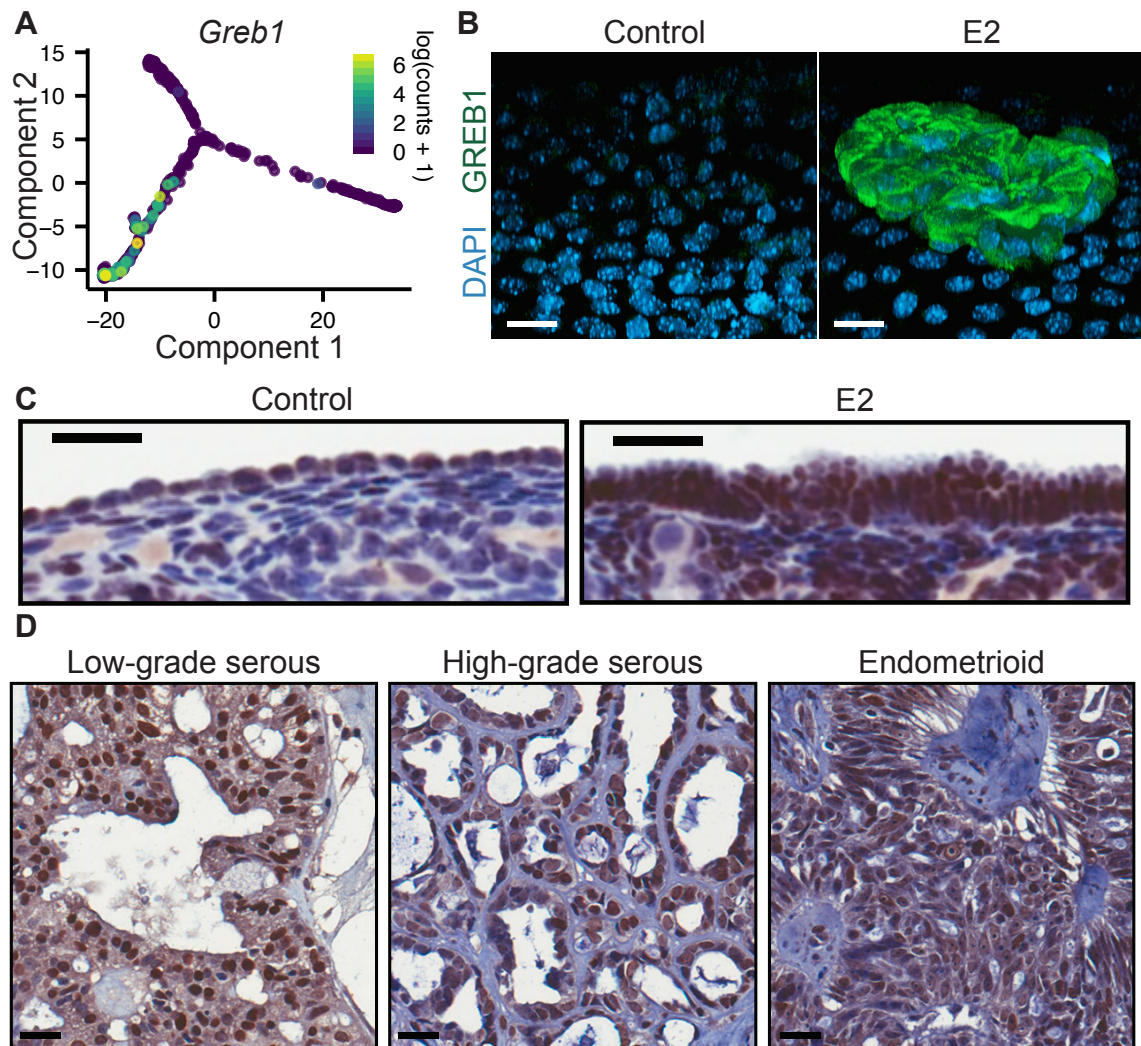


Figure 39: Prolonged E2 exposure leads to up-regulation of GREB1 in stratified OSE cells. (A) Trajectory of OSE cells, coloured by the logged *Greb1* counts. (B) Control and E2 treated OSE cells stained for GREB1 by IF. Scale bars=15 μm . Representative images display z-stack maximal intensity projections of GREB1 and DAPI channels rotated 45° along Y-axis to best display image in XYZ dimension. n=6-8. Images were acquired using a Zeiss LSM800 AxioObserver Z1 system and analyzed using Imaris. (C-D) IHC staining of GREB1 in ovaries from placebo and E2 treated mice (C; n=4) and three subtypes of human ovarian cancer. See **Appendix E** for GREB1 IHC staining controls. (D) Low-grade serous (n=4), high-grade serous (n=16), and endometrioid (n=20). Scale bars= 25 μm .

formation where E2 treated cells first become columnar then progress into stratified OSE that are uniquely GREB1-positive.

To further validate that the trajectory is representative of foci formation *in vivo*, we stained ovaries of E2 treated mice and found increased expression of GREB1 in stratified OSE (**Figure 39C**). If, in humans, similar dysplastic populations give rise to ovarian tumours, a similar gene expression pattern may be expected. We stained 40 human ovarian tumour samples and found detectable GREB1 in 75-85% of each of the various subtypes (**Figure 39D**). Along with the activation of cancer-associated pathways as cells progress through this trajectory, these data support that the molecular profile of dysplastic regions may be consistent with pre-cancerous lesions in humans.

Chapter 4: Discussion

The OSE monolayer has always been documented as a heterogeneous population with multiple morphologies observable on the same ovary (Okamura & Katabuchi 2003). In this study, we have demonstrated that primary cultures of OSE cells seeded on tissue culture plates can replicate many of the characteristics observed with OSE *in vivo* and that this model system can be used to study the molecular mechanisms of OSE biology. Most notably, we developed an *in vitro* model system of E2 induced OSE dysplasia to address the main goal of this thesis: understand the molecular mechanisms by which estrogen therapy increases a woman's risk for ovarian cancer.

4.1: *In vitro* model of E2 induced OSE dysplasia

Primary cultures of OSE cells seeded on plastic adherent culture dishes and maintained in media containing 100 nM E2 for 15 days were able to reproduce the E2 induced dysplasia seen *in vivo*, thereby confirming that the OSE dysplasia observed in E2 treated mice occurs as a direct result of E2 and not because of indirect E2 effects possibly occurring *in vivo* such as altered gonadotropin secretion in the hypothalamic–pituitary–gonadal axis (Laviolette et al. 2010; Laws et al. 2014). Analysis of OSE cell actin structure by confocal microscopy validated that cells in culture become columnar with E2 treatment. Hyperplasia is defined by a benign abnormal increase in number or proliferation of cells. Growth curve experiments and Ki67 staining verified that OSE cultures display both increased number and proliferation of cells in response to prolonged E2 stimulation, thereby confirming that OSE cells in culture also become hyperplastic in response to E2. Visual inspection by phase contrast microscopy showed that E2 stimulated OSE tend to stratify, even in areas of sub-confluence. This ability to continue proliferating after reaching confluence strongly suggests dysregulation of positional cues conferred by polarity proteins and loss of contact inhibition (McClatchey & Yap 2012).

4.2: E2 promotes ovarian cancer by suppressing *Dab2*

4.2.1. E2 suppression of *Dab2* is mediated by the ESR1 pathway

Following E2 exposure, *Dab2* transcripts were significantly decreased within 3 hours and maximal reduction in DAB2 protein was observed after 48 hours. Use of ESR1 antagonists and OSE cells lacking ESR1 consistently showed that E2 suppression of *Dab2* is mediated by ESR1. Moreover, it appears that *Esr1* can also regulate *Dab2* in a

ligand independent manner because in steroid-free conditions, MPP inhibition of ESR1 or knockout of *Esr1* alone was sufficient to increase DAB2 expression. Given the rapid decrease in *Dab2* mRNA after E2 stimulation and the presence of a putative ERE conserved between mouse and humans proximal to the *Dab2* transcription start site, this suggested that ESR1 directly inhibits *Dab2* transcription. ChIP experiments revealed, however, that this ERE is not a *bona fide* ESR1 ERE in mouse ovarian epithelial cells as it does not bind ESR1 following E2 treatment.

4.2.2. E2 responsive miRNAs contribute to *Dab2* suppression and OSE dysplasia

To investigate miRNAs as an alternative mechanism of regulation, we performed microarray analysis and identified 110 miRNA candidates whose expression is altered by E2. By focusing on the most robustly increased miRNAs (>2-fold) and using target prediction, we identified miR-378 as a candidate miRNA capable of targeting *Dab2* in both mouse and human cells. Preliminary validation confirmed that miR-378 is both up-regulated by E2 and its mimics can suppress *Dab2*. Since our goal was to determine if miRNA up-regulation by E2 is a possible mechanism by which *Dab2* can be decreased, we initially only focused on miRNAs that were most robustly increased. It is nevertheless possible that *Dab2* suppression is a combined effort among multiple miRNAs. Using KEGG pathway analysis, we discovered that the E2 regulated miRNAs in MASE are significantly associated with synthesis and metabolism of fatty acids, the Hippo signalling pathway (the main regulator of contact inhibition), and adherens junctions. This is not surprising as E2 is known to play major roles in regulating epithelial morphology, fatty acid metabolism (Torre et al. 2014; Hong et al. 2004), and is consistent

with the results we generated using scRNA-Seq, where E2 regulation of metabolic pathways was frequently observed in OSE. E2 regulation of miRNAs has been studied in multiple E2 responsive tissue types, but little is known about E2 regulated miRNAs in ovarian epithelial cells. Of the miRNAs that were increased >2-fold in our miRNA microarray, only miR-193b had been previously reported to be E2 regulated (in MCF7 breast cancer cells) (Klinge 2015).

4.2.3. miR-378's relevance in ovarian cancer

This is the first time that miR-378 has been reported to be E2 inducible via the ESR1 pathway and a regulator of *Dab2*. So far, the mechanistic actions of miR-378 has only been investigated in the context of lipid metabolism, muscle biology, and angiogenesis (Krist et al. 2015). Since all of these biological processes can be influenced by E2 signalling (D'Eon et al. 2005; Capllonch-Amer et al. 2014; Albrecht & Pepe 2010), it would be interesting to determine if E2 mediates its effects via miR-378 in these systems. Further investigation of the mechanism of action of miR-378 in the context of ovarian epithelial cells would be equally warranted, because miR-378 has been reported to be over-expressed in ovarian cancer patient samples relative to normal ovarian epithelial cells (Chan et al. 2014). Due to miR-378's known role in angiogenesis and clinical observations that its low expression is associated with longer progression-free survival in patients who received bevacizumab, miR-378 has been suggested to serve as a biomarker for response to anti-angiogenic treatment in ovarian cancer (Chan et al. 2014).

4.2.4. E2 induced OSE dysplasia is downstream of *Dab2* suppression

Suppression of DAB2 at the protein level occurs in OSE within 48 hours but no morphological changes were observed under phase contrast microscopy during these early time points, indicating that down-regulation of *Dab2* occurs prior to OSE dysplasia. With continuous E2 exposure, dysplasia of the OSE monolayer occurred, where increased proliferation, formation of foci, and aberrant expression of CDH1 were observed. Using *Dab2*-floxed OSE, we have demonstrated that suppression of *Dab2* alone is sufficient to cause much of the dysplasia observed with E2 stimulation.

Contact inhibition is a behaviour exhibited by epithelial cells to ensure proper formation of an epithelial monolayer by inhibiting cell proliferation. Loss of contact inhibition can result in outgrowth of cells from the epithelial monolayer (McClatchey & Yap 2012), much like the foci of stratified cells observed in our OSE model system. To determine if contact inhibition of cell proliferation was disrupted by prolonged E2 exposure and *Dab2* loss, sub-confluent vs. confluent OSE were stained with Ki67. While *Dab2*-WT and *Dab2*-KO OSE cells showed equal proportions of proliferating cells when sub-confluent, there was a notable difference in confluent cultures where *Dab2*-KO had a higher proportion of proliferating cells relative to *Dab2*-WT OSE. The results suggest that loss of DAB2 does not alter the rate of cell proliferation per se, but DAB2 appears to play an important role in mediating contact inhibition because loss of DAB2 causes a consequent failure to form a quiescent monolayer.

4.2.5. *Dab2* mediates contact inhibition via proper localization of CDH1

In addition to increasing OSE cell proliferation, prolonged E2 exposure resulted in foci of stratified cells that demonstrated loss of apical-basal polarity as demonstrated by loss or mislocalization of CDH1. In areas where E2 treated OSE cells were not stratified, OSE cells had higher expression of CDH1. *Dab2*-KO OSE showed that loss of *Dab2* had no apparent impact on CDH1 protein levels, but was responsible for its mislocalization. Mislocalization of CDH1 due to loss of *Dab2* is likely a contributing factor to OSE dysplasia because cell adhesion mediated by CDH1 has been shown to be an initiator for contact inhibition (St. Croix et al. 1998). It is important to note that loss of DAB2 does not completely mitigate the ability of OSE cells to be contact inhibited, because *Dab2*-KO OSE still show a reduction in Ki67 positive cells as they reach confluence - albeit not to the same extent as *Dab2*-WT OSE. This is not surprising because contact inhibition is mediated by input from multiple pathways where adhesion molecules such as cadherins (i.e.: *Cdh1*) and catenins (i.e.: *Ctnna1*, *Ctnnb1*) can feed into the Hippo pathway by preventing nuclear translocation of YAP to inhibit proliferation. Alternatively, members of the Hippo pathway can directly prevent cell proliferation by deactivating growth factor receptors (i.e.: blocking of EGFR signalling cascade by NF2) (McClatchey & Yap 2012; Curto & McClatchey 2008). KEGG pathway analysis of our E2 treated MASE cell miRNA microarray data showed that E2 can contribute to Hippo signalling and adherens junction via miRNAs. To date, *Dab2* has only been shown to regulate the localization of CDH1 and CTNNB1 (Yang et al. 2007; Hocevar & Howe 2003).

4.2.6. DAB2 is a tumour suppressor

DAB2's tumour suppressive capacity was investigated using *Dab2*-floxed OSE cells over-expressing the oncogene *KRAS*^{G12D}. Results from our *in vitro* assays indicate that loss of *Dab2* alone is not sufficient for transformation and this is not surprising since the tgCAG-TAg mouse model of ovarian cancer has shown that E2 exposure without induction of TAg does not result in tumour development (Laviolette et al. 2010). DAB2's role as a tumour suppressor became clear in the presence of an oncogene as loss of *Dab2* allowed *KRAS*^{G12D} over-expressing OSE cells to bypass oncogene induced senescence and acquire anchorage independent growth, a characteristic feature of transformed cells (Borowicz et al. 2014). *KRAS*^{G12D}-induced senescence in untransformed cells is known to be due to activation of P53 (Serrano et al. 1997); so it may be that DAB2 is able to impair *KRAS*^{G12D}-induced P53 activation. *Dab2* is speculated to have a genetic interaction with *p53* where crosses between *Dab2*(+/-) and *p53*(-/-) mice resulted in sex biased embryonic lethality in females, but the mechanism by which this occurs has not been further investigated (Yang et al. 2006). DAB2's role in preventing anchorage independent growth is better understood as DAB2 has previously been reported to be a mediator of anoikis. Anoikis is a tissue homeostasis mechanism geared to prevent dysplastic or metastatic growth by initiating programmed cell death in the event that epithelial cells detach from their substrate (Taddei et al. 2012). Previous work has shown that over-expression of *DAB2* in breast cancer cells sensitizes them to anoikis and DAB2 is thought to do this by inhibiting ILK activity (Wang et al. 2001). Inhibition of anoikis can also be achieved by increased ERK activity (Taddei et al. 2012). Knockout of *Dab2* results in increased baseline levels of phosphorylated ERK (Tao et al. 2014), providing

an alternative pathway by which DAB2 intervenes with anchorage independent growth.

4.2.7. Physiological relevance of *Dab2* suppression by E2 in healthy OSE

Consistent with the dysplastic OSE phenotype of *Dab2* heterozygous mice wherein the haploinsufficiency causes OSE dysplasia (Yang et al. 2006), a 50% reduction in *Dab2* expression by E2 treatment was sufficient to cause OSE cell dysplasia *in vitro*. This indicates that complete loss of DAB2 is not required for OSE dysplasia to occur. *In vivo*, this change in cell morphology required prolonged suppression of *Dab2*, as OSE dysplasia was not consistently observed in conditional *Dab2* knockout mice until they were 6 months old (Moore et al. 2013). In contrast, mice treated with E2 pellets showed OSE dysplasia by 60 days, perhaps due to additional effects of E2 that are independent of DAB2. In addition to OSE dysplasia, *Dab2* heterozygous female mice have significantly reduced reproductive capacity and, by 6 months of age, they are sterile (Moore et al. 2013). This is likely associated with the uterine dysplasias that develop in these mice, and indicates that DAB2 plays an important role in maintaining epithelial stability in many parts of the female reproductive tract. We speculate its regulation by E2 may be a regular phenomenon during the estrous cycle. Since OSE cell proliferation has been documented to occur only before ovulation (Burdette et al. 2006; Singavarapu et al. 2010), when E2 levels are highest, we speculate that transient *Dab2* suppression during estrous would allow OSE cells to proliferate, despite the contact inhibition of neighbouring cells. Once E2 levels drop following ovulation, re-expression of *Dab2* would play a role in re-establishing a quiescent OSE monolayer.

4.2.8. Physiological relevance of DAB2 in normal FTE biology and dysplastic lesions

Since 70% of high-grade serous ovarian cancers are thought to be derived from the fallopian tubes (Kramer 2013), DAB2 loss observed in 85% of all ovarian cancers (Fazili et al. 1999; Zhang et al. 2014) suggests that loss of *DAB2* is also critical in the initiation of fallopian tube derived tumours. Little is known about the role of DAB2 in normal fallopian tube biology because previous studies investigating *Dab2*-KO mouse models were focused on the ovary (Morris et al. 2002; D. Yang et al. 2006; Moore et al. 2013). In humans, only one study has investigated DAB2 expression in non-malignant FTE cells where they observed that *DAB2* expression is predominantly expressed in secretory cells and DAB2 is decreased in fallopian tube samples collected from women in the luteal phase relative to samples collected in the follicular phase (Tone et al. 2008). These results contrast the findings in our study, but the authors noted that their results were only observable in *BRCA* mutation carriers and *BRCA* has been implicated in regulating steroid receptor activity. Here, we demonstrated that DAB2 is expressed in all cells of the mouse FTE and like the OSE layer, prolonged E2 exposure reduced DAB2 expression and increased areas of FTE dysplasia. The E2 induced FTE dysplasias were comprised of two distinct phenotypes: increased areas of stratified FTE in the infundibulum and hypertrophy of the FTE in the ampulla and isthmus regions (determined by increase in cell layer thickness without increase in proliferation).

4.2.9. E2 may also sensitize FTE to transformation

Our data showed that E2 treatment does not change the proportion of Ki67+ FTE cells in E2 treated mice relative to control. This is consistent with previous findings in

mice and baboons that also showed FTE cells do not proliferate in response to E2 or ovulation (King et al. 2011). However, E2 is thought to play a role in regulating the structure and function of FTE cells where it promotes the differentiation of ciliated cells and promotes secretory cell activity (Okada et al. 2004; Chen et al. 2013). Given that studies observing porcine oviduct epithelial cultures in estrous conditions showed increase FTE cell height and loss of cellular polarity after 2.5 days of high E2 conditions (Chen et al. 2013), it is not surprising that we observed increased FTE cell stratification and hypertrophy after exposing mice to high E2 over 60 days. PAX8 staining showed that the aberrant morphological changes occurred in both PAX8 positive and negative cells, suggesting that E2 can cause dysplasia in both secretory and ciliated FTE cells. To confirm this hypothesis, these samples should be stained with tubulin, a marker of ciliated FTE. Given DAB2's known function in regulating cell polarity, we would predict that the observed reduction of DAB2 in all FTE cells is contributing to their dysplastic changes.

4.2.10. E2 regulation of *DAB2* is conserved in humans

The dysplastic changes associated with loss of DAB2, including loss of contact inhibition and increased proliferation are known to be characteristic of ovarian cancers (Zhang et al. 2014; Yang et al. 2006; Roland et al. 2003; Fazili et al. 1999). Data extracted from human MCF7 cell microarray data (Saleh et al. 2011) showed evidence for *DAB2* regulation by *ESR1*, and by examining human ovarian cancer ascites samples, we found that E2 suppression of *DAB2* is conserved in human cells, suggesting that the results from our mouse study is translatable to humans. Next steps would include

validating this pathway using primary isolations of human OSE. Until then, we tentatively propose that one of the ways by which estrogen therapy increases a woman's risk for epithelial ovarian cancer is by down-regulation of DAB2. Protracted DAB2 suppression leads to epithelial disorganization and increased proliferation resulting in ovarian epithelial dysplasia that renders them more susceptible to transformation.

4.3: Other mechanisms of E2 induced OSE dysplasia

Experiments using *Dab2*-floxed OSE demonstrate that loss of *Dab2* plays a major role in promoting the stratified OSE phenotype and sensitizing OSE to transformation, but loss of *Dab2* is not responsible for all the observed changes seen with E2 treatment such as the increase or decrease of CDH1 protein levels. After our initial focus on *Dab2*, it was our goal to investigate the global impact prolonged E2 exposure had on ovarian epithelial cells. This was made possible by using scRNA-Seq technology as previous attempts using conventional methods to measure transcript and protein expression changes were confounded by the heterogeneous nature of OSE cells. PCA and trajectory analysis of this data set subsequently allowed us to resolve and paint a global mechanistic picture of how E2 disrupts OSE cell biology to ultimately give rise to pre-neoplastic lesions (**Figure 40**).

