Mouse Models of Menopause and Ovarian Cancer Risks

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MOUSE MODELS OF MENOPAUSE AND OVARIAN CANCER RISKS

By

Ying Wang

A DISSERTATION

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MOUSE MODELS OF MENOPAUSE AND OVARIAN CANCER RISKS

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Epithelial ovarian cancer (EOC) is the leading cause of death from gynecological malignancy in Western countries. A better understanding of the etiology and risk factors associated with this disease is crucial for the development of early detection protocols as well as more effective therapies. Epidemiological data has shown that the risks of EOC are highest among peri- or post-menopause women, while increased parity or the use of oral contraceptives is preventive. These data suggest that alterations in reproductive factors are associated with ovarian cancer risks; however, the molecular mechanisms underlying such a link remain to be understood. For decades, EOC was believed to arise from the epithelium that surrounds the ovarian surface, yet this concept fails to explain the morphological resemblance of ovarian epithelial neoplasms with the epithelial cells of the Müllerian-derived female reproductive tract. Alternative ideas have argued that EOC may originate from extra- or para-ovarian tissues such as the fallopian tube and ovarii rete. Studies of the origin of EOC will provide a better understanding of the disease and advance the protocols for early diagnosis. The aims for this thesis are to establish *in vivo* ovarian tumor models based on the germ cell deficient *Wv/Wv* mice that mimicking menopausal physiology. The *Wv* mice harbor a point mutation in *c-Kit*, which reduces its tyrosine kinase activity to about 1%, resulting in a premature loss of ovarian germ cells.
and follicles that recapitulates the initiation of menopause in human. We have developed ovarian tumor models by deleting the tumor suppressor genes p53 or p27kip1 in Wv/Wv mice. We found that both Wv/Wv:p27+/- and Wv/Wv :p27 -/- mice developed ovarian epithelial tumors, which consist of papillary structures lined by hyperchromatic neoplastic cells. Positive Cytokeratin 8 (CK8) staining indicated the epithelial origin of these tumors. In vitro primary cultures of mouse ovarian surface epithelial (MOSE) cells from wildtype, p27 +/- and p27 -/- mice further confirmed the growth advantage caused by p27 deficiency. However, neither p27 +/- nor p27 -/- MOSE cells were transformed in vitro, probably due to the compensatory increase of cyclin dependent kinase inhibitor (CKI) proteins including p21, p16, p19. When p53 was deleted unilaterally in the ovarian surface epithelial cells of Wv/Wv:p53 loxP/loxP mice by single administration of Adenovirus containing Cre activity (Ad-Cre), ovarian tumors developed after long latency. The ovarian tumors were significantly enlarged when compared with the uninfected ovary from the same mouse. However, most of the lesions in Wv:p53 conditional knockout tumors was negative for epithelial and follicular markers. In vitro deletion of p53 in MOSE cells significantly increased the proliferation and passage numbers of these cells. A compensatory increase of the CKI protein p16, as well as the cellular senescence level was also observed in p53 deleted MOSE cells, suggesting that p53 deletion alone was not sufficient to bypass p16- mediated tumor defense mechanisms in MOSE cells. Taken together, single deletion of p27 and p53 significantly amplified the phenotype of benign tubular adenomas in Wv/Wv mouse. However, neither p27 nor p53 deletion was sufficient to induce the development of malignant ovarian carcinomas in Wv/Wv mice, probably due to the up-regulation of CKI family proteins such as p21, p16 or p19.
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<tbody>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>AMH</td>
<td>Anti-Müllerian Hormone</td>
</tr>
<tr>
<td>APC</td>
<td>Anaphase Promoting Complex</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternative Reading Frame</td>
</tr>
<tr>
<td>BRAF</td>
<td>Murine Sarcoma Viral Oncogene Homolog B1</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast Cancer Susceptibility Gene</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>CIP</td>
<td>CDK-Interacting Protein</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin Dependent Kinase Inhibitor</td>
</tr>
<tr>
<td>c-MYC</td>
<td>Cellular Myelocytomatosis Viral Oncogene Homolog</td>
</tr>
<tr>
<td>Cre</td>
<td>Causes Recombination</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial Ovarian Cancer</td>
</tr>
<tr>
<td>Ep-cam</td>
<td>Epithelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactopyranoside</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>FTSEC</td>
<td>Human Fallopian Tube Secretory Epithelial Cell</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>INK4</td>
<td>Inhibitors of Cyclin-Dependent Kinase 4</td>
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<tr>
<td>KIP</td>
<td>Kinase Inhibitor Protein</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>KRAS</td>
<td>Kirsten Rat Sarcoma</td>
</tr>
<tr>
<td>LacZ</td>
<td>Lactose operon Z</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
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<tr>
<td>LMP</td>
<td>Low Malignant Potential</td>
</tr>
<tr>
<td>Lox P</td>
<td>Locus of X-over P1</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double Minute 2</td>
</tr>
<tr>
<td>MIS</td>
<td>Müllerian Inhibiting Substance</td>
</tr>
<tr>
<td>MISRII</td>
<td>Müllerian Inhibiting Substance Receptor II</td>
</tr>
<tr>
<td>MOSE</td>
<td>Mouse Ovarian Surface Epithelial</td>
</tr>
<tr>
<td>OSE</td>
<td>Ovarian Surface Epithelium</td>
</tr>
<tr>
<td>PGC</td>
<td>Primordial Germ Cell</td>
</tr>
<tr>
<td>pRB</td>
<td>protein Retinoblastoma</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>SA</td>
<td>Senescence Associated</td>
</tr>
<tr>
<td>SRC</td>
<td>Schmidt-Ruppin A-2 Viral Oncogene Homolog</td>
</tr>
<tr>
<td>Wv</td>
<td>White Spotting Variant</td>
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Chapter I: Introduction

1.1 Biology of ovarian cancer

By definition, ovarian cancer refers to cancer that arises from the ovaries. The ovaries are female reproductive organs that function to produce and preserve the oocytes (eggs). The human ovary is composed of germ cells, granulosa cells, theca cells, stromal cells and epithelial cells that surround the surface of the ovary. Although epithelial cells comprise only occupied a small percentage of the whole ovary, the most common and deadly ovarian cancers are epithelial carcinomas, which are believed to arise from the surface epithelium of the ovary. Epithelial Ovarian Cancer (EOC) makes up nearly 90% of the adult ovarian neoplasm (Feeley and Wells, 2001; Resta et al., 1993; Scully, 1995), and is the most lethal gynecological cancer among women. Ovarian cancer is known to be a heterogeneous disease that can be histologically categorized into 5 subtypes including serous, endometrioid, mucinous, clear cell and transitional adenocarcinomas (Koonings et al., 1989; Resta et al., 1993). More recently, a new model based on the clinical outcome and molecular studies of ovarian cancer has been proposed. In this model, EOC is categorized into Type I and Type II cancers that potentially rise from two distinct pathways. Type I ovarian cancer usually refers to the low-grade ovarian tumors associated with high free frequency of BRAF/KRAS or PTEN mutations and low level of proliferation potential, p53 mutations, or chromosome instability. Patients diagnosed with low grade type I ovarian cancer usually have a high five year survival rate at approximately 55%. In contrast, Type II ovarian cancer is characterized as either high grade serous carcinoma or undifferentiated mesodermal tumors. The mutation of tumor suppressor gene Trp53 is frequent (~50 to 90%) in this type of ovarian cancer, and the
five year survival rate for patients significantly reduced to around 30% (Shih Ie and Kurman, 2004).

Despite considerable effort made toward the development of EOC, several key questions as to the initiation and progression of this disease remain unanswered: the cellular origin of the ovarian cancer are widely controversial and debated; the causative link between the well-established impact of reproductive factors on ovarian cancer risks has not been satisfactorily explained; and the specific role of various oncogenes during the transformation of ovarian epithelial cells is poorly understood. To answer these questions, the generation of suitable in vitro and especially in vivo mouse models will be necessary and essential. Useful information could be collected from the analysis of these models and advance our understanding of the fundamental questions of EOC, which will eventually benefit the patients that suffered from this deadly disease.

### 1.1.1 Ovarian cancer risks

During the past decade, significant progress has been made in identifying the risks associated with EOC. Age, family history or certain genetic mutations have been well documented as risk factors for ovarian cancers. Among these factors, age remains the most important prediction of the survival of ovarian cancer patients, and most of EOC induced mortality occurs in women over 55 years of age. Most of the non-sporadic ovarian cancers were attributed to the inheritance of the defect in DNA repair genes such as BRCA1/2. The inheritable variation in these two genes are known to increase the risks of breast cancer and ovarian cancer by 60% and 55%, respectively (Barrois et al., 2004; Miki et al., 1994) and are widely accepted as the most common mutant genes for the hereditary breast-ovarian cancer syndrome. However, it should also be noted that the
mutations in BRCA1/2 are rare in sporadic ovarian cancer which accounts for the majority of EOC cases (Bast et al., 2009). Consistently, BRCA1 deletion alone is not sufficient to induce ovarian carcinomas in animal models (Clark-Knowles et al., 2009).

In the past two decades, significant progress has been made towards the understanding of molecular changes that specially associated with EOC (Hamilton et al., 1998; Okuda et al., 2003; Ozols et al., 2004). Unsurprisingly, mutations in the tumor suppressor gene Trp53 is found in more than half of malignant EOC cases, and is characteristic of high grade ovarian serous carcinoma (Aunoble et al., 2000; Feeley and Wells, 2001; Ozols et al., 2004; Shih Ie and Kurman, 2004). The loss of cyclin dependent kinase (CDK) inhibitors such as p27kip1 and p21cip1 has been also found to be associated with the clinical outcome of ovarian cancer patients (Masciullo et al., 2000; Psysrri et al., 2005). In low grade or low malignant potential (LMP) serous carcinomas, BRAF and RAS mutations are frequently detected (Gemignani et al., 2003; Okuda et al., 2003; Shih Ie and Kurman, 2004), whereas increased KRAS activity and mutation of PTEN pathways are prevalent in mucinous and endometrioid carcinomas (Gemignani et al., 2003; Obata et al., 1998; Sato et al., 2000).

Abundant epidemiological data have confirmed the link between the frequency of ovulation and reproductive hormones to ovarian cancer risks (Ozols et al., 2004; Riman et al., 1998). Two major theories have been postulated to explain such a link on the basis of the same epidemiological data (Cramer et al., 1983a; Cramer and Welch, 1983; Ozols et al., 2004). The incessant ovulation hypothesis argues that repeated wounding and repairing processes of ovarian surface epithelial cells during ovulation will lead to uncontrolled proliferation of these cells and ultimately malignant transformation (Godwin
et al., 1992; Schildkraut et al., 1997). As evidence, decreased ovulation frequency, achieved through increased parity or the use of oral contraceptives, has been proven to be preventative against EOC (Gwinn et al., 1990; Ozols et al., 2004; Siskind et al., 2000; Yancik et al., 1993). The competing theory, gonadotropin stimulation hypothesis, postulates that the surges of pituitary gonadotropins during ovulatory event are responsible for the stimulation of proliferation and potential neoplastic changes in ovarian epithelial cell (Cramer et al., 1983a; Cramer et al., 1983b). Since gonadotropins persist at high levels for years after menopause, this theory may explain the dramatic increase of ovarian cancer risks in post-menopause women (Riman et al., 1998). Although these two theories have been proposed for decades, it should be noted that neither has completely explained the etiology of ovarian cancer. Molecular mechanisms underlying the etiology of human ovarian cancers remain to be defined.

1.1.2 Menopause and ovarian cancer risk
In human, ovulation initiates approximately 35 hours after the periodical surge of gonadotropins including luteinizing hormone (LH) and follicle stimulating hormone (FSH). Ovarian stimulation by gonadotropins, together with other cytokines and proteolytic enzymes, is known to cause an inflammatory-like process that leads to the rupture of ovarian surface epithelium and the release of the ovum. Once the ovum is released, estrogen-producing follicle is then converted into a progesterone-producing corpus luteum. The circulating progesterone then initiates a feedback loop and inhibits the further release of pituitary gonadotropins (Espey, 1994; LeMaire, 1989; Tsafiriri and Reich, 1999). In menopausal women, follicle differentiation is completely ceased due to the depletion of germ cells. With the absence of corpus luteum, progesterone levels fall
and the gonadotropin level rises during the menopausal period. This constitutes a hormonal environment that favors tumor formation according to the gonadotropin stimulation hypothesis (Eldridge et al., 1974). Moreover, significant morphological changes are frequently detected in ovaries from peri- or post-menopausal women. These age-dependent changes, also known as ‘ovarian aging’, are presumably caused by repeated wounding and repairing processes during ovulation (Nicosia, 1987). Some of these morphological changes, such as deep invaginations, surface papillomatosis, and inclusion cysts, are presumed to be pre-neoplastic lesions of ovarian cancers (Yancik, 1993; Yancik et al., 1993). Taken together, these facts indicate that the unique hormonal environment and morphological changes in the ovaries of menopausal women may explain the high ovarian cancer incidences among this group. However, due to the complex nature of menopause, the molecular evidence underlying such a link is still missing. The development of ovarian tumor models in laboratory animals, especially those incorporate features of menopausal biology, will be very informative in addressing the causative relation between menopause and increased ovarian cancer risk.

1.1.3 Origin of ovarian epithelial cancer

In humans, ovarian surface epithelial cells are continuous and morphologically indistinguishable from the mesothelium lining the surface of the pelvic and abdominal structures. Therefore, it is reasonable to predict that EOC could share similar morphological features as carcinomas rising from the mesothelial lining of adjacent tissues or organs (mesothelioma). On the contrary, the major subtypes of ovarian epithelial cancers, if not all, are well recognized to display morphological similarities with the epithelial tumors from the extra-ovarian female genital tract (Dubeau, 2008).
Indeed, the most common subtypes of EOC including serous, mucinous and endometrioid EOCs, are actually categorized according to their morphological resemblance to the epithelial cells of fallopian tubes, endocervix and endometrium, respectively (Auersperg et al., 2001; Feeley and Wells, 2001). Recently, those morphological resemblances were further supported by the finding that different subtypes of EOC have similar expression profiles of Hox family genes as the epithelial cells from the corresponding female genital tract (Cheng et al., 2005). Female genital tracts are derived embryologically from Müllerian ducts, while both the ovary and ovarian epithelial cells have completely different precursors (Rodriguez and Dubeau, 2001). Therefore, Müllerian-like morphology of EOCs is inconsistent with the dogma that EOC are derived from ovarian epithelial cells as indicated in the nomenclature.

A more adaptive view to explain the Müllerian-like appearance of ovarian epithelial cancers has argued that ovarian epithelial cells were actually converted into Müllerian-like epithelium before the initiation of neoplastic transformation. This process, known as ‘metaplasia’ is most likely to take place in the invaginating ovarian epithelial cells, which are presumably under the stimulation of hormones released in the ovarian parenchyma (Resta et al., 1993). In support of this idea, Müllerian lining can be detected in the ovarian cortical inclusion cysts even though these structures are clearly originated from the deep invagination of ovarian epithelial cells (Mittal et al., 1993; Scully, 1995). An alternative hypothesis has argued that EOC actually originate from the transformation of the remnant Müllerian tract epithelial cells presented in the ovary, or ‘secondary Müllerian systems’ (Lauchlan, 1994). Müllerian epithelial-lined ducts or small cysts detected in the hilum or deep medulla of the ovary represent the typical secondary
Müllerian systems in the ovary. These structures, also known as rete ovarii, are presumably derived from the proximal region of Müllerian duct during embryological development, and are more likely to form Müllerian-like neoplasms once transformed (Byskov, 1974; Rutgers and Scully, 1988). Moreover, recent evidence also suggested that the epithelium of the fallopian tube might also be an alternative source of EOC (Leeper et al., 2002; Piek et al., 2001). During ovulation, the fimbrial edge of the fallopian tube virtually rubs the ovary to catch the ovum and potentially leaves some of the epithelial cells on the ovarian surface. These adherent cells may be preferably transformed into Müllerian-like EOC under tumor prone physiological conditions such as menopause. Nevertheless, it should be noted that due to the lack of suitable animal models, direct evidence in support of either hypothesis is still missing. The seemingly unnecessary question as to the exact cellular origin of epithelial ovarian cancer is currently under wide debate.

1.2 Mouse models of ovarian cancer

The development of in vivo ovarian tumor models will advance the understanding of human ovarian cancers. Monumental breakthroughs in modern biotechnologies have led to the establishment of several models as briefly reviewed here. First, Orsulic and colleagues introduced potent oncogenes such as K-RAS, V-AKT and V-MYC into the ovarian epithelial cells derived from p53 -/- mice. Those cells were then implanted back into the site of ovary in nude mice and poorly differentiated, malignant ovarian tumors developed within weeks (Orsulic et al., 2002). This model confirmed that ovarian epithelial cells could be at least transformed through genetic manipulations in vitro; however, its biological relevance to the human EOC remains to be defined. Connolly et
al. developed one of the first transgenic ovarian tumor models by generating a transgenic mouse line carrying SV40 T-antigen at the locus of *Misr II*, a gene that presumably has restricted expression in the female reproductive tracts and ovaries. Poorly differentiated ovarian carcinomas were detected bilaterally in over 50% of these transgenic mice, probably due to the concurrently inactivation of *Rb* and *p53* by T-antigen (Connolly et al., 2003). Using the same principle, conditional deletion of *Rb* and *p53* in the ovary epithelial cells has also been proven to be sufficient for the induction of ovarian epithelial carcinomas in vivo (Flesken-Nikitin et al., 2003). These models have provided the direct genetic evidence for EOC formation; however, they both failed to recapitulate the initiation and progression of EOC in human. More recently and significantly, concurrent activation of *K-Ras* and deletion of *PTEN* in mouse ovarian epithelial cells was found to promote the formation of ovarian epithelial carcinomas with apparently similar morphologies as human ovarian endometrioid adenocarcinomas (Dinulescu et al., 2005). Since *K-Ras* and *PTEN* mutations are associated with endometrioid ovarian cancer, this model appears to be the most relevant model to the human disease and is likely to reflect the genetic alterations during the initiation and progression of endometrioid ovarian cancers (Obata et al., 1998). Taken together, the rapid development of ovarian tumor models in the past decade has greatly advanced our understanding of ovarian cancers and will continue to serve as valuable stages for the development and evaluation of novel therapies against this deadly disease.

The general concept behind these ovarian tumor models and generic mouse tumor models is that accumulated genetic mutations or alterations in somatic cells underlie the transformation of the primary cell and the progression of tumor malignancy. Therefore,
most of the mouse tumor models can be referred to as genetic models, since they were
designed to harbor oncogenic mutations associated with specific cancers. It should be
emphasized, however, the effect of the etiological components during tumor development
are usually absent in these models. Despite the significant advance in the development of
mouse EOC models as discussed above, none of them has satisfactorily incorporated any
of the major etiological components associated with human EOC.

1.3 Wv/Wv mouse as a unique model of epithelial ovarian cancer

One unique ovarian tumor model that reflects menopausal physiology is the white
spotting variant (Wv) mice. Wv mice harbor a naturally occurring point mutation in the
kinase domain of the c-kit gene, resulting in the defects in the development of germ cells,
pigment-forming cells, red blood cells and mast cells (Nocka et al., 1990; Reith et al.,
1990; Tan et al., 1990). Homozygous Wv/Wv mice have a normal life-span, but are sterile
due to the loss of germ cells. Before reaching reproductive age at 2 months, female
Wv/Wv mice are completely depleted of oocytes (Nocka et al., 1990). As a result,
ovulation in these mice ceases and gonadotropin levels increase dramatically due to the
lack of progesterone-mediated feedback loop. Since human menopause is initiated by the
completely depletion of germ cells, naturally occurring germ cell deficit Wv/Wv may
therefore serve as an ideal model to study menopausal conditions in human.

The risk of ovarian cancer is considered to be highest in post menopausal women.
Therefore, it is reasonable to predict that female Wv/Wv mice are prone to develop
ovarian cancer. Indeed, pre-neoplastic lesions, defined as tubular adenomas, are detected
in 100% of female Wv/Wv mouse as early as 3 months of age (Murphy and Beamer,
1973). However, this particular type of epithelial ovarian tumor is generally benign and
seldom develops the malignant features observed in human ovarian cancer. This indicates that the menopausal physiology condition alone is not sufficient to induce malignant EOC. Nevertheless, with the completely depletion of germ cells and subsequent increase of gonadotropin levels, \( Wv/Wv \) mice largely mimic the physiological condition of menopausal women and could be further refined into a unique tumor model that incorporates the contribution of etiological factors during the development of EOC.

