Investigating the Effects of Oocytes on Proliferation Rate and Gene Expression of Mouse Ovarian Surface Epithelium-Derived Cancer Lines

Gina Armstrong

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Abstract

The origin of the most common form of ovarian cancer (OC), epithelial OC (EOC), remains a contentious issue. Due to disease heterogeneity, EOC is unlikely to originate from a single progenitor. This research explores an alternative hypothesis for the origin of EOC. During ovarian development, granulosa cells (GC) recruited from the ovarian surface epithelium (OSE) associate with oocytes. During follicular growth, oocyte-secreted growth factors (OSF) facilitate GC phenotype and function. Thus, if oocytes are lost prematurely from non-growing follicles, naïve GC remain. These cells, devoid of their germ cell regulator, may proliferate leading to neoplastic transformation and heterogeneous tumour phenotypes.

This study aimed to elucidate the effects of OSF on (i) proliferation of, and (ii) candidate gene expression in, two mouse OSE-derived cancer cell lines, namely mOSE T2 (*p53*$^{-/-}$/*Akt*/c-*myc*) and BR (*p53*$^{-/-}$/*Brca1*$^{-/-}$/*Akt*/c-*myc)*. The OSF tested were oocyte-secreted media (OSM) containing rat OSF, as well recombinant (rec) porcine (p) BMP15 and pGDF9. Tritiated-thymidine uptake was used as a measure of cell proliferation and quantitative PCR was performed to measure gene expression levels of *Cdh1* (epithelial marker), *Foxl2* (granulosa cell marker), *Dab2* and *Muc16* (cancer markers).

Exposure of mOSE T2 cells to OSM, but not rec pBMP15+pGDF9, resulted in decreased ($P<0.02$) proliferation rate but no change was observed in mOSE BR cells. Additionally, a decrease ($P<0.02$) in *Muc16* mRNA levels was observed only in the T2 cell line incubated with OSM, but not rec pBMP15+pGDF9 and the BR cell line remained unaffected. Interestingly, *Muc16* and *Bmpr2* mRNA levels were lower overall in the mOSE BR, compared to the T2, cell line.

In summary, both proliferation rate and expression levels of the tumourigenesis marker *Muc16* were reduced in the mOSE T2 cell line after the addition of OSF. This supports the alternative hypothesis that proliferation of naïve OSE-derived GC is kept in check by OSF however, upon premature loss of oocytes or more specifically in the absence of OSF, these cells may proliferate and develop into EOC. Importantly, OSF were unable to suppress proliferation rate and *Muc16* mRNA levels in cancer cells with a *Brac1* mutation.

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List of Abbreviations

AFP - α-fetoprotein

Akt – Protein Kinase B

BMP15 – Bome Morphogenetic Protein 15

Bmpr2 - Bone morphogenetic protein receptor type 2

BRCA1/2 – Breast cancer susceptibility gene 1/2

c-myc, - Myc Transcription factor

CA125 – Cancer antigen 125

CC – Cumulus cell

Cdh1 – E-Cadherin

COC – Cumulus cell-oocyte-complex

Dab2 – Disabled 2

Dazla - Deleted in azoospermia-like autosomal gene

Doc2 - Differentially expressed in ovarian carcinoma 2 gene

EOC – Epithelial ovarian cancer

Fancd2 - Fanconi anaemia complementation group D2

FGF8 – Fibroblast growth factor 8

Foxl2 - Forkhead domain/winged helix transcription factor

GC – Granulosa cell

GDF9 – Growth Differentaation Factor 9

hCG - human chorionic gonadotropin

HGSOC – High grade serous ovarian carcinoma

Hox - Homeobox genes

K-ras, - V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

KO – Knock out

MGC – Mural granulosa cell

Muc16 – Mucin 16

NK cells – natural killer cells

OC – Ovarian cancer

OSE – Ovarian surface epithelium

OSF – Oocyte secreted factors

OSM – Oocyte secreted media

Rec - Recombinant

TGF-β - transforming growth factor-β

TP53 - tumor protein p53

TVA - Avian retroviral receptor

1 Introduction

1.1 Ovarian Cancer

Cancers of the ovary remain one of the leading causes of cancer death in New Zealand women (Vaughan et al., 2006), and although survival rates are slowly increasing, the rate of mortality remains remarkably high (Auersperg et al., 2001). According to the latest data an estimated 22,280 new ovarian cancer (OC) cases will be diagnosed in 2016 in the United States, and more than 14,000 US women will die of ovarian cancer (http://www.ovariancancer.org). The Gynaecological Cancer Foundation of New Zealand estimates that 310 New Zealand women will be diagnosed with ovarian cancer every year resulting in approximately 175 deaths (http://rnzcgp.org.nz/). A lack of effective screening methods and sensitive biomarkers for early detection of pre-invasive tumours, coupled with the high heterogeneity of the disease, has meant that ovarian cancer remains the most lethal of the gynaecological malignancies (Bast et al., 2009).

Ovarian cancers of epithelial origin are referred to as epithelial ovarian cancers (EOC) and are by far the most common, making up 75-90% of all ovarian cancer (Jelovac and Armstrong, 2011). EOC can be further subdivided into categories based on its different histotypes. The four main sub-classes of EOC are serous, mucinous, endometrioid and clear cell carcinoma. The sub-types can be classed into two further groupings; type I and type II. Type I EOC are typically low grade tumors of all types that are characterized by specific genetic and epigenetic modifications and are usually confined to the ovary. They are genetically stable and do not generally express mutations in tumor protein p53 (TP53), which is a tumour suppressor protein. Type II EOC are classified as high grade, aggressive tumours. They carry different genetic alterations to those of type I, and are generally genetically unstable with high instances of TP53 mutations (Bast et al., 2009, Kurman and Shih, 2011). The most common form of type II EOC are high grade ovarian serous carcinomas (HGOSCs), which are a particularly invasive and aggressive form of ovarian cancer. The confusing nomenclature of type I and type II OC (or the “dualistic” model of carcinogenisis) gives the impression of a spectrum of disease, from low to high tumour burden. However, there is a growing

body of evidence that these distinctions represent discrete pathologies, with differing origins and therefore different treatment strategies and clinical outcomes (Panici et al., 2014).

There are also other, more rare ovarian cancer derivatives. Germ cell cancers are relatively uncommon, and only constitute about 5% of malignant ovarian cancers (Pectasides et al., 2008). They arise from the primordial germ cells that are yet to undergo complete differentiation, and although malignancies have been detected in extra-gonadal sites, the majority of these cancers arise in the ovary. The germ cell tumour tissues secrete α-fetoprotein (AFP) and human chorionic gonadotropin (hCG), and thus circulating levels of these hormones are used as diagnostic tools throughout all stages of treatment (Low et al., 2012)

Sex cord tumours (also referred to as stromal tumours) arise from the granulosa cells of the ovarian sex cords. Like germ cell tumours, these are relatively rare and constitute approximately 5% of all ovarian malignancies (Gershenson, 1994). Because the granulosa cells undergo neoplastic transformation after differentiation, the tumour tissue itself remains hormonally-active and continues to secrete oestrogen, making these tumours easily distinguishable from EOC, germ cell, and other peritoneal cancers (Pectasides et al., 2008).

1.2 The Origins of Epithelial Ovarian Cancers

The tissue of origin of EOC is still unclear and hotly debated. Historically it was believed that all EOC arise from the ovarian surface epithelium (OSE). However in recent years, evidence supports the notion that some EOC arise from extra-ovarian sites - for example, the fimbria of the fallopian tubes - and are secondarily deposited onto the ovary. Research into the origin of EOC is still on going, and no consensus has been reached.

The ovary is an incredibly dynamic tissue that undergoes constant tissue remodelling processes. For example, when ovulation occurs, the OSE must rupture in order for the oocyte within the preovulatory follicle to be released. Thus, a rupture site appears adjacent to the preovulary follicle and the usually quiescent epithelial cells begin to proliferate to repair the rupture site. Occasionally, the proliferating epithelial cells will form small inclusion cysts within the

invaginations that remain on the ovarian surface following ovulation. These are believed by some research groups to be the precursors of EOC (Burdette et al., 2007). This theory is further supported by evidence that an increased ovulation rate in mice leads to a higher incidence of EOC. Moreover multiple pregnancies, use of hormonal birth control, and late onset ovulation are known to decrease the chances of developing epithelial carcinomas, presumably due to a decrease in the number of ovulations over a lifetime (Burdette et al., 2007). This "Incessant Ovulation Hypothesis" was first predicted by Fathalla in 1971, and has since been extensively evaluated (Fathalla, 1971). To assess the effect of incessant ovulation on OC occurrence in rodents, Testa *et al.* (1994) used a number of rat OSE cell lines to demonstrate that repetitious growth of rat OSE leads to spontaneous malignant transformations (Testa et al., 1994). In support of this, analysis of metadata obtained from a multitude of studies around the world demonstrated that use of oral contraceptives greatly decreases the risk of development of OC when compared to women without any history of oral contraceptive use (Collaborative Group on Epidemiological Studies of Ovarian et al., 2008). This information provides compelling evidence that cells within the OSE maintain potency and may be capable of proliferating to form tumours.

The OSE and other tissues of the ovary, namely Mullerian-derived tissues, are of coelomic epithelial origin and can differentiate along multiple pathways. What is most interesting about EOC histotypes is that serous, mucinous, endometrial and clear cell EOC share features identical to those of epithelial cells found on tissues outside the ovary, such as the fallopian tubes, endocervix, endometrium and vagina, respectively (Bast et al., 2009). Because of the naïve nature of the OSE cells and their relatively undifferentiated state, it is believed that these cells could go on to differentiate into cell types similar to those found outside the ovary, such as the fallopian tubes, via coelomic metaplasia. This is unusual, as a widely accepted model of cancer progression, coined the "dedifferentiation hypothesis" suggests that cells that have undergone neoplastic transformation become less differentiated than their progenitor cells, but still retain some of the characteristics of their tissue of origin (Sell, 1993).

This is in contrast to the "coelomic metaplasia hypothesis". The hypothesis suggests that coelomic epithelial cells (i.e., the cells of the OSE) are triggered by the hormonal environment of the ovary to undergo transformation into other cell types, such as those of Mullerian duct origin e.g., the fallopian tubes. Interestingly, adult OSE cells have been shown to retain a pluripotent phenotype, and many genes that are highly expressed in the OSE have been characterised as genes for the maintenance of "stem-ness". Theoretically, this suggests that OSE cells may potentially differentiate along numerous pathways, and their capacity as OC initiating cells cannot be overlooked (Bowen et al., 2009). For example, the cell surface glycoprotein CA125 is a marker of epithelial differentiation in differentiated epithelium, as well as tumours derived from the Mullerian ducts, but not in normal OSE.

When undergoing neoplastic transformation, OSE cells undergo further differentiation and thus begin to express CA125 (Auersperg et al., 2001). Once differentiation has occurred, the cells undergo neoplastic transformation and become cancerous. Homeobox (*Hox*) genes that are normally expressed in non-ovarian tissues seem to be abnormally re-expressed in these cancers, leading some to believe that the carcinomas are of extra-ovarian origin (Dubeau, 2008). Although it has been shown that forced re-expression of these genes in OSE cells is possible, the true mechanism remains to be elucidated (Bast et al., 2009). More recent studies have also shown that mouse ovaries carrying mutations in *Tp53* gene are capable of developing into HGSOC, the most common and deadly sub-type of EOC. This study also revealed that in the same genetically-modified mice used for this study, carcinomas arose in the fallopian tubes in 100% of the population. This suggested that although the ovarian cells were capable of developing EOC, the fallopian tubes may have been a more dominant or likely source of EOC, or that the tumorigenic potential of fallopian tubes was higher in these mice (Kim et al., 2015).

The other major theory regarding the origin of EOC is that OC originate in extra-ovarian tissues of Mullerian duct origin and are secondarily deposited onto the ovary. Recent evidence suggests that the fimbria of the fallopian tubes, which are in contact with the ovary during ovulation and inflammation, are able to deposit cells into the ovulation rupture sites on the ovarian surface and form inclusion

cysts (Kurman and Shih, 2011). It is possible that these cells then go on to develop into HGSOCs (Dubeau, 2008). The possibility of extra-ovarian origin of OC has important implications for the treatment of OC. EOC has been observed in the peritoneum of females that have undergone oophorectomy. If the true origin of HGSOC is indeed the fallopian tubes, then the ovaries (and thus the fertility) of at-risk women could be spared during treatment (Kurman and Shih, 2011). Although a large portion of the cystic structures in which EOC develop were not found in the fimbria (Dubeau, 2008), ovaries of women with *BRCA1/2* mutations undergoing risk-reducing surgeries have been analysed and found to rarely contain HGSOCs. The genes *BRCA1/2* have been isolated as the genes responsible for increased familial susceptibility to ovarian and breast cancer (Miki et al., 1994). Rather, the fallopian tubes, particularly the fimbria, contained high-grade tubal intraepithelial carcinomas, leading to the notion that at least in cases of familial *BRCA* mutations, the fallopian tubes can not be ruled out as a source of EOC (Piek et al., 2001).

Due to the large array of tumour phenotypes, it is unlikely that all EOC arise from a single tissue of origin or event, and should thus not be considered a single disease entity. More research must be undertaken to elucidate the cell progenitors and markers of each phenotype in order for the success rate to increase following treatment.

1.3 Ovarian Follicular Development

The development of the ovary and the assembly of ovarian follicles is a tightly regulated and complex process. To provide insight into ovarian pathologies, such as ovarian cancers, one must first understand the events leading up to, and during, development of the ovary. Understanding the origin of the different ovarian cell types, as well as the cell-cell interactions that exist, provides an insight into the development of OC, thus allowing clinicians to develop more sensitive and accurate screening techniques and treatments (Bast et al., 2009).

The formation of the ovary begins around Day 23 of foetal life. By this time, primordial germ cells have migrated from the yolk sac to the gonad, which is beginning sexual differentiation (Juengel et al., 2002). By Day 38 of embryonic development, five distinct cell types can be observed in the ovary: surface

epithelial cells, endothelial cells that constitute the blood vessels, stromal cells, pre-granulosa cells (pre-GC) and germ cells (Sawyer et al., 2002). At this stage, most of the oogonia were associated with at least one pre-GC. Whilst the exact origin of the first pre-GC associated with oocytes in the ≤Day 38 embryonic ovary remains unclear, they are probably of mesonephric origin (Sawyer et al., 2002). From Day 45, the oocyte-pre-GC complexes became isolated from the interstitium of the ovary by generation of a basal lamina eventually forming ovigerous cords. These cords were open only to the OSE, a single layer of flat-to-cuboidal cells surrounding the outside of the ovary (Juengel et al., 2002).

Oogonia then began to proliferate from within the ovigerous cords by Day 55, and by Day 75, the maximum number of oogonia was reached. During the time of oocyte proliferation, little or no proliferation was observed in the small number of associated pre-GC cells. Because the ovigerous cords were open only to the OSE until approximately Day 100, it was established that in sheep, most (>95%) of the granulosa cells were recruited the rapidly proliferating OSE, presumably by the oocyte (Sawyer et al., 2002).

Between Days 75 and 90, a large number of oocytes underwent apoptosis. Interestingly, the granulosa cells that were associated with these degenerating oocytes did not undergo apoptosis. Approximately 600,000 healthy granulosa cells were left behind, and it was believed that these excess cells migrated to, and became associated with, living oocytes (Sawyer et al., 2002). The ovigerous cords were maintained throughout follicular formation and were only lost once all follicles were formed (Juengel et al., 2002).

Oocytes that were no longer associated with the ovigerous cords were almost exclusively surrounded by one layer of squamous granulosa cells and entirely enveloped by basal lamina (Sawyer et al., 2002). These newly formed primordial follicles were located at the interface of the cortex and medulla regions of the ovary, each separated from the ovarian interstitium by its own basal lamina (Juengel et al., 2002). By the timing of birth in the mouse, a large number of primordial, non-growing follicles were located adjacent to the OSE (Pitman et al.,

2012). Understanding the process of folliculogenesis may allow for the origins and causes of OC to be better understood.

1.4 Oocyte Secreted Growth Factors and Their Regulation of Somatic Cells

It is now established that the oocyte itself is responsible for orchestrating the overall developmental rate of the follicle (Eppig et al., 2002). This was elegantly demonstrated through the removal and transfer of oocytes from secondary follicles into primordial follicles. These transfers caused the primordial follicles to develop at twice their normal rate in mice (Eppig et al., 2002). This is likely to be due to essential growth factors secreted by the oocyte during follicular development. Two members of the transforming growth factor-β (TGF-β) superfamily secreted by the oocyte, namely growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15), have been identified as regulators of follicle growth (Dong et al., 1996, Susan et al., 2000).

