ROLE OF FOXM1 IN OVARIAN CANCER TUMORIGENESIS AND CHEMORESISTANCE

Submitted by

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B.Sc. (Hons.); M.Phil. HKU

A thesis submitted in partial fulfillment of the requirements for

The Joint Degree of Doctor of Philosophy

at Imperial College London (ICL) and The University of Hong Kong (HKU)

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Ovarian carcinoma is the most lethal gynecological malignancy. The high relapse and mortality rates are attributable to late diagnosis and development of drug resistance. Identifying novel prognostic and therapeutic targets for ovarian carcinoma is crucial for improving patients' long-term survival rate.

Forkhead box protein M1 (FOXM1), which is a widely studied member of the FOX superfamily of proteins, participates in cell proliferation and apoptosis affecting the developmental function of many organs. Recently, there has been emerging evidence supporting the biological significance of FOXM1 in carcinogenesis. Overexpression of FOXM1 has been reported in multiple human malignancies including primary

breast cancer, lung cancer, prostate cancer, etc. However, whether FOXM1 participates in the development of ovarian cancer, with emphasis on the association with clinicopathological parameters and chemoresistance, remains unknown. This study aims at elucidating the functional role of FOXM1 in the tumorigenesis of ovarian cancer.

Immunohistochemical study showed higher nuclear FOXM1 expression was significantly associated with advanced stages of ovarian cancer (P=0.035). Though not reaching statistical significance, FOXM1 overexpression displayed association with serous histologic subtype, high grade cancers (poor differentiation) and chemoresistance. Patients with a low FOXM1 level had a significantly longer overall (P=0.019) and disease-free survival (P=0.014) than those with high FOXM1 expression. Multivariate progression analysis established high expression of FOXM1, advanced cancer stages and poor histological differentiation (high grade) as independent prognostic factors for short overall and disease-free survival. Consistently, *in vitro* Transwell assays demonstrated transient knockdown of FOXM1 was capable of reducing SKOV-3 migration and invasion. Furthermore, paclitaxel treatment down-regulated FOXM1 expression in the sensitive cell line but not the resistant one. Immunofluorescence and flow cytometric analyses demonstrated FOXM1 knockdown could enhance paclitaxel-mediated mitotic catastrophe in ovarian cancer cells.

Recent attention has been drawn to the oncogenic roles of kinesin-like protein KIF2C and p21-activated kinase 4 (PAK4) in human cancers. Interestingly, the expressions of KIF2C and PAK4 altered in a similar pattern to FOXM1 expression upon paclitaxel treatment by displaying down-regulation only in the paclitaxel sensitive cell line but not the resistant one. FOXM1 silencing, qPCR, luciferase reporter assay and chromatin immunoprecipitation confirmed KIF2C and PAK4 to be novel transcriptional targets of FOXM1. Clonogenic assay showed KIF2C knockdown could re-sensitize resistant cell line to paclitaxel treatment. Flow cytometry demonstrated KIF2C silencing was able to increase the number of cells blocked at G2/M cell cycle phase in sensitive cell line and raise the number of apoptotic cells in resistant cell line. Up-regulations of miR-590 and miR-370 were also observed in a panel of drug resistant ovarian and breast cancer cell lines. While ectopic expression of miR-590 reduced FOXM1 expression, FOXM1 also seemed to be able to regulate the expression of miR-590.

In summary, this study showed overexpression of FOXM1 in ovarian cancer correlated with poor survival of patients and paclitaxel resistance. KIF2C and PAK4 were identified as novel transcriptional targets of FOXM1 implicated in chemoresistance.

An abstract of 474 words

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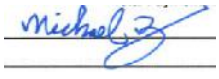
DECLARATION

I declare that this thesis represents my own work, except that where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation, or report submitted to this university or to any other institutions for a degree, diploma, or other qualifications.

Signature



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Journal article

1. Zhao F, Siu MKY, Jiang LL, Tam KF, Ngan HYS, Le XF, Wong OGW, Wong ESY, Bella L, Khongkow P, Lam EW, Cheung ANY. Overexpression of Forkhead box protein M1 (FOXM1) in ovarian cancer correlates with poor patient survival and contributes to paclitaxel resistance. (Under revision)

2. Gomes AR, Zhao F, Lam EW. (2013). Role and regulation of the forkhead transcription actors FOXO3a and FOXM1 in carcinogenesis and drug resistance. *Chin J Cancer*. 32(7): 365-370. Review

3. Zhao F, Lam EW. (2012). Role of the forkhead transcription factor FOXO-FOXM1 axis in cancer and drug resistance. *Front Med*. 6(4): 376-380. Review.