1.4 Summary and Significance

In this thesis, I aim to establish a unique ovarian tumor model that incorporates both etiology components and genetic alterations. Previous epidemiological data have linked the reproductive factors with the risks of ovarian cancer. Since the last century, two major hypotheses, namely incessant ovulation and gonadotropin stimulation, have been proposed to explain the high risk for ovarian cancer during the peri-and post-menopause period. Although supporting epidemiological and biological data could be found for both the theories, there is no direct evidence that could prove either of the theories in vivo. In contrast, most of the existing mouse ovarian cancer models are based on the general concept that cancer rises from the accumulation of oncogenic mutations. Etiological factors that play a pivotal role during tumorigenesis are often omitted from these models. In the case of ovarian cancer, none existing animal models incorporate the contribution of reproductive factors such as ovulation numbers or the effects of gonadotropins in the development of ovarian carcinomas. Our group has successfully characterized the germ cell deficient \( Wv/Wv \) mouse as a unique model mimicking human menopausal conditions. I hypothesize that the combination of menopausal physiology and the genetic manipulations in \( Wv/Wv \) ovaries will lead to the development of ovarian neoplasms that
highly resemble the corresponding human disease. The genetically-modified $W_{v}$ mice will be particularly useful as a tumor model to study the early stage of human ovarian cancer in an in vivo setting, and will also provide insights into the origin of epithelial ovarian cancers. Together, this information will advance our understanding of the pathogenesis of human ovarian cancers and will likely lead to the improvement in both early detection and therapeutic treatment of this complex disease.
Chapter II: The development of genetically modified ovarian cancer models based on germ cell deficient Wv/Wv mice

The Wv/Wv mouse harbors a naturally occurring c-kit mutation that results in the premature depletion of germ cell as well as follicular structures (Fig.1A,B). As shown in our previous results, epithelial lesions as revealed by CK8 staining were observed in the cortex region of the Wv/Wv ovary as early as 2 months of age. As these mice aged, the epithelial lesions were observed throughout the entire ovary. More complex morphological structures such as deep penetration and multiple layers of epithelial cells were also more evident (Fig.1 C-I). These epithelial lesions commonly detected in Wv/Wv mice ovaries were histologically categorized as tubular adenomas, a benign type of ovarian epithelial tumor. However, tumor cells in Wv/Wv ovaries lack malignant features and are not able to expand into larger carcinoma lesions. These findings partially confirmed our hypothesis that menopausal physiology provided a tumor-prone environment for the ovarian surface epithelial cells. However, it should be noted the lack of malignant carcinomas in Wv/Wv ovaries also suggest that the accumulation of more oncogenic mutations are a prerequisite for the rise of EOC. Based on the current understanding of ovarian cancers, genetic and epigenetic mutations remain the most important risk factor (Shih Ie and Kurman, 2004). Therefore, I hypothesize that the benign tumor in the Wv/Wv mouse can be converted into ovarian carcinomas with the introduction of genetic mutations frequently detected in human patients. At this stage, I aim to investigate two separate oncogenic events on the background of germ cell deficient Wv/Wv ovaries: the loss of function mutation of the tumor suppressor genes p27 and Trp53.
Fig. 1 Development of benign tubular adenoma in \( \text{Wv/Wv} \) ovaries. PGC-7 was used to confirm the premature depletion of germ cell in newborn wildtype (A) and \( \text{Wv/Wv} \) (B). Cytokeratin 8 (Troma-1) staining revealed the development of tubular adenomas in \( \text{Wv/Wv} \) aged at 1 Month (C), 2 months (D), 3 months (E), 4 months (F), 5 months (G), 9 months (H). CK8 positive epithelial lesions was only detected in the ovarian surface of a 4 months old wildtype ovary (I,I'). Magnification used were X40 (I) and X100 (A-H,I'); C-H were adapted from previous results in the lab (Yang et al., 2007).
2.1 Ovarian tumor phenotype of Wv:p27 double mutant mice

2.1.1 The role and functions of tumor suppress genes p27 in ovarian cancer

We first selected tumor suppressor p27 as a potential genetic mutation to be introduced into the Wv mouse, p27 is a CKI family protein that prohibits the progression of the cell cycle through its binding with Cyclin dependent kinases. Biochemical studies suggested that the regulation of p27 protein is highly dynamic during cell cycle. The translation of p27 reaches its peak during the G0-G1 transition. During this phase, p27 binds to Cyclin D and CDK4/6 complex and inhibits the assembly and nuclear import of the Cyclin D1-CDK complex and in turn blocks the cell cycle progression (Sherr and Roberts, 1999). Moreover, p27 is also known to have a high affinity for Cyclin E-Cdk2 complex and functionally neutralizes the kinase activities and blocks the G1-S transition. The altered regulation of p27 has been observed in a panel of the most lethal human cancers including lung, prostate, and breast carcinomas. Low p27 expression level has also been widely used as a prediction marker for poor clinical outcome and reduced patients’ survival rates (Chu et al., 2008). Based on the results from several independent multivariate analyses on the cell cycle proteins in epithelial ovarian cancer, reduced p27 expression level was established as an independent prognosis marker for a shorter progression-free interval, and increased risk of mortality while not related to any specific histological subtypes (Masciullo et al., 2000; Milde-Langosch et al., 2003; Psyrri et al., 2005). In these studies, loss of p27 expression was also frequently detected in the late stage of EOC and consistently reported to be associated with poorly differentiated ovarian carcinomas.
Because of its essential role in regulating the cell cycle, p27 deletion had a dramatic effect on the growth and development of mice. Mice with p27 deficiency have a greater body weight probably due to the globally increased cell proliferation. A panel of internal organs including brain, thymus, liver, spleen, and kidney were found to be significantly enlarged in p27 knockout mice generated by two independent groups (Fero et al., 1996; Kiyokawa et al., 1996). Neoplastic growths in the intermediate lobe of the pituitary as well as hyperplasia of hematopoietic progenitors were also frequently detected in p27 knockout mice. Although the follicular activities and gonadotrophin level remains intact in female p27 knockout mice, these mice are infertile due to the defective corpus luteum development (Fero et al., 1996; Kiyokawa et al., 1996).

Although p27 is a well established tumor suppressor gene, no tumor occurrences were reported in p27 nullizygous mice except for the higher frequency of pituitary tumors. Consistently, homozygous deletion of p27 is rarely found in human cancers. On the other hand, low expression level of p27 is commonly associated with the aggressiveness of human cancer and could be used as an independent prognostic marker (Chu et al., 2008). As shown in Fig. 2, p27 expression was detectable in the normal surface epithelial cells of the human ovary. Nuclear localized p27 protein displayed a heterogeneous expression pattern in serous carcinomas, and is completely undetectable in approximately 10% of the tumor cells (Fig. 2 B, C, D). These observations are consistent with previous findings that the loss of p27 protein have a dosage effect on tumor progression (Bamberger et al., 1999; Chu et al., 2008; Jordan et al., 1998). Consistently, p27 heterozygous and nullizygous mice were both reported to be predisposed to multiple tumors after gamma irradiation and chemical carcinogen challenges (Fero et al., 1998). Therefore, p27 is defined as a
haplo-insufficient tumor suppressor gene and decreased p27 expression level is sufficient to promote the tumor malignancy.

Since p27 plays a pivotal role in the regulation of the cell cycle progression and a low p27 expression level is associated with the aggressiveness of a variety human carcinomas including epithelial ovarian cancer (Chu et al., 2008), I first planned to incorporate p27 heterozygous and homozygous deletion into Wv/Wv background and test whether decreased expression of p27 in mouse ovarian surface epithelial (MOSE) cells could lead to the development of malignant carcinomas under a menopause-like environment.

2.1.2 Epithelial lesions in aged p27+/− and p27−/− ovaries

First, I aimed to cross and generate a cohort of Wv:p27 double mutant mice to test whether p27 deficiency directly contributed to the malignancy of ovarian cancer under a menopause-like environment. Female Wv:p27 double mutant mice could potentially serve as a novel ovarian cancer model that incorporates both menopausal physiology and genetic alternations.

It is well documented that p27 heterozygous mice have a normal life span and reproducing activities, while p27 homozygous mice are infertile and prone to pituitary tumors. Incidence of epithelial ovarian cancers was not reported in p27 heterozygous and homozygous mice even after chemical carcinogen or gamma-irradiation radiation challenges (Fero et al., 1996). However, it should be noted that the transformation potential of p27+/- and p27-/- ovarian surface epithelial (OSE) cells has not been thoroughly studied especially in the aged mice. Here, I first analyzed the epithelial composition in the ovaries from wildtype (n=7) and p27+/- mice (n=9) at approximately 6 months old. CK8 staining indicated that the surface epithelium was comparable
Fig. 2 IHC staining of p27 in human ovarian tumor samples. IHC results suggest a heterogeneous expression pattern of p27 in human EOC (A), boxed area was amplified in B-D. A mixed p27 expression pattern was observed in the serous carcinomas (arrowhead B, C), while most of the normal surface epithelial cells (arrow) were positive for p27 staining (D). Magnification: X40 (A), X200 (B-D).
between the wildtype and \( p27^{+/-} \) ovaries at this stage (Fig. 3A, B). Pre-malignant lesions of the ovarian surface epithelium, such as hyperplasia and invagination in the cortical region of the ovary (Fig. 3C), were more frequently observed in the ovaries of aged (12-15 months) \( p27^{+/-} \) mice (7 out of 15) than the wildtype (2 out of 10). These findings suggest that the loss of \( p27 \) have a dosage effect on the proliferation of MOSE cells that is more evident with aging.

Consistent with previous reports, no corpus luteum-like structure could be detected in the ovaries of \( p27^{-/-} \) mice (Fero et al., 1996). Germ cells and surrounding follicular structures appeared to be normal in these ovaries (Fig. 4B, C, D), however, due to the defective corpus luteum development, most of the \( p27^{-/-} \) ovaries appeared to have a distinct morphology from the wildtype ovaries (Fig. 4). Different from \( p27^{+/-} \) ovaries, surface epithelial lesions were less frequently observed in \( p27^{-/-} \) ovaries. The surface epithelium remained a single layer in most of the \( p27^{-/-} \) ovaries (Fig. 4C). Ectopically distributed epithelial cells were frequently detected in the cortical regions close to the surface of the \( p27^{-/-} \) ovaries (11 out of 13), probably as a result of mild invagination of MOSE cells. In a few cases (2 out of 13), uncontrolled proliferation of the surface epithelial cells led to the development of local hyperplasia as well as a massive invagination inside the ovary (Fig. 4D, G, H). In \( p27^{-/-} \) ovaries, cyst structures were commonly observed (10 out of 13) within the ovary or in the extra-ovarian ovarii rete structures (Fig. 4C, D). CK8 staining revealed that most of these cysts were lined with a single layer of flat epithelial cells morphologically indistinguishable from the normal MOSE cells (Fig. 5D, E).

Occasionally, the prominent cyst structures permeated the entire ovary (Fig. 5A-C), and the epithelial cell lining the cyst side took up a more complicated, Müllerian epithelial-
Fig. 3 Pre-neoplastic changes on the surface of aged p27+/- ovaries. Ovaries from wildtype and p27+/- mice at different ages were harvested and stained with CK8. Representative ovaries from young wildtype (n=7), p27+/- (n=9) and aged p27+/- (n=15) mice were shown. The surface epithelium is indistinguishable between wildtype (A,A’) and p27+/- ovaries (B,B’) from 6-month old mice. However, pre-malignant lesions appeared in the aged p27 +/- ovaries. Dysplasia was found on the surface of a 13-month old p27 +/- ovary (C,C’). Magnification: X40 (A-C), X200 (A’-C’).
Fig. 4 Pre-neoplastic changes in p27-/- ovaries. Ovarian tissues were harvested from wildtype and p27-/- mice aged at 5-8 months and subjected to either H&E (A-D) or CK8 staining (E-H). Corpus lutem structures (Arrow) commonly found in wildtype ovaries (A) were absent in p27-/- ovaries (B-D). CK8 staining revealed a normal epithelial layer presented in most of the p27-/- ovaries (E, F). Massive epithelial lesions were occasionally observed in a few cases of p27-/- ovaries (G, H). Magnification: X40 (A-D, E, G), X200 (F, H).
**Fig. 5**

**Metaplastic changes in p27-/- ovarian surface epithelial cells.** Ovarian tissues were harvested from p27-/- mice at 5-8 months and subjected to CK8 (A, B, D, E) or Claudin3 staining (C). Cysts formation was common in p27-/- ovaries (A, D). These cysts were lined with CK8 positive epithelial cells (A, B, D, E). In some cases, more complex epithelial structures were detected in the epithelial cells lining the cyst (B). Claudin3 staining on the same section suggested the potential metaplastic changes in p27-/- MOSE cells (C). Magnification: X20 (A), X40 (D), X200 (B, C, E).
like morphology. The expression of claudin3, a cell junction marker commonly detected in the fallopian tube, endometrial epithelial cells, further confirmed the potential metaplastic changes of p27-/- MOSE cells in vivo (Fig. 5C).

2.1.3 Increased epithelial lesions in Wv:p27 double mutant ovaries

Ovarian pathology, such as hyperplasia of the surface epithelial cells and the formation of intra-ovarian cysts, were readily detectable in the ovaries of p27+/- and p27-/- mice. However, we found no typical EOC of any kind. These data suggested that loss of p27 was not required for the formation of epithelial ovarian cancer but rather pre-disposed the ovarian surface epithelial cells to development of potential tumors. This is also consistent with the previous findings that p27 deficiency alone is not sufficient to promote global tumor formation other than pituitary tumors (Fero et al., 1996; Kiyokawa et al., 1996). On the other hand, widespread tumor development in different tissues could be found in p27+/- and p27-/- mice exposed to gamma radiation (Fero et al., 1998). Collectively, these data suggest loss of p27 is insufficient for the tumor formation but instead significantly promotes tumor progression. This idea is also in line with the heterogeneous pattern of p27 deficiency commonly observed in human ovarian cancer patients.

I next tested whether epithelial ovarian cancer could be achieved in Wv:p27 double mutant mice. As shown previously, the premature depletion of germ cells induced the expansion of epithelial cells in the ovary of Wv/Wv mice. However, malignant carcinomas have not been detected in these mice even after long latency. Histological evaluation of Wv/Wv ovaries suggested that most of the lesions in Wv/Wv ovaries remained as a single layer of flat epithelial cells indistinguishable from the normal MOSE cells. Indeed, the tubular adenomas in these ovaries appeared to be
continuous with the monolayer ovarian surface epithelium. In aged mice, more lesions with a more complex structure developed in the medulla or hillus region of the ovaries; however, these lesions never progressed further into large tumor mass nor metastasized to other tissues. Histologically distinct mitotic features were also rarely observed within the ovarian tumors of \( Wv/Wv \) mice.

The lack of epithelial ovarian tumors in either \( Wv \) or \( p27 \) mutant mice clearly suggested that neither post menopausal physiology nor loss of \( p27 \) alone was sufficient to completely transform ovarian surface epithelial cells. We next asked whether the combination of an extrinsic menopausal-like environment and intrinsic oncogenic mutation could lead to the formation of ovarian neoplasm. Female \( Wv/Wv:p27^{+/} \) or \( p27^{-/-} \) double mutant mice were generated by intercrosses between \( Wv^{+}/p27^{+/} \) breeders. The expected frequency of female \( Wv/Wv, Wv/Wv:p27^{+/-} \) and \( Wv/Wv:p27^{-/-} \) mice is 1:32,1:16 and 1:32, respectively. However, the actually ratio of female \( Wv:p27 \) double knockout mice were much lower than the expected Mendelian ratio due to unknown reasons. Since male \( p27^{-/-} \) are fertile, I had also set up a few breeding cages using male \( Wv^{+}/p27^{-/-} \) as breeders. Using this strategy, the theoretical ratio of female \( Wv:p27 \) double knockout pups was increased to 1:16. \( Wv/Wv:p27 \) double mutant mice were sacrificed at 7 to 12 months of age with \( Wv^{+} \) littermates as parallel controls. The ovaries, as well as attaching uterine horns, were harvested and paraffin embedded. More than 5 serial sections were generated from each ovary, and the largest cross section of the whole ovary was selected as a representative image. As shown by the H&E staining, \( Wv/Wv \) ovaries have a high percentage of infiltrated epithelial ovarian cells; however, the size of the ovarian tumor in \( Wv/Wv \) mice is significantly smaller than the wildtype ovaries,
probably due to the loss of the follicle structures (Fig. 6A, D). Most of the epithelial cells detected in \(Wv/Wv\) ovaries remained flat and obviously originated from the ovarian surface epithelium. In the ovaries from female \(Wv/Wv:p27^{+/−}\) mice, the overall size of the ovarian tumor is significantly enlarged (Fig.6E). The major composition of the epithelial lesions in \(Wv/Wv:p27^{+/−}\) ovarian tumors remained the infiltrated epithelial cells that is continuous with the surface epithelium (Fig.7). Massive formation of CK8 positive papillary structures were observed in some cases of \(Wv/Wv:p27^{+/−}\) ovaries (2 out of 11) (Fig.7D-F). These structures were defined histopathologically as benign papillary ovarian tumors (Fig.7 A-C). \(Wv/Wv:p27^{-/-}\) ovaries also displayed an increased size of ovarian lesion when compared with \(Wv/Wv\) ovarian tumors (Fig.6F). Unexpectedly, these double knockout ovaries appeared to have a similar if not less prominent tumor phenotypes as in \(Wv/Wv:p27^{+/−}\) ovaries. Still, clear morphological differences were evident in some of the \(Wv:p27\) double knockout epithelial cells. As shown in Fig.8, \(Wv/Wv:p27^{-/-}\) MOSE cells were found to adopt either a cubical or columnar shape with obvious cytoplasms (Fig.8 A-C). These structures were rare in \(Wv/Wv\) or \(Wv/Wv:p27^{+/−}\) ovaries, where most of the epithelial cells remained flat (Fig.7 A-C). Branching papillary projections composed of cubical epithelial cells could also be detected in the lining of intra-ovarian cysts (Fig.8A). These advanced epithelial structures displayed a high similarity with the epithelial cells from Müllerian derived female reproductive tracts, such as uterus and the fallopian tube (Fig.8 D, E).

To confirm the increased ovarian tumor phenotype in \(Wv:p27\) double mutant mice, I collected and quantified the area of CK8 positive regions as well as the size of the whole ovarian tumor estimated on the cross sections that reflect the maximal diameter of the
Fig. 6 Increased ovarian tumor phenotype in Wv:p27 double mutant mice. Ovarian tissues were harvested from Wv:p27 double mutant mouse at similar ages (around 8 months) and subjected to &E staining. Representative images were shown for each genotype: Wv/+::p27+/+ (A), Wv/+::p27+- (B), Wv/+::p27-/- (C), Wv/Wv::p27+/+ (D), Wv/Wv::p27+- (E), Wv/Wv::p27-/- (F). Magnification: X20 (A-F).
Fig. 7 Benign epithelial lesions in \( Wv/Wv:p27+/− \) ovaries. Ovaries from \( Wv/Wv:p27+/− \) mice were harvested at 8 months and stained with H&E (A-C) or CK8 (D-E). One case of \( Wv/Wv:p27+/− \) ovarian tumor displayed papillary structure (A-C) similar as benign borderline ovarian tumor in human. These structures are CK8 positive (D-F) and morphologically indistinguishable from the tubular adenoma in \( Wv/Wv \) ovaries. Magnification: X20 (A, D), X100 (B, C), X200 (E, F).
Fig. 8 Metaplastic epithelial lesions in $Wv/Wv$:p27/- ovaries. Ovaries from $Wv/Wv$:p27/- mice were harvested at 6-9 months and subjected to H&E staining (A-C). Cubical epithelial cells could be found in the cyst lining (A), inclusion cyst (B), and glandular structure (C) of the $Wv/Wv$:p27/- ovary (Arrow). These cells also displayed a Müllerian-like morphology as compared with uterine (D) or oviduct (E) epithelial cells (Arrowhead). Magnification: X100 (D, E), X200 (A-C).
ovaries from these mice. Briefly, sections from various ovarian tumors were first astained with CK8 and counter stained with hematoxylin. Lower magnification images (4x) at consistent resolutions (RGB 24 bit setting of ‘MagnaFire’) were then taken to cover as much of the tumor regions as possible. Areas of the brown staining that resulted from the colorimetric development of DAB substrate were selected using Image J as shown in Fig. 9A. The pixel number of the selected regions was automatically measured and converted to units of $\mu m^2$ based on the scale bars at the same magnification (Fig.9A left panel). The whole tumor area was also selected and quantified using the same methodology. CK8 staining in the adjacent oviducts epithelial cells was excluded from this measurement (Fig.9A).

As shown in Fig. 9B, a thin layer of flat, CK8 positive epithelial cells could be only observed on the surface lining of the wildtype ovary. The $p27^{+-}$ ovaries displayed a similar morphology as wildtype, although focal hyperplasia and deep infiltration of the epithelial lesions could be observed in aged mice. As described earlier, $p27^{-/-}$ ovaries took up a distinct morphology, probably due to the defective development of follicles and consequent disruption of hormonal interplay during estrus cycles. Similar to $p27^{+-}$ ovaries, only minor epithelial lesions such as intra-ovarian cysts could be detected in the $p27^{-/-}$ ovaries.