Through the generation of mouse strains with targeted deletions in the second exon of the *Gdf9* gene, it was discovered that homozygous mutant female mice displayed normal primary follicle (i.e. one-layer of granulosa cells) development, but no normal development occurred past this stage, leading to complete infertility (Dong et al., 1996). Interestingly, homozygous mutant mice with no functional copies of the *Bmp15* gene showed no abnormal follicular development and were fertile. In contrast, sheep with natural homozygous mutations for either the X-linked *Bmp15* gene, or the *Gdf9* gene were infertile due to follicular development not continuing after the primary follicle stage (Hanrahan et al., 2004).

These results showed that the BMP15 protein is essential for normal fertility in sheep, but not in mice, whilst GDF9 protein is essential for both species. Interestingly in sheep, half the normal copies of either *Bmp15* or *Gdf9* through heterozygote mutations resulted in an increased ovulation rate, with ovulatory follicles being smaller in size and acquiring LH receptors earlier (Susan et al., 2000). These results are supported by recent observations that rodents express negligible levels of *Bmp15* mRNA in comparison to *Gdf9* mRNA, whilst sheep express similar levels (Crawford and McNatty, 2012). From this information, it is

obvious that GDF9 and BMP15 are essential for early follicular development in most mammalian species, excluding rodents.

During the growth phase of the oocyte, which occurs between activation of primordial follicle growth and antrum formation, the oocyte is associated with granulosa cells, most (95%) of which are of OSE origin. Mork *et al.* also demonstrate in their 2012 study that the majority of granulosa cells in adult mice are of OSE origin (Mork et al., 2012). As the follicle develops, and antrum formation proceeds, the OSE-derived GCs follow two distinct paths of maturation. Those associated most closely with the oocyte develop into cumulus cells (CCs). The CCs have an intimate association with the oocyte, whereby they extrude trans-zonal projections to form gap junctions with the oocyte, eventually forming the cumulus-oocyte complex (COC) (Albertini et al., 2001). GCs lining the wall of the follicle become what are known as mural granulosa cells (MGC), which are involved mainly in steroidogenesis. It is thought that a morphogenic gradient of oocyte-secreted factors maintain the phenotypes of these cells. Moreover, GDF9 and BMP15 have been reported to regulate apoptosis, DNA synthesis and proliferation of GC and CC. The cells located closest to the oocyte (i.e., the cumulus cells), which in theory would be exposed to the highest concentration of OSF, exhibit lowered levels of apoptosis when compared to MGCs located further from the oocyte (Hussein et al., 2005). By regulating the function of cells surrounding it in this way, the oocyte has control over its own microenvironment, allowing for optimal development.

In regards to their role on reproduction, GDF9 and BMP15 are the most extensively studied of the oocyte-secreted factors. However, BMP6 has also been identified as a potential candidate that may influence somatic cell proliferation and function. BMP6 is expressed in oocytes at high levels, but unlike GDF9 and BMP15, it is not expressed in an oocyte-specific manner in mice (Elvin et al., 2000). BMP6 has also been shown to be less important for follicular development when compared to GDF9 and BMP15. BMP6 knockout mouse models show that a lack of BMP6 has no effect on fertility or litter size (Solloway et al., 1998), nor does it have a significant effect on GC proliferation (Juenel et al., 2006). Another candidate, BMP2, has also been investigated as it is localized to the oocyte, but similarly to

BMP6, BMP2 had no effect on sheep GC proliferation, but may play a role in GC function by enhancing steroidogenic activity (Souza et al., 2002). Fibroblast growth factors (FGFs) have also been of some interest, especially FGF8. In mice, *Fgf8* mRNA was localized to oocytes in maturing, but not in primary, follicles. This positive expression of *Fgf8* was concurrent with somatic cell proliferation suggesting a mitogenic effect of FGF8 on GC, but its true effect is still unclear (Valve et al., 1997).

1.5 An Alternative Hypothesis for the Origin of EOC

As the investigations regarding the origin and progression of OC have focused primarily on the OSE- and Mullerian duct-derived tissues, it is possible that the contributions to OC from other cell types within the ovary have been overlooked. For example, as mentioned above, it has been found in sheep that the majority of granulosa cells in primordial follicles are derived from the OSE (Sawyer et al., 2002).

Pitman et al. (2012) hypothesised that the premature loss of the oocyte from the primordial follicle leaves behind naive GC of OSE origin that may retain a propensity for plasticity. Previous studies have shown that in mice that have undergone chemical or radioactive therapies, the resulting loss of the oocytes leads to the development of ovarian tumours (Howell et al., 1954, Guthrie, 1958). Thus it is possible that these naïve GC, devoid of their germ cell regulator, undergo neoplastic transformation and are therefore capable of developing into multiple tumour phenotypes. It has been shown that GC that were no longer exposed to oocyte-secreted growth factors (GDF9 and BMP15) were still able to proliferate and even form solid cyst or cord-like structures (Brawtal et al., 1993, Wu et al., 2004, Pitman et al., 2012).

There are various animal models that can be used to investigate the fate of ovarian cells when oocytes are lost prematurely. *Deleted in azoospermia-like autosomal* gene (*Dazla)* encodes the germ cell cytoplasmic RNA-binding protein Dazla. It has been shown that disruption of the *Dazla* gene at both loci in mice leads to infertility due to a complete absence of oocytes. A lack of the Dazla protein seems to prevent oocytes from completing meiosis and follicles fail to form. Thus it may

be involved in the processing of mRNAs involved in this process (Ruggiu et al., 1997, McNeilly et al., 2000). In this model, oocytes degenerate before folliculogenesis is complete, so it is likely that only a small number of OSE-derived cells were recruited into the ovigerous cords. These cells were maintained in cord-like structures following the loss (apoptosis) of oocytes and remained mostly benign in young mice. However, as these mice aged, the tumours progressed into malignancies (Pitman et al., 2012).

Fanconi anaemia complementation group D2 (Fancd2) is a protein involved in cellular and DNA damage repair and is necessary for ovarian follicular growth.(Houghtaling et al., 2003, Pitman et al., 2012). In the *Fancd2*-knockout (KO) mouse, no follicles were observed past the primordial follicle stage and multiple tumour phenotypes were observed, especially those of epithelial origin. Because follicular formation occurred in this model, degeneration of oocytes in large numbers of primordial and early growing follicles left behind healthy and naive GC of OSE origin. The tumorigenesis that occurred subsequently in the ovaries of these mice may have been due to the uncontrolled growth of these cells lacking the regulatory control imparted by the oocytes (Pitman et al., 2012).

Sheep that are homozygous for a natural mutation in BMP15 are also a useful model for the study of OC development. As mentioned above, a homozygous mutation in *Bmp15* resulted in oocyte degeneration after the primordial follicle stage. As the oocytes degenerated in these naturally-mutated ewes, follicular 'nodules' appeared within the ovaries and the numbers increased with time. Occasionally, these nodules would aggregate and form tumours. The genes expressed in these tumours were similar to those expressed in normal GC, supporting an alternative that OC may develop from naïve GC of OSE origin (Juengel et al., 2000). The study described herein will investigate this alternative hypothesis.

1.6 Genes of Interest

In order to examine the effects of OSF on OSE cells, this study measured expression levels of key regulatory cancer genes in ovarian surface epithelial cells that were

exposed to various OSF. Each of these genes have been implicated in OC and are discussed below.

Mucins are a family of large (0.5-10 mDa), heavily-glycosylated proteins found predominantly at the mucosal interface between epithelial cells and their extracellular milieu (Allen et al., 1982). Although mucins differ in their structure and function, one hallmark of all mucins is that they contain tandem repeats, although the sequence and number of repeats is variable (Gupta and Jentoft, 1989). The normal role of mucins in the body range from protection of human corneal epithelial cells from dehydration and adhesion of pathogens (Blalock et al., 2007), to protection of the lining of the gut from secreted hydrochloric acid (Bhaskar et al., 1992). Mucins are also important in the female reproductive tract, to serve as a barrier from infection as well as facilitating implantation of blastocysts during pregnancy (Carson et al., 1998). Unfortunately, mucins are also implicated in various types of cancer. In 1981, Bast et al. identified a novel cancer antigen CA125 by using a monoclonal antibody (OC125) against an OSE carcinoma cell line OVCA433 (Bast et al., 1981). Since its discovery, circulating CA125 has been widely used as the basis for serum assays to monitor the progression of OC in patients, and has become an indispensible diagnostic tool as a biomarker for late stage EOC and response to new drugs in clinical trials (Duffy et al., 2005). It was not until 2001 that Yin and colleagues, through cloning on CA125, discovered that the CA125 sequence corresponded with a novel mucin species, MUC16 (Yin and Lloyd, 2001). Subsequent studies revealed the location of CA125 as a MUC16 tandem repeat (O'Brien et al., 2001). Since MUC16 and CA125 were first found in cancer studies, the normal biological role of *Muc16* remains elusive. A 2009 study by Cheon et al. suggests that *Muc16* is unnecessary for development, health and reproduction in mice, and homozygous *Muc16*$^{-/-}$ mutants developed normally (at least until one year of age) (Cheon et al., 2009).

In the case of EOC however, the role of *Muc16*/CA125 is more widely understood. *Muc16* is not normally expressed in OSE, but is expressed in the majority of OC (Davis et al., 1986). It has been demonstrated that immortalised OC cells deficient in *Muc16* did not continue to proliferate after confluence was reached, and tumourigenicity *in vitro* was reduced. Moreover, nude mice injected with *Muc16*$^{-/-}$

KO immortalised OC cells did not show any sign of tumour growth. In contrast, mice injected with *Muc16*$^{+/+}$ immortalised OC cells developed subcutaneous tumours. In the same study, cells made to express the MUC16 protein were shown to have increased tumourigenicity both *in vitro* and *in vivo* (Theriault et al., 2011). The silencing of *Muc16* using short hairpin RNA (shRNA) has also been utilized for suppression of cell adhesion, invasiveness and colony formation in both ovarian and breast cancer cells (Reinartz et al., 2012). More recent studies have investigated the effects of *Muc16* on cellular aggregation. These studies have shown that *Muc16*, highly expressed in cells shedding from the original site of the ovarian tumour, inhibits β-catenin degradation, which is involved in regulation of cell adhesion (Giannakouros et al., 2015). The *Muc16* gene has also been shown to prevent natural-killer (NK) cells from forming synapses with ovarian tumours, thus inhibiting lysis of the tumour cells (Gubbels et al., 2010). This information, together with the fact that MUC16 binds via N-linked oligosaccharides to mesothelin, a protein located on cells lining the peritoneal cavity, facilitates metastasis (Gubbels et al., 2006), has meant that *Muc16* has been recognised as an important factor in tumourigenisis and metastasis. If re-introduction of OSF does indeed cause EOC to revert to a more OSE or GC phenotype, then a reduction in the expression of *Muc16* mRNA in immortalised OC cells would be expected.

The gene *disabled-2* (referred to henceforth as *Dab2*, also known as *differentially expressed in ovarian carcinoma-2* or *DOC*-2) has been reported as being down-regulated in ovarian carcinoma cells (Mok et al., 1994), and has been shown to be expressed in numerous tissues, but primarily in breast and ovarian tissue (Sheng et al., 2000). Human *DAB2* was first characterised by Xu *et al.* in 1995 as a 770 amino-acid protein involved in mitogenic signalling, and is homologous to the *Drosophila* gene, *disabled* (Xu et al., 1995). The *Dab2* gene is involved in a multitude of biological processes throughout the body, especially organisation of epithelial cells during embryogenesis and development. The homozygous KO of *Dab2* in mice results in embryonic lethality, and heterozygous-deficient mice developed abnormally (Yang et al., 2002). Upon further characterisation, it was revealed that by embryonic day 5.5 (E5.5), *Dab2*-deficient embryos were lacking in an observable endoderm layer, and it was theorised that the involvement of DAB2

in cellular trafficking aids in the establishment of epithelial polarity, which is crucial for tissue organisation throughout the body (Yang et al., 2007). It is thus no surprise that the aberrant expression of *Dab2* is implicated in cancer. In ovarian tumour cell lines and tissues, *Dab2* expression was either absent or suppressed in those tissues, while staining of normal ovarian tissue showed that DAB2 was most abundant in the OSE layer (Fazili et al., 1999). Interestingly, in the same study it was noticed that epithelial layers located on the periphery of tumour masses occasionally stained positive for *Dab2*, whilst morphologically normal OSE that was located close to or contiguous with tumour masses stained strongly for *Dab2*. It was concluded that as epithelial cells transformed to be more hyper-proliferative, *Dab2* mRNA expression decreased, leading to the hypothesis that loss of *Dab2* mRNA expression occurs early in tumour development (Fazili et al., 1999). It was noted that loss of *Dab2* does not indicate severity or correlate with increased tumour burden, as higher-grade carcinomas did not show a markedly different *Dab2* expression profile than low-grade carcinomas. This reinforces the hypothesis that decreased *Dab2* expression may signify an early, or even initiating, event in the development of EOC (Fazili et al., 1999). Thus, *Dab2* is a likely tumour suppressor in ovarian cancer. A later study reported that *Dab2* mRNA expression was absent in the majority of cases where epithelial basement membrane integrity was lost or compromised in ovarian tumour tissue. Using collagen IV and laminin as membrane markers, it was observed that *Dab2* expression correlated with the presence of an intact membrane structure. Moreover, in cell lines where *Dab2* expression was absent, restoration of expression using a recombinant adenovirus resulted in a reduction of cell death, and reduced adherence to a basement membrane (Sheng et al., 2000). Thus, the overriding theme from these studies was that increased expression levels of *Dab2* mRNA or DAB2 protein in epithelial cells indicated a transition from a more to a less 'cancerous' state. If the addition of OSF to an immortalised OSE cancer cell line *in vitro* does indeed cause the cells to revert to a more OSE-like phenotype, an increase in *Dab2* mRNA expression would be anticipated.

Bone morphogenetic protein receptor type 2 (BMPRII) is a type II serine-threonine kinase receptor for both oocyte-derived growth factors GD9 and BMP15 (Vitt et al.,

2002, Moore et al., 2003). The cellular location of *BmprII* mRNA expression has been reported on GC, CC, theca tissue and oocytes of developing follicles in rats (Shimasaki et al., 1999), sheep (Feary et al., 2007), goats (Silva et al., 2005), and humans (Assou et al., 2006). Upon binding of its ligand, BMPR2 associates with a type I receptor, resulting in the activation of Smad signalling pathways specific to each ligand (Itoh et al., 2000). For the purpose of this study, the *BmpR2* gene is included as being of interest to elucidate if the immortalised cell lines used are in fact capable of responding to the effects of BMP15 or GDF9.

Forkhead domain/winged helix transcription factor (encoded by the *Foxl2* gene) has been reported to play an essential role in GC differentiation and maintenance of normal ovarian function. In a landmark 2009 study, mutations in this gene were implicated as a potential driving force behind the development of GC tumours of the ovary (Shah et al., 2009). In 2004, Schmidt *et al.* developed a homozygous mutant strain of *Foxl2*-deficient mice in order to investigate the extent of the requirement of functional FOXL2 protein. The results showed that without expression of the *Foxl2* gene, murine GC don't differentiate from squamous to cuboidal in shape, a transition which is necessary for oocyte survival. The homozygous mutants thus experienced ovarian-wide follicular atresia, and were completely infertile, with the GC being incapable of proliferation (Schmidt et al., 2004). Expression of *Foxl2* mRNA was found in GC, but not in oocytes (Cocquet et al., 2002), implicating it as a potential GC-specific marker. Thus, the question arises that if OSF were administered to immortalised OSE cancer cell lines *in vitro,* would the cells revert to a more differentiated, and less 'cancerous', phenotype. If the cells differentiated along a more GC-like path, then expression levels of *Foxl2* mRNA would be expected to increase.

The reference gene used (*Rpl19)* is known to be stably expressed in a wide range of ovarian tissue and was thus considered an appropriate reference gene for this study (Crawford and McNatty, 2012).

This study tests the hypothesis that the exposure of OSF to OC cells would cause the cells to revert to a more GC phenotype, away from their potential precursor, the OSE. It is difficult to characterise OSE as it retains both mesothelial and

epithelial characteristics, and, depending on the stimuli, will assume phenotypic characteristics of one or the other. Human OSE has no established tissue-specific markers, and the typical epithelial marker E-cadherin (CDH1) is not present in normal human OSE, apart from in OSE-lined inclusion cysts. However both early and late stage tumours express CDH1 uniformly, signifying metaplasia (Sundfeldt et al., 1997). Species differences do occur however, and the *Cdh1* gene that encodes CDH1 has been reported to be expressed in normal OSE of rodents (Maccalman et al., 1994). This study will measure *Cdh1* mRNA levels in a murine OSE-tumour cell line exposed to OSF to determine whether these cells lose their epithelial marker and differentiate into a more specialised cell type (e.g. GC).

1.7 Study Hypothesis and Aims

The hypothesis of this study was that upon premature loss of oocytes from primordial follicles, the naïve GC that are left behind are of OSE cell origin and due to their limited exposure to OSF, have retained their plasticity characteristics. Thus these cells, devoid of their germ cell regulator, have the capability to undergo prolific transformations into numerous tumour phenotypes. If this hypothesis is true, then an immortalised mouse OSE cancer cell line incubated with OSF, and in particular BMP15 and GDF9, should result in suppression of proliferation and differentiation of the cancer cells into a more specialised cell types such as GC.