4.3.1. E2 promotes proliferation and cell survival pathways

In the *in vitro* model of E2 induced OSE dysplasia, where control and E2 treated OSE cells are expanded in culture over 15 days, we propose that sub-confluent cells from both cultures are phenotypically similar where they are proliferative, metabolically

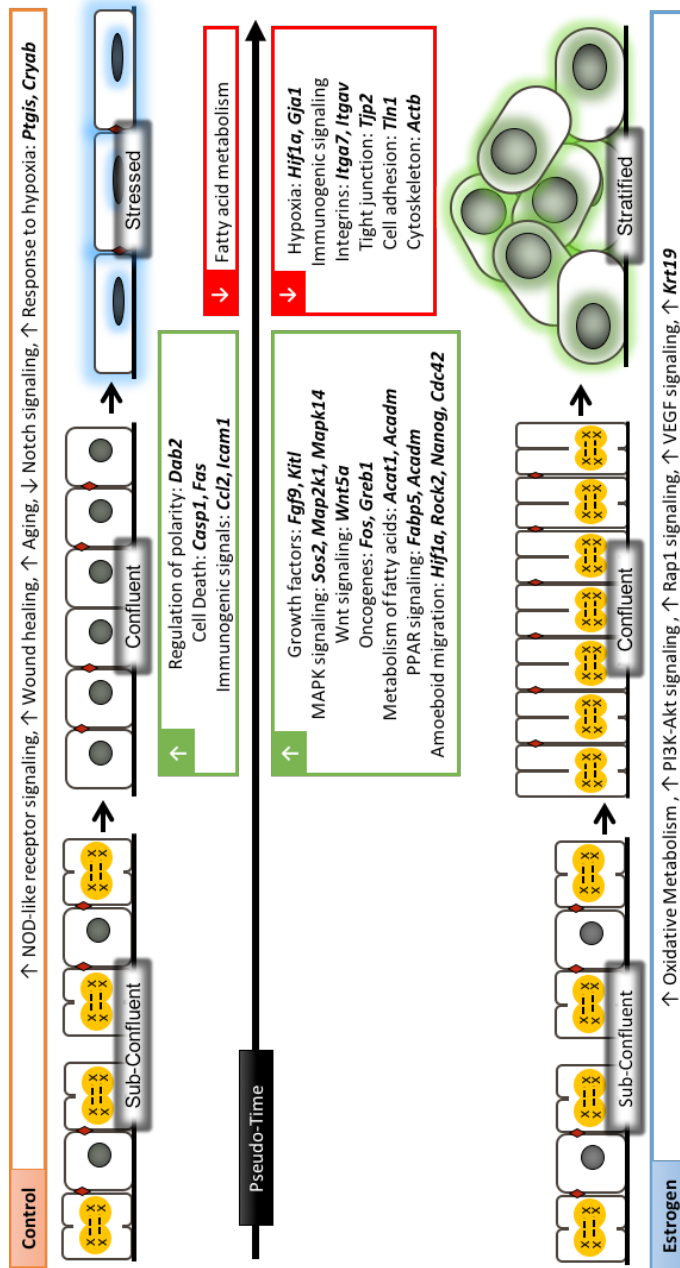


Figure 40: Molecular signaling mechanisms of estrogen-induced dysplasia in OSE. Sub-confluent cells from control and E2 cultures are phenotypically similar where they are proliferative, metabolically active, and cuboidal. With increasing confluence, cell-cell adhesions form between control cells and proliferation is slowed due to contact inhibition mediated by regulators of polarity. Without E2, control cells become stressed with increasing confluence and upregulate genes associated with hypoxia, cell death, and inflammatory responses involving immune cell recruitment through the TNF signalling pathway. E2 treated cells appear to suppress inflammatory immunogenic signals, overcome hypoxia via up-regulation of *Hif1a*, show up-regulation of growth factors, mediators of MAPK/Wnt/PI3K-Akt signaling, and known oncogenes. This increased proliferation is likely sustained by increased metabolism, where E2 treated OSE activate PPAR signaling to promote fatty acid oxidation. With persistent proliferation, columnar cells are first observed to maximally accommodate proliferative cells in an already confluent monolayer. While *Krt19* was consistently higher in E2 treated OSE cells, these cells show loss of epithelial architecture. E2 treated cells upregulate key genes involved in amoeboid migration which has been shown to be the preferred mode of migration of epithelial cancer cells to detach from the ECM and move through dense hypoxic conditions, providing a possible explanation for OSE stratification.

active, and cuboidal. With increasing confluence, cell-cell adhesions form between control cells and proliferation is slowed due to contact inhibition mediated by regulators of polarity, such as *Dab2*. Without E2 however, control cells become stressed with increasing confluence and upregulate genes associated with hypoxia (*Ptgis*, *Cryab*) (van de Schootbrugge et al. 2014), cell death (*Casp1*, *Fas*) (Itoh et al. 1991), and inflammatory responses involving immune cell recruitment through the TNF signalling pathway (*Ccl2*, *Icam1*) (Xu et al. 1996; Marlin & Springer 1987). E2 treated cells appear to overcome hypoxia via up-regulation of E2 responsive *Hif1a* (Yang et al. 2015) and *Connexin 43* (*Gja1*) (Sulkowska et al. 2015; Lin et al. 2003). Inflammatory immunogenic signals were suppressed in E2 treated cells, suggesting that E2 may contribute to an immune-restricted microenvironment, allowing pre-neoplastic lesions to develop and evade immune surveillance (Hanahan & Weinberg 2011). In addition to bypassing stress and apoptosis, E2 treated cells show up-regulation of growth factors (*Fgf9*, *Kitl*) mediators of MAPK signaling (*Map2k1*, *Mapk14*) Wnt signaling (*Wnt5a*) PI3K-Akt signaling, and *Fos*, a known E2 responsive oncogenic driver of proliferation and differentiation (Milde-Langosch 2005; Basu & Rowan 2005). This increased proliferation is likely sustained by increased metabolism, where E2 treated OSE activate PPAR signaling to promote fatty acid oxidation and mitochondrial enzymes (*Acat1*, *Acadm*) that can utilize these fatty acids in the Krebs cycle (Kanehisa et al. 2017).

4.3.2. E2 prioritizes sustained proliferation over maintaining epithelial architecture

Krt19 was consistently higher in E2 treated OSE cells in all analyses performed and no changes for markers of EMT were observed. While this suggests that E2 promotes

epithelial differentiation, components necessary for proper monolayer formation such as integrin (*Itga7*, *Itgav*), tight junction (*Tjp2*), cell adhesion (*Tln1*), and cytoskeleton (*Actb*) genes were all decreased in E2 treated cells. E2 induced columnar cells in confluent areas observed in the axial plane by confocal microscopy consistently showed more cells to be squeezed into a field of view relative to confluent control cells, suggesting that the increased cell height of columnar cells in E2 cultures may be to maximally accommodate proliferative cells in an already confluent monolayer while still allowing attachment to the basement membrane. With increased proliferation associated with progressive loss of epithelial architecture, E2 treated cells also upregulate key genes involved in amoeboid migration [*Rock2* (Jerrell et al. 2017), *Nanog* (Borrull et al. 2012), and *Cdc42* (Gadea et al. 2008)]. This phenotype can be driven by *Hif1a* (Lehmann et al. 2017) and has been shown to be the preferred mode of migration of epithelial cancer cells to detach from the extracellular matrix and move through dense hypoxic conditions, providing a possible explanation for OSE stratification.

4.3.3. Strategies for early detection and prevention of ovarian cancer

Given the lack of strategies for early detection of ovarian cancer and consequent poor patient prognosis, investigating genes upregulated throughout pseudotime in E2 induced areas of dysplasia may inform development of diagnostic and prognostic markers for E2 driven and responsive tumours. *Igfbp5* (Wang et al. 2006), *Enpp2* (Dicks et al. 2017), *Ctsh* (Kiyamova et al. 2012), *Lpl* (Sutphen et al. 2004), and *Tacc1* (Lv et al. 2014) are the top five genes correlated with the E2 branch in pseudotime and all are currently under investigation as cancer biomarkers. Preliminary studies in various human

pathologies have demonstrated that IGFBP5 (Shersher et al. 2011), ENPP2 (Reeves et al. 2015), ENPP2 metabolites (Sutphen et al. 2004) and LPL (Sutphen et al. 2004) are secreted factors detectable in serum. Given that they are up-regulated in pre-neoplastic cells, these are strong candidates to be investigated as markers for the early detection of ovarian cancer.

4.3.4. Investigation of *Greb1*'s role in ovarian cancer initiation

GREB1 is a known E2 responsive gene in breast and ovarian cancer that drives tumour progression (Laviolette et al. 2014), and it is currently being investigated as an alternative prognosis marker for tamoxifen treatment (Hodgkinson & Vanderhyden 2014). The novel finding that *Greb1* can be induced by E2 in untransformed OSE and is uniquely expressed in dysplastic cells suggests that *Greb1* may play a role in the initiation of tumour formation. Given that no one has investigated the role of *Greb1* in normal tissue, *Greb1* would make a strong and interesting candidate to be next pursued in depth using our *in vitro* model system of E2 induced OSE dysplasia to investigate its function in normal OSE biology and how *Greb1* contributes to ovarian cancer initiation.

Conclusions

In the majority of cases, cancer is considered to be a disease of the elderly and in post-menopausal women, estrogen therapy may be considered a double edged sword, for it can promote disease in the context of ovarian cancer, but it is also important for maintaining women's health to reduce the incidence of diabetes, cardiovascular disease,

and osteoporosis (Gupte et al. 2015; Sassarini & Lumsden 2015; Roforth et al. 2014). Using *in vitro* and *in vivo* mouse models, this body of work has taken the first steps in identifying molecular signalling mechanisms to elucidate how estrogen therapy is a risk factor for ovarian cancer.

The initial focus of this study was to determine if estrogen sensitizes normal healthy OSE and FTE to transformation by suppressing *Dab2* and understanding how this occurs. Our results show that *Dab2* is down-regulated by E2 across many cell types, it is an important mediator of maintaining OSE and FTE homeostasis, and that loss of *Dab2* is not a passenger effect of transformation but a key initiating event in perhaps 85% of all ovarian cancers (Fazili et al. 1999; Zhang et al. 2014). Tumourigenesis, however, is a multiple step process that requires accumulation of many abnormal genetic and physiological changes to push a normal cell to transformation. scRNA-Seq was subsequently used to fully appreciate the heterogeneous nature of OSE cells and to fully understand how sustained E2 stimulation leads to OSE dysplasia and sensitizes them to transformation. In doing so, we resolved the asynchronous response to estrogen that OSE cells display and discovered that columnar and stratified OSE are a reflection of one E2 responsive population at different stages of OSE dysplasia, where stratified OSE represent a population of OSE cells poised for transformation. Echoing the “Hallmarks of Cancer” by Hanahan and Weinberg (2011), trajectory analysis reveals that E2 allows ovarian epithelial cells to progressively acquire the ability to resist cell death, sustain proliferative signaling, evade growth suppressors, induce angiogenesis, increase metabolism, avoid immune destruction, and eventually activate invasion and metastasis.

The global molecular signalling mechanisms dysregulated by E2 identified in this study offers a mechanistic explanation for the epidemiologic evidence linking estrogen therapy to increased ovarian cancer risk and revealed potential biomarkers for early detection. Currently, the scRNA-Seq data remains largely correlative and additional biological assays are required to validate the model and delineate cause from effect. Nevertheless, this study is the first in the field of ovarian cancer to use scRNA-Seq to address the initiating events using untransformed primary cells and will no doubt provide a solid foundation for supporting current hypotheses, provide a framework for developing new strategies for ovarian cancer prevention, and mitigate the risk associated with hormone therapy so to better develop strategies in maintaining women's health in the aging female population.

References

- Albrecht, E.D. & Pepe, G.J., 2010. Estrogen regulation of placental angiogenesis and fetal ovarian development during primate pregnancy. *The International Journal of Developmental Biology*, 54(2-3), pp.397–408.
- American Cancer Society, 2016. *Cancer Facts and Figures 2016*, Atlanta: American Cancer Society.
- Auersperg, N., 2002. Early events in ovarian epithelial carcinogenesis: progress and problems in experimental approaches. *International Journal of Gynecological Cancer*, 12(6), pp.691–703.
- Auersperg, N., 2013. Ovarian surface epithelium as a source of ovarian cancers: Unwarranted speculation or evidence-based hypothesis? *Gynecologic Oncology*, pp.1–6.
- Auersperg, N., 2001. Ovarian Surface Epithelium: Biology, Endocrinology, and Pathology. *Endocrine Reviews*, pp.1–34.
- Bai, W. & Nicosia, S.V., 2000. Estrogen Stimulation of Ovarian Surface Epithelial Cell Proliferation. *In Vitro Cellular Developmental Biology - Animal*, 36(10), pp.657–666.
- Barrett, K. et al., 2009. *Ganong's Review of Medical Physiology* 23rd ed., The McGraw-Hill Companies, Inc.
- Basu, A. & Rowan, B.G., 2005. Genes related to estrogen action in reproduction and breast cancer. *Frontiers in Bioscience*, 10, pp.2346–2372.
- Bhagat, R. et al., 2013. Altered expression of β -catenin, E-cadherin, and E-cadherin promoter methylation in epithelial ovarian carcinoma. *Tumour biology*, 34(4), pp.2459–2468.
- Borowicz, S. et al., 2014. The Soft Agar Colony Formation Assay. *Journal of Visualized Experiments: JoVE*, (92), p.51998.
- Borrull, A. et al., 2012. Nanog and Oct4 overexpression increases motility and transmigration of melanoma cells. *Journal of cancer research and clinical oncology*, 138(7), pp.1145–1154.
- Bourdeau, V. et al., 2004. Genome-Wide Identification of High-Affinity Estrogen Response Elements in Human and Mouse. *Molecular Endocrinology*, 18(6), pp.1411–1427.
- Bray, N.L. et al., 2016. Near-optimal probabilistic RNA-seq quantification. *Nature biotechnology*, 34(5), pp.525–527.

Britt, K.L. & Findlay, J.K., 2002. Estrogen actions in the ovary revisited. *The Journal of Endocrinology*, (175), pp.269–276.

Burdette, J.E. et al., 2006. Gonadotropin-induced superovulation drives ovarian surface epithelia proliferation in CD1 mice. *Endocrinology*, 147(5), pp.2338–2345.

Canadian Cancer Society, 2016. *Canadian Cancer Statistics 2016*, Toronto: Canadian Cancer Society.

Capllonch-Amer, G. et al., 2014. Estradiol stimulates mitochondrial biogenesis and adiponectin expression in skeletal muscle. *The Journal of Endocrinology*, 221(3), pp.391–403.

Chan, J.K. et al., 2014. MiR-378 as a biomarker for response to anti-angiogenic treatment in ovarian cancer. *Gynecologic Oncology*, 133(3), pp.568–574.

Chao, A. et al., 2012. Regulation of ovarian cancer progression by microRNA-187 through targeting Disabled homolog-2. *Oncogene*, 31(6), pp.764–775.

Chen, S., Einspanier, R. & Schoen, J., 2013. In vitro mimicking of estrous cycle stages in porcine oviduct epithelium cells: estradiol and progesterone regulate differentiation, gene expression, and cellular function. *Biology of Reproduction*, 89(3), p.54.

Coetzee, S.G. et al., 2015. Cell Type Specific Enrichment of Risk Associated Regulatory Elements at Ovarian Cancer Susceptibility Loci. *Human molecular genetics*.

Collaborative Group on Epidemiological Studies of Ovarian Cancer, 2015. Menopausal hormone uses and ovarian cancer risk: individual participant meta-analysis of 52 epidemiological studies. *Lancet*, 385(9980), pp.1835–1842.

Coscia, F. et al., 2016. Integrative proteomic profiling of ovarian cancer cell lines reveals precursor cell associated proteins and functional status. *Nature communications*, 7, p.12645.

Courtois-Cox, S., Jones, S.L. & Cichowski, K., 2008. Many roads lead to oncogene-induced senescence. *Oncogene*, 27(20), pp.2801–2809.

Couse, J.F. & Korach, K.S., 1999. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocrine Reviews*, 20(3), pp.358–417.

Curto, M. & McClatchey, A.I., 2008. Nf2/Merlin: a coordinator of receptor signalling and intercellular contact. *British journal of cancer*, 98(2), pp.256–262.

D'Eon, T.M. et al., 2005. Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. *The Journal of biological chemistry*, 280(43), pp.35983–35991.

- Dicks, E. et al., 2017. Germline whole exome sequencing and large-scale replication identifies FANCM as a likely high grade serous ovarian cancer susceptibility gene. *Oncotarget*, 8(31), pp.50930–50940.
- Du, L. et al., 2014. miR-93-directed downregulation of DAB2 defines a novel oncogenic pathway in lung cancer. *Oncogene*, 33(34), pp.4307–4315.
- Ellison, T.N.A.L., 2015. *Ovarian cancer: Survival statistics*, Statistics Canada.
- Fazili, Z. et al., 1999. Disabled-2 inactivation is an early step in ovarian tumorigenicity. *Oncogene*, 18(20), pp.3104–3113.
- Ferraro, L. et al., 2012. Effects of oestrogen on microRNA expression in hormone-responsive breast cancer cells. *Hormones and Cancer*, 3(3), pp.65–78.
- Gadea, G. et al., 2008. DOCK10-mediated Cdc42 activation is necessary for amoeboid invasion of melanoma cells. *Current biology: CB*, 18(19), pp.1456–1465.
- Gamwell, L.F., Collins, O. & Vanderhyden, B.C., 2012. The mouse ovarian surface epithelium contains a population of LY6A (SCA-1) expressing progenitor cells that are regulated by ovulation-associated factors. *Biology of Reproduction*, 87(4), p.80.
- Gotfredson, G.S. & Murdoch, W.J., 2007. Morphologic responses of the mouse ovarian surface epithelium to ovulation and steroid hormonal milieu. *Experimental biology and medicine (Maywood, N.J.)*, 232(2), pp.277–280.
- Gulliver, L.S.M. & Hurst, P.R., 2012. Repeat estradiol exposure differentially regulates protein expression patterns for estrogen receptor and E-cadherin in older mouse ovarian surface epithelium: Implications for replacement and adjuvant hormone therapies? *Steroids*, 77(6), pp.674–685.
- Gupte, A.A., Pownall, H.J. & Hamilton, D.J., 2015. Estrogen: an emerging regulator of insulin action and mitochondrial function. *Journal of diabetes research*, 2015, p.916585.
- Hanahan, D. & Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *Cell*, 144(5), pp.646–674.
- Hewitt, S.C. et al., 2010. Biological and biochemical consequences of global deletion of exon 3 from the ER alpha gene. *FASEB Journal*, 24(12), pp.4660–4667.
- Hocevar, B.A. & Howe, P.H., 2003. Regulation of the Wnt signaling pathway by disabled-2 (Dab2). *The EMBO Journal*, 22(12), pp.3084–3094.
- Hodgkinson, K.M. & Vanderhyden, B.C., 2014. Consideration of GREB1 as a potential therapeutic target for hormone-responsive or endocrine-resistant cancers. *Expert opinion on therapeutic targets*, 18(9), pp.1065–1076.

- Hong, S.H. et al., 2004. Expression of estrogen receptor-alpha and -beta, glucocorticoid receptor, and progesterone receptor genes in human embryonic stem cells and embryoid bodies. *Molecules and cells*, 18(3), pp.320–325.
- Huang, D.W., Sherman, B.T. & Lempicki, R.A., 2009a. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research*, 37(1), pp.1–13.
- Huang, D.W., Sherman, B.T. & Lempicki, R.A., 2009b. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols*, 4(1), pp.44–57.
- Ignatov, T. et al., 2013. GPER-1 acts as a tumor suppressor in ovarian cancer. *Journal of ovarian research*, 6(1), p.51.
- Ing, N.H., 2016. Post-transcriptional Regulation of Steroid Hormone Receptors. In P. Menon K M J & P. Goldstrohm Aaron, eds. *Post-transcriptional Mechanisms in Endocrine Regulation*. Post-transcriptional Mechanisms in Endocrine Regulation. Cham: Springer International Publishing, pp. 91–107.
- Itahana, K., Itahana, Y. & Dimri, G.P., 2013. Colorimetric detection of senescence-associated β galactosidase. *Methods in molecular biology (Clifton, N.J.)*, 965(Chapter 8), pp.143–156.
- Itoh, N. et al., 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*, 66(2), pp.233–243.
- Jayson, G.C. et al., 2014. Ovarian cancer. *The Lancet*, 384(9951), pp.1376–1388.
- Jerrell, R.J., Leih, M.J. & Parekh, A., 2017. The ROCK isoforms differentially regulate the morphological characteristics of carcinoma cells. *Small GTPases*.
- Juríková, M. et al., 2016. Ki67, PCNA, and MCM proteins: Markers of proliferation in the diagnosis of breast cancer. *Acta histochemica*, 118(5), pp.544–552.
- Kanehisa, M. et al., 2017. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic acids research*, 45(D1), pp.D353–D361.
- Kantak, S.S. & Kramer, R.H., 1998. E-cadherin regulates anchorage-independent growth and survival in oral squamous cell carcinoma cells. *The Journal of biological chemistry*, 273(27), pp.16953–16961.
- Karst, A.M. & Drapkin, R., 2012. Primary culture and immortalization of human fallopian tube secretory epithelial cells. *Nature protocols*, 7(9), pp.1755–1764.
- King, S.M. et al., 2011. The impact of ovulation on fallopian tube epithelial cells: evaluating three hypotheses connecting ovulation and serous ovarian cancer. *Endocrine-related cancer*, 18(5), pp.627–642.