Conference Report

1. Zhao F, Siu MK, Wong OG, Wong ES, Jiang LL, Tam KF, Ngan HY, Le XF, Lam EW, Cheung AN. Role of FOXM1 in ovarian carcinogenesis. AACR conference Advances in Ovarian Cancer Research: From Concept to Clinic. September 18-21, 2013. Miami, USA. Proceedings of the American Association for Cancer Research. [Abstract accepted]

Related Work:

1. Zhao F, Siu MK, Jiang L, Tam KF, Ngan HY, Le XF, Wong OG, Wong ES, Chan HY, Cheung AN. Overexpression of dedicator of cytokinesis I (Dock180) in ovarian cancer correlated with aggressive phenotype and poor patient survival. *Histopathology.* 2011 Dec;59(6):1163-72.

LIST OF ABBREVIATIONS

FOXM1	Forkhead box protein M1
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
BRAF	V-raf murine sarcoma viral oncogene homolog B1
Cdc42	cell division cycle 42
EMT	epithelial-mesenchymal transition
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
PAK	p-21 activated kinase
PI3K	phosphoinositide 3-kinase
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PTEN	phosphatase and tensin homolog
Rac1	Ras-related C3 botulinum toxin substrate 1
TrkB	tropomyosin-related kinase B

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Chapter 1

Introduction: ovarian cancer

1.1 Epidemiology of ovarian cancer

Ovarian cancer is the fifth and the seventh major cause of cancer deaths among females in the United States and Hong Kong respectively (Hong Kong Cancer Registry, 2013; Reynolds & Moller, 2006). In fact, it is the most lethal among all gynaecological malignancies, with more than 60% of the estimated new cases dying of the disease annually (SEER Cancer Statistics Factsheets, 2013). Although the incidence rate of ovarian cancer has remained fairly steady over 30 years in the United States (Jemal *et al.*, 2009), the rates are on the rise in Hong Kong and the United Kingdom (Hong Kong Cancer Registry, 2013; UK Cancer Registry, 2013). Age-standardized rates of ovarian cancer have also been showing an increasing trend in China and Japan (Marugame & Hirabayashi, 2007).

1.2 Histologic subtypes of ovarian cancer

There are more than 30 different types of ovarian cancer which are identified based on the type of cells from which they originate. Epithelial ovarian carcinomas initiate from cells lining the outer surface of ovary and account for over 90% of all ovarian cancers, while the remaining 10% arises from stromal or germ cells (Auersperg *et al.*, 2001). Epithelial ovarian cancers can be generally classified into five histologic subtypes, namely endometrioid, mucinous, clear cell, serous and undifferentiated

(Rosen *et al.*, 2009).

Serous carcinoma is the most common type of epithelial ovarian cancer, accounting for more than half of the diagnosed cases. Most serous carcinomas display papillary and micropapillary structure with focal slit-like spaces, while glandular, solid and trabecular architecture are also present (Soslow, 2008) (Figure 1.1A). Serous subtype typically demonstrates columnar cells with pink cytoplasm, though polygonal eosinophilic cells, clear cells and signet ring cells can also be observed (Che *et al.*, 2001).

Endometrioid carcinoma contributes to approximately 10% of all ovarian carcinomas (Seidman *et al.*, 2004). Endometrioid ovarian tumours resemble their endometrial counterparts generally displaying tubules, solid, sheetlike growth and papillae (Soslow, 2008) (Figure 1.1B). It has been suggested that most endometrioid cancers can be associated with endometriosis, endometrioid borderline tumour or a synchronous endometrial neoplasm of endometrioid type (Bell & Kurman, 2000; Roth *et al.*, 2003).

Representing approximately 5% of all ovarian carcinomas, the mucinous subtype displays a limited range of histologic features and is among the most difficult ovarian carcinomas for interpretation (Figure 1.1C). Approximately 20% of primary mucinous tumours are borderline tumours, noninvasive or invasive carcinomas with the

remainder being cystadenomas (Hart, 2005). Well-differentiated ovarian mucinous carcinomas often show a labyrinthine pattern evident of expansile invasion (Soslow, 2008).