The overall aim of this study was to investigate the effects of rat OSF or a mix of recombinant (rec) porcine (p) BMP15 and rec pGDF9 on (i) the proliferation rate of, and (ii) expression of key genes in two immortalised mouse OSE-derived cancer cell lines (mOSE T2 and mOSE BR).

2 Methods

2.1 Media Preparation

2.1.1 Collection media – αMEM with HEPES

The bench media into which the COCs were collected was α Minimal Essential Media (αMEM; Life Technologies, NZ) supplemented with 20 mM Hepes and 100 IU/mL Penstrep, and was prepared in the following way. One sachet of αMEM was re-suspended in approximately 450 mL of sterile distilled H_2O (dH_2O). To this, 2.385 g of HEPES (Sigma-Aldrich, USA) was added, the solution was adjusted to pH 7.1 using NaOH, and dH_2O was added to a final volume of 500 mL. The solution was then filter-sterilized with a 0.22 µm filter resulting in a slight increase in pH to 7.2. Finally, 5 mL of 100 IU/mL PenStrep (Life Technologies) was added, and the media was stored at 4°C until required.

2.1.2 Incubation media – αMEM with Bicarbonate

The media in which the COCs were incubated under 5% CO_2 conditions was αMEM supplemented with 26 mM sodium bicarbonate and 100 IU/mL Penstrep, and was prepared in the following way. One sachet of αMEM media was re-suspended in approximately 490 mL sterile dH2O. To this, 1.1 g of sodium bicarbonate was added (Sigma-Aldrich, USA). The pH was adjusted to 7.1 if necessary, and the final volume was corrected with dH_2O to 500 mL. The solution was then filter-sterilized using a 0.22 µm filter, 5 mL of 100 IU/mL PenStrep was added, and the resultant media was refrigerated until required.

2.1.3 Incubation media – MCDB150:M199

The media in which the immortalised cells were incubated under 5% CO_2 conditions was 50% MCDB 105 media (Sigma-Aldrich, USA) and 50% Medium 199 (M199; Life Technologies, NZ) supplemented with 5% fetal calf serum (FCS; Sigma-Aldrich, USA) and 100 IU/mL PenStrep. This media was prepared by mixing 250 mL of MCDB 105 media with 250 mL of M199 media. An aliquot of 25 mL was removed from this solution and replaced with 25 mL of FCS and 5 mL of 100 IU/mL PenStrep. The resultant media was refrigerated until required.

2.1.4 Freezing Media – MCDB150:M199

The media into which the immortalised cells were frozen was 50% MCDB 105 media and 50% M199 supplemented with 40% FCS and 6% dimethyl sulphoxide (DMSO; Sigma-Aldrich, USA). To achieve this, 5.4 mL of 1:1 MCDB 105 media:M199 was added to 4 mL of FCS and 600 μL of DMSO. As DMSO is known to generate heat, the solution was allowed to cool prior to the addition of cells.

2.2 Tissue Collection and Culture

2.2.1 Rat Ovary Collection

Pre-pubertal (21-28 days of age) female Sprague Dawley rats that were excess to the requirements of a breeding programme at Victoria University of Wellington were used in this study. Rats are euthanized by asphyxiation using CO_2 gas in a Perspex chamber. Once chest movements had ceased, rats were removed from the chamber and cervical dislocation was performed to ensure death prior to dissection. Rats were placed onto a sterile surface for dissection and the incision site was cleaned with a 70% ethanol solution. An incision was made immediately anterior to the urogenital opening up the midline, ensuring the incision included both the skin and the abdominal lining. Using forceps to locate the oviduct, the attached ovary on each side was removed using sterile micro-scissors, and placed into approximately 7 mL of pre-warmed (37 °C) collection media for transportation back to the tissue culture laboratory. Once back in the lab, the ovaries were stored in the collection media at 37 °C to keep the tissue at under physiologically optimal conditions until COC collection.

Immediately prior to COC extraction, each ovary was washed in a solution of 1x phosphate buffered saline (PBS, Life Technologies, NZ) containing 1% bleach for ten seconds to rid the ovarian surface of bacteria and other contaminants. The ovaries are then again placed in fresh, warm collection media.

2.2.2 Production of OSF-Enriched Media and recombinant porcine GDF9 and BMP15

Rat ovaries were removed from the collection media and placed into a dry petri 35 mm dish. Using a dissecting microscope, excess fat and tissue were removed from the ovary using micro-scissors. An example of a trimmed ovary is demonstrated in

Figure 1. The trimmed ovaries were then placed in a 35 mm petri dish containing fresh, warmed collection media. Using forceps to hold the ovary in place, every visible follicle on the ovary was lanced with a 20-gauge needle, which liberated the contents of the follicle, including the COC. Healthy COCs, defined as an intact oocyte surrounded by 3 or more CC layers (Figure 2), were transferred into another 35 mm petri dish containing fresh αMEM incubation media for washing. Groups of 200 COCs were then transferred into separate wells of a 96-well plate, taking note of the final transferred volume. Once 200 oocytes had been transferred, the well volume was adjusted to a final volume of 50 µL with αMEM incubation media (4 COCs / µL media). Once all the extracted COCs had been transferred and the final well contained fewer than 200 COC, the final volume of that well was adjusted accordingly to ensure a COC density of 4 COCs / µL media was maintained. To prevent media evaporation from wells containing COCs, 100 µL of 1x PBS or media was added to all the bordering wells of the 96-well plate. The COCs were then incubated for 48 hours in a 37 °C incubator under the conditions of 5% CO_2 and ~96% humidity.

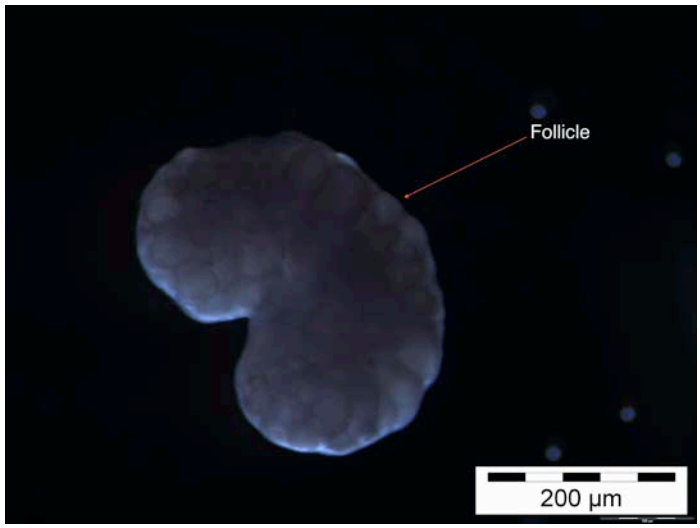

Figure 1. A freshly trimmed, intact rat ovary from a prepubertal rat (20-28 days old).

After the designated 48 hour incubation period, the αMEM incubation media containing the oocyte secreted factors (OSF) was carefully removed using a pipette

and placed into a 1 mL eppendorf tube and stored at -80 °C until required. This media from hereon in will be referred to as oocyte-secreted media (OSM). At the same time, aliquots of αMEM incubation media (control media) were also placed into 1 mL eppendorf tubes and stored at -80 °C to be used for control wells, in the place of OSM.

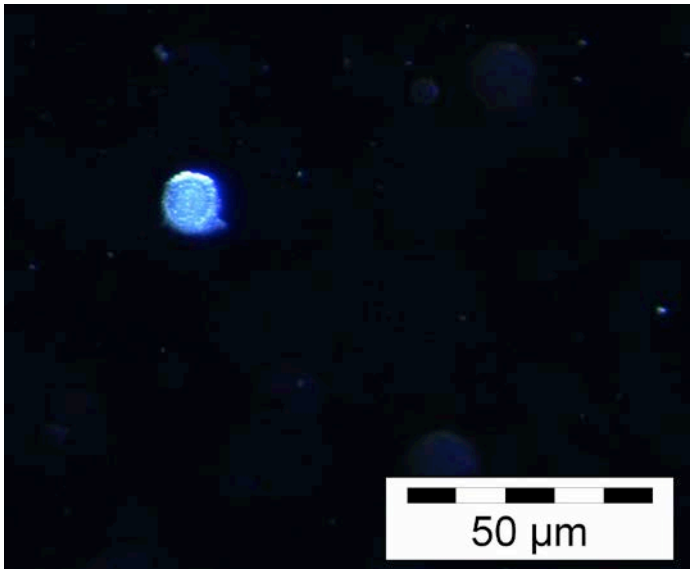

Figure 2. A rat cumulus cell-oocyte complex, defined as healthy due the presence of at least three complete layers of cumulus cells surrounding an intact oocyte.

Recombinant (rec) porcine (p) BMP15 and pGDF9 were generated in-house in the Reproduction Group at Victoria University of Wellington using methodology previously described (McNatty et al., 2005). Their production was not a part of the research presented herein. In short, the coding sequence for porcine BMP15 and GDF9 mature proteins were generated by PCR from porcine oocytes. The resultant cDNA were sub-cloned separately into the pEFIRES-P expression vector and transfected separately into a human embryonic kidney cell line (HEK-293H) by the Fugene 6 transfection reagent. Cells expressing high levels of rec pBMP15 and rec pGDF9 were selected for by increasing the puromycin concentration in DMEM (high glucose) supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. The recombinant protein was produced into serum-free harvesting medium

(DMEM/Ham's F-12 1:1) supplemented with L-glutamine and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin), 0.01 % (w/v) BSA and 100 μg/mL heparin. The proportions (i.e. 1:1 mix) of rec pBMP15 and rec pGDF9 were estimated by Western blotting under reducing conditions using monoclonal antibodies against BMP15 and GDF9 and chemiluminenscence (Janet Pitman, personal communication). Evidence supporting the biological activity of these recombinant proteins included increased proliferation of porcine GC following an 18 hour incubation with rec pBMP15 alone and rec pGDF9, and a synergistic effect was observed when the recombinant proteins were combined (Janet Pitman, personal communication)

2.2.3 Mouse Ovary Collection

Female wild-type and homozygous *Dazl*$^{-/-}$ and *Fancd2*$^{-/-}$ KO mice between the ages of 1-12 weeks were used in this study. Mice were bred for the purpose of this study under the supervision of the Reproductive Laboratory Group at Victoria University of Wellington in accordance with EPA requirements. Mice were euthanized, and their ovaries collected as described above. Once back at the laboratory, ovaries were stored in M199:MCDB150 collection media at 37 °C to keep the tissue at a physiologically optimal temperature until OSE collection.

2.2.4 Mouse Primary OSE Collection and Culture

Due to the delicate nature of the surface epithelium, and the ease at which it is removed, all media in which ovaries were processed was retained, centrifuged at 150 x *g,* resuspended in 2-3 mL of M199:MCDB150 incubation media, and seeded onto a culture dish. All cell populations from each media were kept and grown in separate wells on the same dish. This was to prevent loss of cells from steps other than the scraping of the ovary, such as the Dispase II step. To prevent contamination, all media was first filtered through a 0.2 μm filter, and as many steps as possible were performed in a laminar flow hood. This method is modified from the protocol developed in 2007 by Shepherd *et al.* (Shepherd et al., 2006)

Ovaries were removed from collection media and placed into a 6-well culture dish, in 2-3 mL of pre-warmed (37 °C) fresh, filtered M199:MCDB150 collection/incubation media, careful to keep each genotype separate. In this well,

ovaries were held gently in place using forceps (careful as not to rupture any follicles), and trimmed of fat and oviductal tissue using micro-scissors. The excess fatty tissue was then discarded. Once trimmed, the ovaries were transferred into a new well of a 6-well plate. To this well, 2-3 mL of pre-warmed, filtered Dispase II (Life Technologies, NZ) was added, and the ovaries were then incubated in a 37 °C incubator under the conditions of 5% CO_2 and ~96% humidity for 30 minutes, swirling the dish every ten minutes. Following the 30 minute incubation, ovaries were removed from the Dispase II and placed into another well containing fresh M199:MCDB150 incubation media. Here, ovaries were gently held with forceps while the surface of the ovary was gently scraped with a sterile wire loop. Using a dissection microscope, epithelial cells may be visualised sloughing off from the surface of the ovary, and sheets of OSE clump together (Figure 3). Ovaries were then discarded whilst retaining the OSE cells. The Dispase II solution containing residual cells was transferred into a 15 mL falcon tube and diluted to its entirety with M199:MCDB150 incubation media. As Dispase II is not inactivated by serum present in the media, sufficient dilution is critical to the survival of any OSE cells present. The Dispase II solution was then centrifuged at 100 x *g* for 5 minutes at room temperature and the Dispase II solution carefully aspirated. The OSE pellet was resuspended in 2-3 mL of M199:MCDB150 culture media, and transferred to a new well of a 6-well plate. Because OSE cells are renowned for a poor seeding efficiency and slow growth characteristics, cells were left for 4-5 days prior to the first media change. Any residual red blood cells present were removed in the subsequent media changes.

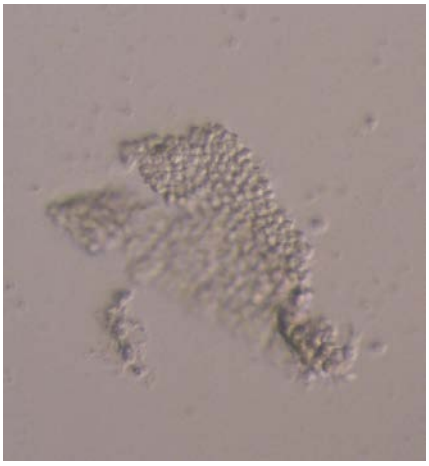

Figure 3 Sheets of primary OSE cells were visualised under a dissecting microscope immediately after the surface of the ovary was scraped with a sterile wire loop.

2.2.5 Primary OSE Passaging

Once the well(s) containing the OSE cells reached 50% confluence, the culture plate was placed on ice for 5-10 minutes. This caused the OSE cells to lift from the bottom of the culture dish without using trypsin or EDTA, which may harm or cause differentiation of the cells. The cells were transferred to a 15mL falcon tube and centrifuged at 100 x *g* for 5 minutes in M199:MCDB150 incubation media. The media was removed and the cell pellet was re-suspended in fresh media and added back into the same well. This was to increase the yield of growing OSE cells. The media was then replaced in the well every 4-5 days with fresh media, until the cells reached 100% confluence. This process took a total time of 2-3 weeks. The cells were then split at a 1:2 ratio and plated into three fresh wells of a 6-well plate at the lower density.

As ovaries do not yield enough OSE cells to use immediately for proliferation assays, growing the OSE cells as described above, should allow for a greater yield of cells and enable the assessment of health by cell morphology.

2.3 Preparation of Immortalised Cell Lines

The two cell lines used in this study were immortalised mouse ovarian surface epithelium (mOSE) T2 and BR obtained from the Orsulic Lab, Memorial Sloan-Kettering Cancer Centre, New York.

2.3.1 Thawing Immortalised Cells

Vials containing immortalised mOSE T2 and mOSE BR cells were removed from liquid nitrogen (~-200°C) and placed in a 37°C water-bath to thaw. Once thawed, the cells were transferred into a 15 mL falcon tube containing 9 mL of MCDB150:M199 incubation media, gently mixed by tube inversion and then centrifuged for 5 minutes at 150 *g*. The media was removed and the pellet was re-suspended in 6 mL of fresh MCDB150:M199 incubation media, before being transferred into a fresh 25 cm^2 cell culture flask. The cells were incubated overnight in a 37°C incubator under conditions of 5% CO2 and 96% humidity. The following day, the old media was replaced with fresh media to remove dead cells not adhered to the flask bottom.

2.3.2 Immortalised Cell Passaging

The MCDB150:M199 incubation media was pre-warmed to 37 °C. Flasks (25 cm^2) containing confluent mOSE T2 or mOSE BR cells were rinsed with 1x PBS, and the PBS was discarded. A 1 mL aliquot of 1x TrypLE Select (Life Technologies, USA) was then added to the flask and incubated at 37°C for 3 minutes to release adhered cells from the bottom of the flask. After the incubation, 9 mL of MCDB150:M199 incubation media was added to the flask to deactivate TrypLE activity. Following gentle rocking of the flask to assist in the re-suspension of cells into the 10 mL media solution, the media was then transferred into a 15 mL falcon tube and centrifuged at 150 *g* for three minutes. The media was aspirated from the tube and the pellet containing the cells were re-suspended in 5 mL of MCDB150:M199 incubation media. Five new 25 cm^2 cell culture flasks containing 1 mL of the cell suspension mix and 5 mL of fresh MCDB150:M199 incubation media were prepared (for a 1:5 split; final volume of 6 mL).

The flasks were then incubated at 37°C under condition of 5% CO_2 and 96% humidity until cells again reached confluence. The cells required passaging approximately every four days.