- Kiselev, V.Y. et al., 2017. SC3: consensus clustering of single-cell RNA-seq data. *Nature methods*, 14(5), pp.483–486.
- Kiyamova, R. et al., 2012. Preliminary study of thyroid and colon cancers-associated antigens and their cognate autoantibodies as potential cancer biomarkers. *Biomarkers: biochemical indicators of exposure, response, and susceptibility to chemicals*, 17(4), pp.362–371.
- Klinge, C.M., 2009. Estrogen Regulation of MicroRNA Expression. *Current genomics*, 10(3), pp.169–183.
- Klinge, C.M., 2015. miRNAs regulated by estrogens, tamoxifen, and endocrine disruptors and their downstream gene targets. *Molecular and Cellular Endocrinology*.
- Kramer, L., 2013. Mixed reviews on removing fallopian tubes to prevent ovarian cancer. *Canadian Medical Association Journal*, 185(9), p.E391.
- Krist, B. et al., 2015. The Role of miR-378a in Metabolism, Angiogenesis, and Muscle Biology. *International journal of endocrinology*, 2015, p.281756.
- Laviolette, L.A. et al., 2010. 17 β -Estradiol Accelerates Tumor Onset and Decreases Survival in a Transgenic Mouse Model of Ovarian Cancer. *Endocrinology*, 151(3), pp.929–938.
- Laviolette, L.A. et al., 2014. 17 β -estradiol upregulates GREB1 and accelerates ovarian tumor progression in vivo. *International journal of cancer*, 135(5), pp.1072–1084.
- Laws, M.J. et al., 2014. Dysregulated estrogen receptor signaling in the hypothalamic-pituitary-ovarian axis leads to ovarian epithelial tumorigenesis in mice. *PLoS genetics*, 10(3), p.e1004230.
- Lee, J.M., 2006. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *The Journal of Cell Biology*, 172(7), pp.973–981.
- Lee, M. & Vasioukhin, V., 2008. Cell polarity and cancer - cell and tissue polarity as a non-canonical tumor suppressor. *Journal of Cell Science*, 121(8), pp.1141–1150.
- Lehmann, S. et al., 2017. Hypoxia Induces a HIF-1-Dependent Transition from Collective-to-Amoeboid Dissemination in Epithelial Cancer Cells. *Current biology: CB*, 27(3), pp.392–400.
- Li, S. et al., 2017. Estrogen receptor α is required for oviductal transport of embryos. *FASEB Journal*, p.fj.201601128R.
- Lin, J.H.-C. et al., 2003. Connexin mediates gap junction-independent resistance to cellular injury. *The Journal of Neuroscience*, 23(2), pp.430–441.

- Lun, A.T.L., Bach, K. & Marioni, J.C., 2016. Pooling across cells to normalize single-cell RNA sequencing data with many zero counts. *Genome biology*, 17(1), p.75.
- Lun, A.T.L., McCarthy, D.J. & Marioni, J.C., 2016. A step-by-step workflow for low-level analysis of single-cell RNA-seq data with Bioconductor. *F1000Research*, 5, p.2122.
- Lv, J. et al., 2014. Prognosis significance of HER2 status and TACC1 expression in patients with gastric carcinoma. *Medical oncology (Northwood, London, England)*, 31(11), p.280.
- MacCalman, C.D., Farookhi, R. & Blaschuk, O.W., 1994. Estradiol regulates E-cadherin mRNA levels in the surface epithelium of the mouse ovary. *Clinical & experimental metastasis*, 12(4), pp.276–282.
- Maniti, O., Carvalho, K. & Picart, C., 2012. Model membranes to shed light on the biochemical and physical properties of ezrin/radixin/moesin. *Biochimie*, pp.1–9.
- Marlin, S.D. & Springer, T.A., 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell*, 51(5), pp.813–819.
- Mählck, C.G., Bäckström, T. & Kjellgren, O., 1988. Plasma level of estradiol in patients with ovarian malignant tumors. *Gynecologic Oncology*, 30(3), pp.313–320.
- McCarthy, D.J. et al., 2017. Scater: pre-processing, quality control, normalization and visualization of single-cell RNA-seq data in R. *Bioinformatics (Oxford, England)*, 33(8), pp.1179–1186.
- McClatchey, A.I. & Yap, A.S., 2012. Contact inhibition (of proliferation) redux. *Current opinion in cell biology*, 24(5), pp.685–694.
- McCloskey, C.W. et al., 2014. A new spontaneously transformed syngeneic model of high-grade serous ovarian cancer with a tumor-initiating cell population. *Frontiers in oncology*, 4, p.53.
- Milde-Langosch, K., 2005. The Fos family of transcription factors and their role in tumourigenesis. *European journal of cancer (Oxford, England: 1990)*, 41(16), pp.2449–2461.
- Mok, S.C. et al., 1994. Molecular cloning of differentially expressed genes in human epithelial ovarian cancer. *Gynecologic Oncology*, 52(2), pp.247–252.
- Moore, R. et al., 2013. Differential requirement for Dab2 in the development of embryonic and extra-embryonic tissues. *BMC Developmental Biology*, 13, p.39.
- Morris, S.M. et al., 2002. Dual roles for the Dab2 adaptor protein in embryonic

development and kidney transport. *The EMBO Journal*, 21(7), pp.1555–1564.

Nilsson, E.E. & Skinner, M.K., 2003. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biology of Reproduction*, 69(4), pp.1265–1272.

Okada, A. et al., 2004. Role of foxj1 and estrogen receptor alpha in ciliated epithelial cell differentiation of the neonatal oviduct. *Journal of molecular endocrinology*, 32(3), pp.615–625.

Okamura, H. & Katabuchi, H., 2003. Cell biology of human ovarian surface epithelial cells and ovarian carcinogenesis. *Med Electron Microsc*, (36), pp.74–86.

Park, S.-H. et al., 2008. Estrogen regulates Snail and Slug in the down-regulation of E-cadherin and induces metastatic potential of ovarian cancer cells through estrogen receptor alpha. *Molecular Endocrinology*, 22(9), pp.2085–2098.

Plaino, L. et al., 2000. Ovarian follicular fluid contains immunoreactive estriol: lack of correlation with estradiol concentrations. *Gynecological endocrinology: the official journal of the International Society of Gynecological Endocrinology*, 14(4), pp.231–235.

Prossnitz, E.R. & Barton, M., 2014. Estrogen biology: new insights into GPER function and clinical opportunities. *Molecular and Cellular Endocrinology*, 389(1-2), pp.71–83.

Qiu, X., Hill, A., et al., 2017. Single-cell mRNA quantification and differential analysis with Census. *Nature methods*, 14(3), pp.309–315.

Qiu, X., Mao, Q., et al., 2017. Reversed graph embedding resolves complex single-cell developmental trajectories. *bioRxiv*, p.110668.

Reeves, V.L. et al., 2015. Serum Autotaxin/ENPP2 correlates with insulin resistance in older humans with obesity. *Obesity (Silver Spring, Md.)*, 23(12), pp.2371–2376.

Reid, B.M., Permuth, J.B. & Sellers, T.A., 2017. Epidemiology of ovarian cancer: a review. *Cancer biology & medicine*, 14(1), pp.9–32.

Roforth, M.M. et al., 2014. Dissection of estrogen receptor alpha signaling pathways in osteoblasts using RNA-sequencing. *PloS one*, 9(4), p.e95987.

Roland, I.H. et al., 2003. Loss of surface and cyst epithelial basement membranes and preneoplastic morphologic changes in prophylactic oophorectomies. *Cancer*, 98(12), pp.2607–2623.

Saleh, Al, S., Mulla, Al, F. & Luqmani, Y.A., 2011. Estrogen receptor silencing induces epithelial to mesenchymal transition in human breast cancer cells. *PloS one*, 6(6), p.e20610.

- Sassarini, J. & Lumsden, M.A., 2015. Oestrogen replacement in postmenopausal women. *Age and ageing*, 44(4), pp.551–558.
- Serrano, M. et al., 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*, 88(5), pp.593–602.
- Shersher, D.D. et al., 2011. Biomarkers of the insulin-like growth factor pathway predict progression and outcome in lung cancer. *The Annals of thoracic surgery*, 92(5), pp.1805–11– discussion 1811.
- Singavarapu, R. et al., 2010. Whole ovary immunohistochemistry for monitoring cell proliferation and ovulatory wound repair in the mouse. *Reproductive biology and endocrinology: RB&E*, 8(1), p.98.
- Soneson, C., Love, M.I. & Robinson, M.D., 2016. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research*, 4, p.1521.
- Song, J. et al., 2005. Estradiol-induced ezrin overexpression in ovarian cancer: a new signaling domain for estrogen. *Cancer Letters*, 220(1), pp.57–65.
- St Croix, B. et al., 1998. E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1). *The Journal of Cell Biology*, 142(2), pp.557–571.
- Stewart, C.A. & Behringer, R.R., 2012. Mouse oviduct development. *Results and problems in cell differentiation*, 55, pp.247–262.
- Sulkowska, U. et al., 2015. Eventual proapoptotic or anti-apoptotic impact of aberrantly expressed Cx43 and Cx26 can depend on ER-alpha overexpression in human endometrioid adenocarcinoma. *Gynecological Endocrinology*, 31(8), pp.604–608.
- Sutphen, R. et al., 2004. Lysophospholipids are potential biomarkers of ovarian cancer. *Cancer epidemiology, biomarkers & prevention*, 13(7), pp.1185–1191.
- Taddei, M.L. et al., 2012. Anoikis: an emerging hallmark in health and diseases. *The Journal of pathology*, 226(2), pp.380–393.
- Taniguchi, F., 2017. New knowledge and insights about the malignant transformation of endometriosis. *The journal of obstetrics and gynaecology research*, 43(7), pp.1093–1100.
- Tao, W. et al., 2014. Hormonal induction and roles of disabled-2 in lactation and involution. *PloS one*, 9(10), p.e110737.
- Terzi, M.Y., Izmirli, M. & Gogebakan, B., 2016. The cell fate: senescence or quiescence. *Molecular biology reports*, 43(11), pp.1213–1220.

- Tone, A.A. et al., 2008. Gene expression profiles of luteal phase fallopian tube epithelium from BRCA mutation carriers resemble high-grade serous carcinoma. *Clinical cancer research*, 14(13), pp.4067–4078.
- Torre, Della, S. et al., 2014. Energy metabolism and fertility - a balance preserved for female health. *Nature Reviews Endocrinology*, 10(1), pp.13–23.
- van de Schootbrugge, C. et al., 2014. Effect of hypoxia on the expression of α B-crystallin in head and neck squamous cell carcinoma. *BMC cancer*, 14(1), p.252.
- van Dijk, D. et al., 2017. MAGIC: A diffusion-based imputation method reveals gene-gene interactions in single-cell RNA-sequencing data. *bioRxiv*, p.111591.
- Vlachos, I.S. et al., 2015. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. *Nucleic acids research*, 43(W1), pp.W460–W466.
- Vrtačnik, P. et al., 2014. The many faces of estrogen signaling. *Biochemia Medica*, 24(3), pp.329–342.
- Wald, F.A. et al., 2008. Atypical protein kinase C (iota) activates ezrin in the apical domain of intestinal epithelial cells. *Journal of Cell Science*, 121(5), pp.644–654.
- Wang, H. et al., 2006. Insulin-like growth factor-binding protein 2 and 5 are differentially regulated in ovarian cancer of different histologic types. *Modern pathology*, 19(9), pp.1149–1156.
- Wang, S.C. et al., 2001. DOC-2/hDab-2 inhibits ILK activity and induces anoikis in breast cancer cells through an Akt-independent pathway. *Oncogene*, 20(47), pp.6960–6964.
- Wang, W.S. et al., 2013. Expression and significance of twist and E-cadherin in ovarian cancer tissues. *Asian Pacific journal of cancer prevention*, 14(2), pp.669–672.
- Webb, P.M. & Jordan, S.J., 2016. Epidemiology of epithelial ovarian cancer. *Best practice & research. Clinical obstetrics & gynaecology*.
- Wong, L. et al., 2007. Endogenous Controls for Real-Time Quantitation of miRNA Using TaqMan. *Applied Biosystems*, pp.1–8. Available at: www.appliedbiosystems.com [Accessed December 29, 2016].
- Xu, L. et al., 1996. Human recombinant monocyte chemotactic protein and other C-C chemokines bind and induce directional migration of dendritic cells in vitro. *Journal of leukocyte biology*, 60(3), pp.365–371.
- Yang, D.H. et al., 2006. Disabled-2 Heterozygous Mice Are Predisposed to Endometrial and Ovarian Tumorigenesis and Exhibit Sex-Biased Embryonic Lethality in a p53-Null Background. *The American Journal of Pathology*, 169(1),

pp.258–267.

Yang, D.H. et al., 2007. Disabled-2 Is an Epithelial Surface Positioning Gene. *The Journal of biological chemistry*, 282(17), pp.13114–13122.

Yang, D.H. et al., 2002. Disabled-2 is essential for endodermal cell positioning and structure formation during mouse embryogenesis. *Developmental biology*, 251(1), pp.27–44.

Yang, J. et al., 2015. Estrogen receptor- α directly regulates the hypoxia-inducible factor 1 pathway associated with antiestrogen response in breast cancer. *PNAS*, 112(49), pp.15172–15177.

Yano, T. et al., 2017. Apical cytoskeletons and junctional complexes as a combined system in epithelial cell sheets. *Annals of the New York Academy of Sciences*, 1405(1), pp.32–43.

Yoshida, J. et al., 2009. Changes in the expression of E-cadherin repressors, Snail, Slug, SIP1, and Twist, in the development and progression of ovarian carcinoma: the important role of Snail in ovarian tumorigenesis and progression. *Medical molecular morphology*, 42(2), pp.82–91.

Zhang, L. et al., 2016. Effects of Kras activation and Pten deletion alone or in combination on MUC1 biology and epithelial-to-mesenchymal transition in ovarian cancer. *Oncogene*, 35(38), pp.5010–5020.

Zhang, L. et al., 2006. Integrative genomic analysis of protein kinase C (PKC) family identifies PKC ζ as a biomarker and potential oncogene in ovarian carcinoma. *Cancer research*, 66(9), pp.4627–4635.

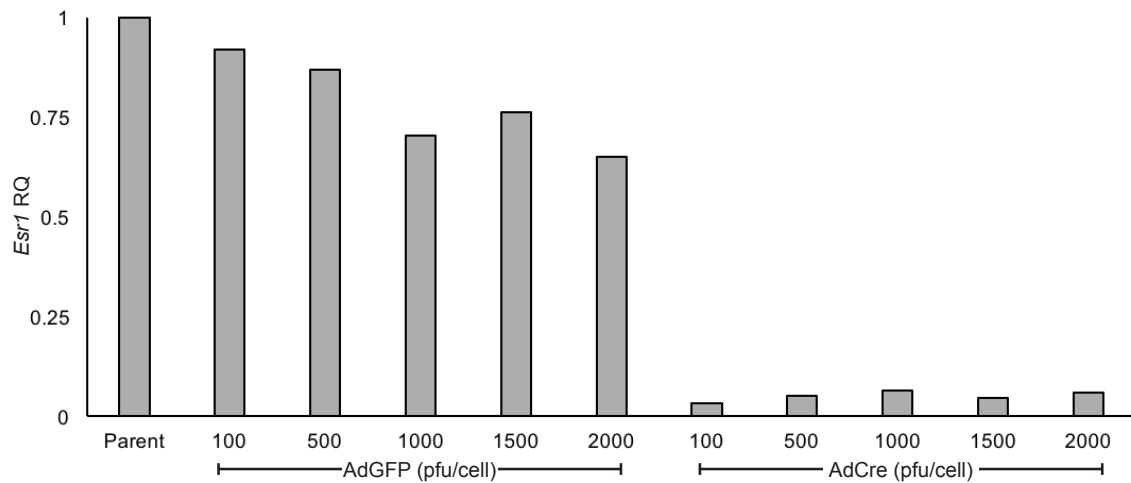
Zhang, Z. et al., 2014. Frequent loss expression of dab2 and promotor hypermethylation in human cancers: a meta-analysis and systematic review. *Pakistan journal of medical sciences*, 30(2), pp.432–437.

Zhou, W. & Slingerland, J.M., 2014. Links between oestrogen receptor activation and proteolysis: relevance to hormone-regulated cancer therapy. *Nature reviews. Cancer*, 14(1), pp.26–38.

Zhu, Y., Nilsson, M. & Sundfeldt, K., 2010. Phenotypic Plasticity of the Ovarian Surface Epithelium: TGF- β 1 Induction of Epithelial to Mesenchymal Transition (EMT) in Vitro. *Endocrinology*, 151(11), pp.5497–5505.

Appendices

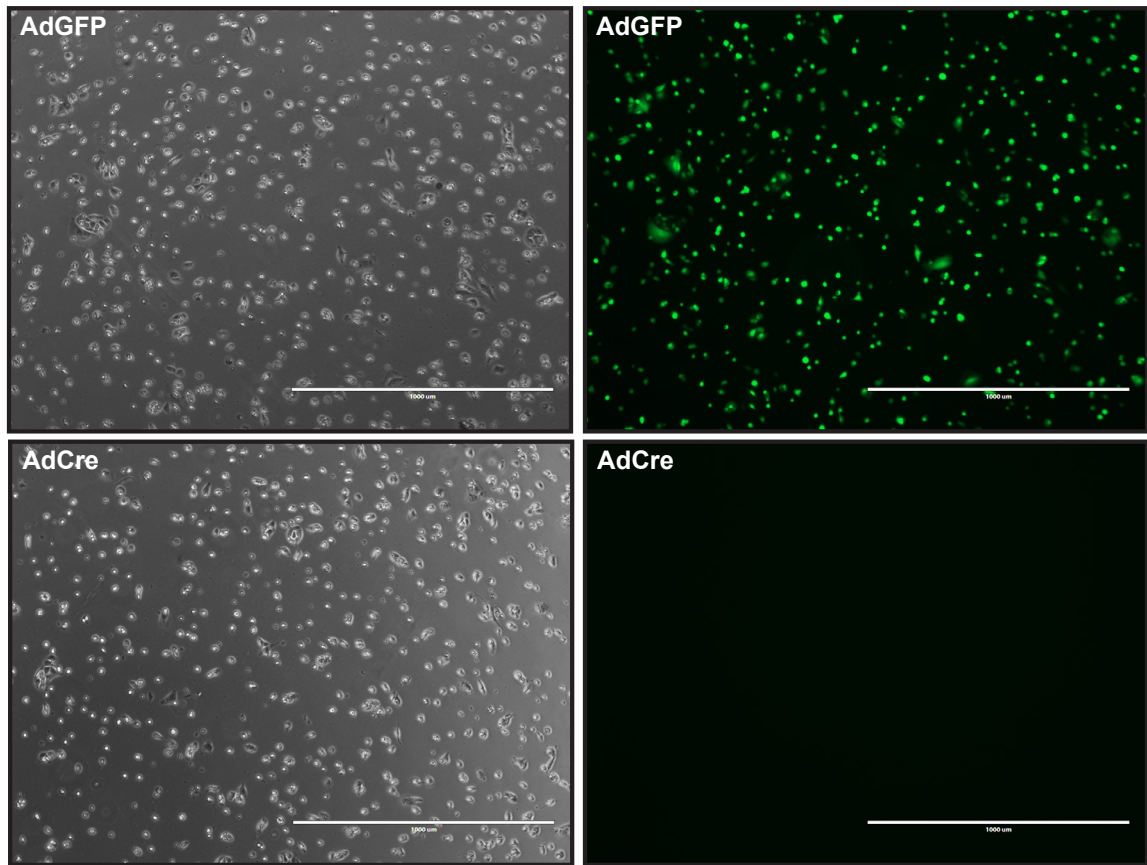
Appendix A: *In vitro* OSE cell adenovirus infection optimization and validation



Esr1-floxed OSE infected with increasing pfu/cell of AdGFP or AdCre to determine optimal concentration (pfu/cell) of adenovirus to infect primary isolations of OSE cultures. Knockout efficiency was measured using qPCR. Mock infected parental cells were used as reference sample and normalized to 1. Efficiency of *Esr1* knockout does not increase with AdCre concentrations while increasing AdGFP concentrations shows a trend towards reducing *Esr1* expression. From this preliminary experiment, it was determined that 100 pfu adenovirus/cell is sufficient for infecting OSE cells in culture to achieve maximal knockout efficiency and minimal off target effects.

Appendix A continued...