Clear cell carcinoma constitutes approximately 5% of all ovarian tumours (Seidman *et al.*, 2004). The clear cell subtype demonstrates a rather limited architecture with the recognition of papillary, tubulocystic and solid varieties. Typical clear cell cancers are characterized by hobnail cells with clear cytoplasm (Soslow, 2008) (Figure 1.1D). It has been reported that more than half of clear cell carcinomas are associated with endometriosis, particularly atypical endometriosis or endometriosis-associated tumours (Soslow, 2008).

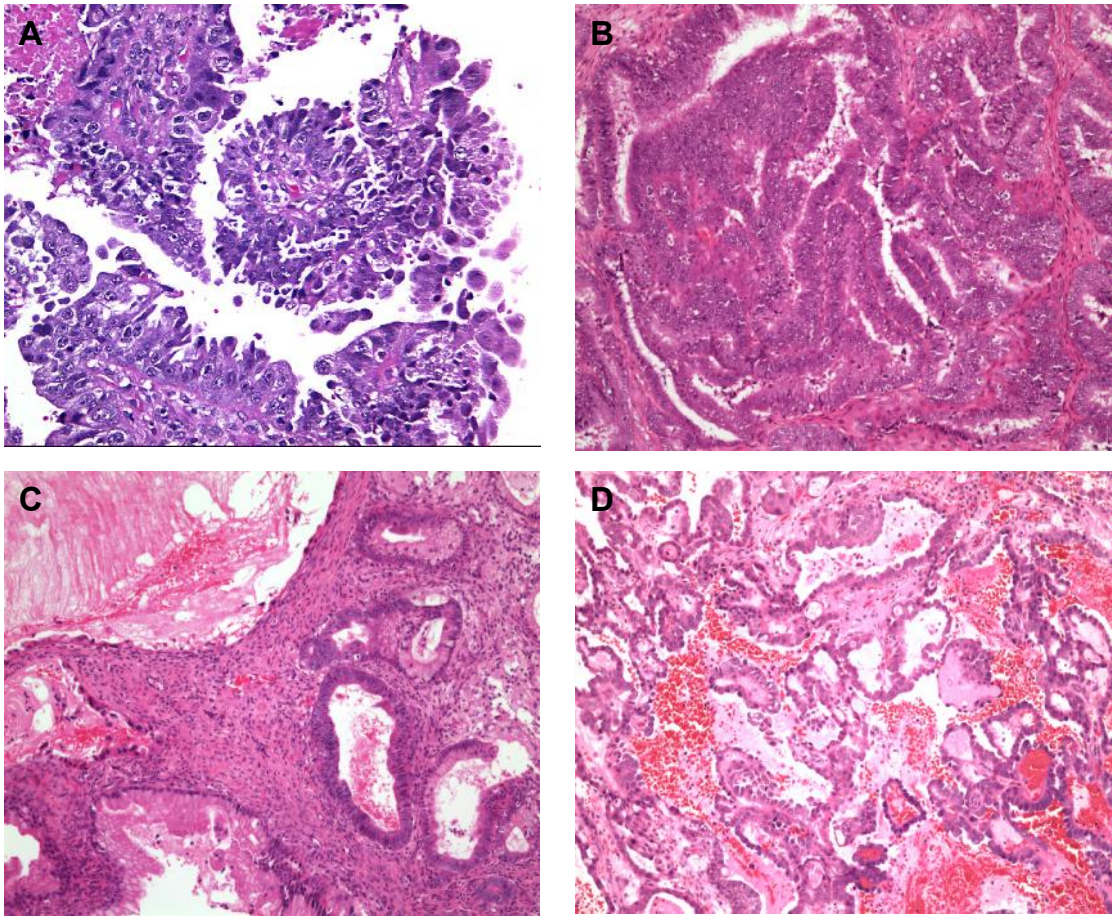


Figure 1.1 Photomicrographs of A) Serous, B) Endometrioid, C) Mucinous and D) Clear cell carcinomas of the ovary. Magnifications X200, haematoxylin-eosin (H&E) stain (Original figure)

1.3 Stages of ovarian cancer

A detailed staging system of ovarian cancer has been outlined by the International Federation of Obstetricians and Gynaecologists (FIGO) in order to identify the spread of ovarian cancer at diagnosis. Briefly, stage 1 describes ovarian tumour confined to one or both ovaries whereas stage 2 tumour involves one or both ovaries and has extended into the pelvis. Stage 3 tumour involves one or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis with or without regional lymph node metastasis. Stage 4 cancer describes distant metastasis of tumour beyond the peritoneal cavity to other body organs such as the liver (UK Cancer Registry, 2012).