2.3.3 Immortalised Cell Freezing

Any mOSE T2 or mOSE BR cells that were excess to requirements were frozen until further use. To prepare the cells for freeze-storage, the pre-described passaging

protocol was followed up to the TrypLE step. Thereafter, instead the cells were re-suspended in 9 mL of the MCDB150:M199 freezing media. Following gentle re-suspension, 1 mL aliquots of the re-suspended cells were transferred into cryostat tubes and stored in liquid nitrogen.

2.4 Proliferation Assay

The pelleted mOSE T2 or mOSE BR cells from the final passage step were re-suspended in 1 mL of MCDB150:M199 incubation media for seeding for proliferation experiments (Day 1). For each replicate experiment, 50 µL was removed and the number of cells was counted using a haemocytometer. The remaining cell suspension was diluted with MCDB150:M199 incubation media such that the final concentration was 200 cells/ µL media. A 96-well plate was prepared with a separate row of five wells containing 50 µL MCDB150:M199 incubation media only (control wells) and three separate rows of five wells, each containing 5,000 cells/well (25 µL cell mix + 25 µL incubation media). The cells were incubated overnight in a 37°C incubator under conditions of 5% CO_2 and 96% humidity for 12 hours.

The following day (Day 2), the media was removed from all the wells containing the cells and the control wells. To media alone row ("no cells" group), 50 µL of fresh MCDB150:M199 incubation media was added. To each of the rows containing 5,000 cells, 25 µL fresh MCDB150:M199 incubation media was added together with either: 25 µL of thawed αMEM incubation media (control media; "cells alone" group); 25 µL of thawed OSM (with secreted factors; "OSM-treated" group); or 20 µL of thawed αMEM and 2.5 µL each of recombinant (rec) porcine (p) BMP15 and GDF9 made in-house (1:1 ratio of rec pBMP15:pGDF9; "pBMP15+pGDF9 treated" group). The cells were incubated for 30 hours in a 37°C incubator under conditions of 5% CO_2 and 96% humidity.

Following a 30-hour incubation period (Day 3), 10 µL tritiated thymidine (~10µCi; SciMed, NZ) was added to all wells. The plate was incubated at 37°C under conditions of 5% CO_2 and 96% humidity for a further 6 hours.

Following the 6-hour incubation period, the cells were transferred onto a filtermat (90 x 120 mm, glass fibre filter; PerkinElmer, USA) using the TOMTEC cell

harvester. The harvester was prepared using an empty 96-well plate as a wash plate, and a pre-printed filtermat. Following two sequential pulse-washes with dH_2O, the wash plate was replaced with the 96-well experimental plate, and the filtermat was replaced with a fresh filtermat. Two more pulse-washes were performed to transfer the radioactive cellular material to the fresh filtermat, which was then removed from the harvester and placed into a microwave to dry on high for three minutes. The dry filtermat was then placed inside a MicroBeta sample bag (PerkinElmer, USA) and sealed using a heat sealer. A small corner section of the sample bag was cut off to allow addition of BetaPlate Scint scintillation fluid (PerkinElmer, USA). The bag was re-sealed and excess plastic was trimmed to allow the filter to fit into a tray. The tray was then placed into a Wallac MicroBeta TriLux Luminescence Counter and radiation levels were quantified in each well and displayed as counts per minute (CPM).

2.5 Gene Expression Analysis

2.5.1 Sample Collection

For every proliferation assay, an identical assay was set up for collection of samples for gene expression analyses (i.e. 15 wells per assay; 5 wells of cells alone, 5 wells of OSM-treated cells, and 5 wells of pBMP15+pGDF9 treated cells). At the cessation of the treatment incubation time (i.e. Day 3), wells containing cells to be collected for gene expression analyses were emptied of their media, and 100 µL of TrypLE was added for a duration of 3 minutes at 37°C under conditions of 5% CO_2 and 96% humidity. After 3 minutes, the TrypLE was removed from each well after gentle pipetting up and down to ensure all of the cells had lifted off the bottom of the wells. The TrypLE solution containing the re-suspended cells was then transferred to an eppendorf tube to which a further 1mL of fresh incubation media was added. The fresh MCDB150:M199 incubation media which contains FCS was added to inactivate the TrypLE. Cells were then centrifuged at 1,000 *g* for 5 minutes. Media was aspirated from the tube carefully, as not to disturb the cell pellet. As a wash step, 1mL of PBS was added and the tube was inverted several times prior to a final centrifugation step (1,000 *g* for 5 minutes). The supernatant solution was removed from the tube and the cells were stored at -80°C until required.

2.5.2 Total RNA Extraction

Total RNA extractions were performed using a MasterPure RNA Purification Kit (MCR85102; Illumina, USA) using a modification to the manufacturer's instructions to accommodate a small sample. This protocol includes a DNase step to remove genomic DNA contamination. In full, a mastermix was prepared such that each sample would receive 0.1 µL Proteinase K in 30 µL of Tissue and Cell Lysis Solution. Following the addition of 30 µL of mastermix to each tube containing cells, the tubes were vortexed for 10 seconds. The tubes were incubated at 67°C for 15 minutes. Following this, samples were placed on ice for 5 minutes before adding 18 µL of MPC Protein Precipitation Reagent to each lysed sample, and vortexed for 10 seconds. Samples were then centrifuged at 12,000 *g* for 7 minutes at 4°C. The pellet was very clearly visible and thus following centrifugation, the supernatant was transferred to a clean microcentrifuge tube and the pellet was discarded. A aliquot of 50 µL of RNAse-free isopropanol was then added to the recovered supernatant, and briefly vortexed. The precipitated RNA was then pelleted by centrifugation at 12,000 *g* for 5 minutes at 4°C. As the pellet was nearly invisible at this stage, it was pertinent to orient the tube so the whereabouts of the pellet in the tube was known. Following centrifugation, all residual isopropanol was aspirated and the pellet is allowed to air dry for 5 minutes. During this time, a DNAse I mastermix solution was prepared such that each sample received 0.5 µL of DNAse I in 20 µL of 1x DNAse buffer. Following the addition of 20µL of the DNAse I solution, each tube was votexed briefly and incubated at 37°C for 30 minutes. Following incubation, 20 µL of double strength (2x) Tissue and Cell Lysis Solution was added to each sample and vortexed for 5 seconds. To this, 20 µL of MPC Precipitation Reagent was added and samples were vortexed for 10 seconds and placed on ice for 5 minutes. Following this, samples were centrifuged at 12,000 *g* for 5 minutes at 4°C. The supernatant containing the RNA was transferred into a fresh microcentrifuge tube and 50 µL of isopropanol was added. The samples were briefly vortexed and the precipitated RNA was then pelleted by centrifugation at 12,000 *g* for 5 minutes at 4°C. Again, the pellet was almost invisible at this stage and thus, care was taken to ensure the location of the pellet. The supernatant was carefully aspirated, so as not to dislodge the pellet. Samples were then rinsed once with RNAse-free 70% ethanol and centrifuged at

12,000 *g* for 3 minutes at 4°C. The ethanol was carefully removed and the pellet was left to air dry for 5 minutes, or until the pellet turned from white to clear. The dried RNA pellet was then re-suspended in 10 µL of UltraPure dH_2O, by a one minute vortex step followed by a 2 minute incubation at 55°C following by a final one minute vortex step. The total RNA samples were then stored at -80°C until required for further processing.

2.5.3 cDNA Synthesis

The synthesis of cDNA from the previously extracted total RNA samples was conducted using a SuperScript VILO cDNA Synthesis Kit (11754-050; Agilent Technologies) following manufacturers instructions. In brief, 10 µL of total RNA, 4 µL of UltraPure dH_2O, 2 µL of 10x SuperScript enzyme mix and 4 µL of 5x VILO Reaction Mix were added to each thin-walled, 0.2mL Corbett PCR tube. Tubes were briefly vortexed and placed into a RotorGene 6000 multiplexing system (Corbett Research, Mortlake, NSW, Australia). Tubes were incubated for 10 minutes at 25°C, then 42°C for 120 minutes, and 85°C for a final five minutes to terminate the reaction. cDNA samples were then stored at -20°C until required.

2.5.4 Primer Selection

All primer sets and Taqman probes (where appropriate) were designed using Beacon Designer software (Premier Biosoft, USA) from sequence information obtained from the National Centre for Biotechnology Information website (*Rpl19* Accession number, NM_009864; *Muc16* Accession number, XM_011242635; *Dab2* Accession number, NM_001008702; *Bmpr2* Accession number, NM_007561; *Foxl2* Accession number, NM_012020; and *Cdh1* Accession number, NM_009864). The primers were synthesised by Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA), and the Taqman probes were manufactured by Sigma Proligo (Proligo-Singapore Pte Ltd, Helios, Singapore). The primer and Taqman probe sequence information is listed in Table 1.

Table 1 Forward and reverse primer sequences, and probe sequences for candidate genes of interest

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe
Rpl19	TCGGACTGAACCATTTTG	GCGATTTCATTGGTCTCA	[Cy5]CTCAGGCTACAGAAGAGGCTTGCC[BHQ3]
Muc16	AGTGGAACTGTCACCAAG	GAGAGGGCAATGTTTGAG	[HEX]CAACACCATCACAGAGTCACAAGAAGT[BHQ1]
Dab2	TCCTCAGCCTATGATGTC	GGTCTAAGCCAGTGAAAG	[ROX]CACACCGCCTCAACCACCTC[BHQ2]
Bmpr2	GTGAGGTCACTCAAGGAA	CACAGACTTGTTTCTCTCC	[6FAM]TCAGCCATCCTCTCCTCAGCA[BHQ1]
Foxl2	GACTTCATTAGGCTGTGTTC	GGATAGGAGTGTCATCTGG	-
Cdh1	AAGCGAATTCTAGGACAG	CTCTCAAAGGTCTTGTCTC	[6FAM]CGTTAGAATGTTCTCTTCCTGCTTCCTG[BHQ1]

2.5.5 qPCR Methodology, Optimisation and Validation

Expression levels of the reference gene *Rpl19,* as well as *Bmpr2, Muc16* and *Dab2* were determined using a Brilliant Multiplex QPCR Master Mix (600553; Agilent Technologies) using an established method. The candidate genes *Foxl2* and *Cdh1,* as well as *Rpl19* were quantified using the Brilliant II SYBR Green QPCR Master Mix kit (600828; Agilent Technologies) using an established method.

Triplex reaction mixes were prepared for the quantification of *Bmpr2*, *Muc16* and *Rpl19* mRNA, whilst singleplex reaction mixes were prepared for the quantification of *Dab2* mRNA. In brief, each reaction mix contained 26 µL Brilliant Multiplex qPCR mastermix, 1.04 µL cDNA sample, primers and Taqman probes at optimal concentrations (see Table 1; see below for details), and UltraPure H_2O was added to a total reaction volume of 52 µL. Following mixing, duplicate aliquots of 25 µL were transferred into 0.1 mL strip tubes (PT1-1000; Qarta Bio).

Singleplex reaction mixes were prepared for *Foxl2, Cdh1* and *Rpl19* mRNA. In brief, each reaction mix contained 26 µL Brilliant SYBR Green qPCR mastermix, 1.04 µL cDNA sample, primers at optimal concentrations (see Table 1; see above for details), and UltraPure H_2O was added to a total reaction volume of 52 µL. Following mixing, duplicate aliquots of 25 µL were transferred into 0.1 mL strip tubes (PT1-1000; Qarta Bio).

The 0.1 mL strip tubes were loaded into a 72-tube gene rotor disc and placed inside a Rotor-Gene 6000 multiplexing system (Corbett Research Ltd) for performing quantitative polymerase chain reactions (qPCR). The samples were amplified using the following conditions: denaturation at 95 ˚C for 10 minutes; and a cycling phase of 95 ˚C for 15 seconds and 60 ˚C for 60 seconds, which was repeated for 40 cycles. For those reactions that included the SYBR green qPCR chemistry, this was followed by a melt phase consisting of a 90 second pre-melt, followed by incremental raises of 0.5 ˚C from 60 ˚C to 95 ˚C, with a 5 second wait between each step.

To assess the optimal primer concentrations for each reaction set, all possible combinations of 100 nM, 200 nM, 300 nM and 500 nM of the forward and reverse primer were tested. To assess the optimal Taqman probe concentrations, reactions were prepared using the pre-optimised primer concentrations to test probe concentrations of 50 nM, 100 nM, 200 nM and 300 nM. Negative controls (i.e., samples with no template) were included in each run to ensure no contamination was present. Optimal concentrations for each primer set and for each Taqman probe were determined by choosing the lowest concentrations that resulted in the lowest CT value.

To assess the amplification efficiency of each gene in the reaction sets, serial dilutions from 1 to 1:128 were prepared from pooled cDNA samples of mouse GC, OSE and mOSE T2. Using optimised concentrations of primers and Taqman probes (where appropriated), reaction mixes that included 1.04 µL of cDNA sample were prepared as described above. Amplification efficiencies for *Rpl19* (singleplex), *Rpl19* (triplex), *Muc16, Dab2, Bmpr2, Foxl2* and *Cdh1* genes were 91%, 98%, 99%, 96%, 94%, 100%, and 96% respectively.

Quantification of all genes in each sample was calculated using the ΔΔCT method (Livak and Schmittgen, 2001). The expression levels of all genes were normalised against the appropriate (singleplex or triplex) reference gene (*Rpl19*). These values were then normalised against, and are relative to, a calibrator sample. The calibrator sample was prepared from a pool of cDNA samples from mouse GC, OSE and mOSE T2, such that mRNA could be measured from all genes. The calibrator

sample was incorporated at the beginning and end of every run, and corrects for inter-assay variation. Controls were incorporated in every run and included reactions that omitted addition of template.

2.5.6 Statistical Analyses

All statistical analyses were conducted using IMB SPSS Statistics 20 software package. Data were subjected to P-P plots to ensure data were distributed normally, and were analysed using a two-way ANOVA for analysis of multiplexed genes, and general linear model for analysis of SYBR Green genes. P values <0.05 were considered statistically significant.

3 Results

3.1 Collection and Culture of Primary Mouse Ovarian Surface Epithelium

Primary mOSE cells were collected using the method described above. Although cells were visualised, and deemed healthy immediately after collection, cultures did not survive after the first passage (~9 days of culture). Following three months of efforts to establish this methodology in our laboratory to obtain sufficient numbers of healthy primary OSE cells from wild-type, *Fancd2*$^{-/-}$ and *Dazl*$^{-/-}$-KO mice, this work was abandoned. Thus, experiments on primary mOSE cells were unable to be performed. As an alternative, immortalised mOSE T2 and BR cell lines were used for all future experiments.

3.2 Effects of Oocyte Secreted Factors on Proliferation Rate of mOSE T2 and mOSE BR Immortalised Cells

The overall rate of cell proliferation in mOSE T2 cells incubated with media alone (control), OSM or rec pGD9+BMP15 (1:1 mix) were measured by H^3-thymidine incorporation and are illustrated in Figure 4. Results are displayed as mean counts per minute (CPM) ±SEM that have been normalised against control values. The addition of OSM to cultured mOSE T2 cells resulted in a 5-fold decrease in proliferation rate compared to those cells cultured in media alone. There were no differences in proliferation rate in cells exposed to rec pGDF9+BMP15 mix compared to control or OSM-treated cells.

The overall rate of cell proliferation in mOSE BR cells incubated with media alone (control), OSM or rec pGD9+BMP15 (1:1 mix) were measured by H^3-thymidine incorporation and are illustrated in Figure 5. Results are displayed as mean CPM ±SEM that have been normalised against control values. There were no differences in proliferation rate between control mOSE BR cells and those incubated with either OSM or rec pBMP15+GDF9 mix.