$Wv/Wv$ ovaries had a phenotype distinct from wildtype ovaries. In these ovaries, the epithelial lesions were significantly amplified and infiltrated into the cortex or medullar regions of the ovaries. As shown in Fig.9C, the epithelial composition of $Wv/Wv$ ovaries is higher than that of the wildtype ovaries ($P<0.001$).
From the analyses of a cohort of $W_v/W_v$ (n=17), $W_v/W_v:p27+/-$ (n=11), and $W_v/W_v:p27-/-$ (n=6) ovaries aged from 7-12 months, I observed a significantly increased level of epithelial lesions and tumor size associated with $p27$ deletion in $W_v/W_v$ mice. As shown in Fig.9B, the loss of one $p27$ allele significantly increased the size of CK8 positive regions from an average of $2.73 \pm 0.32 \times 10^5 \, \mu m^2$ in $W_v/W_v$ ovaries to $8.78 \pm 0.82 \times 10^5 \, \mu m^2$ (p<0.0001) on the cross section that contained the maximal diameter of the ovarian tumor. Homozygous knockout of $p27$ in $W_v/W_v$ ovaries had a similar, if not more subtle, effect on the size epithelial lesions with an average of $6.77 \pm 1.81 \times 10^5 \, \mu m^2$.

We also estimated the size of overall tumor mass by measuring the total ovarian area from the same sections. It should be noted that due to the loss of germ cells and follicle structures, the size of the $W_v/W_v$ ovary is significantly smaller than that of the wildtype ovary. However, benign tubular adenomas are found throughout the $W_v/W_v$ ovaries, while the major compositions of wildtype or $W_v/+\,$ ovaries are follicles and stroma. To avoid the interference of follicular and stromal tissues form the normal ovary, we only defined the whole ovary from $W_v/W_v$ or $W_v/W_v:p27$ mutant strain as the tumor regions excluding the readings from wildtype ovaries. As shown in Fig.9C, loss of $p27$ significantly increased the size of the $W_v/W_v$ ovarian tumor from an average of $1.30 \pm 0.19 \times 10^6 \, \mu m^2$ to $1.93 \pm 0.26 \times 10^6$ and $2.01 \pm 0.64 \times 10^6 \, \mu m^2$ in $W_v/Vv:p27+/-$ and $p27$-/- double mice (P<0.05). The size of the epithelial lesions in $W_v/W_v:p27$-/- ovaries displayed a more sparse distribution pattern when compared with $W_v/W_v:p27+/-$ and $W_v/W_v$ ovaries. This is probably due to the concurrently increased proliferation of the remaining granulosa or other stromal cells as a result of $p27$ deletion.
The data from \(Wv:p27\) ovarian tumors suggest that \(p27\) deficiency is sufficient to induce the expansion of the epithelial lesions defined as benign tubular adenomas that naturally developed in the \(Wv/Wv\) mouse. Loss of one allele of \(p27\) was sufficient to induce a statistically significant increase of both epithelial composition and overall tumor mass. These findings are consistent with the idea that \(p27\) is a haplo-insufficient tumor suppressor gene (Fero et al., 1998). The homozygous deletion of \(p27\) induced a significant yet more subtle increase in the size of epithelial lesion compared with \(Wv/Wv:p27^{+/−}\) ovarian tumors. This could be attributed to the interplay between the epithelial cells and the rapid expansion of remnant ovarian follicles or stromal cells resulted from the loss of \(p27\) (Cipriano et al., 2001). Morphologically, most of the epithelial lesions detected in \(Wv/Wv:p27^{+/−}\) and \(Wv/Wv:p27^{−/−}\) ovarian tumors, are still defined as benign tubular adenomas as the malignant characteristics such as mitotic figures and tumor metastasis are absent.

2.2 Ovarian tumors in \(Wv:p53\) double mutant mice

2.2.1 \textit{Trp53} mutations in human ovarian cancers

As previously described, EOC is a complex disease that is likely to contain a panel of oncogenic mutations and the number of identified genetic mutations involved in the formation and progression of EOC are still accumulating. Among all these genetic mutations, loss-of-function mutation of the tumor suppressor gene \(p53\) is undebatedly the most common and lethal genetic alternation associated with advanced ovarian carcinomas. \textit{Trp53} is the first established tumor suppressor gene, and the loss-of-function mutation of \(p53\) is known to be actively involved in a spectrum of human cancers (Aunoble et al., 2000). \textit{Trp53} promotes the cell growth arrest through the activation of cyclin dependent
**Fig. 9**

A. 

B. 

**Fig.9 Quantification of ovarian tumors \( Wv:p27 \) double mutated mice.** Image J was used to select CK8 positive tumor area as well as and whole tumor mass. The raw pixel reading from the software was converted into \( \mu m^2 \) using the scale bar in the bottom left corner (A). Representative CK8 staining of ovarian tumors from \( Wv/p27 \) mutated mice age at 7-10 months (B). The genotype of each tumor is listed. Scale bar represents 100 \( \mu m \). The quantification of epithelial lesions in \( Wv/p27 \) mutated mice is represented as the size of the CK8 positive region (C) or the whole tumor area (D) in the unit of \( \mu m^2 \). Note the significant increase of epithelial lesions in \( Wv/Wv \) ovaries compared with the wildtype. P value was based on the result of non-paired two-tailed student’s t-test. *, p<0.05 **, P<0.001, ***, P<0.0001. Scale bar equals 50\( \mu m \).
Fig. 9 (Continued)

C.

D.
kinase inhibitor p21 which in turn binds and prohibits the normal function of virtually all of the cyclin-CDK complex essential for cell cycle progression. Once activated, p53 also directly stimulates cell apoptosis through the induction of BAX, PUMA, NOXA and p53AIP (Donehower, 1996). Therefore, p53 functions as a tumor guardian gene that prevents the uncontrolled cell proliferation through the activation of cell senescence and apoptosis pathways.

Mutant $p53$ is most commonly detected in high grade type II serous carcinomas. Previous studies from various groups consistently reported that mutant $p53$ was detectable in 50 to 80% of advanced stage serous ovarian carcinomas (Shih Ie and Kurman 2004). In the case of hereditary epithelial ovarian cancer caused by germ line $BRCA1/2$ mutations, $p53$ was also found to be the most common somatic mutations (69%) in the invasive high-grade serous carcinomas from a cohort of 39 patients with previously confirmed $BRCA1/2$ mutations (Zweemer et al., 1999). Therefore, $p53$ mutation is a common somatic event in both hereditary and sporadic high grade serous epithelial ovarian cancers. As shown in Fig.10, accumulation of p53 was evident in the serous papillomatosis regions of the ovarian cancer. Strong nuclear staining of p53 was ubiquitously detected in the cubical or columnar shaped carcinoma cells while absent in the monolayer of normal surface epithelial cells covering the ovary (Fig.10A,B). Robust nuclear p21 staining could be found in the normal monolayer epithelial cells while lost in the adjacent early neoplastic transition epithelial cells that have a high level of p53 expression (Fig.10C, D). Since p21 is the major downstream target of the p53 signal cascade, the adverse expression pattern of p21 and p53 in the histological transition regions suggests that the function of p53 protein is compromised in the neoplastic cells, probably due to genetic
**Fig. 10**

*Trp53 staining in primary human serous ovarian cancer samples.* IHC staining of p53 (A-C) and p21 (D) was performed on primary human ovarian cancer samples. A strong nuclear staining of p53 was detected in serous carcinoma regions, while absent in normal ovarian surface epithelial cells (A, B). The outlined area in (A) was examined at high magnification in (B). The strong p53 staining in the carcinoma cells is likely to reflect the induction of mutant p53, as p21expression (D) displayed an inverse staining pattern with p53 in sequential tissue sections (C). Magnification: X40 (A), X200 (B-D).
mutations. Indeed, using laser capture microdissection (LCM) and following genomic sequencing, our lab has confirmed the homozygous mutant state of p53 in the p21 negative neoplastic cells adjacent to the normal epithelial tissues (Cai et al., 2009).

Based on the overwhelming evidence correlating the high frequency of p53 mutations with advanced ovarian carcinomas in women, it is plausible to propose that loss-of-function mutation of p53 plays an importantly role for the progression of ovarian carcinomas. However, molecular evidence from in vitro and in vivo studies has clearly suggested that the loss of p53 alone is not sufficient to induce malignant ovarian cancers. Mice with p53 deficiency die within 6-8 months due to a spectrum of simultaneously formed tumors. Among them, lymphoma and sarcomas constitute two of the primary tumors most commonly detected, while the incidence of carcinomas is extremely rare. Cases of epithelial ovarian cancers have never been reported in any strain of p53-/- mice. To circumvent the early lethality that preceded the development of epithelial ovarian cancer in p53-/- mice, Chen et al. transplanted the ovaries from 3-5 weeks old p53 -/- mice into the bursa sac of the ovariectomized wildtype recipient mouse. Surprisingly, only angiosarcomas originating from endothelial cells were observed in the p53-/- ovaries as late as 13 months after the initial transplantation (Chen et al., 2004). This result is in agreement with several other reports suggesting that p53 deficiency alone is not sufficient to induce malignancy in the ovarian surface epithelial cells (Clark-Knowles et al., 2009; Quinn et al., 2009).

**2.2.2 Rescue of ovarian tumor phenotype in Wv/Wv:p53-/- mice**

The incidence of EOC is highest among peri- or post-menopause women, suggesting a causative link between menopausal conditions and ovarian cancer risks. However, this
important biological fact was neglected during the generation of genetic mouse ovarian cancer models. The obvious discrepancy between the high frequency of \( p53 \) mutation in advanced human ovarian carcinomas and the lack of epithelial malignancy in \( p53 \) knockout ovaries led us to hypothesize that \( p53 \) knockout OSE cells may only have a high transformation frequency under a menopause like environment. As described earlier, the \( Wv/Wv \) mouse features the menopause-like condition favorable for the development of epithelial ovarian cancers. Therefore, the combination of \( p53 \) deficiency and germ cell depletion in female \( Wv:p53 \) double knockout mice may lead to the formation of malignant ovarian carcinomas.

Since the status of the \( p53^{-/-} \) OSE cell has never been thoroughly studied in vivo (Chen et al., 2004; Donehower, 1996), the epithelial components from \( p53^{-/-} \) ovaries were first examined. As shown in Fig.11, the gross appearance of six month old \( p53^{-/-} \) ovaries (Fig.11B) was comparable to the wildtype ovaries at the same age (Fig.11A). However, the overall angiogenesis level was elevated as indicated by the increased and irregular distribution of blood vessels and huge hemorrhagic cysts the cortical regions of the ovary (Fig.11B). Follicles of different stages are still presented in \( p53^{-/-} \) ovaries, and the stromal cells appeared to occupy the majority space within the ovary (Fig.11C). IHC staining of cell proliferation marker, Ki-67, revealed an overwhelming high proliferation index among those cells throughout the whole ovary (Fig.11F-H). The ovarian bursa as well as the endometrium/myometrium layer of the smooth muscle was also thickened due to the \( p53 \) depletion-induced proliferation. On the other hand, most of the ovarian surface epithelial cells remained unaffected even in the absence of \( p53 \). Hyperplasia was detected in restricted regions of the ovaries (Fig.11C, D); however, only a low percentage of Ki-67
positive staining indicates the benign nature of the \( p53^-/- \) OSE cells. Moreover, the majority of the surface epithelium remained a flat and single cell layer in contrast to the abundant proliferating stromal cells within in the same ovary (Fig. 11C-E). To test the transformation potential of ovarian surface epithelial cells under menopausal conditions, \( Wv:p53 \) double knockout mice was generated from the intercrosses of \( Wv/+:p53 +/- \) breeders. Unexpected, \( Wv:p53 \) double knockout ovaries displayed a less severe tubular adenoma phenotype when compared with the \( Wv/Wv \) ovaries. As shown in Fig.12, the surface epithelial layer remained intact in \( Wv/Wv:p53 \) double knockout ovaries (Fig.12A-C), although the intra-ovarian epithelial lesions were occasionally evident. Since \( p53 \) depletion was reported previously to preserve the germ cells in male \( Wv/Wv \) mice and restore the infertile phenotypes REF, germ cell marker, PGC7, were used to test whether germ cells were rescued in female \( Wv:p53 \) double knockout mice as well. Indeed, a small number of PGC7-positive oocytes could be observed throughout the ovaries (Fig.12 D-F). Since tubular adenomas in the \( Wv/Wv \) mouse are caused by the gonadotropin stimulation following the premature deletion of germ cells, the presence of oocytes and follicles is in contrast with rationale our menopause ovarian tumor models. Due to this conflict, the generation and further analyses of \( Wv:p53 \) double knockout mice was abandoned.

2.2.3 Latency of ovarian neoplasms in \( Wv/p53 \) conditional knockout mice

To delete \( p53 \) in the ovaries of \( Wv/Wv \) mice, an alternative approach based on the Cre-\( loxP \) system was adopted. Ideally, \( loxP \) flanked genes could be disrupted in a precisely controlled pattern determined by the temporal and spatial distribution of Cre activities. Mouse ovaries are surrounded with a sac-like structure known as the bursa. This anatomically enclosed environment provides an ideal experimental location to test the
Fig. 11 Hyper proliferation of stromal cells in the p53-/- ovary

The ovaries of wildtype (A) and p53-/- (B) were harvested at 6 month of age. Isolated p53-/- ovaries (B) contained hemorrhagic foci with increased angiogenesis levels. Focal hyperplasia was be found in p53-/- ovaries as indicated by CK8 staining (C, D). However, dramatically increased proliferation level was only found in ovarian stromal cells as indicated by Ki-67 staining on the consecutive sections (F, G, H). Magnification: x40 (C, F), X200 (D, E, G, H).
effect of various agents on OSE cells in vivo.

Several groups have successfully delivered Adenovirus over-expressing Cre recombinase (Ad-Cre) in the mouse ovarian bursa sac and conditionally deleted target genes in the surface epithelium. Since somatic $p53$ deletion preserved the germ-cell and rescued the tubular adenomas in the $W_v/W_v$ mouse, $W_v/W_v:p53^{loxP/loxP}$ mice were then generated as a model to study the effects of $p53$ deletion on the progression of ovarian tumors. To deliver Ad-Cre intrabursally, the syringe needle containing Ad-Cre was inserted into the side of the oviducts and pushed through the infundibulum until reaching the bursa sac, where the inoculum was released (Fig.13A). Using this method, successful delivery of the reagents was achieved as indicated by the even distribution of trypan blue surrounding the ovary (Fig. 13B). When $5 \times 10^7$ pfu of Ad-lacZ was administrated intrabursally, strong X-gal staining was detectable only in the ovarian surface epithelium as well as the bursa sac of the injected side (Fig.13C, D). These experiments validated the high efficacy of intrabursal delivery of functional adenovirus.

Ad-Cre and $p53^{loxP/loxP}$ based conditional knockout system were then developed to delete $p53$ in the epithelial cells from $W_v/W_v$ ovaries. Previously, $p53$ conditional knockout strain ($p53^{loxP/loxP}$) was established by inserting two loxP sites at intron 1 and 10 of the gene locus of $p53$. Germ line expression of Cre recombinase led to the deletion of exon 2 to 10 and resulted in a $p53^{A2-10}$ strain that is phenotypically indistinguishable from $p53^{-/-}$ mice. To specifically delete $p53$ in OSE cells in vivo, $p53^{3loxP/loxP}$ allele were introduced into the $W_v/W_v$ mouse and Ad-Cre was later delivered in to OSE cells in vivo through intrabursally injection. Briefly, female $W_v/W_v:p53^{loxP/loxP}$ mice were produced by intercrossing between $W_v/+: p53^{3loxP/loxP}$ breeders and acquired at a frequency of 1:8. At 2
**Fig. 12**

Rescue of germ cells in *Wv/Wv:p53-/- ovaries* IHC staining of CK8 (A-C) and PGC-7 (D-F) was performed on three different ovaries of *Wv/Wv:p53-/-* mice at around 3 months old. Consecutive sections of each ovaries were stained with antibodies against CK8 (A-C) and PGC-7 (D-F), respectively. The presence of germ cells (Arrows, D-F) and lack of epithelial lesions (A-C) hampered the further use of this model. Magnification: X100 (A-F).
months of age, 5 x 10^7 pfu of Ad-Cre were delivered to the ovarian surface epithelial cell through intrabursal injection, while the lateral ovary was left uninjected and served as an internal control. These mice were sacrificed at 10-15 months post Ad-Cre administration. The ovaries were harvested and sectioned, the size and the epithelial lesions in the ovarian tumors were recorded and quantified using the same method as described before. No signs of malignant transformation was detectable in Wv/Wv:p53^{loxP/loxP} ovaries within 10 months after a single Ad-Cre injection. However, after long latency (10-15 months), ovarian lesions characteristic of massive hemorrhage and polycystic tumor masses could be observed in a few (2 out of 9) injected Wv/Wv:p53^{loxP/loxP} ovaries (Fig.14A arrowhead). The control ovary from the same mouse maintained gross morphology common for Wv/Wv ovaries (Fig.14A). In Wv/+:p53^{loxP/loxP} mice, no noticeable difference in gross morphology could be found between Ad-Cre injected and the contralateral control ovary (Fig.14A). To confirm the efficacy of Ad-Cre mediated p53 deletion, part of the Ad-Cre injected ovary was dissected and subjected to genomic PCR. Deletion of p53 was confirmed by the PCR amplification of a 612 bp band that results from Cre mediated excision of p53 allele (Δ 2-10). This recombined band was absent in the uninjected control ovaries and PCR amplification of the non-recombined loxP band was consistently detected (Fig.14B). A panel of paired Wv/Wv:p53^{loxP/loxP} ovaries with or without Ad-Cre injection was then compared and quantified. As shown in Fig.14E, the majority of Ad-Cre injected Wv/Wv:p53^{loxP/loxP} ovaries were significantly larger than the uninjected control ovaries, as estimated from the tumor size on the cross-sections representing the maximal diameter of the ovarian tissues (P< 0.05, paired student’s t-test). Further histological analyses revealed that only part of Wv:p53^{Δ2-10} tumors was
Fig. 13 Efficiency of adenovirus infection in vivo The in vivo delivery of adenovirus was achieved through intrabursal injection (A), successful ovarian delivery was confirmed using trypan blue as a dye (B). The in vivo infection efficacy of adenovirus was confirmed by strong x-gal staining in OSE cells 3 days after injection of Ad-lacZ (C), the boxed area was amplified in (D). Magnification: X20 (C), X200 (D).
positive for epithelial marker CK8. It should be noted that even in the uninjected control ovaries, the percentage of epithelial lesion was also decreased in mice older than 13 months (Fig. 14C upper panel). Intra-ovarian cysts were easily detected in $W_v:p53^{Δ2-10}$ ovaries. Most of these cysts are lined with a single layer of epithelial cells indistinguishable from normal MOSE cells. Although epithelial lesions only covered a relatively small part of the $W_v:p53^{Δ2-10}$ ovaries, the quantification of CK- positive regions suggested significantly amplified epithelial lesions associated with $p53$ depletion (Fig.14D, paired student’s t-test P<0.001). The enlargement of CK8 positive lesions was in proportion with the increment of the overall tumor mass in $W_v:p53^{Δ2-10}$ ovaries, as no significant difference could be found in the percentage of epithelial lesions in the whole ovarian tumor after $p53$ depletion (data not shown).