Appendix A: Continued... (OSE adenovirus infection optimization and validation)

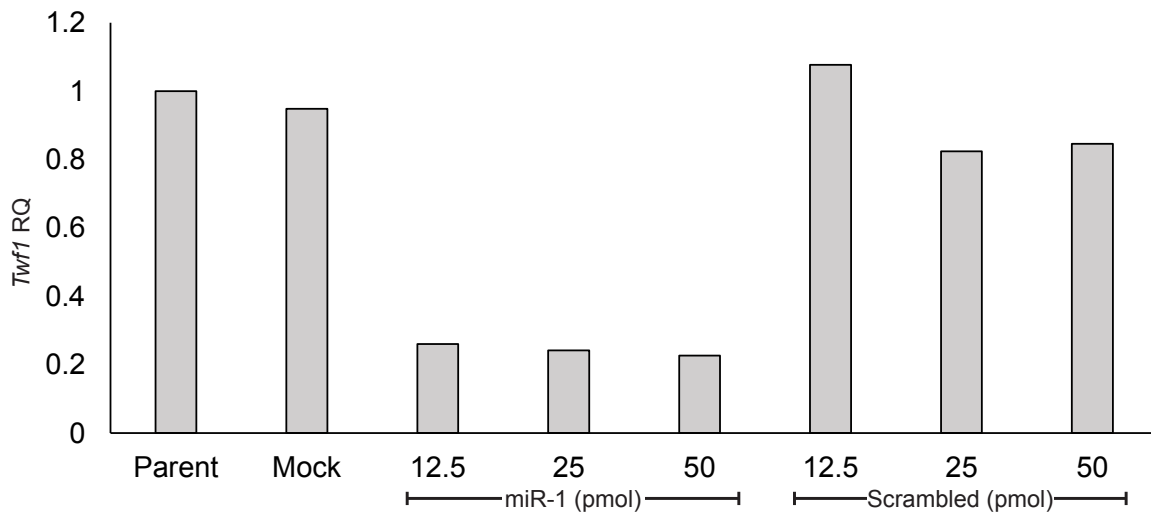


Esr1-floxed OSE 1 day post infection with 100 pfu adenovirus/cell observed under phase contrast and GFP filters using an EVOS FL Cell Imaging System to validate successful adenovirus infection. Representative images shown demonstrates typical infection efficiency of AdGFP. Scale bars= 1000 μm.

Appendix B: miRNA mimic details and optimization experiment

mirVana miRNA mimics acquired from ThermoFisher Scientific.

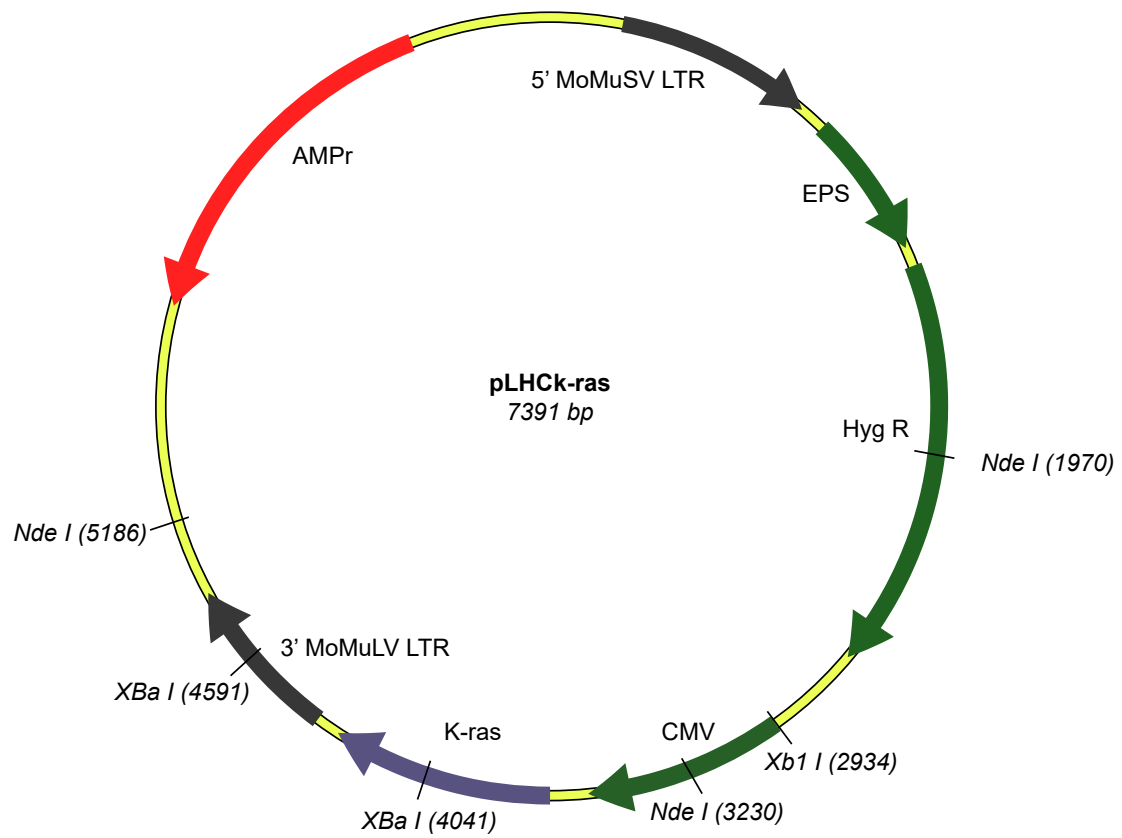
Target	Species	Catalog #	Assay ID
miR-378	Mouse	4464066	MC12581
miR-1 Positive Control	Mouse and Human	4464065	N/A
Scrambled miR Negative Control #1	Mouse and Human	4464058	N/A



Twf1 is a known target for miR-1 in mouse and humans (ThermoFisher Scientific).

To first confirm that the miRNA mechanism is intact in OSE cells, *Twf1* expression was measured by qPCR in OSE cultures transfected with 12.5, 25, and 50 pmols of miR-1 mimic (positive control) or scrambled mimic (negative control). Based on this preliminary experiment, we determined that 5, 10, and 20 pmols of mimic was a good range to use for subsequent experiments to observe any dose response effects of miR-378 with minimal non-specific transcription effects.

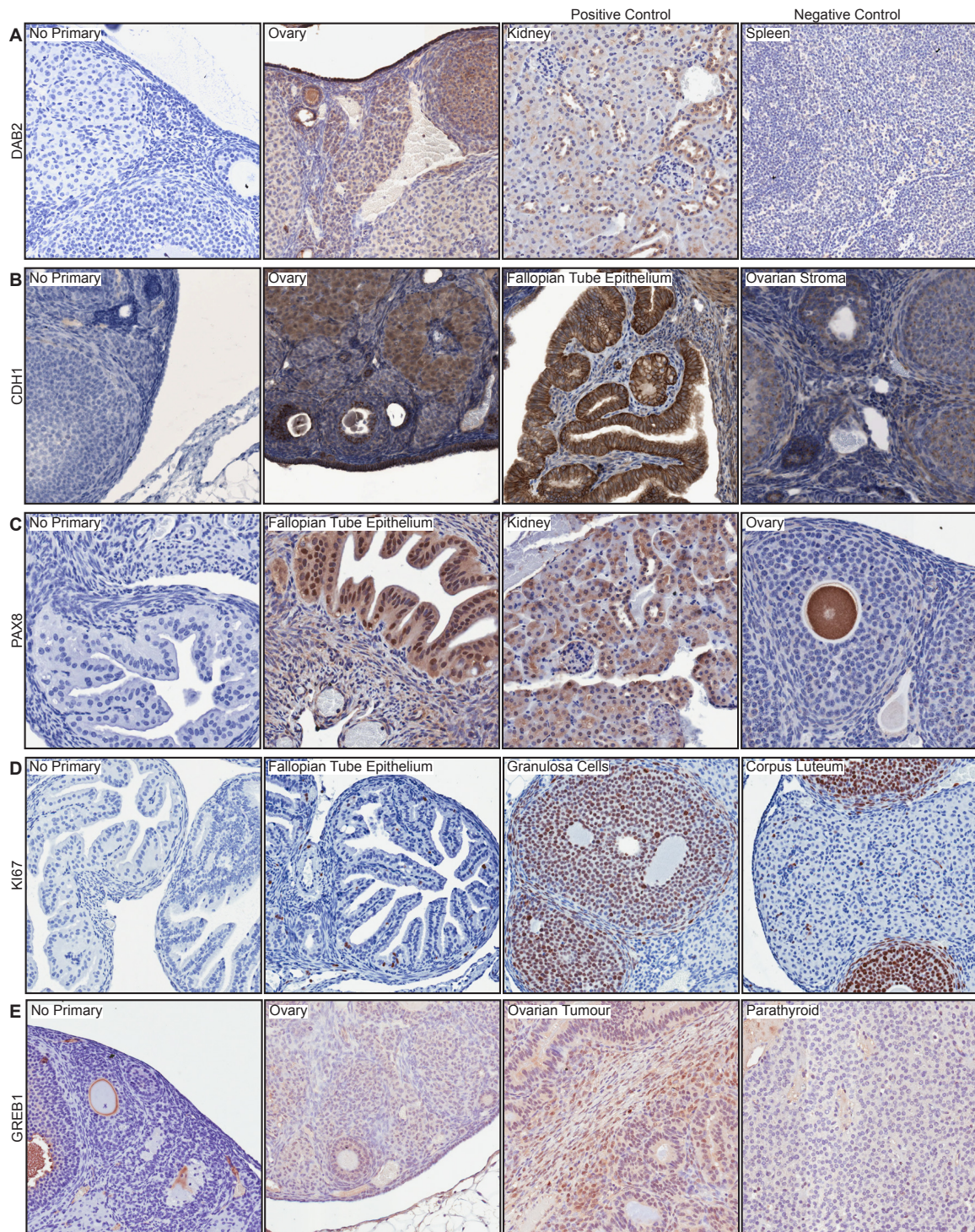
Appendix C: KRAS^{G12D} over-expression vector



Appendix D: List and details of antibodies used for immunohistochemistry staining

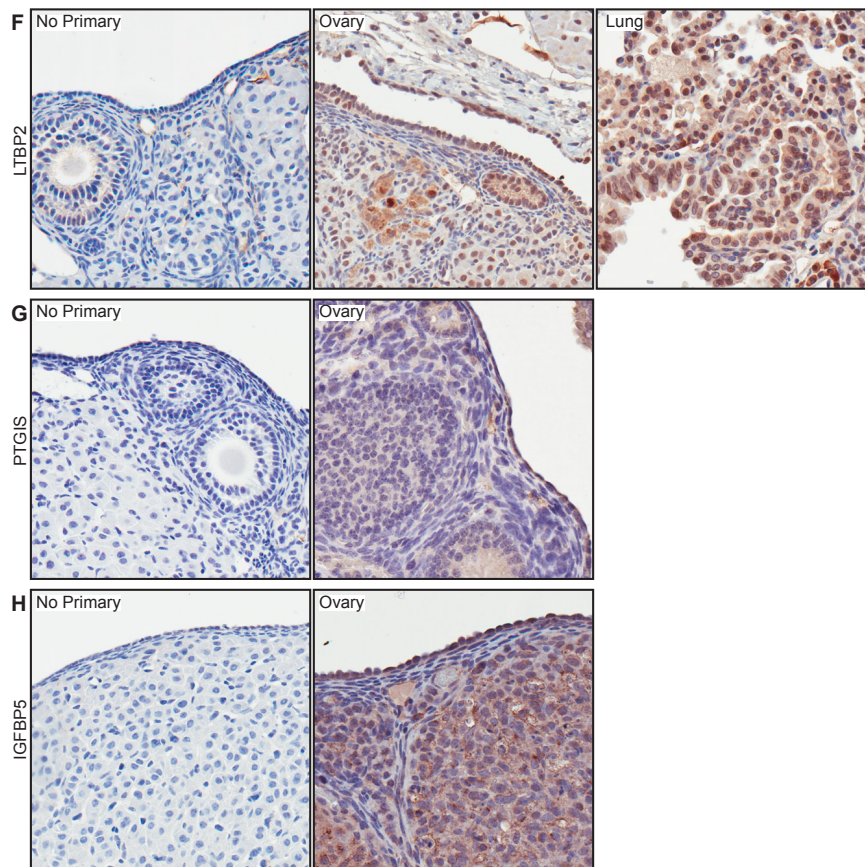
Primary Antibody					Secondary Antibody		
Target	Antibody	Company	Catalog #	Dilution	Antibody	Company	Catalog#
DAB2	anti-DAB2 rabbit polyclonal	Proteintech Group	10109-2-AP	1:100	DAKO EnVision+ System	Dako	K4003
PAX8	anti-PAX8 rabbit polyclonal	Proteintech Group	10336-1-AP	1:500	DAKO EnVision+ System (rabbit)	Dako	K4003
KI67	anti-KI67 rabbit monoclonal	Abcam	ab16667	1:200	DAKO EnVision+ System (rabbit)	Dako	K4003
CDH1	anti-CDH1 mouse monoclonal	BD Biosciences	610181	1:400	M.O.M.™ Biotinylated AntiMouse IgG Reagent, VECTASTAIN® ABC complex	Dako	BMK- 2202
LTBP2	anti-LTBP2 mouse monoclonal	Santa Cruz Biotechnology	sc-166199	1:100	M.O.M.™ Biotinylated AntiMouse IgG Reagent, VECTASTAIN® ABC complex	Vectastain	BMK- 2202
IGFBP5	anti-IGFBP5 mouse monoclonal	Santa Cruz Biotechnology	sc-515116	1:50	M.O.M.™ Biotinylated AntiMouse IgG Reagent, VECTASTAIN® ABC complex	Vectastain	BMK- 2202
PTGIS	anti-PTGIS rabbit polyclonal	ThermoFisher Scientific	PA5-15218	1:50	DAKO EnVision+ System (rabbit)	Dako	K4003
GREB1	anti-GREB1 mouse monoclonal	Millipore Sigma	MABS62	1:100	M.O.M.™ Biotinylated AntiMouse IgG Reagent, VECTASTAIN® ABC complex	Vectastain	BMK- 2202

Appendix E: Immunohistochemistry staining antibody controls



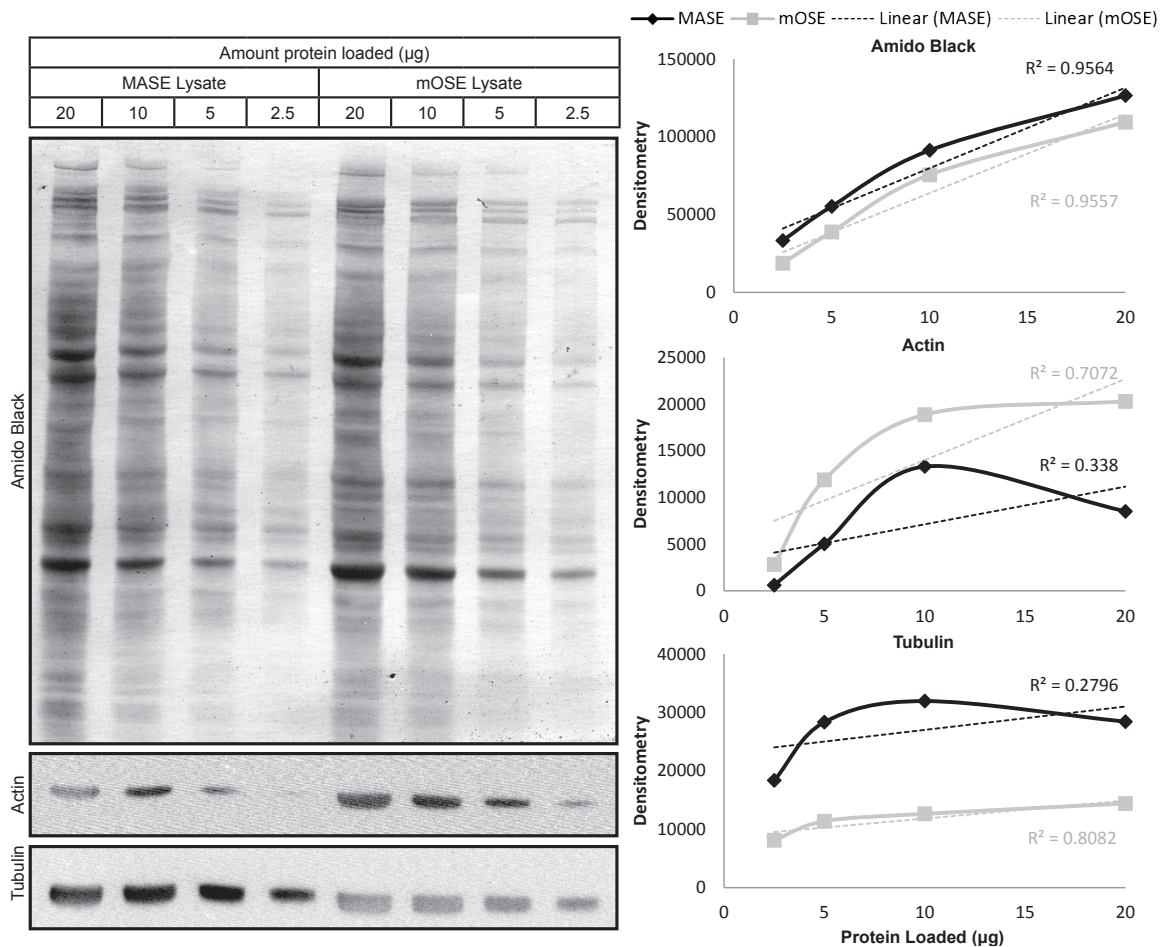
Appendix E continued...

Appendix E: Continued... (Immunohistochemistry staining antibody controls)



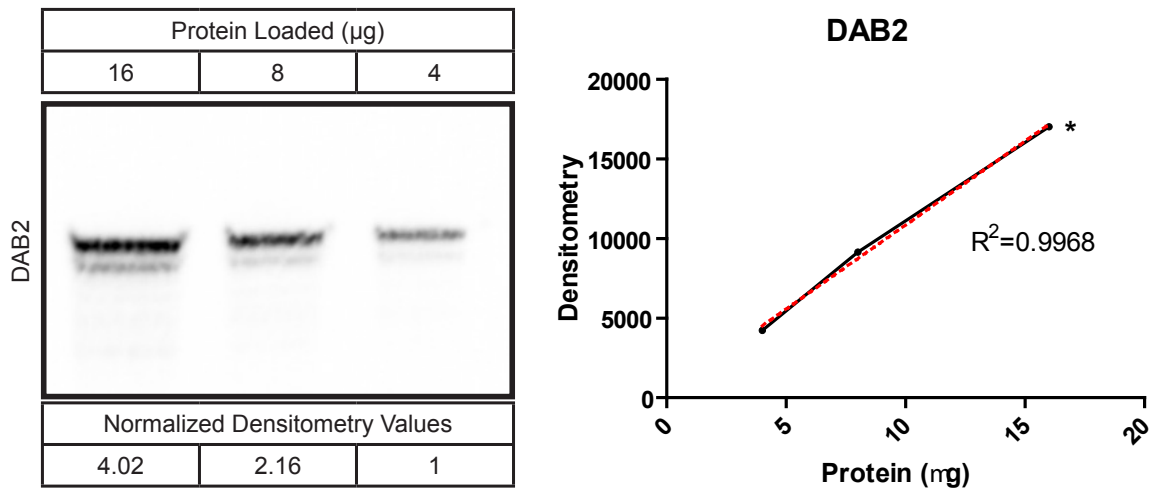
IHC staining controls for DAB2 (A), CDH1 (B), PAX8 (C), Ki67 (D), GREB1 (E), LTBP2 (F), PTGIS (G), and IGFBP5 (H). (F) No Negative control tissue was available for LTBP2. (G-H) No positive or negative control tissue was available for PTGIS and IGFBP5.

Appendix F: Validation of Amido Black as western blot loading control



Untreated MASE and OSE cell lysates were randomly chosen as two independent replicates for validating western blot loading controls. Serial dilution technique was used to load 2.5, 5, 10, and 20 μg protein. Blot was probed for *Actin*, *Tubulin*, then stained with Amido Black last. Linear regression analysis (dotted line) for each loading control show that Amido Black has the best goodness of fit (R^2) value and the only loading control to have a slope significantly different than zero compared to *Actin* and *Tubulin* - two commonly used loading control for western blot analysis (*= p -value<0.05; F-Test).

Appendix G: DAB2 antibody validation for western blot analysis



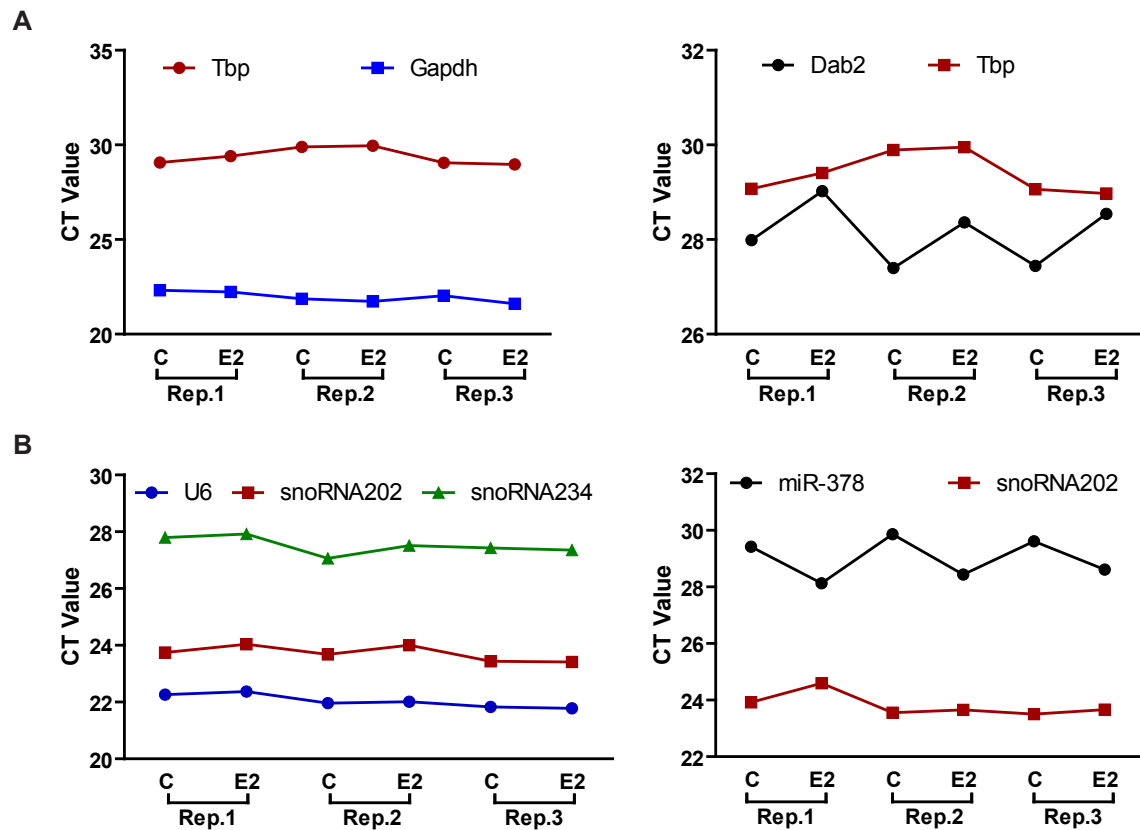
A randomly chosen OSE cell lysate was serially diluted to load 4, 8, and 16 μg of protein. These values encompass the range of protein loaded for western blot analysis shown in this study which range between 5-15 μg. The validation blot was run and probed for DAB2 as described in the Materials and Methods. Densitometric analysis showed that our protocol had been optimized to accurately measure changes in DAB2 levels by western blot and the amount of protein loaded is within the dynamic range of Amido Black, our chosen loading control (**Appendix F**). Linear regression analysis (red dotted line); *=p-value <0.05 (slope is significantly different than zero); F-Test.