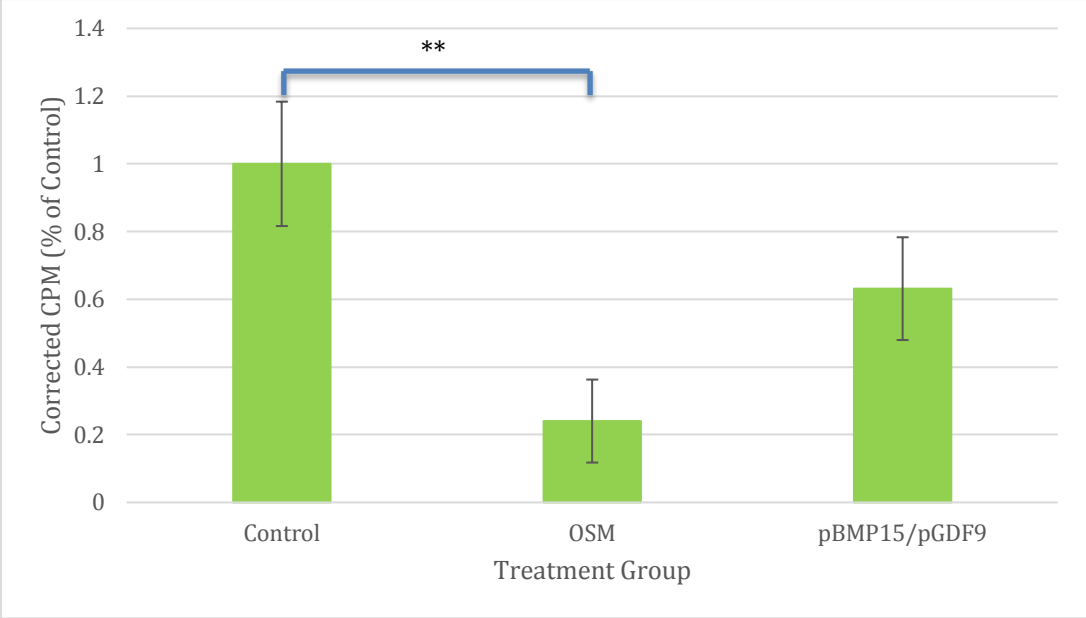

Figure 4 Effects of oocyte-secreted media (OSM), and a 1:1 mix of recombinant (rec) porcine (p) BMP15 and GDF9 on the proliferation rate of immortalised mOSE T2 cells plated at a density of 5000 cells/well, as measured by an H³-thymidine incorporation assay. *Values are mean counts per minute (CPM) ±,SEM that are normalised to the mean value in control cells (incubated in media alone). Treated cells were incubated in media containing either OSM or a 1:1 mix of rec pBMP15+GDF9. Data were normally distributed and analyzed by one-way ANOVA, followed by a Bonferroni post-hoc test. Values that were significantly different from each other are denoted by asterisk (** P=0.015).*

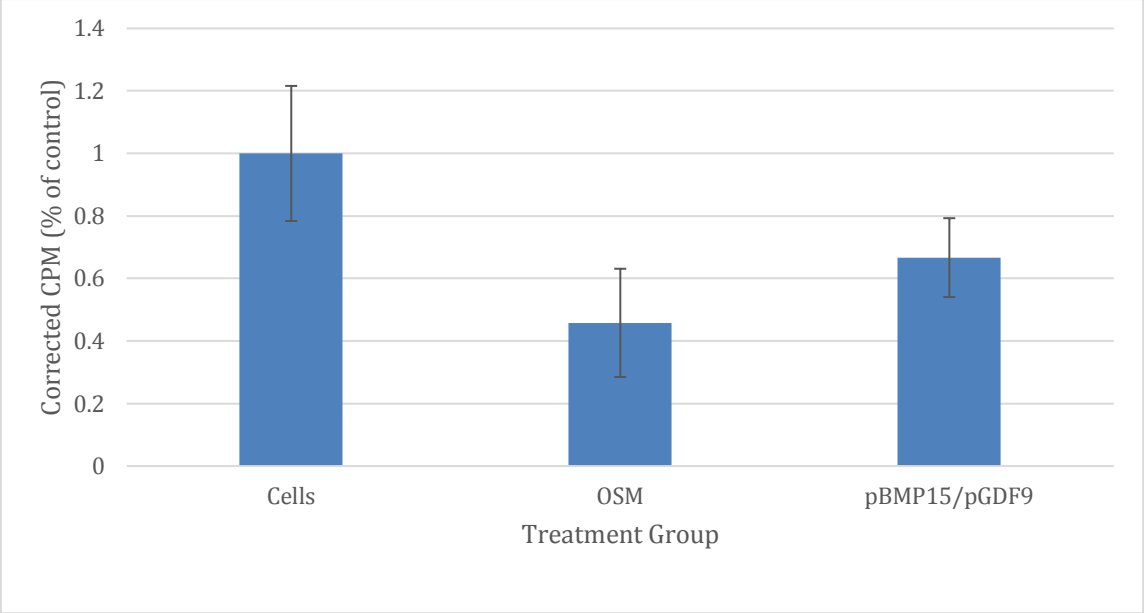

Figure 5 Effects of oocyte-secreted media (OSM), and recombinant (rec) porcine (p) BMP15+GDF9 on the proliferation rate of immortalised mOSE BR cells plated at a density of 5000 cells/well, as measured by a H^3-thymidine incorporation assay. *Values are mean counts per minute (CPM) ± SEM that are normalised to the mean value of control cells (incubated in media alone). Treated cells were incubated in media containing either OSM) or a 1:1 mix of rec pBMP15+GDF9. Data were normally distributed and analyzed by one-way ANOVA. All differences between the treated and control cells had P-values >0.05.*

3.2 Effects of Oocyte Secreted Factors on Candidate Gene Expression Levels in mOSE T2 and mOSE BR Immortalised Cells

The following results revealed the effects of either native (OSM) or recombinant (pBMP15+pGDF9 mix; 1:1 ratio) oocyte secreted factors on overall mRNA expression levels of a range of candidate genes in mOSE T2 and mOSE BR cell lines. All results are displayed as mean mRNA values ±SEM that have been normalised against the reference gene (*Rpl19)* and then against the mean value of control cells.

There were no significant differences in *Bmpr2* mRNA levels between control cells or cells exposed to OSM or rec pBMP15+pGDF9 in either the mOSE T2 and BR immortalised cell line. However, there was a significant difference overall between the two cell lines (Figure 6). The expression levels of *Bmpr2* were lower ($P<0.05$) in the mOSE BR cell line when compared to the mOSE T2 cell line across all treatments.

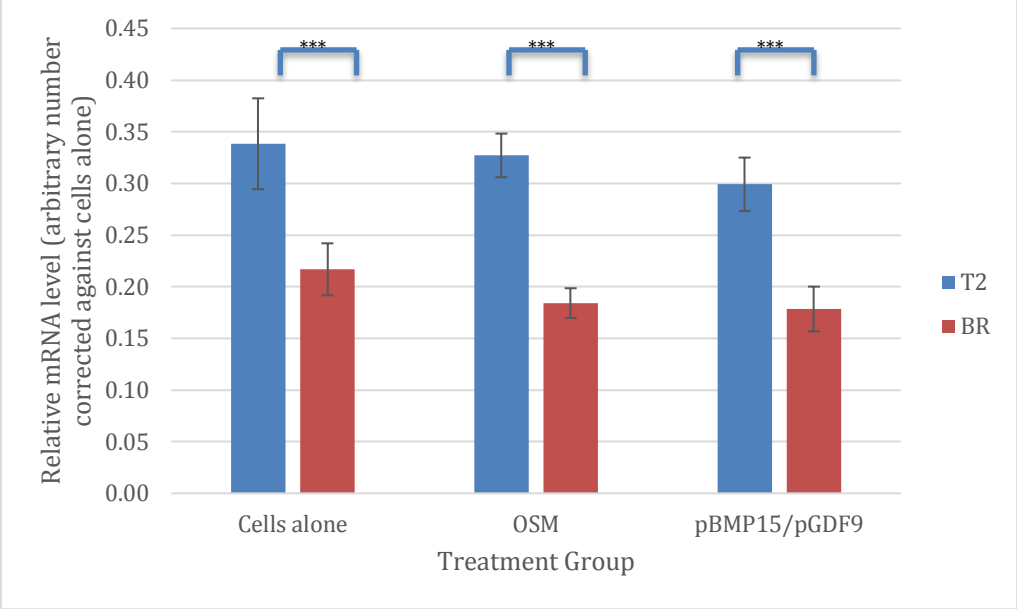

Figure 6 Effects of oocyte-secreted media (OSM), and recombinant porcine BMP15:GDF9 (1:1 mix), compared to media alone (control) on the expression levels of BmpRII mRNA in immortalised mOSE T2 and mOSE BR cells plated at a density of 5000 cells/well. *Mean relative BmpRII mRNA expression levels ± SEM were normalized against Rpl19. Data were normally distributed and analyzed by a two-way ANOVA. None of the P-values calculated were less than 0.05 and thus none of the treatment groups were considered significantly different from each other. The two cell lines were significantly different from each other in the same treatment group (*** P<0.01)*

The addition of OSM, but not rec pBMP15+GDF9, to mOSE T2 cells resulted in lowered (P<0.05) *Muc16* mRNA expression levels, compared to those cells cultured in media alone (Figure 7). There were no differences in *Muc16* mRNA expression levels between control cells and cells exposed to OSM or recombinant porcine BMP15:GDF9 in the mOSE BR cell line (Figure 7). However, an overall difference between cell lines was observed, as levels of *Muc16* were lower (P<0.0???) in the mOSE BR cell line compared to that in the mOSE T2 cell line.

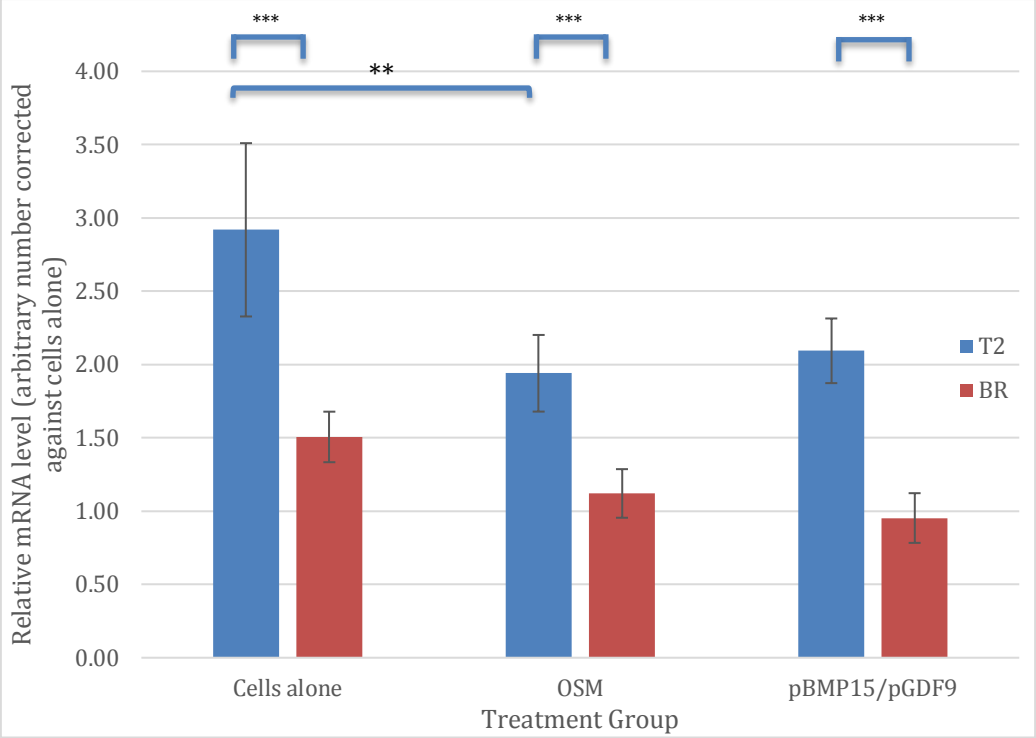

Figure 7 Effects of oocyte-secreted media (OSM), and recombinant (rec) porcine (p) BMP15:GDF9 (1:1 mix), compared to media alone (control) on the expression levels of Muc16 mRNA in immortalised mOSE T2 and mOSE BR cells plated at a density of 5000 cells/well. *Mean relative Muc16 mRNA expression levels ± SEM were normalized against Rpl19. Data were normally distributed and analyzed by a two-way ANOVA. Data were normally distributed and analyzed by a two-way ANOVA, followed by a Bonferroni post-hoc test. Values that were significantly different from each other are denoted by asterisk (** P=0.015). . The two cell lines were significantly different from each other in the same treatment group (*** P<0.01)*

There were no effects of OSM or recombinant porcine BMP15:GDF9 mix on *Foxl2*, *Cdh1* or *Dab2* mRNA expression levels (Figures 8, 9 and 10, respectively) in mOSE T2 and mOSE BR immortalised cell lines. There were also no significant differences detected between the two cell lines in regards to *Foxl2*, *Cdh1* or *Dab2* mRNA expression levels.

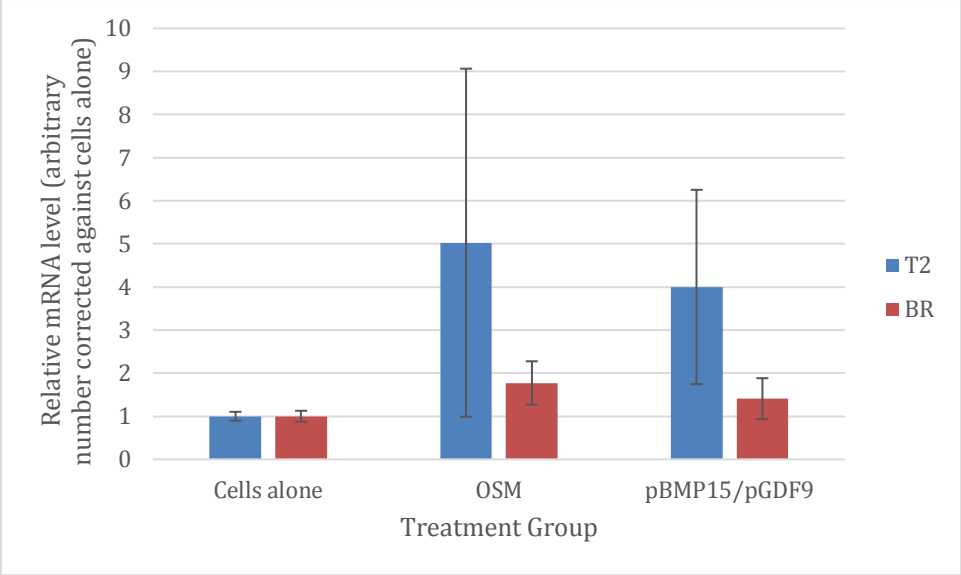

Figure 8 Effects of oocyte-secreted media (OSM), and recombinant (rec) porcine (p) BMP15:GDF9 (1:1 mix), compared to media alone (control) on the expression levels of Foxl2 mRNA in immortalised mOSE T2 and mOSE BR cells plated at a density of 5000 cells/well. *Mean relative Foxl2 mRNA expression levels ± SEM were normalized against Rpl19. Data were normally distributed and analyzed by a two-way ANOVA. Data were normally distributed and analyzed by a two-way ANOVA. None of the P-values calculated were less than 0.05 and thus none of the treatment groups were considered significantly different from each other.*

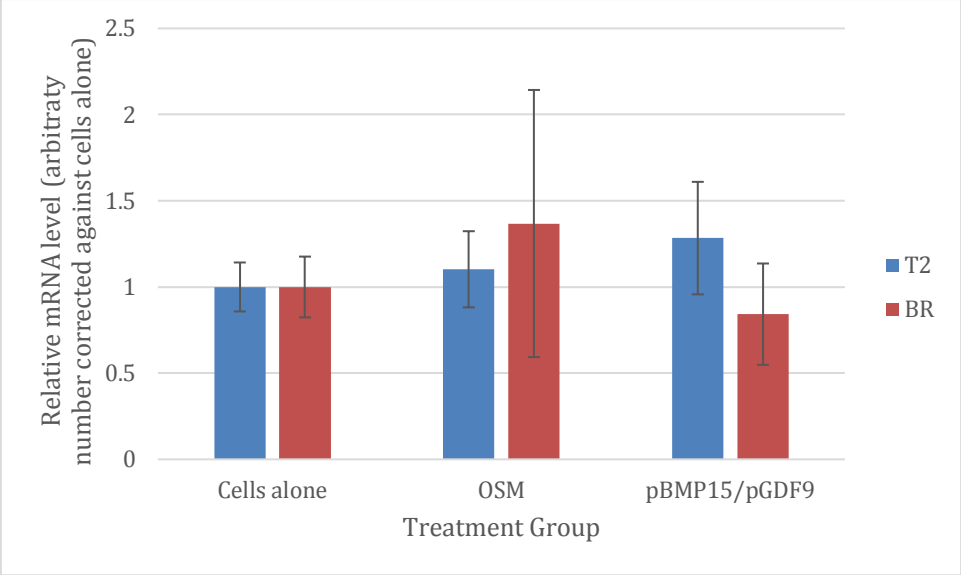

Figure 9 Effects of oocyte-secreted media (OSM), and recombinant (rec) porcine (p) BMP15:GDF9 (1:1 mix), compared to media alone (control) on the expression levels of Cdh1 mRNA in immortalised mOSE T2 and mOSE BR cells plated at a density of 5000 cells/well. *Mean relative Cdh1 mRNA expression levels ± SEM were normalized against Rpl19. Data were normally distributed and analyzed by a two-way ANOVA. Data were normally distributed and analyzed by a two-way ANOVA. None of the P-values calculated were less than 0.05 and thus none of the treatment groups were considered significantly different from each other.*