Early signs of malignant epithelial cells were still detected on the surface of $W_v/W_v$ ovaries 15 months after $p53$ deletion. As revealed by CK8 staining (Fig.15A), these early dysplasia features included local accumulation of disorganized, multiple layers of epithelial cells (left), complex papillary structures composed of neoplastic cells (middle) and intra-peritoneal shedding of the disseminated epithelial clusters (right). Single layers of normal OSE cells were detected on the same tumor section, suggesting that Ad-Cre might not be evenly distributed in the whole ovary. As described before, multiple cysts lined with single layer of epithelial were found frequently in $W_v:p53^{Δ2-10}$ ovaries. Occasionally, the growth of these epithelial cells caused the formation of pre-neoplastic lesions of cystadenocarcinoma structures (Fig.15B). Tubular adenomas, commonly detected in $W_v/W_v$ ovaries, were also found throughout $W_v:p53^{Δ2-10}$ ovaries. The morphology of the epithelial cells within these benign lesions was almost
Fig. 14

(A) Representative image (case #2758, left panel) of ovarian tumor formed in one $W_{v}/W_{v}:p53^{loxp/loxp}$ mouse 15 months after intrabursal Ad-Cre administration, while no significant abnormality were detected in the $W_{v}/+$ control mouse (right panel). Arrowheads indicate the Ad-Cre injected ovary. (B) PCR analyses of Ad-Cre injected and control ovaries confirmed the excision of $p53$ locus from exon 2 to 10. (C) Low magnification images from CK8 staining results indicated the amplification of benign ovarian tumor phenotype (lower panel) in $p53$ deleted $W_{v}/W_{v}$ ovaries compared with the contra-lateral control ovary (upper panel). The case numbers of each set of representative ovaries were labeled under the images. Quantification of epithelial lesions (D) and the overall tumor size (E) in $p53$ deleted and control ovaries from the same mouse ($n=9$) was performed as described in Fig.9A. The case number of each mouse was labeled on x-axis. P value is based on the results of paired two-tailed student’s t-tests. *, $p<0.05$ **, $P<0.001$. Scale bar equals 100µm.
Fig. 14 (Continued)

D.

\[ \text{Wv/Wv; p53}^{laxP/laxP} + \text{Ad-Cre} \]

\[ \text{Wv/Wv; p53}^{laxP/laxP} \]

E.

\[ \text{Wv/Wv; p53}^{laxP/laxP} + \text{Ad-Cre} \]

\[ \text{Wv/Wv; p53}^{laxP/laxP} \]
indistinguishable from the normal OSE cells. Therefore, although some of the tubular
to be the continuous extension of surface epithelium, probably as a result of deep
infiltrations (Fig. 15B, upper panel). Compared with \( W_v/W_v:p53^{\text{loxPloxP}} \) ovaries, tubular
adenomas of \( W_v:p53^{\Delta2-10} \) ovaries tended to be more condensed with a high frequency of
focal hyperplasia. The proliferation index among these tubular adenoma lesions was
evaluated using Ki-67 staining. Although a higher percentage of Ki-67 positive nuclei
could be found within the putative \( p53 \) depleted epithelial cells, the most prominent
dividing cells (as indicated by giant atypical nuclear or obvious mitotic figures) resided
mainly in the stromal tissues that surrounded the tubular adenomas (Fig. 15B, lower
panel). To further characterize the origin of \( W_v:p53^{\Delta2-10} \) ovarian tumor, I performed IHC
staining using inhibin–\( \alpha \) and \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) antibodies, two distinctive
markers for different cell subsets of the ovary. In normal ovary, inhibin–\( \alpha \) is only
expressed in the granulosa cells of the developing follicles (Fig. 16A). In \( W_v/W_v \) ovaries,
only patches of inhibin–\( \alpha \) were present due to the depletion of germ cell and disruption of
follicle structures (data not shown). In \( W_v:p53^{\Delta2-10} \) ovaries, no neoplastic expansion of
inhibin–\( \alpha \) positive cells were observed (Fig. 16C). In normal ovaries, \( \alpha \)-SMA is mainly
expressed in the myofibroblasts located in the external layer of theca cells (theca externa)
or the vascular smooth muscle cells (Fig. 16B). In \( W_v:p53^{\Delta2-10} \) ovarian tumors, a
thickened smooth muscle layer was found beneath the ovarian surface epithelium.
Meanwhile a scattered distribution pattern of \( \alpha \)-SMA positive cells was also present
throughout the ovary (Fig. 16D). However, these cells were not neoplastic as indicated by
the overall normal nuclear morphology and a relatively low proliferation index.
Collectively, we established and characterized a novel ovarian tumor based on a Ad-Cre mediated in vivo conditional knockout. However, $W_v:p53^{Δ2-10}$ ovarian tumors failed to yield overwhelming epithelial malignancy as we had expected. Nevertheless, the epithelial lesions, as well as the overall tumor size, were significantly increased after the conditional deletion of $p53$. In several severe cases, large polycystic and hemorrhagic ovarian tumors developed unilaterally at the site of Ad-Cre injection. In these tumors, pre-neoplastic lesions of the epithelial cells such as focal hyperplasia, papillomatosis were observed. Morphological changes common for $W_v/W_v$ ovaries, such as inclusion cysts and deep invagination were also detected in the $W_v:p53^{Δ2-10}$ ovarian tumors. A modestly higher proliferation index was evident in $W_v:p53^{Δ2-10}$ ovarian epithelial lesions. However, the majority of the aggressively growing cells were CK8 negative stromal cells throughout the ovary. Therefore, our results clearly suggest that $p53$ deletion alone is not sufficient to induce epithelia ovarian tumors even under a menopause-mimicking environment of $W_v/W_v$ mice. These findings, together with the existing literatures on the lack of epithelial ovarian cancers in the transplanted $p53^{-/-}$ ovaries or mice with OSE-specific deletion of $p53$, indicate that additional oncogenic mutation are likely involved in the transformation of ovarian epithelial cells (Chen et al., 2004; Clark-Knowles et al., 2009; Quinn et al., 2009).
Fig. 15

A. 

B. 

**Fig.15 Epithelial lesions in Wv: p53Δ2-10 ovarian tumor.** CK8 (A) and Ki-67 (B) was performed on the ovarian tumor from a Wv: p53Δ2-10 mouse (case #2758). Focal hyperplasia (left), papillomatosis (middle), as well as dissemination of epithelial clusters (right) were detected on the surface of Wv: p53Δ2-10 ovarian tumor (A). Tubular adenomas were also detected in the internal regions of the same ovary (B, upper panel). However, Ki-67 staining of consecutive sections indicated that the rapid proliferating cells were mainly CK8-negative ovarian stromal cells (B, lower panel). Magnification: X200 (A, B)
Fig. 16 Characterization of $Wv:p53^{Δ2-10}$ ovarian tumors IHC staining of inhibin-α (A, C) and α-SMA (B, D) was performed on the ovarian tumor from a $Wv:p53^{Δ2-10}$ mouse. In wildtype ovaries, inhibin-α and α-SMA was detected in the follicles (A) and theca cells (B), respectively. In $Wv:p53^{Δ2-10}$ ovarian tumor (C, D), patched staining pattern of inhibin-α and thickened α-SMA positive layer was observed (D). Magnification X200 (A-D)
Chapter III: In vitro characterization of the transformation potential of mouse ovarian surface epithelial cells

3.1 Pre-neoplastic changes in p27 and p53 mutant MOSE cells

Although this thesis was originally aimed to understand the underlying molecular mechanisms of the causative link between menopausal physiology and ovarian cancer risk in an in vivo setting, the low yield of malignant EOC and the long latency for the tumor formation from \( Wv:p27 \) or \( Wv:p53^{Δ2-10} \) mice hampered further in vivo investigation. Highly malignant epithelial ovarian tumors were not detected in any of the mouse model previously described. However, pre-neoplastic epithelial lesions were evident in both \( Wv:p27 \) or \( Wv:p53^{Δ2-10} \) ovaries. Detailed molecular analyses of the tumor cells generated from these models will still provide valuable insights into the signal cascades involved in the early stages of EOC development.

Primary culture of mouse ovarian surface epithelial (MOSE) cells were first used to exam the oncogenic alterations caused by p27 and p53 mutation in these cells. Briefly, mouse ovarian surface epithelial cells were prepared from ovaries of various mice including: wildtype, p27 +/-, p27-/-, p53 +/- and p53-/-. The growth properties of these cells were measured by the MTT assay. The associated oncogenic changes caused by p27 or p53 deletion were accessed by Western blotting and immunofluorescence staining.

The deletion of p27 in MOSE cells were first confirmed by western blotting (Fig.17C). Due to the lack of a suitable antibody for p53, p53 deletion was indirectly validated by the loss of p21 (Fig.17C), a well established downstream target of p53. As revealed by the MTT assay, the proliferation rate was increased significantly in either p27+/− or p27-/- MOSE cells (Fig.17A). These data are consistent with our previous in vivo findings that findings that loss of one p27 allele was sufficient to increase the epithelial lesions in
Wv/Wv ovaries. On the other hand, only p53-/-, though not p53+/- MOSE cells, had a more robust proliferation potential than wildtype MOSE cells (Fig.17B). Moreover, only p53-/- MOSE cells could survive multiple passages as monolayer cell culture (data not shown). The growth of p27-/- MOSE cells completely ceased at a similar passage number as the wildtype cells, probably due to a complementary induction of CKI proteins: p21, p19 and p16 (Fig.17C, D).

The alterations in the cell cycles proteins of p27 and p53 depleted MOSE cells were further unanalyzed. As shown in Fig.17E and F, cyclin dependent kinase (CDK) 2 and cyclin A were increased in p27 deleted MOSE cells, while CDK2, CDK4 and cyclin A were induced in p53-/- MOSE cells. This, together with the substantially higher expression of the proliferation marker progression cell nuclear antigen (PCNA) in p53 and p27 depleted MOSE cells, suggested that an accelerated cell cycle was induced with the deletion of tumor suppressor genes p27 and p53. In p53-/- MOSE cells, this accelerated cycle was sustained even in later passages (Fig17E, F).

Human MOSE cells are known to have both epithelial and mesenchymal features as an adaptive mechanism against the cyclic and rapid changes in hormonal environment during the menstrual cycle. As a result, mesenchymal specific genes, such as N-cadherin and vimentin, are enriched in ovarian surface epithelial cells, while the expression of epithelial markers including E-cadherin, claudin3 or CA125 are only frequently detected in pre-malignant lesions or benign ovarian neoplasms (Auersperg et al., 1999; Strauss et al., 2011; Sundfeldt et al., 1997; Wong et al., 1999). I next tested whether more epithelial features were associated with the deletion of p27 and p53. As shown by western blots and immunofluorescent staining, the expression level of claudin3 and E-cadherin increased
significantly in both p27-/- and p53-/- MOS cells, while N-cadherin remained a high yet comparable level among wild type, p27-/- and p53-/- MOSE cells (Fig.18). The epithelial features were more obvious in late passages of p53-/- MOSE cells, indicating by the accumulation of E-cadherin and claudin 3 proteins in these cells (Fig.18C). In summary, these data suggest that the deletion of p27 and p53 renders pre-malignant changes in MOSE cells as indicated by increased cellular propagation rate as well as the acquisition of epithelial proteins. However, only p53-/- MOSE cells survived multiple passages in vitro. Similar as wildtype, MOSE cells with p27 deficiency ceased to grow within a few passages, probably due to the complementary accumulation of CKI family proteins.

3.2 Pre-neoplastic changes in Wv/Wv and Wv:p53<sup>Δ2-10</sup> MOSE cells

Since no malignant epithelial tumor is detected in Wv:p53<sup>Δ2-10</sup> ovaries and the activity of the receptor tyrosine kinase c-kit is associated with cell growth, I next tested whether Wv/Wv OSE cells are intrinsically incapable of rapid proliferation. As shown in Fig. 19A, the majority of Wv/Wv MOSE had a typical cobblestone morphology of epithelial cells and rapidly propagated during the early passage. Western blotting as well as IF results suggested that Wv/Wv MOSE cells had readily acquired more intrinsic epithelial features indicated by the increased expression claudin3 and E-cadherin (Fig.20C). The fast growth rate of Wv/Wv MOSE decreased dramatically with repeated passaging in vitro, and most cells ceased proliferation at around 4-5 passages (Fig.19A). Nevertheless, Wv/Wv MOSE cells have a significant growth advantage over wildtype MOSE cells, especially during early passage. Therefore, I excluded the possibility that the lack of the EOC formation in Wv:p53<sup>Δ2-10</sup> mice was attributed to the growth retardation caused by c-kit mutation.
Fig. 17 Increased proliferation of p27-/- and p53-/- MOSE cells The growth rates of wildtype, p27+/-, p27-/- (A) or p53+/-, p53-/- (B) MOSE cells were monitored for 5 to 6 days, and measured by MTT assay (n=3). The loss of p27 and p53 significantly increased the proliferation of MOSE cells (P<0.001). The expression levels of CKI family proteins p27, p21, p16 and p19 were analyzed by western blot in wildtype, p27+/-, p27-/- and early (p.3) or late passage (p.8) of p53-/- MOSE cells (n=4) under the same culturing conditions (C). The results were quantified using Image J, normalized with β-actin and summarized in (D). Two representative samples of each group were shown in (C). The expression level of cell proliferation marker PCNA and cell cycle proteins: cyclin E2, cyclin A, Cdk4, Cdk2 was assayed by western blot (E) and summarized in (F) as described before (n=4). P value is based on the results of student’s t-tests. **, P<0.01 ***, P<0.001. N.D, Not detected.
Fig. 18 Epithelial features of $p27^{-/-}$ and $p53^{-/-}$ MOSE cells in vitro

Mesenchymal marker N-cadherin (red), epithelial marker claudin 3 (green) were co-stained in wildtype, $p27^{-/-}$ and $p53^{-/-}$ MOSE cells. Ubiquitously high level of N-cadherin was observed in all three types of these cells (A), while claudin 3 was profoundly increased in $p27^{-/-}$ and $p53^{-/-}$ deficient cells. This observation was confirmed by western blot from $p27^{-/-}$ (B), and early (p.3) or late passage (p.8) $p53^{-/-}$ MOSE cells (C). The results from western blot were quantified and normalized as described before (n=4). Another epithelial marker E-cadherin was also found to be simultaneously increased in $p27^{-/-}$ and $p53^{-/-}$ MOSE cells (B, C). P value is based on the results of student’s t-tests. *, P<0.05 ***, P<0.001. N.D, Not detected.
As shown previously, $p53^{-/-}$ MOSE cells were found to have a robust proliferation rate and could survive multiple passages in vitro; however, they are not capable of forming malignant tumors in vivo.

To reconcile the discrepancy between the lack of ovarian carcinomas in $p53$ deficiency mice and the prevalent $p53$ mutant found in human ovarian cancer patients, I next switched to an in vitro system using primary culture of mouse ovarian surface epithelial cells to closely monitor the molecular changes associated with $p53$ deletion.

$Wv^{+}: p53^{loxP/loxP}$ and $Wv/Wv: p53^{loxP/loxP}$ MOSE cells were isolated and exposed to one administration of Ad-Cre in vitro at approximately 200 MOI. The deletion of $p53$ was confirmed by the loss of p21 (Fig.21 D). As expected, the proliferation rate of $Wv:p53^{Δ2-10}$ MOSE cells was significantly increased compared with the parental cells (Fig.19D). Moreover, both $Wv/Wv$ and $Wv^{+}: p53^{Δ2-10}$ MOSE cells were capable of surviving multiple passaging in vitro (Fig.19B).

Additional western blotting and IF analyses revealed that $p53$ deletion led to an accelerated cell cycle as indicated by the increase levels of CDK4, cyclin A and PCNA expression (Fig.20C). Moreover, epithelial marker such as E-cadherin and claudin3 was concomitantly increased with deletion of $p53$, while mesenchymal marker, N-Cadherin, remained constant (Fig.20A, B, D).

During the continuous passaging of $p53^{Δ2-10}$ MOSE cells in vitro, increasing number of these cells exhibited characteristic cell senescence features including enlarged and flatten morphology with highly vacuolated cytoplasm (Fig.21B). The classic senescence associated (SA) β-galactosidase assay was then used to test whether the increasing level of cellular senescence was associated with MOSE cells after the deletion of $p53$. 
The accuracy of SA-β gal assay was first validated in primary cultures of young and old mouse embryonic stem fibroblast cells (Fig.21A). In the late passage of Wv/+ and Wv/Wv MOSE cells (Fig.21B), both apoptotic (as indicated by condensation of the cell body) or senescence (as indicated by the blue staining showing the β-gal activities) cells were present. In the p53<sup>Δ2-10</sup> MOSE cells, however, the majority of the cells were much more positive for β-gal activity (Fig.21C), suggesting an overwhelming increase of senescence in MOSE cells as an intrinsic defense mechanism against transformation. Based on current understanding, senescence was mainly stimulated by two independent pathways, p19<sup>Arf</sup>-p53-p21<sup>cip1</sup> and p16<sup>ink4</sup>–Rb (Attardi and Jacks, 1999; Gil and Peters, 2006; Sherr, 2001). Since the function of p21 and p19 are relied on the expression of p53, I first examined whether increased cellular senescence level could result from p16 induction. As shown by western blot, the expression of p16 was dramatically increased upon p53 deletion in both Wv/+ and Wv/Wv MOSE cells (Fig.21D, F). A consistent effect was also observed in p53−/− MOSE cells (Fig.17B). P19 (termed p14 in humans), another cellular senescence maker that is a transcript variant of p16, was simultaneously induced (Fig.21D, F) with p53 deletion. Since the exon 2 and 3 of p16 and p19 shared the same genetic loci, it is possible that the transcription of p16 and p19 are stimulated by the deletion of p53 in a similar mechanism. The induction of p16 and p19 remains constant through the different passages of p53<sup>Δ2-10</sup> MOSE cells, while only a small amount of p16 or p19 protein could be detected even in the later passage of parental cells (Fig.21E).

These data suggest that Wv/Wv MOSE cells readily acquire pre-neoplastic epithelial features compared to the control cells. However, these cells failed to survive continuous passaging in vitro. Ad-Cre mediated p53 deletion significantly improved the cellular life
**Fig. 19** The growth of *Wv/Wv* and *Wv:p53^Δ2-10* MOSE cells in vitro (A) The morphology of *Wv/+* and *Wv/Wv* MOSE cells, both cells ceased to proliferate within 4-5 passages. (B) The morphology of *Wv/+; p53^Δ2-10* and *Wv/Wv: p53^Δ2-10* MOSE cells following Ad-Cre infection. Note the increased endurance for repeated passaging in vitro. (C) The growth curve of *wildtype*, early passage (p.2) and late passage (p.5) *Wv/Wv* MOSE cells were assessed using MTT assay as described before (n=3). (D) Ad-Cre mediated p53 excision in *Wv/Wv* MOSE cells significantly stimulated the growth rate of these cells as determined by MTT assay (n=3). P value is based on the results of student’s t-tests. ***, P<0.001.
span as well as proliferation rate in both \( Wv/+ \) and \( Wv/Wv \) MOSE cells. However, \( p53 \) deletion alone is not sufficient to transform MOSE cells in vitro. This is likely due to the induction of the cellular senescence program mediated by p16, which might also explain the low carcinomas frequency in \( p53^{\Delta2-10} \) ovaries in vivo.
Fig. 20 The acceleration of cell cycle and metaplasia in p53 excised MOSE cells.
Control (A) and p53 depleted (B) wildtype and Wv/Wv MOSE cells were co-stained with CK8 (green), claudin 3 (red), and N-cadherin (white). These results were confirmed by western blot (D). The levels of cell proliferation marker PCNA and cell cycle proteins Cyclin A, Cdk2, Cdk4, before and after Ad-Cre mediated p53 deletion were examined in wildtype and Wv/Wv MOSE cells by western blot (D) These results were summarized as described before (n=3). P value is based on the results of student’s t-tests. ***, P<0.001.
**Fig. 21**

Cell senescence in p53 deleted MOSE cells (A) SA-β-galactosidase assay was validated in young (p.4) and old (p.9) MEFs. (B) Late passage \(Wv/^{+}\) and \(Wv/Wv\) MOSE cells had increased level of senescence as well as cell death. Arrows indicated the condensation of cell body as a result of apoptosis or necrosis. (C) Profound cellular senescence was detected in both \(Wv/^{+}\) and \(Wv/Wv\) MOSE cells after p53 deletion. Two representative areas were shown in each case (B, C). The levels of cell senescence markers: p16 and p19, in \(Wv/^{+}\) and \(Wv/Wv\) MOSE cells immediately (D) or several passages (E) after Ad-Cre mediated p53 deletion were examined by western blot. These results were quantified and normalized as described before (n=3). IF results confirmed the increase of p16 and p19 proteins in \(Wv/Wv\) MOSE cells upon p53 deletion (F). *P value is based on the results of student’s t-tests. ***, *P*<0.001.
Chapter IV: In vivo tracing of the origins of mouse ovarian surface epithelial cells.

One of the dogmas currently under wide debate is whether ovarian carcinomas are derived from the ovarian surface epithelial cells or the Müllerian ducts derived epithelial cell. The ‘ovarian surface epithelium origin’ hypothesis failed to explain the Müllerian like morphology of ovarian cancers despite its wide acceptance. On the other hand, molecular evidence in support of the ‘müllerian-derived’ hypothesis is still missing. During the examination of the ovarian tumors from $\text{Wv}/\text{Wv}$ and $\text{Wv};p27$ double mutant mice, we had detected prominent epithelial lesions arising from the deep medulla and ovarii rete portions of the ovary that have no apparent connection with the surface epithelium. These observations suggest that the epithelia lesions in $\text{Wv}/\text{Wv}$ based tumor models potentially have origins other than the surface epithelium, which partially supports the ‘müllerian derived’ hypothesis.

To determine the contribution of rete ovarii or the remnants of Müllerian in the ovarian tumors of $\text{Wv}/\text{Wv}$ mice, the heterogeneous composition of these tumors were analyzed using an in vivo cell tracing approach based on type II müllerian inhibitory substance receptor II (MISR II)-Cre (Jamin et al., 2002) and MISRII-lacz (Arango et al., 2008) strains acquired from Dr. Richard Behringer (MD Anderson Cancer Center). MIS, also known as anti müllerian hormone (AMH), belongs to the TGFβ family and is essential for the regression of the precursor female reproductive tracts (müllerian duct) during the development of male embryos. MIS deficiency preserved the female reproductive system in the male mouse, thus converted male $\text{MIS}^{-/-}$ mice into hermaphrodites. The expression of MISR II was first found to be restricted within the mesenchymal cells of female reproductive ducts during embryonic development as well as in adult mice.
Moreover, the dynamic expression of MISR II has also been detected in the granulosa cells of preantral and small antral follicles, theca cells, and some stromal cells in the adult ovaries (Arango et al., 2008; Jamin et al., 2002; Jorgez et al., 2004; Kobayashi and Behringer, 2003). Interestingly, MISRII expression could be found in approximately 50-60% of human epithelial ovarian cancer and recombinant MIS prohibits the clonogenic growth of human ovarian cancer lines (Bast et al., 2009; Pieretti-Vanmarcke et al., 2006; Szotek et al., 2006). However, the expression status of MISR II in normal OSE has not been fully characterized and remains controversial. Different groups still have contrasting conclusions from the result of the origin tracing experiments of OSE cells based on MISR II-Cre strains (Arango et al., 2008; Fan et al., 2009; Jamin et al., 2003; Jorgez et al., 2004). Due to the strong expression of MISR II in female reproductive ducts during and after embryonic development, I planned to trace the origin of epithelial lesions in Wv/Wv ovarian tumors using MISR II-Cre or lacZ reporter strains.