Appendix H: List and probing parameters for antibodies used in western blot

analysis

Primary Antibody					Secondary			
Target	Antibody	Company	Catalog #	Dilution	Antibody	Company	Catalog #	Dilution
DAB2	anti-DAB2 rabbit polyclonal	Proteintech Group	10109-2-AP	1:2000	Goat anti-rabbit IgG (H + L) HRP	Bio-Rad	1721019	1:5000
ESR1	anti-ESR1 rabbit polyclonal	Santa Cruz Biotechnology	sc-542	1:100	Goat anti-rabbit IgG (H + L) HRP	Bio-Rad	1721019	1:5000
CDH1	anti-CDH1 mouse monoclonal	BD Biosciences	610181	1:5000	Goat anti-mouse IgG (H + L) HRP	Bio-Rad	1706516	1:5000
KRAS	anti-KRAS mouse monoclonal	Santa Cruz Biotechnology	(F234) sc-30	1:100	Goat anti-mouse IgG (H + L) HRP	Bio-Rad	1706516	1:5000

Appendix I: qPCR endogenous control validation for E2 assays



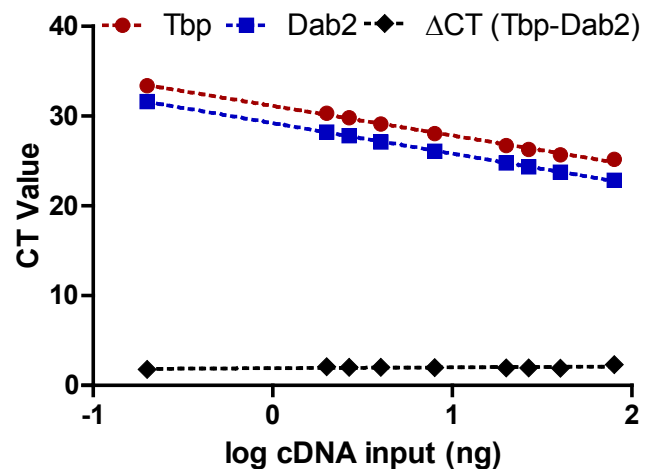
Tbp, *Gapdh*, and *snoRNA202* were confirmed to be not E2 responsive and were used as qPCR endogenous controls for this study. **(A-B)** Histograms show qPCR cycling threshold (CT) values of MASE treated with E2 for 24 hours in 3 biological replicates. **(A)** There is no consistent pattern of CT fluctuation for *Tbp* or *Gapdh* in control vs. E2 treated MASE. In comparison, when the same samples were probed for *Dab2* (with *Tbp* as a plate calibrator), E2 treated MASE consistently showed a higher CT value relative to control. **(B)** U6, *snoRNA202*, and *snoRNA234* are common miRNA endogenous controls and their levels do not fluctuate in MASE cells in response to E2 treatment. For comparison, the same samples were probed for *miR-378* (with *snoRNA202* as a plate calibrator) and E2 samples consistently have a lower CT value than control samples. *snoRNA202* was chosen as an endogenous control for qPCR analysis of miRNAs under the recommendation of Applied Biosystems (Wong et al. 2007).

Appendix J: Probe validation for qPCR analysis

Summary of qPCR probe validation results

Probe	Efficiency	Dynamic Range (CT)	Endogenous control pairing
<i>Dab2</i>	97.50%	23-31	TBP
<i>Esr1</i>	96.50%	24-32	TBP
<i>Twf1</i>	88.01%	22-31	Gapdh
<i>Gapdh</i>	89.85%	17-26	--
<i>TBP</i>	92.13%	26-35	--

cDNA (ng)	log (cDNA)	<i>Dab2</i> CT	<i>Tbp</i> CT	<i>Tbp-Dab2</i> (Δ CT)
80	1.9	22.86	25.17	2.31
40	1.6	23.76	25.67	1.91
26.67	1.43	24.33	26.28	1.94
20	1.3	24.77	26.72	1.95
8	0.9	26.08	28.06	1.99
4	0.6	27.11	29.11	2
2.67	0.43	27.8	29.83	2.03
2	0.3	28.21	30.31	2.1
0.2	-0.7	31.62	33.39	1.77
Slope		-3.3934	-3.2995	0.0939
Efficiency		0.9712	1.0144	0.0432
R2		0.9966	0.9828	0.2649



Dab2 probe validation is shown here to demonstrate how all qPCR probes were validated and matched with their endogenous loading control probe. A pool of RNA from OSE cells were used to generate cDNA. The resulting cDNA was serially diluted then analyzed by qPCR analysis using *Dab2* and *Tbp* probes as described in the Materials and Methods section. Calibration curves for *Dab2* and *Tbp* probes were generated by plotting the cycling threshold (CT) against input cDNA. Linear regression analysis of the calibration curves was performed to determine probe efficiency. On average, *Dab2* and *Tbp* probes had a difference of 4% in amplification efficiency but linear regression analysis of their Δ CT values (*Tbp* CT-*Dab2* CT) demonstrated that within the range of 0.2-80ng of input cDNA, the Δ CT slope was not significantly different than zero. This demonstrates that when *Dab2* and *Tbp* probes are paired, their relative dynamic range is between CT values of 23-31. In this study, between 12.5-25ng of cDNA was loaded for qPCR analysis and CT values remained within 23-31. n=3. *=p-value<0.05 (slope is significantly different

than zero); Linear regression analysis (dotted lines); F-Test. A similar calibration curve was run with *Gapdh* but *Tbp* was chosen as the endogenous control for qPCR analysis of *Dab2* because calibration curves show that *Tbp* probes have a wider relative dynamic range than *Gapdh* probes when they are paired with *Dab2* probes. No protocol for assessing the calibration curves of TaqMan miRNA assays have been established and therefore was not performed.

Appendix K: qPCR probes, primers, miRNA assay sequences

Probes used for qPCR analysis from Integrated DNA Technologies. All probes are for detection of mouse transcripts.

Target	Assay ID	Probe	Forward Primer	Reverse Primer
<i>Dab2</i>	Mm.PT.58.42 543573	FAM/CTGCTGCCA/ZEN/TTCCC TTGAGTTTCAT/3IABkFQ	GCTTGTGTTGTCCCTGAGAG	GTATTGATGATGTGCCTGATGC
<i>Esr1</i>	Mm.PT.51.98 67105	FAM/TCTTTCCGT/ZEN/ATGCC GCCTTTCATCA/3IABkFQ	GCCTTCCAAGTCATCTCTCTG	GCGCAAGTGTTACGAAGTG
<i>Twf1</i>	Mm.PT.58.13 982093	FAM/TGAAGGTGG/ZEN/ACTA CAGGAGCCAAC/3IABkFQ	GGCAAGACAAAGGAGTCGTAG	CCAGAAACGGGAAATACAGACT
<i>Gapdh</i>	Mm.PT.39a.1	FAM/TGCAAATGG/ZEN/CAGC CCTGGTG/3IABkFQ	GTGGAGTCATACTGGAACATGTAG	AATGGTGAAGGTCGGTGTG
<i>Tbp</i>	Mm.PT.39a.2 2214839	FAM/ACTTGACCT/ZEN/AAAGA CCATTGCACTTCGT/3IABkFQ	CCAGAACTGAAAATCAACGCAG	TGTATCTACCGTGAATCTTGGC

Custom designed primers for qPCR analysis acquired from Invitrogen.

Target	Species	Forward Primer	Reverse Primer
<i>Dab2</i>	Human	TGTATCTACCGTGAATCTTGGC	CAAGCAAGTCATTGGCTGAA
<i>TBP</i>	Human	GAACATCATGGATCAGAACAACA	ATAGGGATTCCGGGAGTCAT
<i>KRAS</i>	Human	AGGGTGGTGACTTTACACGC	GATGCCAGGACCGTATGCT
<i>PPIA</i>	Mouse	GGGCATATGTCGACCCGGGAATTCACATAACTGTACACCTTG	CCCGGGATCCACCATGACTGAGTATAAGCTTGT

miRNA assays for qPCR analysis acquired from ThermoFisher Scientific. All miRNA assays have catalog# 4427975.

Target	Species	Assay ID	Sequence
miR-378	Mouse Human	002243	ACUGGACUUGGAGUCAGAAGG
snoRNA202	Mouse	001232	GCTGTACTGACTTGATGAAAGTACTTTTGAACCCTTTTCCATCTGATG
snoRNA234	Mouse	001234	CTTTTGGAACTGAATCTAAGTGATTTAACAAAAATTCGTCACTACCACTGAGA
U6 snRNA	Mouse Human	001973	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAG CATGGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT

Appendix L: List and details of antibodies used for immunofluorescent staining

Primary Antibody					Secondary Antibody		
Target	Antibody	Company	Catalog #	Dilution	Antibody	Catalog #	Dilution
CTNNB1	anti-CTNNB1 rabbit polyclonal	Abcam	ab6302	1:500	Goat Anti-Rabbit IgG H&L	A-11034	1:500
ZO1	anti-ZO1 rabbit polyclonal	Abcam	ab216880	1:100	Goat Anti-Rabbit IgG H&L	A-11034	1:500
DAB2	anti-DAB2 rabbit polyclonal	Santa Cruz Biotechnology	sc-13982	1:100	Goat Anti-Rabbit IgG H&L	A-11034	1:1000
CDH1*	anti-CDH1 rabbit polyclonal	Abcam	ab53033	1:400	Goat Anti-Rabbit IgG H&L	A-11034	1:1000
CDH1**	anti-CDH1 mouse monoclonal	Abcam	ab76055	1:100	Goat anti-Mouse IgG H&L	A-11029	1:1000
KI67	anti-KI67 rabbit monoclonal	Abcam	ab16667	1:100	Goat Anti-Rabbit IgG H&L	A-11034	1:1000
LTBP2	anti-LTBP2 mouse monoclonal	Santa Cruz Biotechnology	sc-166199	1:100	Goat anti-Mouse IgG H&L Superclonal Secondary	A28175	1:500
IGFBP5	anti-IGFBP5 mouse monoclonal	Santa Cruz Biotechnology	sc-515116	1:100	Goat anti-Mouse IgG H&L Superclonal Secondary	A28175	1:500
PTGIS	anti-PTGIS rabbit polyclonal	ThermoFisher Scientific	PA5-15218	1:50	Goat anti-Rabbit IgG H&L Superclonal Secondary	A27034	1:500
GREB1	anti-GREB1 rabbit polyclonal	Sigma Aldrich	HPA024616	1:100	Goat anti-Rabbit IgG H&L Superclonal Secondary	A27034	1:500

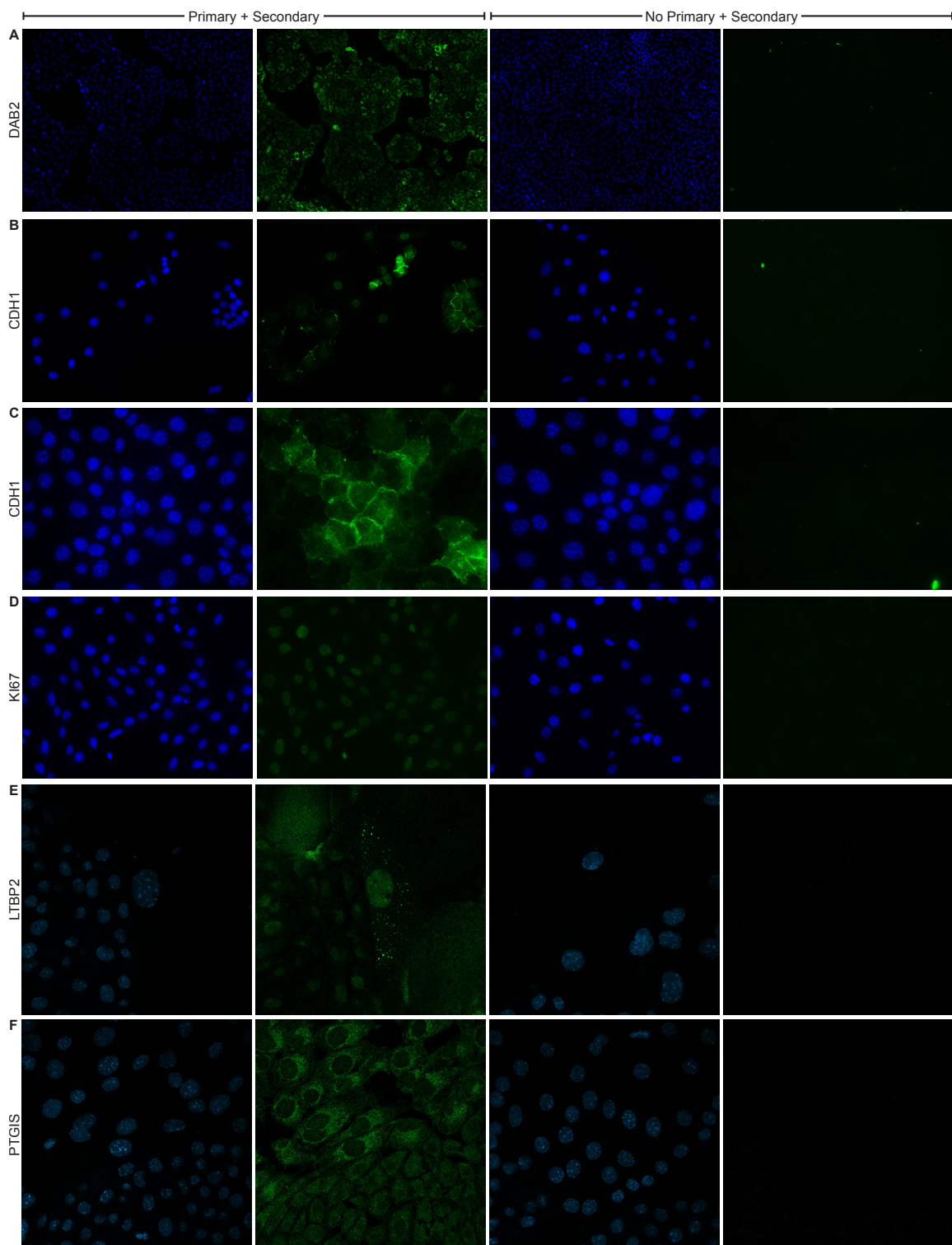
All secondary antibodies were conjugated with Alexa Fluor 488 and purchased from Invitrogen.

Actin staining was performed according to manufacturer's instructions using ActinRed 555 ReadyProbes reagent (ThermoFisher Scientific).

*Discontinued CDH1 antibody used in **Figure 4** and **Figure 11**.

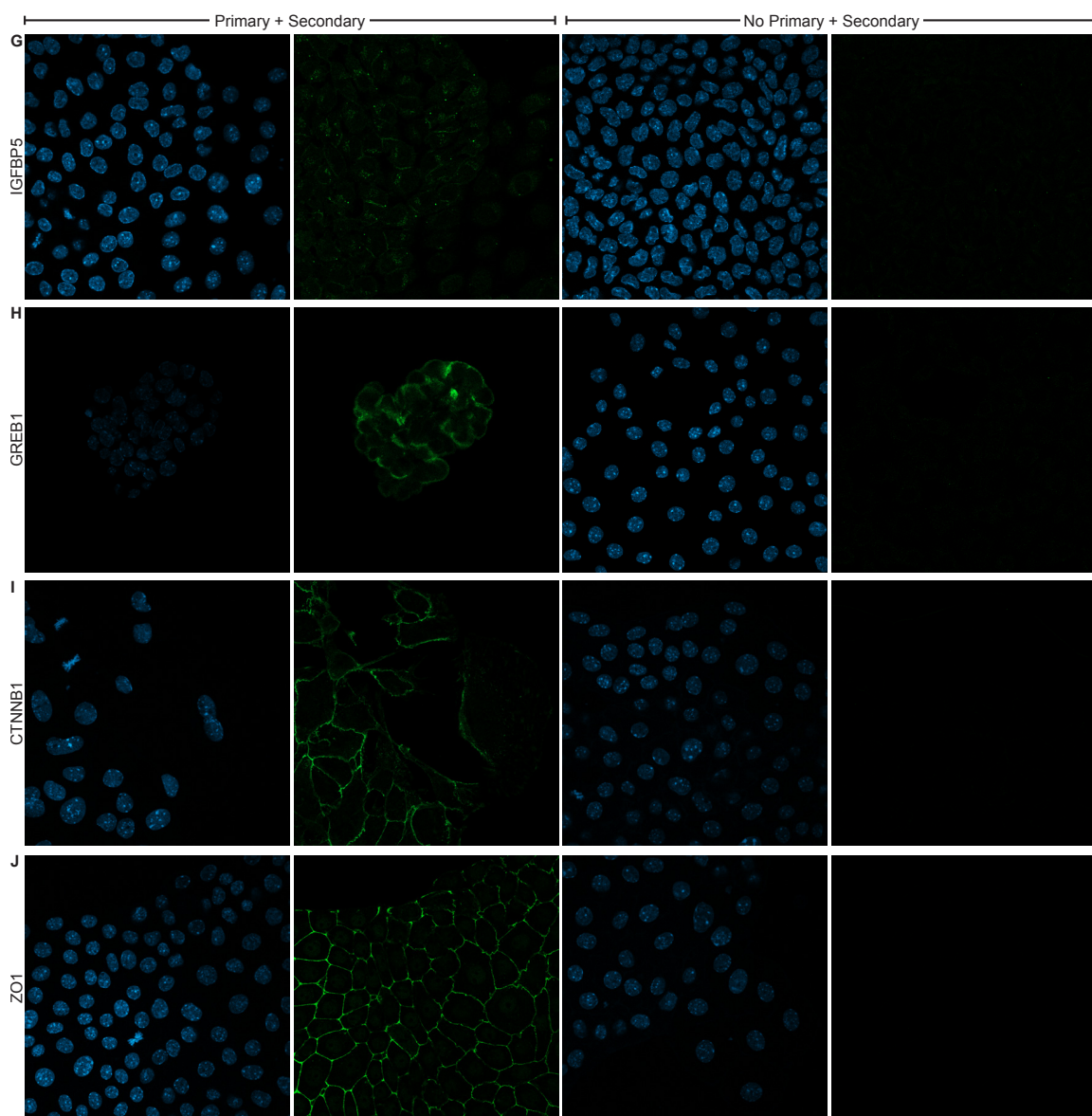
Replacement CDH1 antibody Used in **Figure 20.

Appendix M: Immunofluorescent staining “no primary” controls



Appendix M continued...

Appendix M: Continued... (Immunofluorescent staining “no primary” controls)

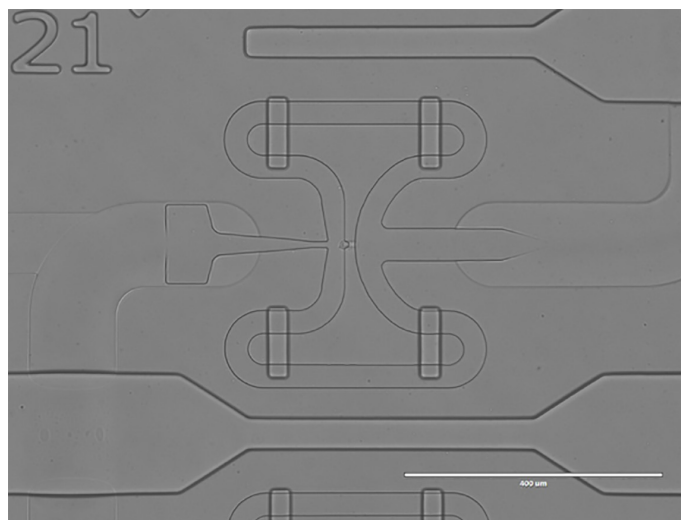


Representative images showing the level of background observed for DAB2 (A), CDH1 (B-C), Ki67 (D), LTBP2 (E), PTGIS (F), IGFBP5 (G), GREB1 (H), BTNNB1 (I), and ZO1 (J) antibodies used for IF. (B) Discontinued CDH1 antibody; ab50033. (C) Replacement CDH1 antibody; ab76055.