1.4 Risk factors for ovarian cancer

1.4.1 Age

The incidence rate of ovarian cancer is highest among women aged between 60 and 64 years (World Health Organization, 2013).

1.4.2 Ethnicity

Not surprisingly, the incidence rates of ovarian cancer vary among ethnic groups. While highest incidence rates are observed in North America, Northern and Western Europe, Asian nations such as China have the lowest incidence rates (Parkin *et al.*, 1999; Pisani *et al.*, 2002). In a large cohort of case-control studies, it has been suggested that white women tend to have higher incidence rates when compared to

black women (John *et al.*, 1993; Whittemore *et al.*, 1992).

1.4.3 Dietary intake

The relationship between dietary intake and risk for contracting cancer has drawn considerable research attention. For instance, high meat consumption may raise the possibility of developing ovarian cancer (La Vecchia *et al.*, 1987). Recently, Di Maso *et al* reported dietary intake of red meat is significantly correlated with the risk of ovarian cancer (Di Maso *et al.*, 2013). However, the association between dietary fat intake and ovarian cancer risk is not supported by other studies (Kushi *et al.*, 1999). It has been found that intake of preserved food is positively associated with the incidence of epithelial ovarian cancer in southern Chinese women (Lee *et al.*, 2013a). Though not significant, intake of sugary drinks has also been suggested to be related to increased risk of ovarian cancer (King *et al.*, 2013). Interestingly, regular tea consumption has been documented to be associated with a reduced risk of ovarian cancer for southern Chinese females (Lee *et al.*, 2013b). Although extensive studies have been carried out to investigate the association between alcohol consumption and ovarian cancer risk, no definitive conclusions can be drawn (King *et al.*, 2013; Kuper *et al.*, 2000; Schouten *et al.*, 2004). Similarly, the relationship between smoking and ovarian cancer is also inconclusive with contradictory findings in different populations (Marchbanks *et al.*, 2000; Modugno *et al.*, 2002; Pan *et al.*, 2004; Terry *et al.*, 2003).

1.4.4 Breastfeeding

Breastfeeding has long been broadly advocated for its beneficial effects to the health of the baby. Emerging findings have indicated breastfeeding may be associated with the risk of ovarian cancer. Gwinn *et al* reported women who adopt breastfeeding enjoy a reduced risk of developing ovarian cancer (Gwinn *et al.*, 1990). This is consistent with recent studies demonstrating an inverse correlation between the duration of breastfeeding and the risk of ovarian cancer based on cohort and case-control studies (Luan *et al.*, 2013). However, contradictory findings have also been shown regarding the duration of lactation and risk of ovarian cancer (Jordan *et al.*, 2007). Clearly, more studies are required in order to determine whether breastfeeding offers protection against the development of ovarian cancer.

1.4.5 Parity

Pregnancy has been associated with the risk of ovarian cancer and it has been documented each delivery could confer a 16% to 22% reduction in the risk of getting ovarian cancer (Zografos *et al.*, 2004). Although it was found that nulliparous females have higher ovarian cancer risk than parous women (Bodelon *et al.*, 2013), Ness *et al* recently reported that anovulatory infertility does not appear to be associated with the risk of ovarian cancer (Ness *et al.*, 2002).

1.4.6 Obesity

Obesity has long been associated with a wide range of diseases including coronary heart disease and diabetes mellitus. Recent studies suggested a positive correlation between obesity and increased risk of ovarian cancer (Leitzmann *et al.*, 2009; Rodriguez *et al.*, 2002). Intriguingly, obesity has been suggested to increase risk of the less common histologic subtypes of ovarian cancer but not that of high-grade invasive serous carcinomas (Olsen *et al.*, 2013).

1.4.7 Oral contraceptives

Several studies have consistently demonstrated a reduced risk of sporadic as well as familial ovarian cancer with the consumption of oral contraception (Walker *et al.*, 2002). The risk reduction is already eminent after a few months of use and the effect persists for years after discontinuation. Although it has been reported oral contraceptives may lower the risk of ovarian cancer in women with pathogenic mutations in *BRCA1* or *BRCA2* gene (Narod *et al.*, 1998), a separate case-control study showed oral contraceptives could reduce the risk of ovarian cancer only in non-carriers but not in carriers of a *BRCA* mutation (Rodriguez *et al.*, 2001).