We have successfully generated female Wv/+ or Wv/Wv: MISR II-Cre strains and further crossed it with a conditional lacZ reporter strain (B6;129S Gtrosa26tm1Sor) from Jackson Lab. To monitor the temporal expression of MISR II, MISR II-LacZ strain was also used as a control. X-gal staining was performed on cryosections of freshly harvested ovarian tissues. The blue MISR II traced cells were mainly mesenchymal cells surrounding the müllerian epithelium in the female reproductive tracts (Fig.22A). In the ovary, granulosa cells, theca cells as well as some of the stromal cells were also positively traced (Fig.22B). These results are consistent with the previous report using the same tracing strategies (Arango et al., 2008; Jamin et al., 2003). Surprisingly, the ovarian epithelial cells displayed a mosaic staining pattern according to MISRII-Cre mediate cell origin.
Fig. 22

A.  
*MISR II-cre:Rosa26*

![Images of Oviduct, Uterus, and Negative control with arrows indicating regions of interest.]

B.  
21 days  |  2 Months  |  5 Months  |  6 Months

![Images showing developmental stages of Oviduct and Uterus with arrows indicating changes over time.]

C.  
*MISR II-lacZ*

![Images showing Young and Adult stages with *MISR II-lacZ* expression.]

D.  
*MISR II-Cre:Rosa26*

![Diagram showing gene expression over development with Young and Adult stages indicated.]
**Fig. 22** Subtypes of MOSE cells revealed by MISR II based tracing assay

(A) X-gal staining in the oviducts (left l) and uterus (middle) from a *MISR II-cre:Rosa26* mouse. The uterus of *Rosa26* strain was used as a negative control (right). Arrowheads indicate the X-gal negative epithelial cells. (B) X-gal staining was performed on ovaries from *MISR II-cre:Rosa26* mice aged at 21 days, 2, 5, and 6 months. Low (4X upper panel) and high magnification (200X middle and lower panel) images revealed the mosaic composition of MOSE cells (arrows). (C) X-gal staining was performed on ovaries from *MISR II-lacZ* mice at 21 days or 6 months old. Low (4X upper panel) and high magnification (200X lower pane) images indicated the weak MISR II expression level in the adult ovary. (D) The experimental strategy of *MISR II-Cre:Rosa26* based original tracing assay of MOSE cells. The absence of X-gal positive cells in the adult *MISR II-lacZ* ovaries suggest that the mosaic X-gal staining pattern in *MISR II-Cre:Rosa26* MOSE cells are likely resulted from the intrinsic properties of these cells rather than the temporal expression of MISR II.
tracing results (Fig. 22B). The mosaic distribution of X-gal positive MOSE cells did not appear to change with age of the mouse (Fig. 22B). Human OSE cells are known to have some mesenchymal features due to the cyclic epithelial-to-mesenchymal stimulation caused by ovulation (Auersperg et al., 1999; Sundfeldt et al., 1997). Since MISR II is strongly expressed in mesenchymal cells of female reproductive tracts, I next tested whether the mosaic pattern in the ovarian epithelium could be caused by the temporal expression of MISR II protein in MOSE cells. X-gal staining was performed on cryosections of the ovaries from MISRII-lacZ stain that reports the endogenous expression level of MISR II (Fig. 22D). As shown in Fig. 22C, the expression of MISR II was silenced in the majority of adult ovarian epithelial cells as well as other ovarian cells. Therefore, the mosaic tracing pattern of MOSE cells likely indicate different origins of these cells rather than the concurrent expression of MISR II protein.

From these preliminary results of MISR II-Cre based tracing strategies, I unexpectedly found that mouse ovarian surface epithelial cells could potentially rise from multiple lineages. Since müllerian ducts (uterus, oviducts, cervix) derived epithelial cells are mostly negative for MISR II tracing, the presence of negatively traced epithelial cells in the ovarian surface epithelium suggest that at least part of the MOSE cells may have the same origin as müllerian duct derived epithelial cells. The lack of malignant ovarian carcinomas in Wv/Wv based ovarian tumor models hampered the further application of MISR II based tracing approach. Nevertheless, the combination of MISRII-Cre strain with more established mouse ovarian cancer models will still provide further insights to the origin of EOC.
Chapter V: Summary and Discussion

5.1 Etiology factors of epithelial ovarian cancer

Most of the current mouse EOC models were established based on the single or multiple genetic alterations in genes such as \textit{BRCA1}, \textit{p53}, \textit{Rb}, \textit{β-catenin} and \textit{APC} (Fong and Kakar, 2009). These models could be categorized as genetic ovarian cancer models. Highly malignant epithelial ovarian cancers were recapitulated in at least some of these models, which provide the direct evidence linking specific oncogenic mutations with different subtypes of epithelial ovarian cancer (Dinulescu et al., 2005; Wu et al., 2007). Although these genetic ovarian cancer models were generally accepted as useful experimental tool to study the tumorigenesis and the treatment of EOC, it should be noted that etiological factors, which play an indispensible role during the development of various human cancers, are commonly neglected in these genetic models. In the case of ovarian cancer, consistent results from epidemiological data clearly link the risk of EOC with reproductive factors. The incidence of ovarian cancer is highest during peri- and post-menopause period, while decreased ovulation numbers achieved through the use of contraceptives or parities have clear protective effects against EOC (Purdie et al., 2003; Smith and Xu, 2008). Through the last century, two major theories, namely the incessant ovulation and gonadotropin stimulation have been proposed to explain the same epidemiological data. Incessant ovulation theory proposes that the repetitive wounding and healing processes during ovulation led to the accumulation of oncogenic mutation in the epithelial cells that eventually resulted in the transformation of OSE over time (Fathalla, 1971). This hypothesis is well supported by an epidemiological study that correlated the occurrence of EOC with the estimated lifetime ovulation in a cohort of 791
ovarian cancer patients (Purdie et al., 2003). In addition, a similar conclusion was drawn from the observation of pre-neoplastic morphological changes in the surgical samples removed from prophylactic oophorectomy of healthy peri-menopausal women (Cai et al., 2006). Although these data persuasively support the incessant ovulation theories, it should be noted that the actually development of EOC is more complex and likely includes multiple factors such as age related accumulation of oncogenic mutations and alternated hormonal regulations (Smith and Xu, 2008). Based on the almost identical epidemiological data, another theory, known as gonadotropin stimulation, was postulated focusing on the special hormonal environment during menopausal period (Cramer et al., 1983a; Cramer and Welch, 1983; Cramer et al., 1983b). In young females, periodical surge of pituitary gonadotropins (LH and FSH) is responsible for the initiation of the ovulation. At the end of ovulation, follicles are differentiated into progesterone producing corpus lutea. Progesterone then forms a feed-back loop to inhibit the release of gonadotropin releasing hormones, and in turn reduces the secretion of LH and FSH from the pituitary gland. During peri- or post-menopausal period, the endocrine feed-back loop was absent due to the depletion of germ cells, follicle structures as well as the corpus lutea. As a result, a persistently high level of gonadotropins was commonly detectable in peri- and post-menopausal women (Cramer and Welch, 1983; Richards et al., 2002). During ovulation, gonadotropins trigger an inflammatory-like process that leads to the rupture of surface epithelium and release of the ovum (Mohle et al., 1985). In menopausal women, the high level of gonadotropins could result in a persistent inflammatory-like environment surrounding the ovaries and cause repetitive wounding and recurring process in surface epithelial cells that eventually leads to malignant
transformation (Richards et al., 2002). This theory is best supported by the fact that lowered circulating pituitary gonadotropin level, achieved through either pregnancy or the use of contraceptives, generally reduces EOC risks (Gwinn et al., 1990; Permuth-Wey and Sellers, 2009; Riman et al., 1998). Incessant ovulation and gonadotropin stimulation theories are not mutually exclusive from each other: persistently elevated gonadotropin levels during peri- and post-menopausal period may foster an inflammatory environment that initiates ovulation-like condition. However, neither of theories provide satisfactory evidence for the causative link between menopausal biology and increased EOC risks. Age and the accumulation of oncogenic mutations remain the most associated risk factors for ovarian cancer. In the setting of experimental animals, gonadotropin treatment in adult rats significantly promoted the proliferation of OSE cells, yet failed to induce any detectable ovarian carcinomas (Stewart et al., 2004). These findings suggest that both incessant ovulation and gonadotropin stimulation predispose OSE cells to a tumor prone status rather than directly stimulate neoplastic transformation.

5.2 $W_v/W_v$ mouse as a unique animal model for EOC etiology

Development of animal models for ovarian cancer is essential for the study of the progression of this deadly disease. Recently, significant progress has been made in the generation of genetic mouse EOC models as described in the introduction. The most impressive epithelial ovarian cancer was derived from the inhibition of p53-Rb signals through the use of T-antigen or concurrently deletion of these two genes in MOSE cells. Moreover, some of the models recapitulated both the genotype (oncogenic mutations) and histopathology of human EOC, thus convincingly provided the causative link between the oncogenic mutations and the formation of certain subtype of human EOC (Dinulescu et
al., 2005; Wu et al., 2007). Nevertheless, the fundamental difference between these animal models and sporadic human EOC is that they fail to explain the high incidence of EOC in peri- and post-menopausal women.

Human EOC is a unique disease developed in accompany with the extended post-reproductive lifespan in modern day women through the last century. For this reason, EOC is rarely found in other mammals with the exception to some laboratory animals. To generate a more relevant and reliable animal model for this disease, the etiological factors of post-reproductive (menopause) physiology should be incorporated. However, due to the extensive time consumption and labor cost of maintaining post-reproductive laboratory animals, few etiological EOC models are available (Fong and Kakar, 2009).

Recent studies using \( Wv/Wv \) mouse have provided some interesting insights into the etiology of EOC. As described before, \( Wv/Wv \) mouse has a naturally occurring \( c\text{-}kit \) mutation that results in a premature loss of germ cells within 2 months. Since menopause is defined with the depletion of oocytes, the rapid loss of germ cells in \( Wv/Wv \) mouse accurately recapitulates the cause of menopause. Moreover, serum gonadotropin levels are also significantly increased in \( Wv/Wv \) mice. Within 6 months, almost all of the female \( Wv/Wv \) mice developed benign ovarian tumors featuring pre-neoplastic morphological changes commonly found in the ovaries from peri- and post-menopause women (Murphy and Beamer, 1973; Yang et al., 2007). Therefore, \( Wv/Wv \) mice recapitulate the cause and biological features of menopause within a short period and represent an exaggerated model to study menopausal biology and its potential effects in the development of ovarian carcinomas. Using this strain, our group and others have revealed the potential
preventative effect of gonadotropin-suppressing and anti-inflammatory reagents against benign ovarian tumors (Blaakaer et al., 1995; Yang et al., 2007).

From the analyses of a large number of aged female $Wv/Wv$ mice accumulated through the years, we rarely found any malignant carcinomas with a high level of mitotic figures and metastatic features. This finding is consistent with the notion that either gonadotropin stimulation or menopausal biology alone is not sufficient to induce malignant EOC but rather provide a tumor prone environment for the transformation of ovarian surface epithelial cells.

The lack of ovarian carcinomas in $Wv/Wv$ mice hampered the further use of these mice as an EOC model; however, it is still plausible to propose that the introduction of additional oncogenic mutations could promote the transformation of OSE cells from $Wv/Wv$ mice and refined $Wv/Wv$ ovarian tumors into a more relevant EOC model.

5.3 Ovarian tumors in $Wv:p27$ double mutant mice

We first incorporated $p27$ deletion in the female $Wv/Wv$ mouse as pioneer experiments to test our hypothesis that the combination of menopausal physiology and oncogenic mutations is sufficient to induce ovarian carcinomas. As a CKI protein, p27 binds and inhibits the catalytic functions of CDK2/Cyclin E complex, thus prohibits the progression of the cell cycle (Sherr and Roberts, 1999). In ovarian cancer patients, a decreased p27 expression level is correlated with the poor prognosis and short overall survival rate (Chu et al., 2008). Our results suggested that morphology of $p27^{+/+}$ and $p27^{-/-}$ ovaries appeared to be normal in younger mice. However, increased incidences of focal hyperplasia appeared in the ovaries from aged $p27^{+/-}$ mice and more sophisticated epithelial structures were evident in $p27^{-/-}$ ovaries. Intra-ovarian cysts lined with cubical
epithelial lesion were frequently detected even in the ovaries from young p27-/− mice. More importantly, some of these epithelial cells took up a morphology similar to the müllerian duct derived epithelial cells. The increased expression level of müllerian epithelium marker claudin3 further supported these morphological observations. Since most of the low grade or differentiated human EOCs are morphologically comparable to different types of Müllerian derived epithelial cells (Dubeau, 2008; Shih Ie and Kurman, 2004), these findings reveal metaplastic transformation potentials associated with the loss of p27 protein. Despite these pre-neoplastic changes, no malignant features were observed in p27+/− or p27-/− ovaries, suggesting that p27 deficiency alone is not sufficient to induce EOC.

These in vivo data were also consistent with the results from the in vitro culture of purified p27+/− or p27-/− MOSE cells. Both of these cells had a slightly increased proliferation rate than the wildtype cells, yet they failed to either transform simultaneously or survive repeated passaging in vitro. Interestingly, I did not notice any apparent morphological difference between p27 +/− and p27-/− MOSE cells in vitro, suggesting that the complex epithelial structures found in p27-/− ovaries could also be attributed to the abnormal ovarian environment in p27-/− mice that caused by the lack of progesterone-producing corpus luteum structures (Fero et al., 1996; Kiyokawa et al., 1996).

When p27 deficiency was incorporated into female Wv/Wv mice, a statistically significant increases of epithelial lesions was detected in both Wv/Wv:p27+/− and Wv/Wv:p27-/− ovaries. This observation partially confirmed our hypothesis that the addition of oncogenic mutations will increase the malignancy of benign tubular adenomas in Wv/Wv
ovaries. However, the majority tumors detected in Wv:p27 double mutant mice were benign tubular adenomas with few mitotic figures or metastatic features. As revealed by H&E and CK8 staining, the morphology of the epithelial lesions in Wv/Wv:p27+/- ovaries were indistinguishable from that of Wv/Wv mice. Papillae structure commonly detected in human benign or borderline ovarian tumors (Vang et al., 2009) were observed in a few cases of Wv:Wv:p27+- ovaries. Both of these papillary structures were lined with a single layer of apparent normal OSE cells. On the other hand, the epithelial cells from Wv/Wv:p27-/- ovaries occasionally took up a more sophisticated structures that is similar as müllerian derived epithelial cells. However, the size of epithelial lesions in these mice appeared to be lower and exhibited a high variation when compared with Wv/Wv:p27+/- ovarian tumors. This phenotype could be attributed to several reasons: First, p27-/- mice are prone to the tumor formation in pituitary gland that is responsible for the secretion of gonadotropin (Fero et al., 1998; Kiyokawa et al., 1996). Therefore, the size variation in of Wv/Wv:P27-/- ovarian tumors could reflect the dynamic gonadotropin level in these mice. Secondly, few cancer cases were reported in p27-/- mice other than pituitary tumors, indicating that a compensatory tumor defense mechanism could be activated with the deletion of p27. Indeed, a elevated level of CKI family protein p21 was reported in p27 deficient hepatocytes and maintained quiescent status of these cells (Kwon et al., 2002). Induction of p21 was also detected in isolated p27-/- MOSE cells, suggesting similar tumor defense mechanisms might restrain the transformation potential of p27-/- MOSE cells. Thirdly, strong p27 expression was detected in the granulosa cells of the differentiating follicle and p27 deficiency dramatically accelerated the progression of granulosa tumors in Inhibin-α knockout mice (Cipriano et al., 2001). These data suggest
that $p27$ protein is essential for the regulation granulosa cell and follicle differentiation. The uncontrolled proliferation of remnant granulosa cells in $W_v/W_v: p27^{-/-}$ ovaries could also competitively prohibit the further expansion of epithelial lesions.

As the first attempt to refine $W_v/W_v$ ovarian tumor model, $p27$ deficiency in $W_v/W_v$ background yielded increased size of epithelial lesions. Moreover, homozygous deletion of $p27$ rendered some OSE cells a more complex müllerian epithelial morphology. These findings partially proved our hypothesis that menopausal physiology and oncogenic mutations have a synergic effect on the tumorigenesis of EOC. However, no carcinomas were observed in the ovaries of $W_v/p27$ double mutant mice. These findings suggest that $p27$ deficiency alone is not sufficient to stimulate the transformation of OSE cells, even under a menopause-like environment. In human EOC, loss of $p27$ was mainly found in the advanced stage of EOC and rarely detected in any specific histological subtypes of low grade EOC (Duncan et al., 2010; Masciullo et al., 2000). These data, together with our in vivo results, suggest that $p27$ deficiency facilitates the progression of advanced EOC and is dispensable or at least insufficient for the initiation of ovarian carcinomas under menopausal environment.

5.4 Ovarian tumor in $W_v:p53$ double mutant mice.

The amplified ovarian tumor phenotype in $W_v:p27$ double mutant ovaries reaffirmed us of the transformation potential of OSE cells derived from $W_v/W_v$ mice. Using the same strategy, I next aimed to establish mouse EOC models through the combination of $W_v/W_v$ and $p53$ deletion. Loss of function mutant of $p53$ could be found in approximately 50-70% of overall human EOC and is the most prevalent mutation of type II high grade serous carcinomas (Shih Ie and Kurman, 2004). Nevertheless, epithelial ovarian cancer is rarely
detected in $p53$-/- ovaries (Chen et al., 2004). The obvious discrepancy between the clinicopathological findings and the experimental animal data could be explained by the absence of peri- or post-menopause-like environment in $p53$ deficient mouse models. With the deletion of $p53$ in $Wv/Wv$ mice, it is plausible to predict that malignant lesions similar as high grade serous carcinoma will develop in the ovary of these double mutant mice. However, the feasibility of this experiment setting suffered from two major drawbacks: first, $p53$ is a potent tumor suppressor gene and $p53$-/- mice usually ceased within 6 months (Donehower et al., 1992). This early lethality may prevent further transformation of the neoplastic lesions in the ovaries of $Wv/p53$ double mutant mice. Secondly, $Wv/Wv$ mouse are infertile and the breeding of female $p53$-/- mice are known to be a challenge due to the early lethality caused by the overgrowth of neuronal tubes in mid-brain regions (Sah et al., 1995). Therefore, the yield of female $Wv:p53$ double mutant mice was extremely low.

The germ cells were unexpectedly detected in $Wv/Wv:p53$-/- ovaries. In some cases, granulosa cells were found to surround the germ cells and formed follicle like structures (data not shown). This finding is consistent with the previous report showing that the loss of $p53$ preserved the sperms and rescued the infertility of male $Wv/Wv$ mice (Jordan et al., 1999). Due to the preservation of germ cells, the epithelial lesions from $Wv:p53$ double mutant females were even milder than that of $Wv/Wv$ mice at the same age. Although it was not fully determined whether the rescue of tubular adenomas by $p53$ deletion was directly caused by the restoration of the normal hormonal environment, the preservation of germ cells and the low yield of female $Wv:p53$ double mutant mice hampered the further use of this model.
Cre-loxP based in vivo excision of $p53$ was then adopted through the intrabursal administration of Ad-Cre in the ovaries of $Wv/Wv:p53^{loxP/loxP}$ mice. This method was widely used to conditionally delete various genes in MOSE cells in vivo (Clark-Knowles et al., 2007; Clark-Knowles et al., 2009). Ovarian tumors were developed in Ad-Cre injected ovaries of female $Wv/Wv:p53^{loxP/loxP}$ mice after long latency (>14 months) without affecting the survival of the tumor bearing mice. A small fraction of Ad-Cre injected ovaries (2 out of 9) converted into polycystic and hemorrhagic cancerous tissues. However, further histological analyses in these tumors only yield a small percentage of epithelial lesions. A slightly higher level of ki-67 positive cells was observed in $p53$ deleted MOSE cell in vivo, while the majority of highly proliferative and pleiomorphic cells were found within the stromal cells surrounding epithelial lesions. The reason behind the lack of the ovarian carcinomas in $Wv:p53^{Δ2-10}$ ovaries remains unclear. The high infection rate of adenovirus was determined by lacZ staining from Ad-LacZ injected ovaries. The efficacy of Cre mediated $p53$ deletion was confirmed by the PCR amplification of the excised $p53$ fragment ($p53^{Δ2-10}$). These results rule out the potential technological artifacts from the delivery of Ad-Cre in vivo. The unexpected hyper-proliferation rate and tumorigenesis potential in the stromal regions of $Wv:p53^{Δ2-10}$ ovaries were likely caused by the accidentally leaking of Ad-Cre into the intra-ovarian regions during the procedure of intrabursal injection. This observation raises the possibility that different ovarian cell types might have distinct susceptibility for $p53$ deficiency induced transformations. Indeed, rapid proliferating cells were more frequently observed in the stromal regions of $p53^{-/-}$ ovary, while rarely detected in the ovarian epithelium. This finding is also consistent with several other
reports showing the spontaneous development of angiosarcoma or leiomyosarcoma in p53-/-
or p53:Brca1 conditional knockout ovaries (Chen et al., 2004; Clark-Knowles et al.,
2009; Donehower et al., 1992; Quinn et al., 2009). On the other hand, the most
impressive serous ovarian carcinomas developed after the concurrent deletion of p53 and
pRb in OSE cells (Connolly et al., 2003; Flesken-Nikitin et al., 2003), suggesting that
additional oncogenic mutations are involved in the formation of high grade serous EOC
other than p53 deletion.