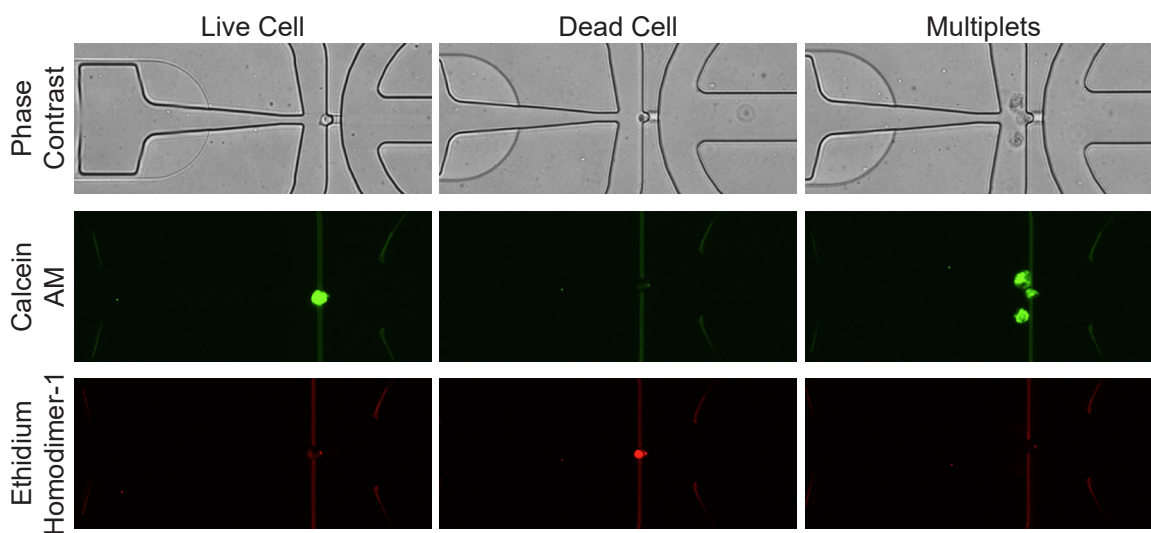
Appendix N: Primer sequences used for chromatin immunoprecipitation of ESR1

Target	Sequences	
<i>Dab2</i> ERE	Forward	GTGTTACGCAAGAACAGACAG
	Reverse	TGCTGACTTCAGGAGAAGGGT
<i>Grebl</i> ERE1	Forward	ACCGCAAACGTATCAGTGG
	Reverse	CCAGCTGCCACAATTAGAAAC
<i>Grebl</i> ERE2	Forward	ACAAACTGTCCTCACCCACAG
	Reverse	ACATGGGAAGCCACGATAAG
<i>Gapdh</i>	Forward	TACTCGCGGCTTTACGGG
	Reverse	TGGAACAGGGAGGAGCAGAGAGCA

Appendix O: Single cell capture quality control



A representative phase contrast image of a single cell capture site on the Fluidigm integrated fluidic circuit.



Representative image showing how individual capture sites were scored for later data filtering. Calcein AM (green) is used to identify live cells and Ethidium Homodimer-1 (red) is used to identify dead cells. Images were acquired using the phase contrast, GFP, and RFP filter sets on the EVOS FL Cell Imaging System.

Appendix O continued...

Appendix O: Continued... (Single cell capture quality control)

Score of each capture site on IFC.

	Control											E2									
	1	2	3	4	5	6	7	8	9	10		11	12	13	14	15	16	17	18	19	20
● Live Cell																					
● Dead Cell																					
✿ Ambiguous																					
1	-	●	●	-	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
2	●	●	✿	●	●	●	●	●	●	●		●	●	●	●	●	●	-	●	●	●
3	-	●	●	●	●	●	●	●	●	-		●	●	●	●	●	●	●	●	●	●
4	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
5	●	●	●	●	●	●	-	●	●	●		●	●	●	●	●	●	●	●	●	-
6	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
7	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
8	●	●	●	●	●	●	●	●	●	●		-	●	✿	●	●	●	●	●	●	●
9	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
10	●	●	●	●	●	●	●	●	●	●		-	●	●	●	●	●	●	●	●	●
11	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
12	●	●	●	●	●	●	●	●	●	●		-	●	-	●	●	●	●	●	●	●
13	●	●	●	●	●	●	●	●	●	●		●	●	●	✿	●	●	-	●	●	●
14	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
15	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
16	●	●	●	●	●	●	●	✿	●	●		●	●	●	●	●	●	-	●	●	●
17	●	-	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
18	●	●	●	●	●	●	●	●	●	●		●	●	-	●	●	●	●	●	●	●
19	●	●	●	●	●	●	●	●	●	●		✿	●	●	●	-	●	-	●	-	●
20	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
21	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
22	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
23	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
24	●	●	✿	●	●	●	✿	●	●	●		●	●	●	●	●	●	●	●	●	●
25	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
26	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
27	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
28	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
29	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
30	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
31	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
32	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
33	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
34	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
35	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
36	●	●	●	●	●	●	●	●	●	●		-	●	●	●	●	●	●	●	●	●
37	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
38	●	●	●	●	●	●	●	●	●	●		✿	●	●	●	●	●	●	●	●	●
39	●	●	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●
40	●	●	●	●	●	●	●	●	●	●		✿	✿	●	✿	●	●	●	-	●	●

Appendix P: Control vs. E2 microRNA array results

MicroRNA microarray results from MASE cells treated with or without E2 for 24 hours (n=3). Paired one-way repeated measure ANOVA was used and only significantly changed miRNAs are shown. Data are sorted by fold change (FC).

miRNA Name	FC	P.Value	miRNA Name	FC	P.Value	miRNA Name	FC	P.Value
miR-6984-5p	5.524	5.00E-02	mir-181d	1.295	5.00E-02	mir-3084-1	0.806	2.20E-02
miR-7007-5p	4.875	4.30E-02	miR-707	1.294	2.20E-02	miR-5107-3p	0.798	3.40E-02
miR-5121	4.698	4.50E-02	miR-7646-5p	1.292	4.10E-02	miR-6912-3p	0.791	1.70E-02
miR-6368	3.52	4.10E-02	mir-99a	1.282	9.70E-03	miR-1945	0.787	3.00E-02
miR-1894-5p	3.248	2.70E-03	mir-6900	1.278	2.50E-02	miR-1197-3p	0.787	1.60E-02
miR-7082-5p	2.761	4.80E-02	mir-1894	1.277	4.20E-02	miR-669c-3p	0.787	3.50E-02
miR-1231-5p	2.661	2.90E-02	miR-466n-5p	1.271	5.00E-02	miR-3112-5p	0.785	1.80E-02
miR-7016-3p	2.325	2.60E-02	miR-670-5p	1.27	3.60E-02	miR-7224-3p	0.777	3.80E-02
miR-193b-3p	2.134	5.50E-05	mir-1933	1.269	4.10E-02	miR-6408	0.774	2.40E-02
miR-378c	2.072	9.90E-05	mir-690	1.269	1.40E-02	miR-6962-3p	0.767	1.10E-02
miR-378d	2.065	5.80E-05	miR-6359	1.263	4.50E-02	miR-6481	0.766	1.30E-02
miR-378a-3p	2.005	1.30E-04	miR-7234-5p	1.25	4.20E-02	mir-7027	0.764	3.90E-02
miR-24-1-5p	1.95	2.30E-02	miR-207	1.248	1.50E-02	miR-194-5p	0.758	5.00E-03
miR-378b	1.923	2.40E-05	miR-541-5p	1.242	3.10E-02	miR-6397	0.756	1.80E-02
miR-5622-3p	1.765	3.10E-02	miR-3971	1.237	4.20E-02	miR-547-5p	0.755	6.60E-03
miR-7115-5p	1.755	3.50E-02	mir-5623	1.236	2.40E-02	miR-3106-3p	0.752	7.70E-03
miR-1907	1.732	3.70E-02	miR-147-5p	1.231	2.80E-02	miR-466d-5p	0.743	4.40E-03
miR-466i-5p	1.701	2.30E-02	mir-1952	1.23	3.20E-02	mir-212	0.741	6.90E-03
mir-5121	1.66	4.70E-03	miR-7074-3p	1.229	3.30E-02	miR-7051-5p	0.729	2.80E-03
miR-5128	1.657	3.30E-02	miR-7232-5p	1.217	2.40E-02	miR-322-5p	0.723	3.70E-02
miR-365-2-5p	1.654	1.20E-02	miR-6918-3p	1.217	2.30E-02	miR-466p-5p	0.709	4.30E-03
miR-6942-5p	1.598	7.80E-04	mir-7662	1.216	3.30E-02	miR-376c-3p	0.708	4.70E-02
miR-185-3p	1.569	2.40E-02	mir-1839	1.212	4.40E-02	miR-7079-5p	0.7	1.20E-02
miR-15a-3p	1.567	3.70E-02	miR-6985-3p	1.209	4.30E-02	miR-344d-1-5p	0.678	4.20E-02
mir-3113	1.539	2.90E-02	miR-6949-3p	1.208	4.40E-02	miR-1947-3p	0.667	2.40E-02
miR-125b-2-3p	1.485	3.20E-02	mir-1195	1.206	2.80E-02	miR-331-5p	0.653	2.90E-03
miR-7001-5p	1.463	3.00E-02	mir-3069	1.204	4.00E-02	miR-6236	0.649	1.60E-02
miR-425-3p	1.461	3.60E-02	miR-3070b-3p	1.201	3.90E-02	miR-339-3p	0.536	1.90E-02
mir-7684	1.445	4.20E-02	mir-3475	1.189	4.60E-02			
miR-6993-5p	1.425	1.30E-02	mir-192	1.167	4.90E-02			
miR-3092-5p	1.392	2.40E-02	miR-216b-3p	1.167	4.90E-02			
miR-7031-5p	1.389	1.10E-02	miR-7021-3p	0.841	3.30E-02			
miR-6958-5p	1.347	2.70E-02	miR-7089-5p	0.838	5.00E-02			
miR-293-3p	1.326	2.70E-02	miR-370-5p	0.828	4.70E-02			
miR-133c	1.326	7.30E-03	mir-6925	0.823	2.80E-02			
miR-299a-5p	1.324	1.40E-02	mir-7057	0.823	4.30E-02			
miR-3572-3p	1.316	5.00E-02	miR-7220-3p	0.822	4.00E-02			
mir-16-2	1.306	3.10E-02	mir-301a	0.818	4.90E-02			
miR-672-5p	1.304	4.90E-02	miR-489-3p	0.813	2.60E-02			
mir-3971	1.299	1.50E-02	miR-7233-3p	0.812	3.30E-02			
miR-692	1.299	1.40E-02	miR-190b-5p	0.811	3.40E-02			

Appendix Q: Differential expression results between Clusters 1 and 2 (k=2)

Differentially expressed genes (q-value<0.05) in cluster 1 (E2 unresponsive) relative to 2 (E2 responsive) in k=2 groupings that have log fold change (logFC) > 0.5 and < -0.5.

Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC
Col1a2	5.759	Myof	1.3	Capza2	1.004	Htra2	0.866	Clip1	0.762	Ankra2	0.683	Taldo1	0.64
C1s1	3.54	Camk1	1.297	Kcmf1	1.001	Hnrnpf	0.865	Vcp	0.76	Casd1	0.681	Npnt	0.64
Tm4sf1	3.124	Myh10	1.269	Tspan9	1	Wdsub1	0.864	Ogn	0.759	Rasa1	0.68	Mob1a	0.64
Msln	2.924	Bex2	1.257	Fam60a	0.988	Smim10l1	0.864	Card19	0.759	Nars	0.679	Rpl5	0.638
Cyp1b1	2.883	Ahnak2	1.244	Ybx3	0.981	Nedd9	0.857	Satb1	0.758	Fam198b	0.679	Irf5	0.637
C1s2	2.728	Gm6166	1.225	Gsto1	0.981	Tia1	0.856	Calu	0.758	Dennd5a	0.679	Dek	0.637
Ltbp2	2.558	Chil1	1.203	Gng12	0.979	Cdc42ep3	0.856	Tfcp2l1	0.757	Fam178a	0.678	Dctn1	0.636
Lrrfip1	2.489	Snmp27	1.199	Ahr	0.975	Ppp4r2	0.854	Cobl	0.757	Ddit4l	0.678	Srsf11	0.635
Mmp2	2.468	Rasgrf2	1.191	Usp9x	0.974	Ccln5	0.854	Atp11a	0.757	Usp2	0.675	Ppig	0.635
Gpc3	2.339	Ide	1.189	Met	0.965	Csgalnact2	0.853	Rab7	0.756	Tpst2	0.675	Ctu2	0.635
Axl	2.242	Cul1	1.181	Hal	0.963	Epn3	0.851	Polr1a	0.755	Gm15500	0.672	Smc5	0.633
Tmsb10	2.133	B2m	1.179	Fndc3b	0.963	Picalm	0.85	Rhpn2	0.754	Col4a6	0.672	Rps15a	0.633
Nt5e	2.089	Sox12	1.177	Fez2	0.963	Pcdh7	0.85	Coro1c	0.753	Fam118b	0.67	Depdc7	0.633
Igfbp3	2.032	Cyr61	1.176	Ptcd3	0.962	Rnf128	0.849	Prdx4	0.752	Sfxn3	0.668	Hacd4	0.632
Cald1	1.975	Anxa1	1.168	Serinc5	0.96	Cbx3	0.848	Mtus1	0.751	Ramp1	0.668	Gnai1	0.632
Star	1.955	Gabapapl1	1.167	Tmem106b	0.958	Klhl30	0.841	Lrp6	0.75	Ttc39b	0.667	Scamp1	0.631
Pappa2	1.949	Pcolce	1.159	Ubqln2	0.954	Pole4	0.835	Dtx4	0.75	Flna	0.666	Kif5b	0.631
Rbm28	1.896	Vnn1	1.157	Cd2ap	0.952	Ssfa2	0.834	Rsu1	0.749	Esf1	0.666	Fuom	0.631
Itpr1	1.896	Arf5	1.153	Foxp1	0.945	Ubn2	0.833	Prune2	0.749	Aldh1a1	0.666	Fis1	0.631
Exoc6b	1.881	Cgnl1	1.146	Tcf4	0.943	Itgav	0.832	Atp6ap2	0.748	H2afz	0.665	Sord	0.63
Tmem176a	1.829	Jade1	1.14	Serbp1	0.94	Fhod3	0.831	Ttc14	0.746	Arglu1	0.664	Etnk1	0.629
C1ra	1.762	Cpe	1.138	Fat1	0.939	Atp6v1f	0.826	Bex3	0.744	Arhgef26	0.663	Parva	0.628
Bex4	1.761	Pgrmc1	1.131	Tmem151a	0.937	Sec13	0.824	Ndufa4	0.742	Scd1	0.659	Fgfr1op2	0.627
Abca1	1.727	Dcaf12l1	1.13	Kank1	0.937	Isyna1	0.823	Ctgf	0.742	Fundc2	0.658	Chmp4b	0.627
Sbsn	1.659	Arhgef17	1.128	Ap1s2	0.928	Hmgn5	0.823	Brk1	0.742	Armxc2	0.657	Copg1	0.626
Dctn6	1.632	Setd7	1.123	Pcsk6	0.927	Plxna1	0.821	Inha	0.738	Rabl6	0.656	Pmaip1	0.625
Nod1	1.63	F2r	1.12	Wbp1	0.923	Kdm5a	0.815	Ap2a2	0.737	Ppp1r9a	0.656	Araf	0.624
Krt18	1.619	Smo	1.109	Nfib	0.922	Tmem64	0.81	Wbp11	0.736	Zfand5	0.654	Ypel3	0.623
Rin2	1.587	Hnmpa2b1	1.109	Ptpa	0.92	Alpk2	0.807	Suc1g1	0.734	Naa15	0.654	Washc2	0.622
Lama1	1.571	Egr1	1.107	Dlg3	0.919	Rmnd5a	0.805	Uba2	0.731	Ari8b	0.654	Vhl	0.621
Lgals3	1.562	Wasl	1.099	Ipo8	0.913	Agrt1a	0.804	Rabggtb	0.731	Slc7a1	0.653	Pak3	0.621
Ak5	1.562	Tax1bp1	1.091	Pdia4	0.912	Kdm7a	0.803	Rab2a	0.73	Rhobtb3	0.653	Hmgb2	0.619
Hk2	1.56	Prss23	1.09	Nuak1	0.909	Rif1	0.801	Wwc1	0.725	Usp33	0.652	Ctps2	0.619
Thbs1	1.533	Cct7	1.087	Bola3	0.908	Srp2k	0.798	Tsc22d2	0.723	Mdfic	0.652	Cltc	0.619
Fabp5	1.5	Krcc1	1.072	Pnrc1	0.907	Pls3	0.793	Fads1	0.721	Kras	0.652	Adgr3	0.619
Frm4b	1.498	Fmr1	1.061	Cpne3	0.907	Mtpn	0.792	Spcs3	0.718	H2afj	0.652	Irak1	0.615
Pmepa1	1.49	Zfp618	1.054	Chd3	0.899	Tshz1	0.791	Gzf1	0.718	Ms4a2	0.651	Spr	0.613
Htra1	1.484	Ltbp4	1.052	Creb3l2	0.896	Ltbr	0.79	Me3	0.715	Tmem126a	0.65	Hsd17b12	0.613
Ahnak	1.484	Sort1	1.049	C1rb	0.896	Pmp22	0.787	Stx3	0.712	Sec62	0.65	Cyb5a	0.613
Chmp3	1.474	Calm2	1.046	Stard5	0.894	Akr1c18	0.785	Ube2r2	0.71	Slc39a8	0.649	Aebp2	0.613
Maf	1.467	Amfr	1.042	Lbh	0.891	Phf14	0.783	Prkab2	0.707	Trpm7	0.648	Wdfy3	0.612
Tmem176b	1.457	Sem1	1.035	Megf9	0.89	Mkln1	0.782	Dnajc5	0.705	Pias2	0.647	Hnrnp2	0.612
Abcg2	1.43	Arhgef5	1.035	Tpm4	0.888	Prmt3	0.781	BC005537	0.705	Gsn	0.647	Echdc3	0.61
Plxdc2	1.397	Irak2	1.033	Tor1b	0.888	Fgf8	0.781	Cops7a	0.702	Slc44a1	0.646	Ran	0.608
Akr1b8	1.397	Lrrn4	1.032	Bcl2l13	0.886	Atp6v1e1	0.78	Kdm3a	0.701	Ier3ip1	0.646	Mlt3	0.608
Tgln1	1.394	Exoc4	1.029	Serpinb6a	0.885	Tceal9	0.777	Ccn1	0.698	E330009J07Rik	0.646	Aldh1a3	0.608
Peg3	1.393	Tnfrsf1a	1.028	Scd2	0.878	Amotl1	0.776	Eef1g	0.696	Sf3b3	0.645	mt-Nd6	0.606
Atrnl1	1.392	Mrps33	1.028	Dusp16	0.878	Hoxa7	0.775	Park2	0.695	Ddx39b	0.645	Ep400	0.606
Hoxd8	1.372	Eps8	1.023	Mrpl51	0.877	Alad	0.775	Rpl39	0.694	Setd1b	0.644	Adam9	0.605
Lmo4	1.371	Mmd	1.022	Spred1	0.873	Ssbp1	0.773	Iqsec1	0.694	Fhl2	0.642	Celf1	0.604
Mfge8	1.365	Mrps25	1.02	Immt	0.873	Tra2a	0.772	Gabbr1	0.692	Etf1	0.642	Tnc	0.601
Wnk1	1.346	Fkbp9	1.018	Emp3	0.871	Rpl32	0.767	Med13l	0.69	Anapc1	0.642	Gabrb1	0.601
Ermp1	1.336	Id2	1.008	Nckap1	0.868	Tmem2	0.765	Anapc4	0.688	Pon2	0.641	Cebpz	0.601
St3gal5	1.323	Slc25a5	1.004	Aes	0.868	Osbpl3	0.763	Mbnl3	0.687	Tjp2	0.64	Zrsr2	0.6

Appendix Q continued...