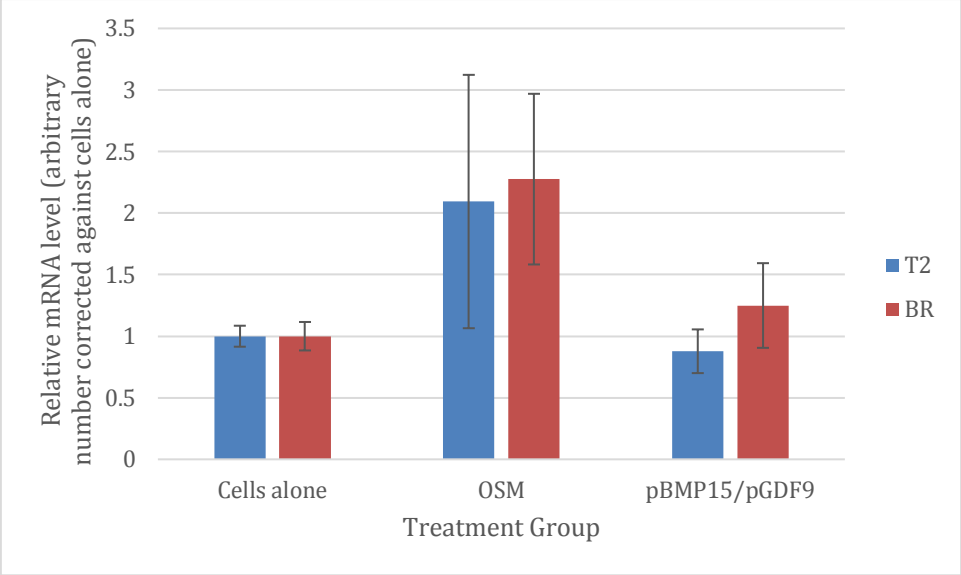

Figure 10 Effects of oocyte-secreted media (OSM), and recombinant (rec) porcine (p) BMP15:GDF9 (1:1 mix), compared to media alone (control) on the expression levels of Dab2 mRNA in immortalised mOSE T2 and mOSE BR cells plated at a density of 5000 cells/well. *Mean relative Dab2 mRNA expression levels ± SEM were normalized against Rpl19. Data were normally distributed and analyzed by a two-way ANOVA. Data were normally distributed and analyzed by a two-way ANOVA. None of the P-values calculated were less than 0.05 and thus none of the treatment groups were considered significantly different from each other.*

4 Discussion

This study was undertaken to test the hypothesis that exposing an ovarian surface epithelial-derived cancer cell line to OSF would have a negative effect on attributes associated with cancer such as cell proliferation and expression of cancer marker genes. This hypothesis was based on the theory that cells surrounding the oocyte are of OSE origin, and that although they may retain some plasticity, the germ cell they are associated with ultimately keeps their proliferation and phenotype in check. Theoretically, the premature loss of this germ cell control may lead the OSE-derived GC to undergo neoplastic transformation, leading to a variety of ovarian cancer phenotypes. The results of the experiments described herein revealed that culturing the immortalised mOSE T2 cell line with media containing native rat OSF leads to a significant decrease in cancer cell proliferation rate, thus supporting the hypothesis. Interestingly, the same results were not observed when the same cells were incubated with rec pGDF9+BMP15 suggesting that an OSF other than GDF9 and BMP15 may be responsible for the anti-proliferative effects. Gene expression analysis was also performed on a number of key genes, but *Muc16* mRNA expression levels were affected by OSF in the mOSE T2 cell line only. Incubation of the mOSE BR cell line with any of the forms of OSF tested failed to elicit any effects on the parameters measured herein.

During follicular development in sheep, oocytes within the ovigerous cords, which are open only to the OSE, recruit large numbers of epithelial cells, which will eventually become the majority of the GC of the primordial follicle (Sawyer et al., 2002). It is well documented that at this time, as well as when damage has occurred due to ovulation, the cells of the OSE are highly proliferative (Auersperg et al., 2001, Sawyer et al., 2002). The effect of TGF-β on the proliferation of the OSE has been extensively studied and it was found that this growth factor inhibits proliferation as well as invasiveness in normal surface epithelium (Berchuck et al., 1992, Rodriguez et al., 2001). In the case of studies investigating the role of TGF- β on epithelial cancer, and OSE-derived cell lines, the results were more varied, but usually the consensus was that transformed and immortalised cell lines don't show a significant decrease in proliferation rate following exposure to TGF- β (Hurteau et al., 1994). In the case of early stage carcinomas however, TGF-β does decrease

proliferation rate, similar to that observed in normal OSE. These divergent observations may be due to the downstream loss of cell cycle inhibitory control in late-stage, but not early-stage, cancers (Kretzschmar et al., 1999). The proteins GDF9 and BMP15 are part of the TGF-β superfamily, and share many structural motifs and similarities to TGF-β. The effect of oocytes, or many of their secreted growth factors including BMP15 and GDF9, on OSE/OSE-derived cancer proliferation has not yet been fully elucidated. However, if these proteins act in a similar manner, it could offer an explanation as to why the addition of OSF to an OSE-derived cancer cell lines would inhibit proliferation in the same way as early stage carcinoma, or normal OSE.

Oocytes also have an effect of the proliferation and differentiation of the follicular somatic cells (GC and CC) within the follicle, and these effects are much more widely documented. It has been observed in multiple studies that factors secreted by the oocyte act as potent mitogens that promote GC proliferation (Vanderhyden et al., 1992, Li et al., 2000, Gilchrist et al., 2001, Gilchrist et al., 2003). In mouse pre-antral follicles in which oocytes have been removed from COCs through oocytectomy (OOX), the follicles showed a decrease in size compared to control follicles. Moreover, when OOX pre-antral follicles are treated with OSM from denuded oocytes, this effect was reversed (Vanderhyden et al., 1992). When more differentiated GC (mural; MGC) are cultured with OSM from denuded oocytes, an increase in proliferation was observed when compared to MGC cultured in media alone. Moreover, CC from OOX complexes exhibited a reduction in H^3-thymidine uptake, a phenomenon that was reversed upon addition of OSM from denuded oocytes. In this case, the H^3–thymidine uptake in CC returned to normal levels observed in COCs (Vanderhyden et al., 1992). The CC and MGC have different physiological roles within the follicle, but work in concert to support the growth and developmental competency of the oocyte. MGCs are phenotypically different from CC in that they more steroidogenic, and are directly involved in follicular growth. The CCs are primarily responsible for aiding oocyte maturation. Their functional and phenotypic differences are maintained and predominantly regulated by growth factors secreted by the oocyte throughout follicular development, thus the oocyte is able to control its own development and

microenvironment (Li et al., 2000). When oocytes were removed from bovine COCs, the CC of the OOX complex begin to lose their CC phenotype and become progressively more similar to MGCs. In particular, the CCs within the OOX complex exhibited decreased proliferation and became more steroidogenically active, with increased progesterone secretion. With re-addition of OSF to these OOX complexes, progesterone production decreased and proliferation rates returned to pre-OOX levels. This suggests that the OSF themselves are responsible for the maintenance of the CC phenotype in the COC. MCGs, on the other hand, react differently to exposure to OSF. Progesterone secretion decreases, indicating a switch to a more CC phenotype. This evidence suggests that GCs in the growing follicle will revert to the MGC phenotype by default, but it is the gradient of exposure to the secreted factors from the oocyte that maintains the two distinct phenotypes (Li et al., 2000). Husein et al. (2005) demonstrated that the effects of the OSF do indeed act along a gradient by conducting an experiment that showed that cells closest to the oocyte and thus the OSF in the bovine COC had a lowered apopotic activity when compared to those on the outside of the COC. In OOX complexes, reintroduction of denuded oocytes into the culture media led to a decrease in apoptotic activity on the cells on the periphery of the OOX. Together, these results indicate that the proximity of the cells within the follicle to the oocyte has an effect on their activity (Hussein et al., 2005).

In regards to the hypothesis for an alternative origin of EOC; if the cells left behind upon premature loss of oocytes were more likely to differentiate along an epithelial phenotype, then one may assume reintroduction of OSF would lead to a decrease in proliferation rate, such as that observed when normal OSE are exposed to TGF-β. Conversely, if the cells differentiate along a more GC-like pathway, then reintroduction of OSM may have the opposite effect and lead to an increase in proliferation. The cells used in this experiment were immortalised mouse OSE cells, which may not follow the true pathway of progression from normal to neoplastic cells as occurs *in vivo.* As these cells are of epithelial origin, and not from cells that have been recruited from the OSE by the oocyte during development of the follicle, they may not be a true representation of the cells involved in the progression of OC following oocyte loss. From the proliferation data, it would seem

as if the cells in this experiment may have retained their epithelial phenotype, and thus proliferation is reduced upon introduction of OSF.

Of all candidate genes studied, only *Muc16* was differentially expressed in the mOSE T2 cell line when treated with OSM. If the alternate hypothesis for the origin of EOC presented herein is correct, it would not be surprising that exposure of mOSE-derived cancer cells to OSF led to a decrease in *Muc16* mRNA levels. As mentioned above, this gene has been implicated in tumourigenesis and cancer metastases. Epithelial mesenchymal transition (EMT) was first described in 1982 by Greenburg and colleagues when, whilst investigating the stability of epithelial phenotypes, they found that adult epithelial cells cultured in collagen gel solutions assume a morphology which could not be distinguished from motile mesenchymal cells (Greenburg and Hay, 1982). Since then, EMT, which is critically important during embryonic development, has been implicated as a promoting factor in cancer cell metastases (Thiery, 2002). In 2004, Rump *et al* demonstrated that MUC16/CA125 binds to mesothelin, a membrane-bound protein that facilitates cell adhesion (Rump et al., 2004). This observation, together with other findings implicating other mucins in cell adhesion and motility (Satoh et al., 2000) would lead one to assume that over-expression of *Muc16* would play such a role in ovarian cancer, and contribute to metastatic processes. Confusingly, a 2011 study found that knock-down of CA125/MUC16 not only promoted cellular migration and invasion in an ovarian cancer cell line, but EMT markers were significantly altered and EMT was induced (Comamala et al., 2011). To explain this finding, the authors suggested that MUC16 associates with E-cadherin, thereby localising it to the surface of the cell and thus allowing contact with other cells to suppress invasion. Thus, knock-down of MUC16 prevented E-cadherin from reaching the cell surface, and facilitated the increased cellular invasion and motility observed. Obviously these confounding results suggest that the true function of MUC16, both in cancer and in development, remains to be fully elucidated.

In the case of this study, decreased *Muc16* expression in the cancer cell line mOSE T2 could mean one of two things; a) that the mOSE T2 cells underwent EMT, or b) that the cells became less tumorigenic. With the variability in the expression of E-Cadherin in this study, as well as no information regarding any post-translational

modifications of any of the mRNA products studied, the decrease in *Muc16* expression up to OSF reintroduction is but a small stepping stone in the path to a greater understanding of the true effects of MUC16 in cancer progression.

Hitherto, it seemed possible that any observed changes in proliferation or gene expression due to OSF may be specifically due to GDF9 and BMP15. In this experiment, COCs were cultured in media, which was then used as a source of OSM. The OSM produced was pooled and aliquoted such that all experiments were subjected to the same batch of OSM. It was deemed preferable to standardise the OSF concentrations rather than using oocytes directly as this method has produced widely-varied results in the past (Janet Pitman, personal communication). Recent studies have demonstrated that *Gdf9* and *Bmp15* mRNA are specific to the oocyte and neither is produced by the closely-associated CCs (Duson and Crawford, 2012). Whilst the amount of OSF bound to the extracellular matrix or to receptors present on cumulus cells was unknown, COCs (rather than denuded oocytes) were still used as a source of OSF as it was unclear whether denuded oocytes would continue to secrete OSF into the medium over an extended incubation period. It is more likely that they would have undergone spontaneous meiotic resumption, after which time *Gdf9* and *Bmp15* mRNA expression levels decline (Mester et al., 2015). Recombinant porcine proteins were used in this study as a source of BMP15 and GDF9 to determine the combined effects of these growth factors, without the presence of other oocyte-secreted growth factors or proteins secreted from the COC. Pigs and rodents are both poly-ovulatory animals. A link has been reported between litter size and the ratio of *Gdf9*:*Bmp15* mRNA detected in the oocyte. Rodents express ~3 to 5-fold more *Gdf9* mRNA than *Bmp15* mRNA (Crawford and McNatty, 2012), and BMP15 secretion from rat oocytes is negligible compared to GDF9 secretion (Lin et al., 2012). Of the mono-ovulatory mammals compared to date, such as the sheep, cow and deer, *Bmp15* mRNA levels were higher than that observed in the rodent with *Gdf9*:*Bmp15* mRNA ratios of ~1.2, 0.24 and 0.10, respectively. Pigs, however, did not follow this trend with a *Gdf9:Bmp15* mRNA ratio of 0.51 revealing a high expression of *Bmp15* mRNA (Crawford and McNatty, 2012). Thus, one would deduce that to investigate the effects of both GDF9 and BMP15 on rodent OSE, pig recombinant proteins are most appropriate. As seen

above, addition of these proteins did not have a significant effect on the proliferation or gene expression in either cell line. This may seem to suggest that the effects observed are not due to GDF9 and BMP15 after all, but some other factor present in the OSM instead. This is supported by the fact that secreted media from rat oocytes, presumably containing negligible BMP15, were capable of eliciting an effect on one of the mOSE-derived cancer cell lines. It is important to note that species differences exist in the onset of expression of *Bmp15* and *Gdf9.* In sheep, *Gdf9* expression is observed at early stages of development, when GC can still be considered naïve in nature, whereas *Bmp15* expression occurs later, closer to birth, where the GC are more differentiated (Mandon-Pepin et al., 2003). This evidence, as well as evidence mentioned above that rodents express negligible levels of *Bmp15,* suggest that perhaps introducing both factors to the mOSE-derived cancer lines was no appropriate, and that *Bmp15* would probably have a negligible effect on the differentiation of GC compared to *Gdf9*.

The mouse cell lines used for this study were developed to replicate EOC development and progression in humans. These cell lines were developed using an avian retroviral gene delivery method for introduction of one or multiple oncogenes and marker genes into mouse OSE. Avian retroviral receptors, *Tva*, were ectopically expressed on the surface of mouse cells, allowing for the delivery of other avian derived vectors carrying coding regions of oncogenes or marker genes. These were delivered to the cell via the TVA receptor. Thereafter, the viral genome was then replicated by the host, allowing for sustained expression of the newly-introduced gene(s). For this study, *Keratin5-tva* transfected cells were used, as expression of *Tva* was restricted to the OSE and no other ovarian cell types. The *Tva*-transgenic mice were crossed with *p53*-knockdown mice to obtain a preliminary *K5-Tva/p53*$^{-/-}$ mouse strain. As stated earlier, Type II EOC, especially HGSOC, contain high instances of p53 mutations. Other oncogenes involved in cellular growth and death pathways in EOC were also induced in various combinations to elucidate their effects on their own and in combination with the *p53* mutations. Regardless of p53 status, expression of vectors carrying only one oncogene (c-*myc,* K-*ras,* or *Akt*) did not lead to the development of tumours from ovarian cells. However, when two or three of the oncogenes were expressed in

combination with *p53* mutations, tumours did form. Thus it was concluded that to investigate the progression of OSE-derived cancers, cells derived from mice with mutations in the tumour suppressor gene *p53*, as well as at least two of the aforementioned oncogenes must be used (Orsulic et al., 2002). Accordingly, the mOSE T2 cell line harbours a *p53*$^{-/-}$/*Akt*/c-*myc* phenotype.

Women with mutations in *BRCA1* have an increased susceptibility to development of reproductive cancers such as breast cancer and OC, including EOC (Easton et al., 1995). A 1999 study concluded that *Brca1* most likely acts as a 'caretaker gene', and that loss of function of *Brca1* leads to increased rates of mutation for genes which are important for cancer development and progression (Xu et al., 1999). The same study also concluded that mutations in *p53*, located on the same chromosome as *Brca1,* play a large role in the development of tumours. By generating *Brca1*:*p53* mutant mice, accelerated tumour formation was observed. Thus, the mOSE BR cell line was generated in the same way as the mOSE T2 line, but with the addition of a *Brca1* mutation. The *Brca1* mutation consisted of a deletion of exon 11, which includes approximately 60% of the BRCA1 protein (Orsulic et al., 2002). BRCA1 and p53 are also known to co-precipitate, and BRCA1 regulates *p53* dependent gene expression by interacting with the p53 C-terminus, which is also crucial for p53 DNA binding (Ouchi et al., 1998). This cell line was used in this study as well as mOSE T2 in order to encompass a wider range of EOC possibilities and outcomes.