The lack of EOC formation in Wv:p53Δ2-10 ovaries could also be explained by a relatively
new concept that argues human EOCs, especially high grade serous carcinomas may
actually arise from the epithelial cells of the distal end of the fallopian tube (tubal
fimbriae). For years, the high similarity between EOC and müllerian derived epithelium
has fostered the speculation that EOC have alternative precursors rather than the ovarian
surface epithelial cells. Through the analyses of ovarian samples from prophylactic
oophorectomies, several groups consistently found overall strong and stabilized p53
protein levels at the site of distal fallopian tubes mucosa while absent in the ovarian
surface epithelium (Folkins et al., 2008; Lee et al., 2007; Semmel et al., 2009). Since p53
mutation is the prevalent mutation of serous ovarian carcinomas, this phenomenon,
termied as ‘p53 signature’ has underlined the possibility that fimbriae of the fallopian tube
could be another plausible candidate as the origin of high grade EOC (Levanon et al.,
2008). One of the pioneer studies to support this concept was carried out recently
through the modeling of the transformation potential in human fallopian tube secretory
epithelial cells (FTSEC). In this study, primary FTSECs were first transformed in vitro
with the addition of a panel of various oncogenes including T-antigen, C-MYC, RAS, or
mutant CDK4 and then inoculated in the nude mice. Strikingly, the xenograft of transformed FTESCs gave rise to serous carcinomas with a highly similar histological and genomic profile as human EOC (Karst et al., 2011). Based on these data and the common müllerian-like morphology of human EOC cells, it is intriguing to propose that ovarian carcinomas may have alternative origins other than OSE cells. The lack of EOC in \( p53 \) deficient ovaries could also be explained by the idea that ovarian carcinomas are actually derived from müllerian epithelium adjacent to the ovary rather than the ovarian surface epithelium.

5.5 Metaplastic potential of \( p27^-/- \) and \( p53^-/- \) MOSE cells

Emerging evidence suggests that ovarian surface epithelium cells favored a mesenchymal rather than epithelial status, probably due to the periodical wound and healing process during menstrual cycle (Auersperg et al., 1999; Auersperg et al., 2001; Liu et al., 2004). Cell adhesion molecules of advanced and complex epithelial structure (i.e. E-Cadherin, claudin3, Ep-Cam) are less frequently detected in normal OSE cells both in vivo and in vitro (Auersperg et al., 2001; Wong et al., 1999). Consistent with this notion, stabilized level of mesenchymal transition marker N-Cadherin (the common form of cadherin in mesodermal, neural cells) was uniformly found at the surface of MOSE cells under monolayer culture condition. The expression level of E-cadherin and claudin3 was relatively weak in wildtype MOSE cells as indicated by western blot. Immunofluorescent results also displayed a patched staining pattern of claudin3 on the surface of wildtype MOSE cells cultures. Interestingly, the expression levels of E-cadherin and Claudin3 protein were significantly increased in \( p27^-/- \) and \( p53^-/- \) MOSE cells as revealed by western blot and IF. These results suggested that \( p53 \) or \( p27 \) deficiency could lead to the
‘epithelial switch’ in MOSE cells. In humans, epithelial markers such as E-cadherin and claudins3 are usually absent in the normal OSE cells, while ubiquitously expressed in the epithelium of female reproductive ducts. The expression of these proteins also significantly increased in benign and borderline ovarian tumors (Sundfeldt et al., 1997). Our results from the primary cultures of p27-/- and p53-/- MOSE cells support the idea that increased expression of epithelial marker in OSE cells could be attributed to the intrinsic oncogenic mutations. The complex epithelial structures found in Wv/Wv:p27-/- and Wv:p53Δ2-10 ovaries further confirmed the metaplastic potential of these mutant OSE cells in vivo. Interestingly, Wv/Wv MOSE cells readily displayed epithelial features as indicated by elevated E-cadherin and claudin3 expression levels. This raised the possibility that the constant stimulation of ovulation-like process could also lead to the metaplastic changes in OSE cells. At this point, it is not fully understood whether these metaplastic changes directly contribute to the transformation of OSE cells during the early stage of EOC. Over-expression of E-cadherin and caudin-3 reversed the mesenchymal-like morphology of human OSE in vitro (Agarwal et al., 2005; Auersperg et al., 1999). However, the proliferation index was minimally affected in these epithelialized human OSE cells (Agarwal et al., 2005; Auersperg et al., 1999). Pilot experiments that specifically knock down the expression of these epithelial proteins in pre-neoplastic Wv/Wv, p27-/-, and p53-/- MOSE cells will further elucidate the role of metaplasia during early tumorigenesis stage of EOC.

5.6 Compensation of CKI proteins associated with p27 and p53 deletion

Wv/Wv mice have a naturally occurring mutation in c-kit kinase, which is also an oncogene that partially regulates the P-Erk and P-Akt signals through cross-talks with Src
or Ras (McLaughlin and McIver, 2009) pathways. I first hypothesized that the lack of ovarian carcinomas in \(Wv/Wv\) mice could be attributed to the defective cell proliferation caused by the intrinsic c-kit mutation. However, the results from in vitro cell proliferation assay results suggested that \(Wv/Wv\) MOSE cells had an even higher in vitro propagation rate than the wild type MOSE cells despite that the growth advantage of \(Wv/Wv\) MOSE cells quickly lost upon repeated passaging in vitro. Nevertheless, these data excluded the potential interference of c-kit mutation against tumor formation.

Since p27 belong to the family of cyclin depend kinase inhibitor proteins, I next hypothesized that the spontaneous compensation of other CKI family members might prevent the transformation of \(p27^{-/-}\) MOSE cells. Indeed, the cooperation of CKI proteins was found to prohibit cell cycle in a broad spectrum of tissues: p27 and \(p19^{ink4d}\) functioned together to cause the exit of cell cycle in postnatal neuronal cells (Zindy et al., 1999), whereas p21 and p57 cooperatively prevented the cellular division and further differentiation of skeletal muscle and lung alveoli (Zhang et al., 1999). Unlike the more potent tumor suppressor gene \(p53\), the activities of CKI proteins usually demonstrate certain level of functional redundancy. As listed above, the abnormal cell cycle exit are usually achieved through the disruption of multiple CKI proteins, whereas mice deficient of a single CDK inhibitor gene usually have a low level of tumor occurrences (Deng et al., 1995; Franklin et al., 1998; Franklin et al., 2000; Sharpless et al., 2001; Zhang et al., 1997).

Applying this notion in our scenario, it is plausible to speculate that the lack of EOC in our ovarian tumor models results from the functional overlapping of CKI in MOSE cells. Indeed, \(p27^{-/-}\) MOSE cells had a significant induction of another important CKI family
protein p21$^{\text{cip1}}$. This result is consistently with the previous report that p21 expression level was elevated in multiple tissues from p27$^{-/-}$ mouse (Kwon et al., 2002). Moreover, increased expression level of another two CKI family proteins, p16 and p19, were simultaneously observed in p27$^{-/-}$ MOSE cells. The accumulation of p21, p16 and p19 proteins may presumably take up the role of p27 in p27$^{-/-}$ MOSE cells and prevent further tumorigenic events.

Mutation of p53 was detected in a panel of human cancers including the most common type of EOC-serous ovarian carcinomas (Attardi and Jacks, 1999; Aunoble et al., 2000). However, p53$^{-/-}$ ovaries rarely developed any epithelial ovarian cancer. A similar CKI compensatory mechanism may also underlie the low frequency of tumorigenesis in p53$^{-/-}$ MOSE cells. Interestingly, we found significant accumulations of both p16$^{\text{INK4a}}$ and p19$^{\text{ARF}}$ proteins from the INK4a-ARF locus. P16$^{\text{INK4a}}$ belongs to the CKI family and played a central role in initiating cellular senescence program against hyper-proliferation stimuli (Sharpless et al., 2001; Sherr, 2001). In the current paradigm, P16$^{\text{INK4a}}$ binds with CDK4 or CDK6 and blocks the catalytic activities of Cyclin D-CDK4/6 complex and the phosphorylation of pRb (Gil and Peters, 2006). Since the phosphorylation of pRb and subsequent release E2f-1 is essential for the G1-S transition during the cell cycle, the activity of p16 induced a pRb dependent cell cycle arrest. Different from p19, p16 is considered to be a p53 independent tumor suppressor gene and the genetic loss of p16 is evident in a panel of human cancers (Caldas et al., 1994). In the case of human EOC, however, the complete depletion of p16 locus is rare (Marchini et al., 1997) while the expression of p16 is commonly reduced or suppressed, accounting for approximately 30-40% among all ovarian tumors (Havrilesky et al., 2001; McCluskey et al., 1999; Sui et al.,
In contrast to p27, p16 exhibits a histological subtype dependent expression pattern in human EOC. Interestingly, over-expression of p16 was preferably found in high grade serous ovarian carcinomas compared to other EOC subtypes (Milde-Langosch et al., 2003). Since serous carcinomas are characterized by the frequent loss-of-function mutation of p53, this observation is consistent with our findings from the primary culture of p53-/- MOSE cells.

The activation of p16 plays a central role in the induction of cellular senescence program against various mitogenic stimuli (Serrano et al., 1997). However, over-expression of p16 does not necessarily promote the cellular senescence as malfunctioned pRb protein will bypass the tumor suppression effect of p16. This is especially true in ovarian carcinoma cell lines, as the ectopic expression of p16 only induced growth arrest in cancer cells with intact pRb functions (Todd et al., 2000). Here, the result of SA-β-gal assay in wildtype and Wv/p53Δ2-10 MOSE cells clearly induced an elevated level of cellular senescence as a result of p16 induction. These suggestive results potentially attribute the lack of EOC in p53-/- ovaries to the compensatory stimulation of p16 and subsequent cellular senescence. Interestingly, serous ovarian carcinomas could be induced through the concurrently inhibition of both p53 and Rb pathways (Flesken-Nikitin et al., 2003), indicating that the barrier of p16 mediated cell senescence must be overridden before the formation of ovarian carcinomas.

The induction of p19Arf protein could also be simultaneously observed with the deletion of p53, probably due to an epigenetic mechanism as shown in p53-/- mouse embryonic fibroblast (MEF) cells. The main anti-proliferative function of p19 is achieved through antagonizing p53 inhibitor protein Mdm2 and subsequent activation of p53. Since p53 is
readily deleted in the experimental MOSE cells, I did not any expect any inhibitory
effects of p19 against the transformation of p53-/- MOSE cells. However, the unexpected
induction of p19 in p27-/- MOSE cells might account for the complementary induction of
p53-p21 signals in these cells.

5.7 Tracing the origin of OSE cells

Despite its wide acceptance, the ‘surface epithelium origin’ hypothesis is constantly
challenged by the müllerian like morphologies of EOC cells. Recent reports suggested
that genetically manipulated MOSE cells could develop into müllerian ovarian tumors in
vivo (Dinulescu et al., 2005; Wu et al., 2007), which strongly supported the idea that
müllerian like appearance of EOC results from the metaplasia of OSE cells. However, it
should be noted that most of these mouse ovarian tumor models are genetically
manipulated and may not reflect the spontaneously developed EOC in humans.
Meanwhile, no satisfactory biological evidence is available to support the ‘müllerian
derived’ hypothesis due to the limited knowledge of the biology of müllerian derived
epithelium and the remnant müllerian derived tissues (secondary müllerian system)
surrounding the ovary.

In \(Wv/Wv\) mice, prominent epithelial lesions arise from deep medulla and rete ovarii
portions of the ovary that have no apparent connections with the surface epithelium.
These observations suggest that ovarian epithelia tumor may have different origins other
than the surface epithelium, which partially supports the ‘müllerian derived’ hypothesis.
MISR II-Cre strain was used to trace the origin of mouse surface epithelium, since MISR
II was originally reported to be exclusively expressed in the female reproductive ducts
while absent in ovarian surface epithelial cells (Arango et al., 2008; Jamin et al., 2002;
Jorgez et al., 2004; Kobayashi and Behringer, 2003). However, more detailed studies suggested that müllerian derived epithelium was negatively traced using this approach (Fan et al., 2009), which sabotages the usefulness of the tracing experiments based on this methodology. MISR II expression could be detected in the cancer cells from ascites of EOC patients (Masiakos et al., 1999). Several ovarian cancer cell line derived from human patients and mouse ovarian cancer models displayed detectable level of MISR II expression and were sensitivity to the recombinant müllerian inhibitory substance (MIS) (Pieretti-Vanmarcke et al., 2006; Szotek et al., 2006). These reports clearly suggested that at least part of EOC could derive from a MISR II positive lineage.

The in vivo cellular tracing experiment using MISR II-Cre strain consistently revealed that mouse OSE cells have a mosaic composition of at least two distinct subpopulations. The use of MISR II-lacZ reporter strain further validated these tracing results. The mosaic composition of MOSE cells explains the inconsistent result from previous literature using the same strain. Since müllerian derived epithelial cells were not traced in MISR II-Cre based system, the presence of the positively traced epithelial lesions in Wv/Wv mouse partially supports the ‘surface epithelium origin’ hypothesis. The expression of MISR II is common in human and mouse ovarian cancer cell lines. Thus our result also raise the possibility that MISR II positively traced OSE cells might represent the subpopulation of epithelial cells prone to the development of ovarian cancer. Nevertheless, before jumping to any conclusions, the successful generation of EOC mouse models based on MISR II-Cre strain is prerequisite for the validation of these preliminary claims and will ultimately shed light on the origin of EOC cells.
5.8 Concluding Remarks

Emerging epidemiology evidence has linked the high occurrence of epithelial ovarian cancer with menopausal physiology. However, direct biological evidence supporting the causative role of menopausal physiology during EOC formation is still missing. In this thesis, I aimed to develop mouse epithelial ovarian cancer models focusing on the etiological factor of this disease. \textit{Wv} mouse has a natural occurring \textit{c-kit} mutation that resulted in a premature deletion of germ cells. In female \textit{Wv/Wv} mice, oocytes and follicle structures were completely depleted within 2 months, accompanied with an elevated gonadotropin level and the formation of benign ovarian tubular adenoma. Thus, female \textit{Wv/Wv} mice may be used as an exaggerated model of menopause. I had refined \textit{Wv/Wv} mice into a more relevant EOC models by the deletion of mutation of tumor suppressor genes \textit{p27kip1} or \textit{p53}. Both \textit{Wv/Wv:p27+/-} and \textit{p27 -/-} mice develop epithelial ovarian tumor consists of papillary structures lined by hyperchromatic neoplastic cells. CK8 staining confirmed the epithelial identities of these tumors. However, neither mitotic figures nor metastatic lesions were detected in \textit{Wv/p27} double mutant ovarian tumors. In vitro primary cultures of MOSE cells from wildtype, \textit{p27+/-} and \textit{p27 -/-} mice confirmed the growth advantage and metaplastic changes upon \textit{p27} deletion. However, neither \textit{p27 +/-} or \textit{-/-} MOSE cells were transformed in vitro, probably due to the compensatory increase of CKI proteins p21, p16 and p19. When \textit{p53} was unilaterally deleted in the ovarian surface epithelial cells of \textit{Wv/Wv:p53^{loxp/loxP}} mice, ovarian tumors developed after long latency. Both the size and the epithelial lesions in these tumors were significantly increased compared to the uninfected ovary from the same mouse. However, the main lesions found in \textit{Wv:P53^{A2-10}} tumors were negatively
stained for epithelial markers, which hampered the further use of these mice as a model of ovarian carcinoma. In vitro deletion of \( p53 \) significantly increased the proliferation rate and passage numbers of both wildtype and \( Wv/Wv \) MOSE cells. However, a compensatory increase of tumor suppressor genes p16 or p19 was accompanied with \( p53 \) deletion in both cells. As a result, high senescence level was evident in \( p53 \) depleted MOSE cells. These in vitro and in vivo data suggest that \( p53 \) deletion alone is not sufficient to induce malignant ovarian cancer even under a menopause-like environment.

Taken together, \( p27 \) or \( p53 \) deletion significantly increased the level of epithelial lesions in the otherwise benign tubular adenomas of \( Wv/Wv \) mouse. However, either \( p27 \) or \( p53 \) deletion alone is not sufficient to induce transformation of MOSE cells, probably due to the compensatory up-regulation of CKI family proteins such as p21, p16 and p19. To generate a more relevant and malignant \( Wv/Wv \) EOC model, additional oncogenic mutations are required.
Chapter VI: Material and Methods

Experimental Animals

All inbreeding colonies of the Wv ovarian tumor models were established by crossing Wv/+ mice with p27+/-, p53+/-, p53\textsuperscript{loxp/loxp}, MISRII-Cre and MISRII-lacZ mice. The double transgenic mice were inbred for at least five generations with the wildtype C57BL/6J strain. Wv strain was obtained from Jackson Laboratory (Bar Harbor, ME).

The genotypes of the Wv progeny litters were determined by the coat color: white, black or agouti with random distributed white spots, and pure black/agouti represent \textit{Wv/Wv}, \textit{Wv/+}, or wildtype respectively. The \textit{p27+/-} mouse was a kind gift from Dr. Andrew Koff (Kiyokawa et al., 1996). The following primer sets were used for PCR genotype to amplify the wildtype and targeted mutant \textit{p27} allele 5'-AGGTGAGAGTGTCTAACGG-3'; 5'-AGTGCTTCTCCAAGTCCC-3'; 5'-GCGAGGATCTCGTCTCGTGAC-3'. All three primers were used simultaneously in the PCR reaction, yielding a 130 bp wildtype and/or 450 bp \textit{p27} mutant band. The \textit{p53+/-} mice were purchased from Taconic (Hudson, NY).

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The following primer sets were used for PCR genotype to amplify the wildtype and \textit{loxp} flanked intron 1 of \textit{p53} allele: Forward primer: 5'-CACAAAAAC AGGTAAACCCAG-3' and Reverse primer: 5'-AGCACATAGGAGGCAGAGAC-3', yielding a 288 bp band for wildtype, and a 370 bp band for \textit{loxp} flanked intron 1 of \textit{p53},
respectively. Another primer sets were also used to amplify the wildtype and \textit{loxP} flanked intron10 of the $p53$ allele: Forward primer: 5$^\prime$-AAGGGGTATGAGGGA CAAGG-3$^\prime$ and Reverse primer 5$^\prime$-GAAGACAGAAAAGGGGAGGGG -3$^\prime$, yielding a wildtype band at 431 bp and a \textit{loxP} flanked intron10 band at 584 bp. To confirm Cre-mediated excision of exon 2-10 of $p53$ allele, the forward primer of intron 1 and reverse primer of intron 10 were used in the PCR reaction, yielding a 612 bp band for $p53^{\Delta 2-10}$ allele. \textit{MISRII-Cre} and \textit{MISRII-lacZ} strains were kind gifts from Dr. Richard Behringer (Arango et al., 2008; Jamin et al., 2003). Generic Cre and LacZ primer sets were used to determine the genotype of these mice.

**Primary culture of MOSE cells**

Primary culture of MOSE cells was generated using a slightly modified protocol as described in previous literatures (Clark-Knowles et al., 2009; Flesken-Nikitin et al., 2003). Ovaries from adult wildtype, $p27^{-/-}$, $p53^{-/-}$ and $p53^{\text{loxP/loxP}}$ mice were carefully dissected using sterilized surgical instruments. These ovaries were then washed with PBS and incubated in 0.25% Trypsin solutions (Invitrogen) at 37°C for one hour.

Disassociated MOSE cells were then pelleted and cultured on 6 well plates previously coated with 0.1% gelatin. MOSE cells were maintained as monolayers in high glucose DMEM/F12 medium supplemented with 10% fetal bovine serum, 1 mM glutamine, epidermal growth factor (10 ng/ml), 500 ng/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin. MOSE cells from Wv/Wv ovaries were isolated by the incubation with DMEM/F12 medium containing 0.5% collagenase IV (Invitrogen) for 2 hours at 37 °C and cultured under the same conditions as wildtype MOSE cells.
Cell growth assay

The growth of MOSE cells was analyzed using the MTT based Wst-1 kit from Roche. Briefly, 2 x 10⁴ MOSE cells of various genotypes were sub-cultured on 96-well plates previously coated with 0.1% gelatin. These cells were allowed to grow from 24 to 96 hours. At each 24 hour interval, the cell culture medium was substituted with freshly prepared reaction buffer composed of DMEM/F12 media containing 10% of Wst-1 solution. These wells were then incubated at 37°C to allow the accumulation of the soluble formazan dyes produced by the viable cells. The reaction solution was aspirated after one hour and the levels of cell growth were estimated by the amount of soluble formazan dye measured at an absorbance of 450 nm using a standard multi-well spectrophotometer.