1.4.8 Hereditary ovarian cancer

Family history of ovarian cancer remains the most significant risk factor though hereditary ovarian cancer only accounts for about 10% of the cases (Reynolds & Moller, 2006). The lifetime risk estimate for individuals who have one first degree

relative with ovarian cancer is much higher than the average population risk (Stratton *et al.*, 1998; Thompson & Easton, 2002). Individuals with more than one affected relative have an estimate of 3 to 23% (Stratton *et al.*, 1998; Thompson & Easton, 2002). Two types of ovarian cancer susceptibility genes have been identified: the breast and ovarian cancer tumour suppressor genes (*BRCA1* and *BRCA2*) and the mismatch repair genes associated with Hereditary Nonpolyposis Colorectal Cancer (HNPCC) syndrome (Daniilidis & Karagiannis, 2007).

BRCA1 and *BRCA2* are tumour suppressor genes that play important roles in transcriptional regulation, homologous recombination and DNA damage repair (Turner *et al.*, 2005). Not surprisingly, insertions or deletions and missense alterations of *BRCA1* and *BRCA2* generating truncated protein product could eventually lead to tumorigenesis (Honrado *et al.*, 2005). Mutations in the *BRCA1* gene are estimated to confer a 30% lifetime risk of ovarian cancer up to the age of 60, while mutations in *BRCA2* are estimated to confer a risk of 27% up to the age of 70 (Breast Cancer Linkage Consortium, 1999; Thompson & Easton, 2002).

While mutations in *BRCA* genes account for approximately 90% of hereditary ovarian cancer cases, HNPCC syndrome is responsible for the remaining (Aarnio *et al.*, 1999). The mismatch repair genes confer an increased lifetime risk of ovarian cancer of approximately 9 to 12% in addition to an elevated risk of endometrial cancer (Aarnio *et al.*, 1999; Farrell *et al.*, 2006).

1.4.9 Other factors

Regular physical activity is beneficial to the well being of an individual and has been demonstrated to protect against a wide range of diseases. Although it is hypothesized that physical activity may reduce the risk of ovarian cancer, Bertone *et al* reported a small, insignificant decrease in the risk only for the highest category of vigorous activity (Bertone *et al.*, 2002).

Animal experiments suggest that circadian disruption may be associated with ovarian cancer. In an attempt to evaluate potential association between nightshift work and risk of ovarian cancer, Bhatti *et al* recently performed a population-based case-control study and found that nightshift work is associated with an increased risk of invasive and borderline tumours among women aged 50 or above (Bhatti *et al.*, 2013).

Although it has been documented non-steroidal anti-inflammatory drugs enhance apoptosis of ovarian cancer cell lines (Rodriguez-Burford *et al.*, 2002), whether there exists an association between commonly used anti-inflammatory drugs and risk of ovarian cancer remains elusive due to a lack of observational studies. Among the few related findings, aspirin consumption has been shown to be inversely associated with epithelial ovarian cancer (Akhmedkhanov *et al.*, 2001).

Hormone replacement therapy (HRT) has also been linked to ovarian cancer. Recently, several studies consistently demonstrated an elevated risk of ovarian cancer for

long-term users of oestrogen replacement therapy (Cramer *et al.*, 2001; Riman *et al.*, 2002).

1.5 Genetic bases of epithelial ovarian cancer

Approximately 90% of all epithelial ovarian cancers belong to sporadic tumours involving amplifications and mutations of a myriad of tumour suppressor genes and oncogenes (Auersperg *et al.*, 2001). Selected molecular signatures of ovarian cancer will be discussed as follows.

1.5.1 *BRCA1 and BRCA2*

In spite of original thoughts suggesting an involvement of *BRCA* genes only in the tumorigenesis of inherited ovarian tumours, accumulating evidence points to a role of *BRCA* genes in sporadic ovarian cancers. For instance, it has been demonstrated in a population-based study that promoter hypermethylation of *BRCA1* may serve as an alternative to mutation in causing the inactivation of *BRCA1* in sporadic ovarian cancer (Baldwin *et al.*, 2000). Mutations in *BRCA1* and *BRCA2* have also been implicated to be involved in the tumorigenesis of sporadic ovarian cancers via distinct molecular pathways (Jazaeri *et al.*, 2002). Furthermore, Khoo *et al* reported somatic mutations of *BRCA1* can be observed in sporadic ovarian tumours implying a tumour suppressor function of *BRCA1* in sporadic cancers (Khoo *et al.*, 1999).