In this study, when both cell lines were treated with OSM, a significant decrease in proliferation was observed for the mOSE T2, but not mOSE BR, cell line. As mentioned above, the *Brca1* gene has been reported to be a potent suppressor of cellular proliferation. Transfection of a normal *BRCA1* gene into human ovarian and breast cancer cell lines, or mouse breast tumors, in which the protein had lowered, aberrant or absent translation resulted in a significant inhibition of proliferation (Holt et al., 1996). Similarly, inhibition of *BRCA1* in primary mammary epithelial cells, as well as in established cell lines, led to a marked increase in cellular proliferation. Therefore as the mOSE BR cell line expresses a mutated *Brca1* gene*,* it is not surprising that the addition of factors that may inhibit proliferation would have no significant effect. The fact that the *p53* gene is also

mutated in the mOSE BR cell line would further compound this effect as BRCA1 has been shown to play a crucial role in the p53-mediated growth inhibitory pathway. That is, BRCA1 potentially enhances *p53*-dependent transcription of DNA, as well as physically interacting with p53 itself, to enhance the anti-proliferative, tumour inhibition of p53 (Zhang et al., 1998). Thus, the fact that the mOSE T2 cell line had a fully functional *Brca1* gene, whilst the mOSE BR cell line did not, may explain the proliferation differences observed following incubation with OSM. In other words, a fully-functional *Brca1* gene inferred susceptibility to the proliferative inhibitory effects of OSM.

It was also observed in this study that both *Bmpr2* and *Muc16* were expressed at significantly lower levels in the mOSE BR, compared to the mOSE T2, cell line. Overexpression of TGF-β1 exhibited a negative effect on mammary tumour development and *Tgfb1* expression was clearly lower in cancer tissue (Pierce et al., 1995). If the assumption was made that the mOSE BR cell line is similar to epithelial familial cancers of the breast and ovary, it would be expected that *Bmpr2,* involved in TGF-β signalling, would be expressed at lower levels than the mOSE T2 line, as was the case. Whilst an association between *Brca1* expression and Smad signalling in samples of hereditary breast cancer tissue was not observed (Xie et al., 2002), suppression of the co-SMAD SMAD4 confirmed a role for SMAD signalling in the actions of TGF- β in a rat mammary adenocarcinoma cell line (Giampieri et al., 2009) thus an explanation for the lowered expression of *Bmpr2* in the mOSE BR cell line remains elusive. Similarly, *Muc16* expression was decreased in the mOSE BR cell line. However due to the fact that down-regulation of *Muc16* expression has been reported to be associated with both a lessened and a heightened cancer-like phenotype, a meaningful explanation for the lower expression levels of *Muc16* in the BR cell line is difficult.

5 Summary

In summary, both proliferation rate and expression levels of the tumourigenesis marker *Muc16* were reduced in the mOSE T2 cell line after the addition of OSF. This supports the alternative hypothesis that proliferation of naïve OSE-derived GC is kept in check by OSF however, upon premature loss of oocytes or more

specifically in the absence of OSF, these cells may proliferate and develop into EOC. Importantly, OSF were unable to suppress proliferation rate and *Muc16* mRNA levels in cancer cells with a *Brac1* mutation.

6 Future Directions

Overall, the results of this study indicate that OSM, presumably containing the OSFs GDF9 and BMP15, result in a decreased level of proliferation in the mOSE T2 cell line, but not the mOSE BR cell line. To gain a more thorough understanding of the true reason for the observed results, these experiments should be repeated on GC cell lines from rodents, which are readily available. Perhaps the best and most telling information would come from the use of primary OSE and GC from wild-type mice, and KO mice, such as the *Dazl*$^{-/-}$ and *FancD2*$^{-/-}$ strains, which lose their oocytes prematurely. As mentioned above, an appropriate culture method for these cells was unfortunately not achieved in this study.

In this study, OSM was used as a standard treatment to investigate the effects of oocytes on the aforementioned cell lines. But which factors are the cause of the effects seen? To investigate this, the next step would be to check for the presence or absence of various proteins using immunoblotting with antibodies against each protein of interest. Once identified, these proteins (recombinant or native) would be added to cell culture individually, and their effects investigated. In this study, porcine recombinant proteins were used to elucidate the effects of GDF9 and BMP15 on the proliferation and gene expression of rodent epithelium. This may not have been appropriate, as downstream signalling in response to these growth factors varies depending on species of growth factor and target cell (Reader et al., 2011). Thus, use of recombinant rodent growth factors in place of porcine ones would have been more useful and appropriate to elucidate the true effects of these OSF. As mentioned above, the level of BMP15 in the rodent follicle is negligible (Crawford and McNatty, 2012), thus it may not have been appropriate at all to use recombinant BMP15 in this study. Repeating the experiment with denuded oocytes from different species and comparing any differences could also be of interest to future investigation into the influence of CCs in these studies.

Of all the genes investigated, the only change in expression as a result of treatment was a decreased *Muc16* expression in the mOSE T2 cell line as a result of treatment with OSM. As a deeper investigation of the literature revealed, this could mean either a decrease or increase in cancer-like phenotype, and requires further investigation.

Finally, future studies should repeat these experiments in order to investigate any roles or interactions *Brca1* has with these genes and their products in order to elucidate why the mOSE BR cell line behaved differently from the mOSE T2 cell line.

Bibliography

Albertini, D. F., C. M. Combelles, E. Benecchi and M. J. Carabatsos (2001). "Cellular basis for paracrine regulation of ovarian follicle development." Reproduction **121**(5): 647-653.

Allen, A., A. Bell, M. Mantle and J. P. Pearson (1982). "The Structure and Physiology of Gastrointestinal Mucus." Advances in Experimental Medicine and Biology **144**: 115-133.

Assou, S., T. Anahory, V. Pantesco, T. Le Carrour, F. Pellestor, B. Klein, L. Reyftmann, H. Dechaud, J. De Vos and S. Hamamah (2006). "The human cumulus-oocyte complex gene-expression profile." Human Reproduction **21**(7): 1705-1719.

Auersperg, N., A. S. T. Wong, K. C. Choi, S. K. Kang and P. C. K. Leung (2001). "Ovarian surface epithelium: Biology, endocrinology, and pathology." Endocrine Reviews **22**(2): 255-288.

Bast, R. C., M. Feeney, H. Lazarus, L. M. Nadler, R. B. Colvin and R. C. Knapp (1981). "Reactivity of a Monoclonal-Antibody with Human Ovarian-Carcinoma." Journal of Clinical Investigation **68**(5): 1331-1337.

Bast, R. C., B. Hennessy and G. B. Mills (2009). "The biology of ovarian cancer: new opportunities for translation." Nature Reviews Cancer **9**(6): 415-428.

Berchuck, A., G. Rodriguez, G. Olt, R. Whitaker, M. P. Boente, B. A. Arrick, D. L. Clarke-Pearson and R. C. Bast, Jr. (1992). "Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor-beta." Am J Obstet Gynecol **166**(2): 676-684.

Bhaskar, K. R., P. Garik, B. S. Turner, J. D. Bradley, R. Bansil, H. E. Stanley and J. T. Lamont (1992). "Viscous Fingering of Hcl through Gastric Mucin." Nature **360**(6403): 458-461.

Blalock, T. D., S. J. Spurr-Michaud, A. S. Tisdale, S. R. Heimer, M. S. Gilmore, V. Ramesh and I. K. Gipson (2007). "Functions of MUC16 in corneal epithelial cells." Investigative Ophthalmology & Visual Science **48**(10): 4509-4518.

Bowen, N. J., L. D. Walker, L. V. Matyunina, S. Logani, K. A. Totten, B. B. Benigno and J. F. McDonald (2009). "Gene expression profiling supports the hypothesis that human ovarian surface epithelia are multipotent and capable of serving as ovarian cancer initiating cells." BMC Med Genomics **2**: 71.

Brawtal, R., K. P. Mcnatty, P. Smith, D. A. Heath, N. L. Hudson, D. J. Phillips, B. J. Mcleod and G. H. Davis (1993). "Ovaries of Ewes Homozygous for the X-Linked Inverdale Gene (Fecx(1)) Are Devoid of Secondary and Tertiary Follicles but Contain Many Abnormal Structures." Biology of Reproduction **49**(5): 895-907.

Burdette, J. E., R. M. Oliver, V. Ulyanov, S. M. Kilen, K. E. Mayo and T. K. Woodruff (2007). "Ovarian epithelial inclusion cysts in chronically superovulated CD1 and Smad2 dominant-negative mice." Endocrinology **148**(8): 3595-3604.

Carson, D. D., M. M. DeSouza, R. Kardon, X. H. Zhou, E. Lagow and J. Julian (1998). "Mucin expression and function in the female reproductive tract." Human Reproduction Update **4**(5): 459-464.

Cheon, D. J., Y. Wang, J. M. Deng, Z. Lu, L. Xiao, C. M. Chen, R. C. Bast and R. R. Behringer (2009). "CA125/MUC16 Is Dispensable for Mouse Development and Reproduction." Plos One **4**(3).

Cocquet, J., E. Pailhoux, F. Jaubert, N. Servel, X. Xia, M. Pannetier, E. De Baere, L. Messiaen, C. Cotinot, M. Fellous and R. A. Veitia (2002). "Evolution and expression of FOXL2." Journal of Medical Genetics **39**(12): 916-921.

Collaborative Group on Epidemiological Studies of Ovarian, C., V. Beral, R. Doll, C. Hermon, R. Peto and G. Reeves (2008). "Ovarian cancer and oral contraceptives: collaborative reanalysis of data from 45 epidemiological studies including 23,257 women with ovarian cancer and 87,303 controls." Lancet **371**(9609): 303-314.

Comamala, M., M. Pinard, C. Theriault, I. Matte, A. Albert, M. Boivin, J. Beaudin, A. Piche and C. Rancourt (2011). "Downregulation of cell surface CA125/MUC16 induces epithelial-to-

mesenchymal transition and restores EGFR signalling in NIH:OVCAR3 ovarian carcinoma cells." British Journal of Cancer **104**(6): 989-999.
Crawford, J. L. and K. P. McNatty (2012). "The ratio of growth differentiation factor 9: bone morphogenetic protein 15 mRNA expression is tightly co-regulated and differs between species over a wide range of ovulation rates." Mol Cell Endocrinol **348**(1): 339-343.
Davis, H. M., V. R. Zurawski, R. C. Bast and T. L. Klug (1986). "Characterization of the Ca-125 Antigen Associated with Human Epithelial Ovarian Carcinomas." Cancer Research **46**(12): 6143-6148.
Dong, J., D. F. Albertini, K. Nishimori, T. R. Kumar, N. Lu and M. M. Matzuk (1996). "Growth differentiation factor-9 is required during early ovarian folliculogenesis." Nature **383**(6600): 531-535.
Dubeau, L. (2008). "The cell of origin of ovarian epithelial tumours." Lancet Oncology **9**(12): 1191-1197.
Duson, S. M. and R. S. Crawford (2012). "The role of endografts in the management of type B aortic dissections." Perspect Vasc Surg Endovasc Ther **24**(4): 177-183.
Easton, D. F., D. Ford, D. T. Bishop, N. Haites, B. Milner, L. Allan, D. F. Easton, B. A. J. Ponder, J. Peto, S. Smith, D. Ford, M. Stratton, S. A. Narod, G. M. Lenoir, J. Feunteun, H. Lynch, A. Arason, R. Barkdardottir, D. V. Egilsson, D. T. Bishop, D. M. Black, D. Kelsell, N. K. Spurr, P. Devilee, C. J. Cornelisse, H. Varsen, J. M. Birch, M. S. Santibanezkoref, M. D. Teare, M. Steel, D. Porter, B. B. Cohen, A. Carothers, E. Smyth, B. Weber, M. Boehnke, F. S. Collins, L. A. Cannonalbright, D. Goldgar and M. Skolnick (1995). "Breast and Ovarian-Cancer Incidence in Brcai-Mutation Carriers." American Journal of Human Genetics **56**(1): 265-271.
Elvin, J. A., C. N. Yan and M. M. Matzuk (2000). "Oocyte-expressed TGF-beta superfamily members in female fertility." Molecular and Cellular Endocrinology **159**(1-2): 1-5.
Eppig, J. J., K. Wigglesworth and F. L. Pendola (2002). "The mammalian oocyte orchestrates the rate of ovarian follicular development." Proceedings of the National Academy of Sciences **99**(5): 2890-2894.
Fathalla, M. F. (1971). "Incessant ovulation--a factor in ovarian neoplasia?" Lancet **2**(7716): 163.
Fazili, Z., W. P. Sun, S. Mittelstaedt, C. Cohen and X. X. Xu (1999). "Disabled-2 inactivation is an early step in ovarian tumorigenicity." Oncogene **18**(20): 3104-3113.
Feary, E. S., J. L. Juengel, P. Smith, M. C. French, A. R. O'Connell, S. B. Lawrence, S. M. Galloway, G. H. Davis and K. P. McNatty (2007). "Patterns of expression of messenger RNAs encoding GDF9, BMP15, TGFBR1, BMPR1B, and BMPR2 during follicular development and characterization of ovarian follicular populations in Ewes carrying the Woodlands FecX2(w) mutation." Biology of Reproduction **77**(6): 990-998.
Gershenson, D. M. (1994). "Management of Early Ovarian-Cancer - Germ-Cell and Sex Cord-Stromal Tumors." Gynecologic Oncology **55**(3): S62-S72.
Giampieri, S., C. Manning, S. Hooper, L. Jones, C. S. Hill and E. Sahai (2009). "Localized and reversible TGF beta signalling switches breast cancer cells from cohesive to single cell motility." Nature Cell Biology **11**(11): 1287-U1249.
Giannakouros, P., M. Comamala, I. Matte, C. Rancourt and A. Piche (2015). "MUC16 mucin (CA125) regulates the formation of multicellular aggregates by altering beta-catenin signaling." American Journal of Cancer Research **5**(1): 219-230.
Gilchrist, R. B., M. P. Morrissey, L. J. Ritter and D. T. Armstrong (2003). "Comparison of oocyte factors and transforming growth factor-beta in the regulation of DNA synthesis in bovine granulosa cells." Molecular and Cellular Endocrinology **201**(1-2): 87-95.
Gilchrist, R. B., L. J. Ritter and D. T. Armstrong (2001). "Mouse oocyte mitogenic activity is developmentally coordinated throughout folliculogenesis and meiotic maturation." Developmental Biology **240**(1): 289-298.
Greenburg, G. and E. D. Hay (1982). "Epithelia Suspended in Collagen Gels Can Lose Polarity and Express Characteristics of Migrating Mesenchymal Cells." Journal of Cell Biology **95**(1): 333-339.

Gubbels, J. A. A., J. Belisle, M. Onda, C. Rancourt, M. Migneault, M. Ho, T. K. Bera, J. Connor, B. K. Sathyanarayana, B. Lee, I. Pastan and M. S. Patankar (2006). "Mesothelin-MUC16 binding is a high affinity, N-glycan dependent interaction that facilitates peritoneal metastasis of ovarian tumors." Molecular Cancer **5**.
Gubbels, J. A. A., M. Felder, S. Horibata, J. A. Belisle, A. Kapur, H. Holden, S. Petrie, M. Migneault, C. Rancourt, J. P. Connor and M. S. Patankar (2010). "MUC16 provides immune protection by inhibiting synapse formation between NK and ovarian tumor cells." Molecular Cancer **9**.
Gupta, R. and N. Jentoft (1989). "Subunit Structure of Porcine Submaxillary Mucin." Biochemistry **28**(14): 6114-6121.
Guthrie, M. J. (1958). "Tumorigenesis in ovaries of mice after X irradiation." Cancer **11**(6): 1226-1235.
Hanrahan, J. P., S. M. Gregan, P. Mulsant, M. Mullen, G. H. Davis, R. Powell and S. M. Galloway (2004). "Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries)." Biol Reprod **70**(4): 900-909.
Holt, J. T., M. E. Thompson, C. Szabo, C. RobinsonBenion, C. L. Arteaga, M. C. King and R. A. Jensen (1996). "Growth retardation and tumour inhibition by BRCA1." Nature Genetics **12**(3): 298-302.
Houghtaling, S., C. Timmers, M. Noll, M. J. Finegold, S. N. Jones, M. S. Meyn and M. Grompe (2003). "Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice." Genes & Development **17**(16): 2021-2035.
Howell, J. S., J. Marchant and J. W. Orr (1954). "The Induction of Ovarian Tumours in Mice with 9-10-Dimethyl-1-2-Benzanthracene." British Journal of Cancer **8**(4): 635-&.
Hurteau, J., G. C. Rodriguez, R. S. Whitaker, S. Shah, G. Mills, R. C. Bast and A. Berchuck (1994). "Transforming Growth-Factor-Beta Inhibits Proliferation of Human Ovarian-Cancer Cells Obtained from Ascites." Cancer **74**(1): 93-99.
Hussein, T. S., D. A. Froiland, F. Amato, J. G. Thompson and R. B. Gilchrist (2005). "Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins." J Cell Sci **118**(Pt 22): 5257-5268.
Itoh, S., F. Itoh, M. J. Goumans and P. ten Dijke (2000). "Signaling of transforming growth factor-beta family members through Smad proteins." European Journal of Biochemistry **267**(24): 6954-6967.
Jelovac, D. and D. K. Armstrong (2011). "Recent Progress in the Diagnosis and Treatment of Ovarian Cancer." Ca-a Cancer Journal for Clinicians **61**(3): 183-203.
Juenel, J. L., K. L. Reader, A. H. Bibby, S. Lun, I. Ross, L. J. Haydon and K. P. McNatty (2006). "The role of bone morphogenetic proteins 2, 4, 6 and 7 during ovarian follicular development in sheep: contrast to rat." Reproduction **131**(3): 501-513.
Juengel, J. L., L. D. Quirke, D. J. Tisdall, P. Smith, N. L. Hudson and K. P. McNatty (2000). "Gene expression in abnormal ovarian structures of ewes homozygous for the Inverdale prolificacy gene." Biology of Reproduction **62**(6): 1467-1478.
Juengel, J. L., H. R. Sawyer, P. R. Smith, L. D. Quirke, D. A. Heath, S. Lun, S. J. Wakefield and K. P. McNatty (2002). "Origins of follicular cells and ontogeny of steroidogenesis in ovine fetal ovaries." Molecular and Cellular Endocrinology **191**(1): 1-10.
Kim, J., D. M. Coffey, L. Ma and M. M. Matzuk (2015). "The ovary is an alternative site of origin for high-grade serous ovarian cancer in mice." Endocrinology **156**(6): 1975-1981.
Kretzschmar, M., J. Doody, I. Timokhina and J. Massague (1999). "A mechanism of repression of TGF beta/Smad signaling by oncogenic Ras." Genes & Development **13**(7): 804-816.
Kurman, R. J. and I. M. Shih (2011). "Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer-Shifting the paradigm." Human Pathology **42**(7): 918-931.
Li, R., R. J. Norman, D. T. Armstrong and R. B. Gilchrist (2000). "Oocyte-secreted factor(s) determine functional differences between bovine mural granulosa cells and cumulus cells." Biology of Reproduction **63**(3): 839-845.