Appendix Q: Continued... (Differential expression results between Clusters 1 and 2)

Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC
Slc25a39	0.599	Nolc1	0.566	Actb	0.531	Gatad1	0.504	Os9	-0.526	Cpox	-0.557	Gramd1b	-0.607
Mbp	0.599	Ldhd	0.566	Shroom3	0.53	Elavl1	0.504	Col12a1	-0.526	Ethel	-0.558	Fkbp11	-0.607
Ccni	0.599	Gabra4	0.566	Skap2	0.528	Gm10499	0.503	Car2	-0.526	Capzb	-0.558	Samp	-0.608
Rpl36a	0.598	Cenpb	0.566	Cul4b	0.528	Mtmr12	0.502	Tenm4	-0.527	Faf1	-0.56	Ehd1	-0.608
Kctd5	0.598	Kif13a	0.565	Ruvbl1	0.526	Adrm1	0.502	Srgap2	-0.527	Smim1	-0.561	Zcchc11	-0.609
lkbkap	0.598	Dusp11	0.565	Cep120	0.526	Abhd5	0.502	Mmp17	-0.527	Csf1	-0.562	Ebna1bp2	-0.609
Cux1	0.598	Bmp4	0.563	Rsrc2	0.524	Xrn2	0.501	Fbxo6	-0.527	Tmem9	-0.564	Pcyt1a	-0.611
Hoxb9	0.597	Sppl2a	0.562	Hs6st2	0.524	Wisp1	0.5	Maged2	-0.529	Ndufa6	-0.564	Ifitm2	-0.611
Ywhah	0.596	Lima1	0.562	Rpl7a	0.523	Prss12	-0.5	Wnt5a	-0.53	Map1b	-0.564	Cox6b2	-0.612
Nr1d1	0.596	Suds3	0.561	Rest	0.523	Hspb11	-0.5	Gm12222	-0.531	Arhgef12	-0.564	Pxk	-0.613
Rac1	0.595	Pus7	0.561	Lrfl1	0.523	Brwd1	-0.5	Fzd2	-0.531	Sh3bp4	-0.566	Hpcal1	-0.613
Rabgap1	0.595	Atp2a2	0.561	Arpc1a	0.523	Zbp1	-0.501	Stx8	-0.532	Gm15114	-0.567	Cnpy2	-0.614
Pdcd5	0.595	Tmem168	0.56	Anp32b	0.523	Pla2g12a	-0.501	Flrt1	-0.533	Akr1c13	-0.567	Aqp5	-0.614
Npr1	0.595	Mex3c	0.56	Med21	0.522	Mrps16	-0.502	Thy1	-0.534	Lyplal1	-0.568	Cdk11b	-0.615
Gas6	0.595	Luc7l2	0.559	Gfpt1	0.522	Tagap	-0.504	Ppp1r13b	-0.534	Gstt1	-0.568	Pgm2	-0.617
Msl1	0.593	Crbn	0.559	Tnks2	0.521	Endod1	-0.504	Mien1	-0.536	Slc16a9	-0.569	Abcb1a	-0.617
Erbin	0.592	Acsf5	0.559	Aup1	0.521	Cystm1	-0.504	Pnrc2	-0.537	Atp1a3	-0.569	Macf1	-0.618
Tnpo3	0.591	Ubr3	0.558	Bri3	0.52	Abat	-0.505	Gm12191	-0.537	Zfp560	-0.571	Gstm2	-0.619
Gul1	0.591	Ndufa2	0.557	Zfp185	0.519	Micu2	-0.506	Azin1	-0.538	Srp9	-0.571	Mettl26	-0.621
Eif4g2	0.591	Samd9l	0.555	Mospd2	0.519	NA	-0.507	Ube2l3	-0.539	Gspt1	-0.576	Ilvbl	-0.621
Ddc	0.591	Phb2	0.554	Hmgxb4	0.519	Nipbl	-0.508	Trip11	-0.54	Escl	-0.576	Commdb	-0.622
Gm8624	0.59	Asns	0.554	Fam126a	0.519	Lonp1	-0.508	Gsk3b	-0.54	Tom1l2	-0.579	Cggbp1	-0.622
Srf	0.589	Zfhx3	0.553	Ccdc91	0.519	Cygb	-0.508	Gm8430	-0.54	Plpp2	-0.579	Cd68	-0.622
Rps17	0.589	Nfatc3	0.553	Arnt2	0.519	Ap2m1	-0.508	Gm	-0.541	Mycbp	-0.579	Rbpms	-0.623
Fbxl5	0.589	Gars	0.553	Sf3b2	0.518	Syk	-0.509	Kitl	-0.542	Ifi35	-0.579	Gm10177	-0.623
Snrpd2	0.588	Hras	0.552	Serpind1	0.518	Fndc3a	-0.51	Psat1	-0.543	Tcf20	-0.58	Cav1	-0.623
Slc3a2	0.588	Arl2bp	0.551	Rnf170	0.518	Tbcl1d23	-0.511	Dab2	-0.543	Mpc2	-0.58	Oasl2	-0.624
Sh3bgrl	0.588	Ube2h	0.55	Ptprr	0.518	Smpd2	-0.511	Ywhaq	-0.544	Fmc1	-0.584	D1Ert622e	-0.624
Fiz1	0.587	Ube2d2a	0.55	Dnaja2	0.518	Spns2	-0.512	Pfkl	-0.544	Gm2a	-0.586	Txndc9	-0.625
Pdcd11	0.586	Uba3	0.549	Prpf40a	0.517	Ppil3	-0.512	Oxa1l	-0.544	Nhp2	-0.587	Trabd2b	-0.63
Rlim	0.584	Znrf2	0.548	Il1rl2	0.517	Pdia6	-0.513	H2afv	-0.544	Fam189a2	-0.588	Atp5h	-0.63
Nt5c3	0.584	Pten	0.547	Eogt	0.517	P3h4	-0.513	Tfg	-0.545	Rsl1d1	-0.589	Sorsb2	-0.633
Dnajc10	0.584	Mtch1	0.544	Rab31	0.516	Bzw2	-0.513	Mrpl57	-0.545	Gm10288	-0.589	Fdft1	-0.633
Apc	0.583	Map3k7	0.544	Tle4	0.514	Cox6c	-0.514	Cyc1	-0.545	Sash1	-0.59	B4galt4	-0.633
Tbl1x	0.582	Cnbp	0.544	Dynl1	0.512	Gm10273	-0.515	Adam33	-0.545	Pgk1	-0.59	Gm13532	-0.635
Sema3f	0.581	Myrf	0.543	Usp47	0.51	Csgalnact1	-0.516	Prrg3	-0.546	Thrap3	-0.593	BC002163	-0.635
Rnf14	0.581	Golt1b	0.543	Vamp8	0.509	Kcna1	-0.517	Map2k1	-0.546	Ogdh	-0.593	Rai14	-0.636
Rpl13	0.58	Saa3	0.542	Hoxd9	0.509	Plec	-0.518	Trim25	-0.547	Il4ra	-0.594	Park7	-0.636
Mgea5	0.579	Gria3	0.542	B4galt6	0.509	Top2b	-0.519	Jup	-0.547	Syt1	-0.595	Xbp1	-0.637
Pcdhgc4	0.577	Usp14	0.541	Ankrd10	0.509	Nkiras2	-0.519	Rbms2	-0.548	Gabarap	-0.595	Fam162a	-0.64
Odc1	0.575	Pygb	0.541	Iscu	0.508	Ntan1	-0.52	Lims1	-0.548	Guk1	-0.596	Ddt	-0.64
Smpd1	0.574	Mrgprc2-ps	0.54	Ctbp1	0.508	Ndufa10	-0.52	Sdhb	-0.549	Fam180a	-0.596	Zfp622	-0.641
Chrb1	0.574	Selenot	0.539	Fam107b	0.507	Adgrl3	-0.52	Nap1l1	-0.549	Sesn1	-0.597	Imp3	-0.641
Kbtbd2	0.573	Ifi122	0.539	Bms1	0.507	Tmem53	-0.521	Ginm1	-0.551	Uchl3	-0.598	Tkfc	-0.642
Ier2	0.573	Gtpbp4	0.539	Aggf1	0.507	Atf3	-0.521	Dhx40	-0.551	Mrpl12	-0.598	Cmc1	-0.642
Ak7	0.573	Pde4dip	0.538	Rps6	0.506	Txlna	-0.522	Manf	-0.552	Colec12	-0.598	Abrac1	-0.643
Slc25a51	0.572	Hoxc8	0.538	Herc2	0.506	Thsd4	-0.522	Smtn	-0.553	Lrrc58	-0.599	Slc30a1	-0.647
Gbf1	0.572	Farp1	0.538	Agl	0.506	Rpl19-ps11	-0.522	Palm	-0.553	Glrx5	-0.6	Slc1a5	-0.647
Rassf8	0.57	Atraid	0.537	Vmp1	0.505	Mrpl55	-0.522	Slit3	-0.554	Mrpl13	-0.601	St3gal6	-0.649
Ppfbp1	0.569	Rad21	0.536	Nsmaf	0.505	Stx7	-0.524	Dixdc1	-0.554	Ptma	-0.602	Skp1a	-0.649
M6pr	0.569	Tgfb2	0.535	Npepl1	0.505	Ifi27l2a	-0.524	Yipf1	-0.556	P4hb	-0.604	Zdbf2	-0.651
Cpz	0.569	Srrm2	0.534	Cnot7	0.505	Cbr3	-0.524	Stk10	-0.556	Hmox2	-0.606	Mrps24	-0.651
Fam84a	0.567	Xiap	0.532	Cicn4	0.505	Tmem255a	-0.525	Rpl35a	-0.557	Sugt1	-0.607	Gpx7	-0.651
Fam32a	0.567	Nr1d2	0.532	Hibadh	0.504	Gm20721	-0.525	Ppp2r2a	-0.557	Pdlim4	-0.607	Mtss1	-0.653

Appendix Q continued...

Appendix Q: Continued... (Differential expression results between Clusters 1 and 2)

Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC
Rer1	-0.654	Gm8797	-0.694	Cct8	-0.766	Abcb1b	-0.831	Aldoc	-0.945	ApoE	-1.115	Soat1	-1.619
Rpl35a-ps6	-0.655	Clybl	-0.694	Rpl15	-0.767	Jun	-0.834	Lgals2	-0.949	Gucy1a3	-1.124	Itga6	-1.638
Rpl35a-ps4	-0.655	Filip1l	-0.695	Tec	-0.768	Ifi47	-0.834	Mmachc	-0.964	Fgf9	-1.128	Ndr2	-1.661
Rpl35a-ps3	-0.655	Zmym6	-0.696	NA	-0.768	Ndufb9	-0.835	Sparc	-0.965	Affa	-1.131	Csrp2	-1.674
Rpl35a-ps2	-0.655	Tspan7	-0.696	Nrxn2	-0.769	Uqcr10	-0.836	Sdpr	-0.965	S100a4	-1.143	Alcam	-1.687
Gm10243	-0.655	Hspb8	-0.699	Gm1821	-0.77	Sdf2	-0.838	Ctsb	-0.965	Idh1	-1.146	Clu	-1.699
Egfr	-0.655	St3gal4	-0.7	Sat1	-0.771	Ptprf	-0.838	Chchd1	-0.965	Mbnl2	-1.172	Igf2	-1.75
Cdc42ep1	-0.655	Aprt	-0.7	Pgd	-0.773	Ndufs5	-0.839	App	-0.967	Tmem205	-1.176	Ccdc80	-1.81
Fuca1	-0.659	Plscr3	-0.704	Pcbp2	-0.774	Mif	-0.839	Islr	-0.971	Tshz2	-1.177	Atf5o	-1.866
Gm17711	-0.66	Slc6a6	-0.705	Dbi	-0.778	Got1	-0.839	Pim1	-0.974	Dad1	-1.18	Serpinf1	-1.927
Copz1	-0.66	Klf4	-0.706	Rdh10	-0.779	Dap	-0.846	Rab11a	-0.976	Zfp3612	-1.188	Il1r1	-1.927
Acadvl	-0.66	C130026121Rik	-0.706	Ptk2b	-0.779	Wasf2	-0.853	Col6a1	-0.977	Minos1	-1.189	Id3	-1.938
Psmbl2	-0.661	Zfp503	-0.707	Eny2	-0.783	Fads6	-0.857	Fam84b	-0.979	Ptgs1	-1.192	Fxyd1	-2.063
NA	-0.662	Tle3	-0.708	Pnpo	-0.784	Ybx1	-0.861	Nedd8	-0.988	Ptgr1	-1.195	Pkhd11l	-2.094
Gm4070	-0.662	Fcf1	-0.709	Procr	-0.785	Celf2	-0.861	Rcn3	-0.992	Atf1f1	-1.205	Ndr1	-2.094
Ly6e	-0.663	Rgs3	-0.71	Gm10175	-0.785	Gsta3	-0.862	Mrpl30	-0.993	Col6a2	-1.206	Hspb1	-2.127
Tonsl	-0.665	Derl1	-0.71	Rpl36a	-0.787	Syne2	-0.863	Nupr1	-0.995	Anxa6	-1.212	Ptgis	-2.152
Vldlr	-0.666	Pacsin2	-0.713	Fbln1	-0.787	Zbtb20	-0.864	Cyp2s1	-0.996	Ifitm3	-1.213	S100a10	-2.205
Gm9964	-0.666	Ccser2	-0.714	Ndufb4	-0.788	Lama4	-0.866	Slc48a1	-0.999	Hand2	-1.216	Fos	-2.22
Copa	-0.667	Ssu72	-0.715	Anapc13	-0.788	Sqstm1	-0.867	Ghitm	-0.999	Cybrd1	-1.217	Cd55	-2.235
Cox17	-0.668	Arf4	-0.719	Rap2a	-0.791	Bst1	-0.867	Golgb1	-1.004	Dhrs3	-1.219	Angptl7	-2.308
Col1a1	-0.668	Neur1a	-0.721	Ctf	-0.792	Rpl36a-ps3	-0.871	Acaa2	-1.006	Sp100	-1.223	Ctsh	-2.502
Rpl24	-0.67	Snhg11	-0.722	Litaf	-0.793	Heg1	-0.871	Fosb	-1.01	Rbm5	-1.245	Pcp41l	-2.562
Plscr2	-0.67	Man1a	-0.722	Rtn1	-0.794	Cldn3	-0.871	Gbp2	-1.011	Rspo1	-1.257	Nlrc3	-2.853
Cttnb1	-0.67	Nr4a3	-0.723	Pfdn5	-0.796	Gm5436	-0.873	Ndufs7	-1.013	Atf5j	-1.27	Krt19	-2.87
Ccdc36	-0.671	Fh1	-0.725	Podxl	-0.797	Ptfr	-0.875	Hmgcs1	-1.017	Zfp972	-1.271	Fn1	-2.878
Acyp1	-0.672	Ppib	-0.727	Ndufaf1	-0.799	Uqcrc1	-0.879	Cldn15	-1.021	Ppp2r5a	-1.277	Igfbp5	-2.893
Syf2	-0.673	Tmem59	-0.735	Unc119	-0.8	Ubb	-0.881	Tsc22d1	-1.023	S100a1	-1.285	Creg1	-2.995
Bnip3l	-0.674	Ssbp3	-0.736	Tmc6	-0.8	Mpl2l	-0.881	Rpl22	-1.023	Ktn1	-1.287	Loxl1	-3.147
Pkm	-0.675	Akr1a1	-0.738	Oaz2	-0.8	Atp5d	-0.882	Dusp1	-1.023	Pank3	-1.3	Lgals1	-3.298
Ckmt1	-0.675	Syng1	-0.74	Esf3	-0.801	Celf4	-0.887	Zfp975	-1.025	Enpp2	-1.3	Prph	-3.564
Zfp280d	-0.676	Atp5g2	-0.74	Cox7a2	-0.801	Pef1	-0.89	Pcnx2	-1.028	St3gal1	-1.33	Trf	-3.8
Sdh	-0.676	Smpd3	-0.741	Pfdn2	-0.809	Mpl52	-0.892	Tmbim4	-1.052	Psmc1	-1.33	Mt2	-4.01
Tmed4	-0.677	Cxadr	-0.741	Tspan8	-0.813	Igfbp6	-0.892	Ifi27	-1.053	Emilin2	-1.33	Igfbp4	-4.185
Tnk2	-0.678	Ccnd3	-0.741	Cct2	-0.813	Psmc2	-0.894	Ptce1	-1.054	Prss35	-1.333	Mt1	-4.507
Thra	-0.678	Psmbl10	-0.742	Gm11966	-0.814	Cox14	-0.897	Mat2a	-1.06	Casp1	-1.363	Crip1	-4.594
Itm2c	-0.678	Pdhb	-0.742	Cox5b	-0.814	Cd9	-0.897	Mat2a	-1.06	Ncf2	-1.37		
Gnl2	-0.679	Icam2	-0.742	Camta1	-0.816	Selenop	-0.898	Bgn	-1.062	Cdc42	-1.374		
Gvin1	-0.681	Bsg	-0.743	Sec14l1	-0.817	Nnmt	-0.899	Sh3bp1	-1.063	Gm2830	-1.401		
Gm6265	-0.681	Fermt2	-0.749	Il11ra1	-0.817	Prdx6	-0.901	Scp2	-1.063	Prdx1	-1.408		
Fbxo2	-0.682	Aldh1a2	-0.749	Pear1	-0.818	Flrt2	-0.902	Selenok	-1.068	Uqcrh	-1.428		
Agap1	-0.683	Arhgap29	-0.752	Acot1	-0.819	Sulf1	-0.906	Sod1	-1.071	Eno1	-1.43		
Spcs1	-0.684	Tmco1	-0.754	Ube2l6	-0.821	Trim44	-0.91	Elf3g	-1.073	Tgm2	-1.435		
Slc1a3	-0.684	Slc38a2	-0.755	Deptor	-0.822	Rbp1	-0.913	Fam213a	-1.08	Ecm1	-1.441		
Mdh1	-0.684	Cxcr2	-0.756	Ica1l	-0.824	Kdr	-0.914	Gm15280	-1.083	Itm2b	-1.451		
Tpm1	-0.685	Tpt1	-0.757	Arhgdib	-0.824	Creb3l1	-0.917	Mapkapk2	-1.085	Ildr2	-1.474		
Thoc7	-0.685	Bloc1s1	-0.757	Tspo	-0.825	Rhou	-0.918	Tgfb3	-1.091	Tacc1	-1.494		
Cd47	-0.686	Tmem108	-0.761	Ndufa7	-0.825	Prr13	-0.918	Zfp579	-1.096	Rars	-1.503		
Gm3244	-0.687	Espn	-0.761	Gria2	-0.825	Sdhc	-0.921	Mettl7a1	-1.097	Uchl1	-1.529		
Lrrc17	-0.688	Ddah2	-0.761	Megf6	-0.826	Esam	-0.928	Col3a1	-1.097	Ier3	-1.544		
Ddr1	-0.688	Vps25	-0.763	Cryl1	-0.828	Npc2	-0.929	Gm8113	-1.098	Me2	-1.546		
Smim4	-0.689	Nudc	-0.763	Morf4l1	-0.83	Laptn4b	-0.932	Snx6	-1.105	Amhr2	-1.564		
Lox	-0.691	Slc39a10	-0.764	Bicd1	-0.831	Sp140	-0.934	Prkg2	-1.112	Rnase4	-1.61		
Hacd3	-0.691	Nenf	-0.765	Adgrg2	-0.831	Ndufb3	-0.936	Ift20	-1.112	Rpap3	-1.613		

Appendix R: Differential expression results between Clusters 2 and 3 (k=3)

Differentially expressed genes (q-value<0.05) in cluster 3 (E2) relative to cluster 2 (control) in k=3 groupings that have log fold change (logFC) > 0.5 and < -0.5.

Gene	logFC	Gene	logFC	Gene	logFC	Gene	logFC
Igfbp5	2.954	Map2k1	0.841	Tec	0.529	Rps12-ps3	-0.71
Ctsh	2.457	Gpcpd1	0.81	Pcca	0.509	Mmp14	-0.73
Lpl	2.222	Hat1	0.791	Rnf149	0.502	Wdr77	-0.73
Enpp2	2.197	Rhoc	0.772	Tmem173	-0.5	BC022687	-0.73
Pank3	1.945	Hcfc1r1	0.769	Ormdl3	-0.5	Ptgis	-0.73
Tacc1	1.855	Angptl4	0.768	Rhbdf1	-0.5	Hdac7	-0.76
Creg1	1.651	Smoc2	0.765	Sp140	-0.51	Pla2g16	-0.76
Cd55	1.613	Ccdc85a	0.76	Arel1	-0.51	Arid5b	-0.76
Slc25a12	1.612	Sh3bp1	0.754	Mmp11	-0.51	Sdpr	-0.79
Tmem255a	1.531	Ndufa7	0.748	Adh7	-0.51	Ifi202b	-0.8
Lgals1	1.506	Aldh1a2	0.745	Tspan9	-0.52	Farp1	-0.8
Il4ra	1.49	Adgrg1	0.732	Zfp771	-0.52	Nfkbia	-0.8
Cd320	1.486	Mpzl1	0.723	Arhgef17	-0.52	Ptpa	-0.8
Gm6311	1.481	Adgrf4	0.713	Fam134a	-0.53	Tgm2	-0.81
Rars	1.466	Cryl1	0.695	Lgmn	-0.54	Ndrp1	-0.82
Tagap	1.433	Kitl	0.683	Ctpe	-0.54	Trf	-0.82
Idh1	1.357	Rbbp7	0.678	Ctge	-0.55	Hist2h2aa2	-0.83
Rpl10a-ps1	1.356	Mesdc1	0.675	Saa3	-0.55	Thy1	-0.83
Rps15a-ps7	1.32	Hmgb1	0.671	Epn3	-0.56	Cxcl16	-0.83
Bicd1	1.263	Tmem5	0.658	Casp1	-0.56	Tnfrsf8	-0.84
Fgf9	1.242	Btbd3	0.658	Isyna1	-0.56	Pear1	-0.85
Rgs3	1.227	Ica1l	0.657	Ccl2	-0.56	Myk	-0.88
Soat1	1.127	Greb1	0.644	Bgn	-0.58	Kank1	-0.88
Atp6v1b2	1.067	Fut8	0.64	Gsdmd	-0.58	Ahnk2	-0.89
Ncam1	1.055	Plin2	0.634	Fbxo6	-0.59	Nbl1	-0.9
Mapkapk2	1.045	Sfrp1	0.627	Zfp579	-0.59	Sema3f	-0.92
Gm20721	1.029	Ago3	0.623	Prph	-0.59	Efrp2	-0.93
Krt19	1.026	Esyt2	0.612	Oser1	-0.61	Cd63	-0.95
Flrt1	1.006	Col6a1	0.603	Fam32a	-0.61	Tspan8	-0.95
Fosb	0.993	Cd9	0.599	Tmem132a	-0.61	Cryab	-0.97
Pim1	0.953	Ankrd12	0.594	Alcam	-0.62	Maf	-0.98
Gcsh	0.931	Arhgef3	0.592	Mboat7	-0.62	Sorbs3	-0.99
Adgrg2	0.927	Slc25a5	0.582	Unc119	-0.63	C3	-0.99
Ndrp2	0.921	Jak1	0.578	Synp	-0.63	Mgp	-1.01
Cystm1	0.919	Utp14b	0.572	Usp2	-0.63	Nrp2	-1.01
Ctsl	0.919	Dcl1	0.57	Syn3	-0.64	Plxn2	-1.02
Cdc42	0.915	Msr2	0.57	Tjp2	-0.65	Arhgap18	-1.04
Col6a2	0.912	Timm23	0.566	Gm4617	-0.65	Igfbp6	-1.07
Rdh10	0.893	Ppa2	0.563	Hspb1	-0.66	Sbsn	-1.08
Stard5	0.893	Twsg1	0.559	Timp1	-0.67	Tns2	-1.18
Il1r2	0.882	Sdhb	0.554	Inpp5a	-0.67	Gmpr	-1.22
Pkp4	0.878	mt-Nd4l	0.55	Il11ra1	-0.67	Angptl7	-1.23
St3gal6	0.876	Dcbl2	0.55	Tgtp2	-0.68	Timp2	-1.24
Arhgap29	0.857	Dsel	0.546	Cpz	-0.68	C1s1	-1.3
Emilin2	0.856	Pfipb1	0.54	Nr4a1	-0.69	Tnfrsf2	-1.31
Cfdp1	0.856	Prkd3	0.533	Cpe	-0.7	Psap	-1.42
Pcp4l1	0.851	Adgrg6	0.529	Col5a2	-0.71	Tspan4	-1.53

Appendix S: Pseudotime branch-dependent gene expression results

Cluster 1 ranked interaction coefficient with E2 pseudotime branch (Corr.E2).