In vivo X-gal staining

Adult MISRII-Cre:Rosa26 or MISRII-lacZ mice sacrificed by neck snapping were immediately perfused with 4% paraformaldehyde in PBS. Ovarian tissues including the attached uterine horns were dissected and washed in PBS. Freshly prepared PBS containing 0.25% glutaraldehyde was used to fix these tissues overnight at 4°C. Fixed ovarian tissues were then incubated in 30% sucrose solution for 6 hours and subjected to cryosectioning. Freshly cut ovarian slides were allowed to dry at room temperature for at least ten minutes and then incubated with X-gal staining solution (5 mM potassium ferricyanide crystalline, 5 mM potassium ferricyanide trihydrate, 2 mM magnesium chloride, 0.01% sodium deoxycholate, 0.02% NP-40 in PBS) containing 1 mg/ml X-gal at 37 °C or room temperature. The reaction was allowed to proceed for a minimum of 12 hours. After X-gal staining, the slides were rinsed three times in PBS, counter-stained
with nuclear fast red, dehydrated through increased gradient of ethanol and xylene, and permanently mounted in Permount before microscopic examination.

**SA-β-gal staining**

Primary culture of MOSE cells in 6-well plates were quickly washed with cold PBS two times and fixed with PBS containing 0.25% glutaldehyde for 5 minutes at room temperature. Excess fixatives were then washed away by PBS. SA-β-gal staining working solution (5 mM potassium ferricyanide crystalline, 5 mM potassium ferricyanide trihydrate, 2 mM magnesium chloride in PBS, pH adjusted to 6.0) containing 1mg/ml of X-gal were freshly prepared each time. Fixed MOSE cells were incubated with SA-β-gal staining working solution overnight at 37 °C. X-gal staining results were checked under conventional microscope. The stained cells were overlaid with 70% glycerol and kept at -20°C for long term storage.

**Western blot and antibodies**

Live MOSE cells were washed twice with cold PBS and immediately collected in RIPA buffer (20 mM Tris pH 7.5, 50 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate). The protein samples were then lysed in SDS gel loading buffer and boiled for 10 minutes. The proteins were separated on 8 or 10 % SDS polyacrylamide gels depending on the size of the target protein. Immunoblotting was performed according to standard procedures. Primary antibody used in the western blotting includes: polyclonal anti-p27, p16, p19, CDK2, CDK4 and cyclin A antibodies, monoclonal anti-PCNA, Cyclin E2 antibodies from Santa Cruz; monoclonal anti-p21, E-cadherin, N-cadherin antibodies from BD Bioscience; monoclonal anti-β-actin antibodies from Sigma and polyclonal anti-claudin3 antibodies from Invitrogen.
**Immunofluorescence Microscopy**

MOSE cell of various genotypes were seeded onto gelatin-coated coverslips at $2 \times 10^5$ cells per well in 12-well plates. For immunofluorescence staining, the cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes, permeabilized with 0.5% triton X-100 for 5 minutes and blocked with 5% bovine serum albumin (BSA) in PBS for 30 minutes. The primary antibodies were diluted in 3% BSA and incubated with the slides for 1 hour at room temperature. The antibodies and working dilutions were: anti-CK8 (Developmental Studies Hybridoma Bank, Ames, IA) at 1:500; anti-E-cadherin or N-cadherin (BD Bioscience) at 1:200; anti-claudin3 (Invitrogen) at 1:200; anti-p16 and anti-p19 at 1:400 (Santa Cruz). The cover slips were then washed three times with PBS and incubated with either Alexa Fluor 488- or 596-conjugated secondary antibodies (Molecular Probes) diluted in 3% BSA for 1 hour. The unbound secondary antibodies were washed 4 times with PBS, and the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes. The coverslips were mounted in anti-fade Prolong Gold (Invitrogen) according to the manufacturer's directions. Immunofluorescence staining was viewed with 20X (dry) or 63X (oil) objective lens on a Zeiss Axio Observer Z1 microscope linked to Zeiss AxioCam MR R3 camera. Images were acquired and processed using AxioVision software.

**Immunohistochemistry**

The entire ovaries well as the attached uterine horns from $W_v$ ovarian tumor models were dissected, fixed in 10% formalin, paraffin-embedded, and sectioned into 6 μm thick slices collected on the glass slides. The sections were dewaxed in xylene and rehydrated through decreased gradient of ethanol. Antigen retrieval was performed by boiling the
sections submerged in antigen retrieval buffer (Dako) in a plastic slides holder for 20 minutes. The endogenous peroxidase activity was quenched by the incubation with 3% H₂O₂ for 15 minutes. The slides were then blocked with 5% BSA at room temperature for 30 minutes, and subsequently incubated with the following antibodies: monoclonal CK8 antibodies at 1:600, anti-α-SMA antibodies (Invitrogen) at 1:50, and anti-Ki-67 antibodies (Dako) at 1:100, in 5% BSA overnight at 4°C. On the next day, the slides were washed and incubated with appropriate secondary antibodies conjugated with horseradish peroxidase polymers for 1 hour at room temperature. For CK8 and Ki-67 staining, polyclonal rabbit anti-rat “bridging” antibodies (DAKO) at 1:500 dilutions were used before HRP conjugated antibodies. Diaminobenzidine (DAB) substrate (Vector laboratories) was used as chromogens for the immunoperoxidase reaction. The developed slides were then counterstained with hematoxylin, dehydrated through the increased gradient of ethanol and xylene and permanently mounted in Permount before microscopic examination.
Appendix: Ectopic expression of GATA6 bypasses requirement for Grb2 in primitive endoderm formation

The following work studying Grb2-SOS-Erk signals during early embryogenesis was carried out during the Ph.D. period as a side project parallel to the main body of the thesis that focused on mouse ovarian tumors models. It is written as an appendix because of the completely independent nature.

Overview:
Gene knockouts in mice have shown that Grb2 and GATA6 are essential for the formation of primitive endoderm in blastocysts. Here, we found that implanted Grb2-null blastocysts lack primitive or extraembryonic endoderm cells either at E4.5 or E5.5 stages. We analyzed the relationship between Grb2 and GATA6 in the differentiation of embryonic stem (ES) cells to primitive endoderm in embryoid body models. Upon transfection with a GATA6 expression vector, Grb2-null ES cells underwent endoderm differentiation as indicated by the expression of the extraembryonic endoderm markers Dab2 and GATA4. When GATA6-transfected Grb2-null ES cells were allowed to aggregate, fragments of an endoderm layer formed on the surface of the spheroids. The results suggest that GATA6 is downstream of Grb2 in the inductive signaling pathway and the expression of GATA6 is sufficient to compensate for the defects caused by Grb2 deficiency in the development of the primitive and extraembryonic endoderm.
**Background and Introduction**

The primitive endoderm is one of the earliest cell lineages arising from the inner cell mass in early embryogenesis. Recent studies indicate that primitive endoderm cells originate within the interior of the pluripotent cells of the inner cell mass, and the differentiated cells sort and position on the surface to form an epithelial layer covering the inner cell mass that is referred to as the epiblast in the later stage of the blastocysts (Chazaud et al., 2006; Rula et al., 2007; Plusa et al., 2008; Meihac et al., 2009). Dab2 is required for sorting of the newly derived primitive endoderm cells to the surface to form an epithelial layer (Yang et al., 2002; 2007). The sorting and surface positioning of the primitive endoderm cells are thought to be determined by the ability of the primitive endoderm cells to generate apical polarity, rather than differential adhesive affinity (Yang et al., 2007; Gerbe et al., 2008; Moore et al., 2009).

Oct3/4 and Nanog are markers of pluripotency for the cells of the inner cell mass and epiblast (Pesce and Scholer, 2001; Cavaleri and Scholer, 2003; Mitsui et al., 2003; Chambers et al., 2007). Expression of Oct3/4 and Nanog is lost and several other genes are induced upon differentiation, and laminin, GATA4, GATA6, and Dab2 are common markers for the primitive endoderm lineage (Rossant and Tam, 2004; Cai et al., 2008). Primitive endoderm forms at the time of blastocyst implantation (Gardner, 1982). Parietal endoderm cells are subsequently derived from the primitive endoderm cells and migrate out to cover the surface of the blastocoels (Gardner, 1989). The parietal endoderm cells retain the expression of GATA4 and GATA6, and actively produce extracellular matrix components such as laminin to form a thick basement membrane, the Reichert’s membrane (Cai et al., 2008). The remaining primitive endoderm cells
covering the epiblast mature into visceral endoderm cells, which retain GATA4 expression but lose GATA6 expression (Cai et al., 2008).

Characterization of mutant embryos from genetic knockout mice identified GATA6 as an essential gene for the development of extraembryonic endoderm (Koutsurakis et al., 1999; Morrisey et al., 1998), and GATA6 is required at the step of commitment to primitive endoderm fate (Cai et al., 2008). GATA family proteins consist of six transcription factors that bind “A/G GATA A/G” core sequence and regulate the development of various cell lineages and organs (Patient and McGhee, 2002). Among them, GATA4, in addition to GATA6, plays a role in the differentiation of primitive endoderm in embryoid bodies (Soudais et al., 1985). Unlike wildtype cells, GATA4-null embryonic stem (ES) cells do not spontaneously differentiate and form an endoderm layer upon aggregation as embryoid bodies (Soudais et al., 1985). However, the requirement of GATA4 is bypassed by addition of retinoic acid (Bielinska and Wilson, 1997; Capo-chichi et al., 2005). In contrast, GATA6 is essential for primitive endoderm differentiation both in vivo and in vitro (Cai et al., 2008).

Another essential gene for primitive endoderm development is Grb2 (Cheng et al., 1998; Chazaud et al., 2006). This conclusion was reached in the studies of Grb2 null blastocysts in vitro, however the phenotype of Grb2-null blastocysts implanted inside uterine has not been reported (Cheng et al., 1998; Chazaud et al., 2006). Grb2, an adaptor protein that links receptor tyrosine kinase (RTK) to Sos, is a crucial component of the RTK-Grb2-Sos-Ras-MEK-Erk signaling cascades. Several studies support a pivotal role of this Ras/MAPK pathway in the differentiation of primitive endoderm (Cheng et al., 1998; Chazaud et al., 2006; Yamanaka et al., 2010; Nichols et al., 2009).
Targeted disruption of fibroblast growth factor receptor 2 or its ligand fgf4, which activate the Ras/MAPK signal pathway in the cells of the inner cell mass, also abrogates primitive endoderm differentiation (Feldman et al., 1995; Arman et al., 1998). Suppression of Ras/MAPK pathway preserves pluripotency of the inner cell mass (Nichols et al., 2009). Furthermore, a constitutive active mutant of either Ras or MEK is sufficient to promote primitive endoderm differentiation of murine ES cells in culture (Verheijen et al., 1999). Activation of Ras/MAPK pathway is believed to suppress Nanog expression (Hamazaki et al., 2006), which subsequently releases its repression of GATA6 expression, since putative Nanog binding motif has been predicted in the promoter region of GATA6 (Hamazaki et al., 2006). Thus, it is thought that the differentiation of primitive endoderm is initiated through the repression of Nanog expression, which is expressed with GATA6 in a “salt and pepper” pattern in the inner cell mass of blastocysts as early as 3.5 days post coitum (Chazaud et al., 2006).

Additional crucial genes affecting primitive endoderm development are Nanog and Oct-3/4. These are transcription factors required for the maintenance of pluripotency of the epiblasts and their expression is expected to subside upon differentiation (Nichols et al., 1998; Cavalieri and Scholer, 2003; Chambers et al., 2007). In implanting blastocyst, the expression of Nanog is mutually exclusive with GATA4 and GATA6-positive cells that are markers for primitive endoderm (Chazaud et al., 2006). In vitro, Nanog deficient ES cells take up parietal endoderm fate (Mitsui et al., 2003; Chambers et al., 2003). In contrast, Oct3/4 is present initially in the primitive endoderm cells at the early stage of blastocyst development, and gradually subsides upon further differentiation (Nichols et al., 1998; Niwa et al., 2000; Pesce and Schöler, 2001). Nevertheless, suppression of
Oct3/4 in ES cells in culture leads to a trophectoderm rather than primitive endoderm fate (Hough et al., 2006).

In this study, we further compared the mutant phenotypes of Grb2 and GATA6 knockout embryos, and analyzed the relationship between Grb2 and GATA6 genes in ES cell differentiation and primitive endoderm development.
Material and Methods

Mutant Mice and Embryos

Two mating pairs of Grb2 (+/-) mice (129 Grb2<tm1Paw>) (Cheng et al., 1998) were given by the Toronto Centre for Phenogenomics (Toronto, Ontario) with agreement by Dr. Anthony Pawson (Samuel Lunenfeld Research Institute, Toronto, Canada) to establish a breeding colony at the University of Miami. The Grb2 (+/-) mice were used for timed matings to obtain embryos of the desired age. For the analysis of E4.5 and E5.5 embryos, the entire uterine horns were harvested for histology. The blocks were sectioned and one in every 5 adjacent slides were examined by H&E staining to identify the implanted embryos.

Since the early embryonic lethality of Grb2-null pre- and post- implanted blastocysts has been well established (Cheng et al., 1998; Chazaud et al., 2006), we used the abnormal morphology and the lack of staining for the primitive endoderm markers Dab2 and GATA4 to distinguish Grb2-null embryos. Littermates containing an endoderm layer that are positive for Dab2 and GATA4 staining were identified as wildtype or Grb2 (+/-). GATA6-null embryos were used for comparison (Cai et al., 2008).

Culture and Maintenance of Mouse ES Cells and Embryoid Bodies

RW4 (Capo-chichi et al., 2005) and Grb2 (-/-) (Cheng et al., 1998) mouse embryonic stem (ES) cells were used in experiments. A frozen vial of the original Grb2 (-/-) ES cells was given by Dr. Pawson (Samuel Lunenfeld Research Institute, Toronto, Canada). These mouse ES cells were maintained on a layer of irradiated mouse embryonic fibroblasts in ES medium consisting of DMEM with 15% FBS, 1 mM glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, and 1,000 U/ml leukemia
inhibitory factor (LIF). Three days prior to the experiment, the ES cells were trypsinized and seeded in ES culture medium containing LIF on plates coated with 0.1% of gelatin. All-trans-retinoic acid (RA) dissolved in DMSO was added 24 hours after plating at a final concentration of 1 μM to induce differentiation. An equal volume of DMSO vehicle was added to the control cells.

To form embryoid bodies, dispersed ES cells were allowed to aggregate in ES culture medium without LIF in non-adhesive poly-hema (Sigma) coated petri dishes. In some cases, RA was added 24 hours after the initiation of ES cell aggregation. After 4-7 days in suspension culture, the ES cell aggregates/spheroids were collected by brief centrifugation and then were used for analysis.

Cell Transfection

Wildtype mouse ES cells RW-4 and Grb2 (-/-) cells were seeded at 2.5X10⁵ on gelatin coated 6-well plates. Plasmid DNA was purified using Qiagen Maxiprep columns. GATA4 and GATA6 expression constructs were described previously (Capo-chichi et al., 2005). Lipofectamine LTX (Invitrogen) reagent was used for transfection according to the manufacturer’s protocols. Empty vector or H2BGFP expression plasmid was used as control and as an indicator of transfection efficiency. ES cells were collected for analysis by Western blot or immunofluorescence microscopy 72 hours after transfection.

Western Blot Analysis and Antibodies

Following treatment, ES cells were washed twice with cold PBS and collected in RIPA buffer (20 mM Tris pH 7.5, 50 mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate). The samples were then lysed in SDS gel loading buffer and boiled for 10 minutes. The proteins were separated on 8% SDS-polyacrylamide gels, and immunoblotting was
performed according to standard procedures and the signals were detected using the GE-Amersham ECL Western blotting kits. Monoclonal anti-Dab2, Grb2, and ERK1 antibodies were purchased from BD Sciences. Polyclonal pan laminin and monoclonal β-actin antibodies were from Sigma. Polyclonal anti-phospho-Erk antibodies were purchased from Cell Signaling Technology. Polyclonal anti-Nanog antibody was obtained from EMD Bioscience. Monoclonal anti-GATA4 and Oct3/4 antibodies were from Santa Cruz Biotechnology. Anti-GATA6 rabbit polyclonal antibodies were produced and characterized as detailed previously (Cai et al., 2008).

Small Interfering RNA (siRNA)

Pre-designed Mission siRNA against mouse Nanog (SASI_Mm02_00333133) and Oct3/4 (SASI_Mm01_00091228) were purchased from Sigma. The siRNA oligonucleotides were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) in serum-reduced Opti-MEM medium in accordance to the manufacturer's recommendation. Transfection efficiency was determined by the cellular uptake of fluorescein-labeled dsRNA oligomer from Invitrogen. The typically efficiency was 90%. The cells were maintained in full ES cell culture media and analyzed 2-3 days following siRNA transfection.

Immunofluorescence Microscopy

Wildtype RW4 and Grb2 (-/-) mouse ES cells were first seeded onto gelatin-coated cover slips at 0.5X10^5 cells per well in 12-well plates. These cells were differentiated with 1 µM RA for 3 days or transfected with expression plasmids. For immunofluorescence microscopy, the cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes, permeabilized with 0.5% Triton X-100 for 5 minutes, blocked with 5% bovine
serum albumin (BSA) in PBS for 30 minutes at room temperature. The primary antibodies were diluted into 3% BSA in PBS and incubated with the slides for 1 hour. The dilutions were: anti-GATA6 and anti-Nanog (Calbiochem) at 1:1,000; anti-Dab2 at 1:2,000; anti-GATA4 and anti-Oct3/4 at 1:400. The cover slips then were washed three times with PBS and incubated with either Alexa Fluor 488- or 596-conjugated secondary antibodies (Molecular Probes) in 3% BSA at room temperature for 1 hour. The unbound secondary antibodies were washed away with PBS for four times, and the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes. The cover slips were mounted in anti-fade media Prolong Gold (Invitrogen) according to the manufacturer's directions. Immunofluorescence staining was viewed with 20X or 63X (oil) objective lens on a Zeiss Axio observer Z1 microscope linked to Zeiss AxioCam MR R3 camera. Images were acquired and processed using AxioVision software.

**Histology and Immunohistochemistry**

Following timed matings of Grb2 (+/-) parents, uteri containing embryos were harvested at E4.5 or E5.5. The entire uterine horns were fixed in 10% formalin, paraffin-embedded, sectioned into 6 μm slices, and adhered to positively charged slides. The sections were dewaxed in Xylene and hydrated through graded ethanol. Antigen retrieval was performed by boiling the sections submerged in antigen retrieval buffer (Dako) in a glass slide holder for 20 minutes in a kitchen steamer. The endogenous peroxidase activity was quenched by incubation with 3% H₂O₂ for 15 minutes. The slides were then blocked with 5% BSA at room temperature for 30 minutes, and subsequently incubated with antibodies: anti-GATA4 (1:2,000), anti-Dab2 (1:1,000), anti-Nanog (1:1,000), rat monoclonal cytokeratin 8/Troma-1 antibodies (Developmental Studies Hybridoma Bank,
Ames, IA) (1:600), in 5% BSA at 4°C overnight. Anti-Activated caspase-3 was from Cell Signal, Inc. Anti-Ki-67 antibodies were from Dako. The slides were then washed and incubated with appropriate secondary antibodies conjugated with polymer horseradish peroxidase for 1 hour at room temperature. For CK8 staining, polyclonal rabbit anti-rat “bridging” antibodies (DAKO) at 1:500 dilution were used. Diaminobenzidine (DAB) was used as chromogen for the immunoperoxidase reaction. The slides were counterstained with hematoxylin, dehydrated in xylene and mounted in Permount.
Results

Failure of Primitive Endoderm Epithelial Layer Formation in Grb2-Null Embryos

Previously, Grb2 homozygous knockout embryos were found to die at an early step of mouse embryonic development, at around E5-6 stages (Cheng et al., 1998). In vitro study of blastocysts suggested that Grb2 null blastocysts fail to undergo differentiation to form a primitive endoderm layer (Chazaud et al., 2006). We further examined the phenotype of Grb2 mutant embryos implanted on uterine wall. Following timed mating of Grb2 (+/-) parents, whole uterine horns were harvested at approximate 4.5 days post coitum and processed for histological analysis (Fig. 23A). In the sections, morphologically normal implanted blastocysts containing a Nanog-positive inner cell mass and Dab2- and GATA4-positive primitive endoderm layer were identified and assigned as wildtype or Grb2 (+/-). On the same sections, littermates containing Nanog-positive inner cell mass but lacking Dab2- and GATA4-positive primitive endoderm cells were observed, and these abnormal blastocysts were designated as the presumptive Grb2-null embryos (Fig. 23A). The Grb2-null embryos were found to constitute approximately the expected ratio of one-fourth of the total embryos: 4 mutant E4.5 embryos were found among a total of 13 E4.5 embryos analyzed. Thus, we confirmed that Grb2 is required for primitive endoderm formation in implanted blastocysts.