Lin, J. Y., J. L. Pitman-Crawford, A. H. Bibby, N. L. Hudson, C. J. McIntosh, J. L. Juengel and K. P. McNatty (2012). "Effects of species differences on oocyte regulation of granulosa cell function." Reproduction **144**(5): 557-567.
Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.
Low, J. J. H., A. Ilancheran and J. S. Ng (2012). "Malignant ovarian germ-cell tumours." Best Practice & Research Clinical Obstetrics & Gynaecology **26**(3): 347-355.
Maccalman, C. D., R. Farookhi and O. W. Blaschuk (1994). "Estradiol Regulates E-Cadherin Messenger-Rna Levels in the Surface Epithelium of the Mouse Ovary." Clinical & Experimental Metastasis **12**(4): 276-282.
Mandon-Pepin, B., A. Oustry-Vaiman, B. Vigier, F. Piumi, E. Cribiu and C. Cotinot (2003). "Expression profiles and chromosomal localization of genes controlling meiosis and follicular development in the sheep ovary." Biol Reprod **68**(3): 985-995.
McNatty, K. P., J. L. Juengel, K. L. Reader, S. Lun, S. Myllymaa, S. B. Lawrence, A. Western, M. F. Meerasahib, D. G. Mottershead, N. P. Groome, O. Ritvos and M. P. Laitinen (2005). "Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function." Reproduction **129**(4): 473-480.
McNeilly, J. R., P. T. K. Saunders, M. Taggart, M. Cranfield, H. J. Cooke and A. S. McNeilley (2000). "Loss of oocytes in Dazl knockout mice results in maintained ovarian steroidogenic function but altered gonadotropin secretion in adult animals." Endocrinology **141**(11): 4284-4294.
Mester, B., L. J. Ritter, J. L. Pitman, A. H. Bibby, R. B. Gilchrist, K. P. McNatty, J. L. Juengel and C. J. McIntosh (2015). "Oocyte expression, secretion and somatic cell interaction of mouse bone morphogenetic protein 15 during the peri-ovulatory period." Reproduction Fertility and Development **27**(5): 801-811.
Miki, Y., J. Swensen, D. Shattuckeidens, P. A. Futreal, K. Harshman, S. Tavtigian, Q. Y. Liu, C. Cochran, L. M. Bennett, W. Ding, R. Bell, J. Rosenthal, C. Hussey, T. Tran, M. Mcclure, C. Frye, T. Hattier, R. Phelps, A. Haugenstrano, H. Katcher, K. Yakumo, Z. Gholami, D. Shaffer, S. Stone, S. Bayer, C. Wray, R. Bogden, P. Dayananth, J. Ward, P. Tonin, S. Narod, P. K. Bristow, F. H. Norris, L. Helvering, P. Morrison, P. Rosteck, M. Lai, J. C. Barrett, C. Lewis, S. Neuhausen, L. Cannonalbright, D. Goldgar, R. Wiseman, A. Kamb and M. H. Skolnick (1994). "A Strong Candidate for the Breast and Ovarian-Cancer Susceptibility Gene Brca1." Science **266**(5182): 66-71.
Mok, S. C., K. K. Wong, R. K. W. Chan, C. C. Lau, S. W. Tsao, R. C. Knapp and R. S. Berkowitz (1994). "Molecular-Cloning of Differentially Expressed Genes in Human Epithelial Ovarian-Cancer." Gynecologic Oncology **52**(2): 247-252.
Moore, R. K., F. Otsuka and S. Shimasaki (2003). "Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells." Journal of Biological Chemistry **278**(1): 304-310.
Mork, L., D. M. Maatouk, J. A. McMahon, J. J. Guo, P. Zhang, A. P. McMahon and B. Capel (2012). "Temporal differences in granulosa cell specification in the ovary reflect distinct follicle fates in mice." Biol Reprod **86**(2): 37.
O'Brien, T. J., J. B. Beard, L. J. Underwood, R. A. Dennis, A. D. Santin and L. York (2001). "The CA 125 gene: An extracellular superstructure dominated by repeat sequences." Tumor Biology **22**(6): 348-366.
Orsulic, S., Y. Li, R. A. Soslow, L. A. Vitale-Cross, J. S. Gutkind and H. E. Varmus (2002). "Induction of ovarian cancer by defined multiple genetic changes in a mouse model system." Cancer Cell **1**(1): 53-62.
Ouchi, T., A. N. A. Monteiro, A. August, S. A. Aaronson and H. Hanafusa (1998). "BRCA1 regulates p53-dependent gene expression." Proceedings of the National Academy of Sciences of the United States of America **95**(5): 2302-2306.
Panici, P. B., C. Marchetti, L. Salerno, A. Musella, L. Vertechy, I. Palaia, G. Perniola, I. Ruscito, T. Boni, R. Angioli and L. Muzii (2014). "Dualistic Classification of Epithelial Ovarian Cancer: Surgical and Survival Outcomes in a Large Retrospective Series." Annals of Surgical Oncology **21**(9): 3036-3041.

Pectasides, D., E. Pectasides and D. Kassanos (2008). "Germ cell tumors of the ovary." Cancer Treatment Reviews **34**(5): 427-441.
Pectasides, D., E. Pectasides and A. Psyrri (2008). "Granulosa cell tumor of the ovary." Cancer Treatment Reviews **34**(1): 1-12.
Piek, J. M. J., P. J. van Diest, R. P. Zweemer, J. W. Jansen, R. J. J. Poort-Keesom, F. H. Menko, J. J. P. Gille, A. P. M. Jongsma, G. Pals, P. Kenemans and R. H. M. Verheijen (2001). "Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer." Journal of Pathology **195**(4): 451-456.
Pierce, D. F., A. E. Gorska, A. Chytil, K. S. Meise, D. L. Page, R. J. Coffey and H. L. Moses (1995). "Mammary-Tumor Suppression by Transforming Growth-Factor-Beta-1 Transgene Expression." Proceedings of the National Academy of Sciences of the United States of America **92**(10): 4254-4258.
Pitman, J. L., A. S. McNeilly, J. R. McNeilly, L. E. Hays, G. C. Bagby Jr, H. R. Sawyer and K. P. McNatty (2012). "The fate of granulosa cells following premature oocyte loss and the development of ovarian cancers." International Journal of Developmental Biology **56**(10-12): 949-958.
Reader, K. L., D. A. Heath, S. Lun, C. J. McIntosh, A. H. Western, R. P. Littlejohn, K. P. McNatty and J. L. Juengel (2011). "Signalling pathways involved in the cooperative effects of ovine and murine GDF9+BMP15-stimulated thymidine uptake by rat granulosa cells." Reproduction **142**(1): 123-131.
Reinartz, S., S. Failer, T. Schuell and U. Wagner (2012). "CA125 (MUC16) gene silencing suppresses growth properties of ovarian and breast cancer cells." European Journal of Cancer **48**(10): 1558-1569.
Rodriguez, G. C., C. Haisley, J. Hurteau, T. L. Moser, R. Whitaker, R. C. Bast and M. S. Stack (2001). "Regulation of invasion of epithelial ovarian cancer by transforming growth factor-beta." Gynecologic Oncology **80**(2): 245-253.
Ruggiu, M., R. Speed, M. Taggart, S. J. McKay, F. Kilanowski, P. Saunders, J. Dorin and H. J. Cooke (1997). "The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis." Nature **389**(6646): 73-77.
Rump, A., Y. Morikawa, M. Tanaka, S. Minami, N. Umesaki, M. Takeuchi and A. Miyajima (2004). "Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion." Journal of Biological Chemistry **279**(10): 9190-9198.
Satoh, S., Y. Hinoda, T. Hayashi, M. D. Burdick, K. Imai and M. A. Hollingsworth (2000). "Enhancement of metastatic properties of pancreatic cancer cells by MUC1 gene encoding an anti-adhesion molecule." International Journal of Cancer **88**(4): 507-518.
Sawyer, H. R., P. Smith, D. A. Heath, J. L. Juengel, S. J. Wakefield and K. P. McNatty (2002). "Formation of ovarian follicles during fetal development in sheep." Biology of Reproduction **66**(4): 1134-1150.
Schmidt, D., C. E. Ovitt, K. Anlag, S. Fehsenfeld, L. Gredsted, A. C. Treier and M. Treier (2004). "The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance." Development **131**(4): 933-942.
Sell, S. (1993). "Cellular origin of cancer: dedifferentiation or stem cell maturation arrest?" Environ Health Perspect **101 Suppl 5**: 15-26.
Shah, S. P., M. Kobel, J. Senz, R. D. Morin, B. A. Clarke, K. C. Wiegand, G. Leung, A. Zayed, E. Mehl, S. E. Kalloger, M. Sun, R. Giuliany, E. Yorida, S. Jones, R. Varhol, K. D. Swenerton, D. Miller, P. B. Clement, C. Crane, J. Madore, D. Provencher, P. Leung, A. DeFazio, J. Khattra, G. Turashvili, Y. J. Zhao, T. Zeng, J. N. M. Glover, B. Vanderhyden, C. Q. Zhao, C. A. Parkinson, M. Jimenez-Linan, D. D. L. Bowtell, A. M. Mes-Masson, J. D. Brenton, S. A. Aparicio, N. Boyd, M. Hirst, C. B. Gilks, M. Marra and D. G. Huntsman (2009). "Mutation of FOXL2 in Granulosa-Cell Tumors of the Ovary." New England Journal of Medicine **360**(26): 2719-2729.
Sheng, Z. J., W. P. Sun, E. Smith, C. Cohen, Z. Sheng and X. X. Xu (2000). "Restoration of positioning control following Disabled-2 expression in ovarian and breast tumor cells." Oncogene **19**(42): 4847-4854.

Shepherd, T. G., B. L. Theriault, E. J. Campbell and M. W. Nachtigal (2006). "Primary culture of ovarian surface epithelial cells and ascites-derived ovarian cancer cells from patients." Nat Protoc **1**(6): 2643-2649.
Shimasaki, S., R. J. Zachow, D. M. Li, H. Kim, S. Iemura, N. Ueno, K. Sampath, R. J. Chang and G. F. Erickson (1999). "A functional bone morphogenetic protein system in the ovary." Proceedings of the National Academy of Sciences of the United States of America **96**(13): 7282-7287.
Silva, J. R. V., R. van den Hurk, H. T. A. van Tol, B. A. J. Roelen and J. R. Figueiredo (2005). "Expression of growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and BMP receptors in the ovaries of goats." Molecular Reproduction and Development **70**(1): 11-19.
Solloway, M. J., A. T. Dudley, E. K. Bikoff, K. M. Lyons, B. L. M. Hogan and E. J. Robertson (1998). "Mice lacking Bmp6 function." Developmental Genetics **22**(4): 321-339.
Souza, C. J. H., B. K. Campbell, A. S. McNeilly and D. T. Baird (2002). "Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry." Reproduction **123**(3): 363-369.
Sundfeldt, K., Y. Piontkewitz, K. Ivarsson, O. Nilsson, P. Hellberg, M. Brannstrom, P. O. Janson, S. Enerback and L. Hedin (1997). "E-cadherin expression in human epithelial ovarian cancer and normal ovary." International Journal of Cancer **74**(3): 275-280.
Susan, M. G., P. M. Kenneth, M. C. Lisa, P. E. L. Mika, L. J. Jennifer, T. S. Jokiranta, J. M. Robert, L. Kaisu, G. D. Ken, W. M. Grant, E. B. Anne, H. D. George and R. Olli (2000). "Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner." Nature Genetics **25**(3): 279-283.
Testa, J. R., L. A. Getts, H. Salazar, Z. M. Liu, L. M. Handel, A. K. Godwin and T. C. Hamilton (1994). "Spontaneous Transformation of Rat Ovarian Surface Epithelial-Cells Results in Well to Poorly Differentiated Tumors with a Parallel Range of Cytogenetic Complexity." Cancer Research **54**(10): 2778-2784.
Theriault, C., M. Pinard, M. Comamala, M. Migneault, J. Beaudin, I. Matte, M. Boivin, A. Piche and C. Rancourt (2011). "MUC16 (CA125) regulates epithelial ovarian cancer cell growth, tumorigenesis and metastasis." Gynecologic Oncology **121**(3): 434-443.
Thiery, J. P. (2002). "Epithelial-mesenchymal transitions in tumour progression." Nature Reviews Cancer **2**(6): 442-454.
Valve, E., T. L. Penttila, J. Paranko and P. Harkonen (1997). "FGF-8 is expressed during specific phases of rodent oocyte and spermatogonium development." Biochemical and Biophysical Research Communications **232**(1): 173-177.
Vanderhyden, B. C., E. E. Telfer and J. J. Eppig (1992). "Mouse Oocytes Promote Proliferation of Granulosa-Cells from Preantral and Antral Follicles Invitro." Biology of Reproduction **46**(6): 1196-1204.
Vaughan, M., P. Sykes, C. Johnson, B. Fitzharris and o. b. of the New Zealand Gynaecologic Cancer Group (2006). "Improving outcomes in ovarian cancer." The New Zealand Medical Journal (Online) **119**(1242): U2171.
Vitt, U. A., S. Mazerbourg, C. Klein and A. J. W. Hsueh (2002). "Bone morphogenetic protein receptor type II is a receptor for growth differentiation factor-9." Biology of Reproduction **67**(2): 473-480.
Wu, X. M., L. Chen, C. A. Brown, C. N. Yan and M. M. Matzuk (2004). "Interrelationship of growth differentiation factor 9 and inhibin in early folliculogenesis and ovarian tumorigenesis in mice." Molecular Endocrinology **18**(6): 1509-1519.
Xie, W., J. C. Mertens, D. J. Reiss, D. L. Rimm, R. L. Camp, B. G. Haffty and M. Reiss (2002). "Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: A tissue microarray study." Cancer Research **62**(2): 497-505.
Xu, X. L., K. U. Wagner, D. Larson, Z. Weaver, C. L. Li, T. Ried, L. Hennighausen, A. Wynshaw-Boris and C. X. Deng (1999). "Conditional mutation of Brca1 in mammary epithelial cells

results in blunted ductal morphogenesis and tumour formation." Nature Genetics **22**(1): 37-43.
Xu, X. X., W. N. Yang, S. Jackowski and C. O. Rock (1995). "Cloning of a Novel Phosphoprotein Regulated by Colony-Stimulating Factor-1 Shares a Domain with the Drosophila Disabled Gene-Product." Journal of Biological Chemistry **270**(23): 14184-14191.
Yang, D. H., K. Q. Cai, I. H. Roland, E. R. Smith and X. X. Xu (2007). "Disabled-2 is an epithelial surface positioning gene." Journal of Biological Chemistry **282**(17): 13114-13122.
Yang, D. H., E. R. Smith, I. H. Roland, Z. J. Sheng, J. Q. He, W. D. Martin, T. C. Hamilton, J. D. Lambeth and X. X. Xu (2002). "Disabled-2 is essential for endodermal cell positioning and structure formation during mouse embryogenesis." Developmental Biology **251**(1): 27-44.
Yin, B. W. T. and K. O. Lloyd (2001). "Molecular cloning of the CA125 ovarian cancer antigen - Identification as a new mucin, MUC16." Journal of Biological Chemistry **276**(29): 27371-27375.
Zhang, H. B., K. Somasundaram, Y. Peng, H. Tian, H. X. Zhang, D. K. Bi, B. L. Weber and W. S. El-Deiry (1998). "BRCA1 physically associates with p53 and stimulates its transcriptional activity." Oncogene **16**(13): 1713-1721.