Gene	Corr.E2	Gene	Corr.E2	Gene	Corr.E2	Gene	Corr.E2
Grhpr	-0.436	Fas	-0.506	Aldh1a1	-0.642	Upk1b	-1.304
Nt5c2	-0.438	Ctsa	-0.507	Capns1	-0.642	Tspan4	-1.517
Irf2bp2	-0.438	Ltbp3	-0.507	Gm4617	-0.642		
Sfxn3	-0.439	Klc1	-0.509	Sars	-0.647		
Araf	-0.439	Basp1	-0.515	Acadl	-0.651		
Fam76a	-0.439	Icam1	-0.517	Rpl34	-0.655		
Rpl27	-0.449	Gm6563	-0.52	Oser1	-0.663		
Ppp2r3c	-0.451	Cst3	-0.526	Cebpb	-0.667		
Myo1c	-0.453	Cysltr1	-0.526	Pcyox1	-0.673		
Myo1d	-0.453	Raph1	-0.532	Scyl1	-0.675		
Zfp106	-0.456	Cltb	-0.532	Tmem151a	-0.675		
Fgfr1	-0.458	Krt7	-0.535	Rab24	-0.68		
2610507B11Rik	-0.459	Gdpd1	-0.535	Fmo1	-0.681		
Atp5k	-0.461	Nub1	-0.537	Atg12	-0.687		
Zfp329	-0.462	Atf4	-0.54	Gm11942	-0.689		
Schip1	-0.464	Rcn1	-0.545	Rpl38-ps2	-0.706		
Ano6	-0.465	Fip111	-0.547	Rpl38	-0.714		
Mras	-0.465	Stat1	-0.548	Ctps	-0.734		
Rpl30	-0.465	Shc1	-0.548	Inpp5a	-0.747		
Uqcrrs1	-0.465	Mbp	-0.549	Trim56	-0.751		
Ier5l	-0.471	Cp	-0.549	Tcf25	-0.77		
Gm10075	-0.471	Cnn2	-0.55	Farp1	-0.773		
Nipa2	-0.474	Eif3b	-0.552	Ptpa	-0.773		
Fdxr	-0.475	Tbl1x	-0.553	Synpo	-0.774		
Cd151	-0.475	Rpl23	-0.553	Wdr77	-0.778		
Ddb1	-0.476	Pcdhgc4	-0.555	Slc46a1	-0.781		
Epb41l2	-0.477	Tpd52l2	-0.557	Mboat7	-0.782		
Fah	-0.479	Aplp1	-0.557	Timp1	-0.792		
2310030G06Rik	-0.479	Mgarp	-0.558	Igsf3	-0.804		
Vat1l	-0.484	Rpl31	-0.559	Igfbp7	-0.818		
Rprd2	-0.484	Rbms1	-0.56	Cpz	-0.838		
Btg1	-0.485	Mmp11	-0.56	Isyna1	-0.838		
Palld	-0.486	Efna5	-0.562	Ccl2	-0.848		
Use1	-0.486	Itga7	-0.562	Arpc1b	-0.857		
Snx10	-0.486	Rpl12	-0.565	Col5a2	-0.857		
Jade2	-0.491	Becn1	-0.575	Klf9	-0.886		
Mfsd1	-0.492	Ap2s1	-0.581	Mylk	-0.918		
Folr1	-0.492	Gtf3c1	-0.583	Rgs10	-0.951		
Peak1	-0.494	Aebp1	-0.591	Ddx6	-0.966		
Tuba1b	-0.496	Gimp	-0.592	Plxnb2	-0.993		
Slx1b	-0.497	Saa3	-0.594	Nbl1	-1.023		
Atp13a1	-0.498	Atp2b1	-0.602	Cryab	-1.056		
Kif1c	-0.499	Eif3k	-0.614	Ifi202b	-1.06		
Exosc4	-0.5	Ypel3	-0.617	Nrp2	-1.075		
Zfp991	-0.502	Ctsd	-0.622	Mgp	-1.081		
Slc9a3r1	-0.502	Sgip1	-0.625	Efemp2	-1.112		
Rpl41	-0.503	Stom	-0.626	Nfkb1a	-1.188		
Ddx17	-0.505	Nme7	-0.628	Cd63	-1.195		
Csnk1g2	-0.505	Appbp2	-0.63	Gmpr	-1.281		

Appendix S continued...

Appendix S: Continued... (Pseudotime branch-dependent gene expression results)

Cluster 2 ranked interaction coefficient with E2 pseudotime branch (Corr.E2).

Gene	Corr.E2	Gene	Corr.E2	Gene	Corr.E2	Gene	Corr.E2	Gene	Corr.E2
Hmgn1	-0.437	Kdm6b	-0.486	Btg2	-0.539	Sde2	-0.632	Pla2g16	-1.02
Trib1	-0.438	Nupr1	-0.488	Prdx6	-0.543	Cxadr	-0.639	Tns2	-1.105
Rnf213	-0.438	2210016F16Rik	-0.49	Nosip	-0.544	Cd38	-0.64	Sorbs3	-1.148
Atp6v1a	-0.439	Sgk1	-0.49	Itpril2	-0.546	Son	-0.645	Cxcl16	-1.162
Trove2	-0.44	1700025G04Rik	-0.491	Josd1	-0.547	Rab11a	-0.647	Mt2	-1.166
Coasy	-0.441	Tns1	-0.491	Ubl5	-0.547	Lgmn	-0.648	Igfbp6	-1.169
Os9	-0.442	Got2	-0.491	Ptov1	-0.548	Tapbp	-0.65	Ndrp1	-1.184
Rpl10a	-0.442	Arf3	-0.492	Hist1h2bc	-0.551	Gm5244	-0.651	Trf	-1.19
Plscr2	-0.445	Grb2	-0.492	Insig2	-0.552	Nr4a1	-0.654	C3	-1.254
Tuba1c	-0.446	Mpzl2	-0.493	Zdhc20	-0.552	Arel1	-0.658	Angptl7	-1.345
Uqcr11	-0.446	Osmr	-0.495	Hs2st1	-0.555	Unc119	-0.659	Timp2	-1.348
Lats2	-0.446	Cfh	-0.495	Lrrc47	-0.556	Arid5b	-0.66	Tnfrsf2	-1.459
Tuba4a	-0.449	Cog4	-0.497	Zfp771	-0.557	Gpx4	-0.661	Psap	-1.619
Dusp3	-0.449	Tmbim1	-0.499	Acaa1a	-0.56	Aamp	-0.672		
Etv5	-0.449	Dnajc15	-0.499	4930529C04Rik	-0.561	Ctdspl	-0.698		
Mrps16	-0.449	Supt6	-0.5	Slc48a1	-0.564	BC022687	-0.704		
Taok1	-0.45	Pkm	-0.502	Lonp1	-0.564	Usp50	-0.706		
Gngt2	-0.452	Tmbim4	-0.502	Rap2a	-0.565	Noct	-0.707		
Pdpn	-0.453	Zfp36	-0.503	Sfpq	-0.565	Ephx1	-0.714		
Pdpn	-0.453	Pabpn1	-0.504	Ppp3ca	-0.568	Casp1	-0.715		
Dym	-0.453	Rps25	-0.506	Pawr	-0.571	Il11ra1	-0.74		
Arhgdib	-0.453	Ptpn9	-0.508	Eef1d	-0.575	Hdac7	-0.742		
Ecm1	-0.453	Cnppd1	-0.508	Gstt1	-0.577	Fbxo6	-0.768		
Polr2e	-0.454	Map2k2	-0.511	Syn3	-0.578	Tgtp2	-0.771		
Sec24d	-0.455	Ndufs2	-0.513	Ubr4	-0.579	Flrt2	-0.774		
Dab2	-0.455	Tmem132a	-0.516	Parp4	-0.579	Mical2	-0.775		
Ets2	-0.456	Bloc1s1	-0.516	S100a4	-0.58	Tgm2	-0.775		
Arpc2	-0.458	Islr	-0.517	Grn	-0.581	Thy1	-0.778		
Tagln2	-0.459	Sparc	-0.517	Tmem173	-0.581	Espn	-0.783		
Fam207a	-0.461	Pold4	-0.517	Il17re	-0.583	Bgn	-0.786		
Sbds	-0.462	Pold4	-0.517	Nmt1	-0.584	Mt1	-0.786		
Usp19	-0.463	Pin4	-0.518	Adh7	-0.598	Rps12-ps3	-0.791		
Chchd10	-0.463	Anxa7	-0.52	Eef1b2	-0.598	Lars2	-0.794		
Lgals3bp	-0.464	S100a6	-0.52	Psme1	-0.602	Ifitm3	-0.797		
Rab34	-0.468	Atp1b1	-0.521	Ilvbl	-0.602	Alcam	-0.805		
Rplp1	-0.469	Snta1	-0.527	Atpif1	-0.605	Npc2	-0.81		
Echdc2	-0.471	Spats2l	-0.527	Zfp579	-0.608	Tnfrsf8	-0.819		
Lpp	-0.471	Sdf4	-0.527	Psen2	-0.61	Hspb1	-0.853		
Rpl37a	-0.473	Exoc6	-0.53	Fkbp1a	-0.611	Ptgis	-0.856		
Pnp	-0.477	Rps29	-0.53	Sp140	-0.611	Clu	-0.868		
Nrxn2	-0.479	Sugt1	-0.531	Gm14681	-0.613	Mrpl52	-0.893		
Sod1	-0.479	Eva1b	-0.532	Mmp14	-0.614	Hist2h2aa2	-0.894		
Atp2b4	-0.482	Vcl	-0.533	Grina	-0.617	Gbp2	-0.91		
Sirt2	-0.482	Spag9	-0.535	Col1a1	-0.619	Psmb10	-0.917		
Gnb1	-0.482	Prnp	-0.537	Gm15501	-0.623	Arhgap18	-0.942		
Ssr4	-0.483	Ifi47	-0.538	Slc16a9	-0.624	Pear1	-0.981		
Acsl4	-0.485	Ormdl3	-0.539	Oaz2	-0.628	Tspan8	-0.993		
Pabpc1	-0.485	Zdhc17	-0.539	Gsdmd	-0.629	Prph	-1.005		
Wdr26	-0.485	Ckmt1	-0.539	Spsb1	-0.63	Sdpr	-1.012		

Appendix S continued...

Appendix S: Continued... (Pseudotime branch-dependent gene expression results)

Cluster 3 ranked interaction coefficient with E2 pseudotime branch (Corr.E2).

Gene	Corr.E2	Gene	Corr.E2
Ahnak	-0.436	Cnot1	-0.563
Tmem2	-0.436	Ltbp4	-0.563
Pon2	-0.438	Cops7a	-0.569
Gm12469	-0.44	Ube2z	-0.571
Nab1	-0.442	Emp3	-0.578
Coro1c	-0.443	Tjp2	-0.58
Megf8	-0.446	Msln	-0.581
Ddc	-0.446	Gsta4	-0.583
Rplp2	-0.446	Bmp4	-0.586
Pcolce	-0.452	Actb	-0.587
Fam168b	-0.453	Bri3	-0.589
Rnf187	-0.459	Myof	-0.602
Suds3	-0.467	Anxa1	-0.604
Rpl21	-0.471	Ccdc86	-0.606
Igfbp3	-0.473	Aes	-0.625
Gm2000	-0.476	Arhgef17	-0.63
Vamp8	-0.477	Ing1	-0.632
Lrrfip1	-0.482	Cpe	-0.635
Ide	-0.484	Usp2	-0.648
Ctgf	-0.484	Rhpn2	-0.648
Rps17	-0.485	Serpib6a	-0.653
Fau	-0.486	Mmd	-0.655
Rpl34-ps1	-0.489	Uap111	-0.669
Pdk3	-0.491	Lima1	-0.681
Wdr89	-0.498	1810037117Rik	-0.684
Rpl27a	-0.498	Tmem176a	-0.686
Egr1	-0.498	C1ra	-0.686
Tspan9	-0.499	Htra1	-0.688
Cyr61	-0.5	Ltbr	-0.69
Tomm7	-0.504	Msn	-0.692
Fam84a	-0.511	Rps27rt	-0.693
Gm10275	-0.516	C1s2	-0.697
Nectin2	-0.516	Itgav	-0.707
Rps15a	-0.517	Tnfrsf1a	-0.742
Rpl13a	-0.52	Atp6v1e1	-0.744
Immt	-0.52	Ldha	-0.748
Rsrc2	-0.52	Tpm3	-0.76
Dynl1	-0.523	Tmem176b	-0.8
Vcp	-0.527	Krt18	-0.812
Rpl17-ps8	-0.529	B2m	-0.813
Gabarapl1	-0.53	Kank1	-0.864
Ndufa9	-0.534	Sema3f	-0.925
Epn3	-0.547	Maf	-0.926
Pmepa1	-0.548	Ahnak2	-0.956
Top1	-0.558	Gsn	-0.983
Ndufa2	-0.56	Sbsn	-1.221
Rps27	-0.56	C1s1	-1.503
Tln1	-0.562		
Itpr1	-0.563		

Appendix S continued...

Appendix S: Continued... (Pseudotime branch-dependent gene expression results)

Cluster 4 ranked interaction coefficient with E2 pseudotime branch (Corr.E2).

Gene	Corr.E2	Gene	Corr.E2	Gene	Corr.E2	Gene	Corr.E2
Igfbp5	2.693	Csrp2	0.699	Cd9	0.468	Zfp503	0.372
Enpp2	2.36	Fos	0.691	Kitl	0.464	Tec	0.37
Ctsh	1.956	Rhoc	0.686	Gm5436	0.462	Fxyd3	0.37
Lpl	1.956	Ccdc85a	0.684	Gja1	0.462	Xpr1	0.368
Tacc1	1.61	Hat1	0.662	Ndufa7	0.461	Nanog	0.367
Tmem255a	1.581	Ndrp2	0.646	Palm	0.459	Rnaset2b	0.367
Pank3	1.509	Mpzl1	0.642	Akr1c14	0.459	Aff4	0.361
Slc25a12	1.475	Cdc42	0.641	Map7	0.454	Dvl3	0.361
Creg1	1.465	Prkd3	0.612	Cbx5	0.451	Lamp2	0.359
Cd320	1.404	Map2k1	0.605	Atp2c1	0.451	Srgap1	0.359
Lgals1	1.387	Mettl7a1	0.6	Socs2	0.447	Gria2	0.356
Tagap	1.28	Utp14b	0.597	Cdk2ap2	0.443	Gm9840	0.355
Fgf9	1.269	Zfp560	0.588	Cdk2ap2	0.443	Ddr1	0.353
Rgs3	1.233	Cryl1	0.581	1700017B05Rik	0.439	Bphl	0.353
Il4ra	1.225	Ago3	0.576	Cfdp1	0.432	Wdr75	0.352
Cd55	1.174	Arhgap29	0.574	Scrg1	0.431	Col12a1	0.351
Rpl10a-ps1	1.152	mt-Nd4l	0.574	BC002163	0.424	Rps13-ps2	0.348
ldh1	1.059	Twsg1	0.568	Slc18a1	0.423	Ubb	0.347
Rars	1.047	Arhgef3	0.561	Mpdz	0.423	Prps1	0.346
Rps15a-ps7	1.016	Rock2	0.559	Col6a1	0.421	Dlx2	0.346
Pim1	1.004	Pqlc1	0.558	Dynl1f	0.421	Adcy2	0.346
Atp6v1b2	0.998	Cygb	0.549	Dynl1c	0.417	Adprh	0.345
Bicd1	0.997	Greb1	0.548	Cbfa2t3	0.413	Nsdhl	0.345
Soat1	0.996	Fut8	0.548	Plekhh1	0.413	Mgst1	0.344
Adgrf4	0.984	Slc39a10	0.547	Ssr3	0.411	Tsc22d3	0.343
Ncam1	0.984	Stx8	0.545	Sptssa	0.409	Ssbp3	0.343
Col6a2	0.932	Gstm2	0.542	Mapk14	0.409	Agap1	0.342
Krt19	0.916	Snrbp2	0.541	Mbnl2	0.407		
Emilin2	0.874	Sdha	0.54	Cgn	0.407		
Mapkapk2	0.867	Sfrp1	0.533	Gvin1	0.401		
Adgrg2	0.856	Tmem5	0.53	Tnfrsf21	0.398		
Sh3bp1	0.829	Sos2	0.524	Hif1a	0.397		
Rdh10	0.81	St3gal4	0.521	Gm6265	0.397		
Galnt1	0.81	Ica1l	0.518	Nhp2	0.396		
Gpcpd1	0.809	Dclk1	0.511	Utp11	0.396		
Angptl4	0.799	Ago1	0.506	Wnt5a	0.393		
Flrt1	0.796	Jak1	0.503	Pknx2	0.393		
Cystm1	0.783	Btbd3	0.503	Timm23	0.393		
Hcfc1r1	0.782	S100a10	0.498	Syk	0.393		
Adgrg1	0.772	Cybrd1	0.498	G6pc3	0.391		
Fosb	0.771	Ano4	0.498	Senp6	0.391		
St3gal6	0.77	Adgrg6	0.493	Armc9	0.385		
Gm20721	0.765	Csad	0.493	Esyt2	0.376		
Pkp4	0.763	Sat1	0.492	Glo1	0.376		
Ilidr2	0.742	Msrp2	0.49	Azin1	0.375		
Smoc2	0.734	Rnaset2a	0.482	Dsel	0.374		
Aldh1a2	0.721	Ppa2	0.479	Wdr46	0.373		
Plin2	0.701	Tgfb3	0.477	Ncoa1	0.373		
Isoc1	0.701	Pcp4l1	0.477	Gucy1a3	0.373		

Appendix S continued...

Appendix S: Continued... (Pseudotime branch-dependent gene expression results)

Cluster 5 ranked interaction coefficient with E2 pseudotime branch (Corr.E2).

Gene	Corr.E2	Gene	Corr.E2
Gm6311	1.587	Sec13	0.362
Gcsh	0.885	Zfp945	0.36
Ctsl	0.881	Hjurp	0.358
Stard5	0.826	Ptdss1	0.355
Dpy30	0.742	Sppl3	0.355
Mesdc1	0.718	Washc4	0.35
2810474O19Rik	0.701	Ube2f	0.35
Ndufv2	0.697	Ndufaf7	0.35
Zbtb7c	0.67	Bhlhe40	0.349
Ermp1	0.604	Gm13841	0.347
Cadm1	0.603	Gm8210	0.347
Slc16a1	0.598	Acat1	0.347
Dcbld2	0.58	Ppp1ca	0.347
Rbbp7	0.561	Ppp1ca	0.347
Ankrd12	0.541	Mea1	0.345
Scd2	0.526		
Rnf149	0.516		
Gtf2a2	0.503		
Pdcd5	0.5		
Vapa	0.495		
Smpd4	0.494		
Clk1	0.483		
Slc25a5	0.454		
Mdh2	0.429		
Magi1	0.42		
Ogt	0.417		
Spast	0.415		
Acadm	0.414		
Ppfibp1	0.413		
Fam234a	0.413		
Prkacb	0.407		
Jagn1	0.404		
Rps16-ps2	0.403		
Ndufb10	0.402		
Gm5514	0.394		
Hnrnpf	0.391		
Krt15	0.388		
Gorasp2	0.387		
Qsox1	0.387		
Fabp5	0.384		
Mrpl23-ps1	0.381		
Serf1	0.378		
Atad1	0.376		
Afdn	0.375		
Cdc42se1	0.372		
Insr	0.371		
Guf1	0.366		
Rpl23a-ps3	0.366		
Abcd2	0.364		