Grb2-null embryos persist to E5.5 stage, showing deformed embryos lacking a Dab2- and GATA4-positive extraembryonic endoderm observed in wildtype or Grb2 (+/-) littermates (Fig. 23B). Similar to the normal embryos, trophectoderm cells (indicated by cytokeratin 8 staining) are present, scattered around the implanted site of the presumptive Grb2-null E5.5 embryos (Fig. 23C). Noticeably, large numbers of the Grb2-null
embryonic cells are also positive for cytokeratin, suggesting possible differentiation of
the Grb2-null inner cell mass to trophectoderm lineage (Fig. 23C). In the mutant
embryos, Nanog-positive embryonic cells are still observable at E5.5, at which stage
normal embryos no longer contain Nanog-expressing cells (Fig. 23D). In comparison,
the Grb2-null embryos at E4.5 stage are indistinguishable from those of GATA6-null
embryos (Fig. 23E), which also lack a primitive endoderm layer (Cai et al., 2008).
Although both Grb2- and GATA6-null embryos lack cell organization and structure at
E5.5 stage, the Grb2-null embryos (Fig. 23B) are significantly smaller than the GATA6
null embryos (Fig. 23E). We used staining of ki67 and activated caspase-3 to monitor
proliferation and apoptotic cell death respectively in the embryos (Fig. 24). At E5.5,
cells within the normal/wildtype embryos and the deciduas are highly proliferative, but
the lack of cell proliferation in the Grb2 (-/-) embryo is apparent, though deciduas
carrying the defective embryo are positive for ki67 staining (Fig. 24). Little caspase-3
activation was observed in either wildtype or Grb2-null embryos (Fig. 24). Cell stained
for activated caspase-3 were observed in other location in the same slides (ovaries and
uteri), serving as a positive control (not shown). Thus, like GATA6, Grb2 is essential for
primitive endoderm differentiation; however, unlike GATA6, Grb2 is also required for
cell proliferation and the expansion of the inner cell mass. Grb2 appears to be not
required for the differentiation and development of trophectoderm.

Grb2-Null ES Cells Are Incompetent in Differentiation into Primitive Endoderm Lineage

ES cells differentiate towards extraembryonic lineages following retinoic acid treatment
or cell aggregation to form embryoid bodies (Capo-chichi et al., 2005). We further
studied the propensity of Grb2 (-/-) ES cells to undergo differentiation in culture. Like
Fig. 23 Absence of primitive endoderm epithelial layer in Grb2 KO embryos. Embryos enclosed in uterine horns from timed matings of Grb2 (+/-) parents were harvested, fixed, and embedded in paraffin. Adjacent sections were immunostained for Nanog, Dab2, and GATA4. Embryos containing Dab2 and GATA4-positive endoderm cells are assigned as either wildtype or Grb2 heterozygous. Abnormal embryos lacking endoderm markers were presumptive Grb2 (-/-).  

A: Representative E4.5 implanted embryos from timed matings between Grb2 (+/-) parents are shown. Littermates are compared between a wildtype or Grb2 heterozygous and a presumptive Grb2 (-/-) embryo.  

B: Representative E5.5 embryos from timed matings between Grb2 (+/-) parents are shown, comparing Dab2 and GATA4 immunostaining of a wildtype or Grb2 heterozygous with a presumptive Grb2 (-/-) embryo.  

C: Representative cytokeratin 8 immunostaining of E5.5 embryos from timed matings between Grb2 (+/-) parents are shown, comparing a wildtype or Grb2 heterozygous with a presumptive Grb2 (-/-) embryo.  

D: Representative wildtype or Grb2 heterozygous and a presumptive Grb2 (-/-) E5.5 embryos are shown, of immunofluorescence staining of Dab2, cytokeratin 8 (CK), Nanog, and DAPI.  

E: Representative Dab2 immunostaining of E4.5 and E5.5 implanted, presumptive GATA6-null embryos from timed matings between GATA6 (+/-) parents are shown for comparison. Scale bars are provided next to the images.
Reduced proliferation of Grb2-null embryos. E5.5 embryos enclosed in uterine horns from timed matings of Grb2 (+/-) parents were harvested, fixed, and embedded in paraffin. Adjacent sections were stained with markers for Nanog, Dab2, and GATA4 to assign genotypes to be either wildtype or Grb2 heterozygous, or presumptive Grb2 (-/-). Examples of adjacent sections of a wildtype and a Grb2-null embryo stained with either ki67 or activated/cleaved caspase-3 are shown.
wildtype RW4 ES cells, prior to treatment, Grb2-null ES cells ubiquitously express pluripotent markers Oct3/4 and Nanog (Fig. 25A). Upon treatment with retinoic acid, Oct3/4 and Nanog expression were reduced in a large fraction of Grb2 null ES cells as judged by immunofluorescence microscopy (Fig. 25A). Both Nanog and Oct3/4 were reduced to a comparable extent in both wildtype and Grb2-null ES cells assayed by Western blot (Fig. 25B). However, unlike RW4 cells, Grb2-null ES cells did not undergo endoderm differentiation since the markers, GATA4, GATA6, and Dab2, were not induced (or greatly reduced) as assayed by both immunofluorescence staining and Western blot (Fig. 25C,D). Nevertheless, Grb2-null ES cells were capable of inducing cytokeratin 8 expression upon retinoic acid treatment (Fig. 25D). Retinoic acid was much stronger than the withdrawal of LIF in inducing differentiation of RW4 ES cells, but retinoic acid still did not stimulate a significant induction of primitive endoderm markers in Grb2-null ES cells. We observed that retinoic acid induced an increased MAPK activation in both RW4 and Grb2 (-/-) ES cells (Fig. 25E), suggesting activation of MAPK pathway may play a role in ES cell differentiation, but MAPK activation is not sufficient to induce the primitive endoderm lineage.

Unlike RW4 ES cells, Grb2 null ES cells did not undergo endoderm differentiation upon aggregation to form embryoid bodies either with or without retinoic acid (Fig. 26). Consistent with previous results (Capo-chichi et al., 2005), spontaneous differentiation of RW4 embryoid bodies to form an outer shell was readily observed after 5 days in suspension culture, while an outer shell was absent in all embryoid bodies formed from aggregation of Grb2-null ES cells (Fig. 26A). Immunofluorescence staining with
Retinoic acid induces differentiation of ES cells in cultures. Following withdrawal of LIF from medium or addition of retinoic acid (1 µM) for 4 days in culture, the differentiation of RW4 wildtype and Grb2 (-/-) ES cells was monitored by the loss of pluripotent markers Oct3/4 and Nanog, and the induction of endoderm lineage markers Dab2, GATA4, and GATA6, by both immunofluorescence microscopy and Western blots. A: Double immunostaining of Oct3/4 and Nanog was performed in RW4 wildtype and Grb2 (-/-) Es cells with or without treatment with retinoic acid. DAPI staining reveals the nuclei. B: The loss of Oct3/4 and Nanog expression following retinoic acid treatment was assayed by Western blots. C: Double immunostaining of Dab2 and GATA4, Dab2 and GATA6, or Dab2 and Nanog, was performed in RW4 wildtype and Grb2 (-/-) ES cells treated with retinoic acid. DAPI staining reveals the nuclei. D: The induction of extra-embryonic endoderm markers Dab2, laminin, GATA6, and GATA4, and trophectoderm marker cytokeratin 8, was assayed by Western blots. E: The activation of MAPK was assayed by Western using phospho-specific antibodies to phosphorylated ERK1/2.
primitive endoderm markers GATA4 and Dab2 confirmed that the aggregates of Grb2 null ES cells lack primitive endoderm cells or layers, compared to wildtype RW4 embryoid bodies (Fig. 26B). Addition of retinoic acid to the cell aggregates exaggerated the endoderm differentiation of RW4 embryoid bodies, but failed to stimulate endoderm differentiation in the aggregates of Grb2-null ES cells (Fig. 26B). While RW4 embryoid bodies consist of an endoderm shell enclosing the interior cells positive for both Oct3/4 and Nanog, the Grb2-null cells in the aggregates are uniformly positive for both Oct3/4 and Nanog (Fig. 26C). Retinoic acid treatment decreased the fraction of Oct3/4 and Nanog-positive cells in the aggregates from either RW4 or Grb2-null ES cells (Fig. 26C). We observed an increased MAPK activation in the course of aggregation of RW4 ES cells, with a peak on day 5 (Fig. 26D). However, MAPK activation was much weaker in the aggregations of Grb2-null ES cells (Fig. 26E).

Collectively, these results suggest that retinoic acid can induce Grb2-null ES cell differentiation by suppressing pluripotent markers Oct3/4 and Nanog; however, the cells differentiated into lineage(s) other than the primitive endoderm.

Suppression of Nanog Is Insufficient for Endoderm Differentiation of Grb2-Null ES Cells

It has been suggested that ES cells undergo endoderm differentiation following transcriptional suppression of Nanog through Grb2-mediated MAPK activation (Hamazaki et al., 2006). We used RNA interference to suppress Nanog expression in Grb2-null ES cells and investigated endoderm differentiation (Fig. 5). In RW4 ES cells, suppression of Nanog induced endoderm differentiation, indicated by the induction of markers such as laminin, Dab2, GATA4, and GATA6, consistent with previous reports.
**Fig. 26** Grb2-null ES cells are unable to form primitive endoderm in embryoid bodies. RW4 wildtype and Grb2 (-/-) ES cells were allowed to aggregate in non-adherent culture flasks to form embryoid bodies both in the presence and absence of 1 µM retinoic acid. **A**: Representative day 5 embryoid bodies viewed in bright field under a microscope show an outer layer for wildtype embryoid bodies and absence of an outer layer in Grb2 (-/-) cell aggregates. **B**: Embryoid bodies were harvested on day 5 and processed for histology. Immunofluorescence microscopy shows the presence of an outer endoderm layer stained with endoderm markers Dab2 (green) and GATA4 (red) in RW4 but not in Grb2 (-/-) embryoid bodies. Nuclei were stained with DAPI. **C**: The embryoid bodies were stained with Nanog and Oct3/4. **D**: Western blot was used to determine the time course of MAPK activation during the formation of embryoid bodies. **E**: Embryoid bodies were harvested on day 5 of the cultures and were assayed for MAPK activation by Western blots.
(Hamazaki et al., 2006). However, similar suppression of Nanog in Grb2-null ES cells failed to induce endoderm differentiation (Fig. 27A). Suppression of Nanog did not appear to influence Oct3/4 level (Fig. 27A); however, suppression of Oct3/4 reduced Nanog in both wildtype and Grb2 null ES cells (Fig. 27B). Oct3/4 suppression did not significantly induce endoderm differentiation, but did cause trophectoderm differentiation, indicated by the induced expression of cytokeratin 8 in both RW4 and Grb2-null ES cells. We found that Nanog suppression did not significantly alter MAPK activation either in RW4 or Grb2 (-/-) ES cells (Fig. 27C). Thus, these data further confirm that Grb2 deficiency blocks the formation of primitive endoderm lineage, but does not interfere with differentiation into trophectoderm lineage.

**Ectopic Expression of GATA6 or GATA4 Is Sufficient to Restore Endoderm Differentiation in Grb2-Null ES Cells in Culture**

Both Grb2 and GATA6 are required for primitive endoderm differentiation and the mutant embryos exhibit similar phenotypes, suggesting that the two genes act in the same differentiation pathway. To analyze the relationship between GATA6 with Grb2, we next tested whether ectopic expression of GATA6 is sufficient to rescue the defective endoderm differentiation of Grb2-null ES cells. In RW4 wildtype ES cells, transfection and expression of GATA4 or GATA6 induced each other and endoderm differentiation (Fig. 28), consistent with previous reports (Fujikura et al., 2002; Capo-chichi et al., 2005). Ectopic expression of GATA4 or GATA6 in Grb2-null ES cells induced endoderm markers laminin, Dab2, GATA4 or GATA6 in Grb2-null ES cells to a similar degree as in RW4 ES cells (Fig. 28). Thus, forced expression of GATA4 or GATA6 is sufficient to override the requirement of Grb2 in endoderm differentiation.
Fig. 27 Suppression of Oct3/4 or Nanog in Grb2-null ES cells does not induce endoderm differentiation. RW4 wildtype and Grb2 (-/-) ES cells were treated with siRNA to Nanog, Oct3/4, or scrambled controls for 3 days in LIF-containing ES cell culture medium. The cells were then collected and subjected to Western blot analysis to determine cell differentiation and lineages. A: The ES cells were treated with control or siRNA to Nanog. Western blot was performed for endoderm markers, laminin, Dab2, GATA6, GATA4; and pluripotent markers Nanog and Oct3/4. B: The cells were treated with control or siRNA to Oct3/4. Western blots were performed for the endoderm markers: laminin, Dab2, GATA6, GATA4; the trophectoderm marker cytokeratin 8; and the pluripotent markers Nanog and Oct3/4. C: MAPK activation was assayed in Nanog siRNA treated and control cells by Western blot using phospho-specific antibodies.
Ectopic GATA6 Expression Enables Grb2-Null ES Cells to Form Primitive Endoderm in Embryoid Bodies

The transfection efficiency in both RW4 and Grb2-null ES cells was estimated to be about 40% based on transfection controls using histone H2B expression plasmids, and a similar degree of GATA4 and GATA6 expression was observed in the mixture of differentiated and undifferentiated ES cells (Fig. 28). When we allowed the GATA4 or GATA6-transfected and non-transfected mixture of ES cells to aggregate and form embryoid bodies, the differentiated extraembryonic endoderm cells sorted to the surface to form endoderm layer, as established earlier (Rula et al., 2007; Moore et al., 2009). Similar to the aggregates from RW4, endoderm markers were assayed positive by Western blots in the aggregates from the mixture of transfected Grb2-null ES cells (Fig. 29A). Dab2-positive extraembryonic endoderm outer layers were observed by immunofluorescence microscopy in aggregates from either GATA4- or GATA6-transfected Grb2-null ES cells (Fig. 29B). No significant difference was observed between embryoid bodies formed from either GATA4- or GATA6-transfected Grb2-null ES cells. The transfected Grb2-null ES cells marked by Dab2 expression (green) formed a partial endoderm layer with a distinctive laminin (red) basement membrane (Fig. 29C), as shown in a higher magnification (Fig. 29D). Comparing to embryoid bodies from RW4 cells, embryoid bodies formed by GATA4 or GATA6-transfected Grb2-null ES cells had a much thicker basement membrane. The endoderm layer from the GATA4- or GATA6-transfected Grb2-null ES cells resembles the Reichert’s membrane forming parietal endoderm cells. Thus, expression of either GATA4 or GATA6 can bypass the requirement for Grb2 in the formation of extraembryonic endoderm. We conclude that Grb2 is upstream of GATA6 signals during early embryogenesis (Fig. 30).
Fig. 28

Fig. 28 Ectopic expression of GATA6 or GATA4 is sufficient to restore the endoderm differentiation in Grb2 (-/-) ES cells in monolayer cultures. RW4 or Grb2 (-/-) ES cells were transfected with expression vectors for GATA4, GATA6, H2B-GFP, or vector control. Following transfection for 3 days, the cells were harvested for analysis by Western blots and immunofluorescence microscopy. A: Western blot analysis was performed for laminin, Dab2, GATA6, and GATA4 in the transfected ES cells. B: Immunostaining for GATA6 (green), GATA4 (red), and Dab2 (white) was performed simultaneously in the transfected cells. DAPI (blue) was used to mark the nuclei.
Fig. 29 Ectopic expression of GATA6 or GATA4 enables Grb2 (-/-) ES cells to form endoderm layer in embryoid bodies. Embryoid bodies were produced from aggregation of RW4 or Grb2 (-/-) ES cells transfected with expression vectors for GATA4, GATA6, and histone H2B-GFP (control). The 3-day embryoid bodies were processed for analysis by Western blot and immunofluorescence microscopy. A: Western blot was performed to compare markers of the cell aggregates. B: Representative embryoid bodies show the presence of partial endoderm layer from Grb2-null ES cells transfected with GATA4 or GATA6. The endoderm cells were stained with Dab2 (green), and GATA4 (red) in GATA4-transfected cells, and GATA6 (red) in GATA6-transfected cells. DAPI marks the nuclei. C: Representative embryoid bodies show the presence of partial endoderm layer from Grb2-null ES cells transfected with GATA4 or GATA6, compared to embryoid bodies from RW4 ES cells. The endoderm cells are stained with Dab2 (green), and laminin (red) for basement membrane. DAPI marks the nuclei. D: The stainings for Dab2 (green) and laminin (red) are shown in a higher magnification for the presence of partial endoderm layer from Grb2-null ES cells transfected with GATA4 or GATA6, comparing to embryoid bodies from RW4 ES cells.
Summary and Discussion

Primitive endoderm and trophoderm are the earliest cell lineages arising from the inner cell mass during early embryogenesis. Primitive endoderm initially covers pluripotent cells of the inner cell mass (epiblast) and is further developed into polarized visceral endoderm lining what is now the primitive ectoderm and parietal endoderm covering the blastocoel. Trophoderm lining the outer layer of the blastocysts eventually forms the fetus part of placenta. Neither primitive endoderm nor trophoderm contributes directly to the formation of embryo lineage and is therefore considered as extra-embryonic tissues. However, increasing evidence has suggested that those tissues represent as the nutritional and signal source during early embryogenesis and are actively involved in various regulatory processes essential for the growth of the fetus as well as the proper axis patterning of the embryo.

Gene knockout studies showed that Grb2 (Cheng et al., 1998; Chazaud et al., 2006) and GATA6 (Cai et al., 2008) are essential for the differentiation and formation of the primitive endoderm in early embryos. Here, we found that implanted Grb2-null blastocysts contained no primitive endoderm cells and resembled the mutant GATA6-null embryos at E4.5 stage. We showed that ectopic expression of GATA6 is able to restore the ability of Grb2-null ES cells to undergo endoderm differentiation and form endoderm layer in embryoid bodies. Thus, we establish that GATA6 is downstream of Grb2 in a signaling pathway to induce primitive endoderm differentiation in the early embryos.

Previously, the general model is that Grb2-mediated Ras/MAPK activation suppresses Nanog transcription, and a reduction of nanog level will release its suppression of GATA4 and GATA6 expression, leading to primitive endoderm differentiation from the
cells of the inner cell mass (Hamazaki et al., 2006). However, our experiments of suppressing Nanog in Grb2-null ES cells indicate that suppression of Nanog is insufficient to induce endoderm lineage, and thus Grb2 is involved in a pathway independent of Nanog to induce primitive endoderm differentiation. Likewise, suppression of Oct3/4 also leads to a reduction of Nanog, but the ES cells differentiated into trophectoderm instead of endoderm, and Grb2 is not required for trophectoderm differentiation of ES cells. Although Ras/MAPK pathway also promotes trophectoderm lineage differentiation (Lu et al., 2008), Grb2 is not essential for the formation of trophectoderm in early mouse embryos as well as in ES cell differentiation in to trophectoderm-like cells. Consistently, the appearance of trophectoderm was also observed in blastocysts cultured in the presence of inhibitors for Ras/MAPK pathway (Nichols et al. 2009).

The rescue experiments suggest that the critical role of Grb2 is to induce GATA4 or GATA6 in extraembryonic endoderm differentiation. The observed difference between endoderm from the wildtype and the transfected Grb2-null ES cells is the thickened basement membrane. Endoderm of the wildtype embryoid bodies has a thin basement membrane, which resembles that of the visceral endoderm; however the endoderm basement membrane of embryoid bodies formed by GATA4- or GATA6-transfected Grb2 null ES cells is much thicker, and resembles the multilayered Reichert’s membrane produced by parietal endoderm cells. It is known that GATA6 expression declines after primitive endoderm differentiation and further maturation into visceral endoderm cells, while GATA6 expression persists in parietal endoderm cells (Cai et al., 2008). This suggests that persistent expression of GATA6 either due to forced ectopic expression, or
induced by expression of the transfected GATA4 may stimulate parietal endoderm lineage of the transfected Grb2-null ES cells.

In summary, in the current study we have established that Grb2-mediated pathway mainly stimulates GATA6 expression in the primitive endoderm differentiation of the early embryos. In addition, Grb2 is also required for proliferation and expansion of the inner cell mass. These data also suggest additional complexity in the cellular pathway in primitive endoderm differentiation.
Fig. 30 Schematic diagram of Grb2-Erk pathway during early embryogenesis. The previous models suggest that external mitogenic signals (such as FGF4) stimulate Grb2/SoS/RAS pathways and inhibit Nanog expression at transcription level. The reduced Nanog expression level leads to the release of primitive endoderm determining factors: Gata6 and Gata4 and result in the differentiation of endodermal lineage cells. In this study, we further characterized the signal cascades during early embryogenesis and found that Nanog inhibition was not sufficient to induce primitive endoderm differentiation under a Grb2 defective background. Our results suggested that mitogenic signals and the stimulation of Ras pathway might exert more functions (potentially through the activation of Gata factors) for the differentiation of endoderm cells other than reducing Nanog expression.
References


**Reference for appendix**