1.5.2 *TP53*

TP53 encodes the tumour suppressor protein p53 which is crucial for protection against neoplasia by inducing apoptotic cell death, DNA damage repair and cell cycle arrest (Levine *et al.*, 1991). Given the pivotal role of p53 in safeguarding the cell against a wide range of stresses, it comes with no surprise that mutations of *TP53* have been implicated in the malignant progression of multiple types of cancers including tumours of the ovary (Kupryjanczyk *et al.*, 1993).

1.5.3 *PTEN* and *PI3K*

The phosphatase and tensin homolog (*PTEN*) gene is a tumour suppressor that dephosphorylates phosphatidylinositol 3,4,5-triphosphate (PIP3) and inhibits the phosphatidylinositol 3-kinase (PI3K) signaling pathway (Maehama *et al.*, 2001). Mutations of *PTEN* resulting in constitutively active PI3K signaling could lead to enhanced cell proliferation and resistance to apoptosis which are prerequisites of oncogenic transformation (Manning & Cantley, 2007). Frequent somatic *PTEN* mutations have been detected in endometrioid ovarian carcinoma, indicating a role of *PTEN* in the development of endometrioid subtype of ovarian cancer (Obata *et al.*, 1998). Mutations in the p110 subunit of PI3K, namely *PI3KCA*, have also been reported to contribute to activation of the PI3K signaling pathway and such mutations are detected in 12% of ovarian cancers (Levine *et al.*, 2005).

1.5.4 *KRAS* and *BRAF*

KRAS belongs to a family that regulates cell growth, survival and differentiation through activation of downstream effector pathways including PI3K and RAF (Schubbert *et al.*, 2007). Mutations in *KRAS* or *BRAF* genes are common in low-grade serous carcinomas and serous borderline ovarian tumours, but are rarely observed in high-grade ovarian tumours (Wong *et al.*, 2010).

1.5.5 *Hox*

The *HOX* family of homeobox genes, originally widely investigated in *Drosophila* and is responsible for the modulation of normal axial and spatial development, has been implicated in lineage differentiation of epithelial ovarian cancer (Rosen *et al.*, 2009). Cheng *et al* reported that the *HOX* genes are not expressed in normal ovarian surface epithelium, but indeed are observed in different histologic subtypes of epithelial ovarian cancer (Cheng *et al.*, 2005). It was further demonstrated that ectopic expression of different members of *HOX* genes could give rise to tumours resembling serous, endometrioid and mucinous subtypes of epithelial ovarian cancer, implying a pivotal role of *HOX* members in specifying regional identity in the reproductive tract (Cheng *et al.*, 2005).

1.5.6 *c-Myc*

The proto-oncogene *c-Myc* encodes phosphoproteins that play important roles in the regulation of a wide range of biological processes including cell proliferation,

apoptosis and differentiation. Although *c-Myc* overexpression has been documented in epithelial ovarian cancers, further elaborative studies are necessary to elucidate whether *c-Myc* participates in the tumorigenesis of ovarian carcinoma (Chen *et al.*, 2005; Obaya *et al.*, 1999).

1.5.7 *TrkB*

Tropomyosin-related kinase B (TrkB) mediates multiple functions in the development of nervous system including neuronal differentiation and survival. Recently, TrkB overexpression has been documented in several human cancers (Lewin & Barde, 1996). Intriguingly, TrkB and its ligand, brain-derived neurotrophic factor (BDNF), have been reported to contribute to ovarian carcinogenesis and may serve as potential prognostic and therapeutic target of ovarian cancer (Au *et al.*, 2009).

1.6 Models of ovarian carcinogenesis

1.6.1 Hypotheses on ovarian tumorigenesis

Several hypotheses have been proposed to explain the mechanism of ovarian carcinogenesis. First proposed by Fathalla in 1971, the long-standing incessant ovulation hypothesis argues that repeated cycles of ovulation-induced trauma and the subsequent repair of ovarian surface epithelial cells at the site of ovulation contribute to the development of ovarian cancer (Fathalla, 1971; Risch, 1998). According to this hypothesis, recurrent rupture, repair and proliferation of ovarian surface epithelial cells at the ovulation site may accumulate and propagate genomic instability, which

predisposes this cell layer to tumorigenesis (Ho, 2003). Findings based on experimental evidence provided support to this hypothesis. For instance, oxidative DNA damage, expression of p53 and apoptosis occur among the epithelial cells located within the formative sites of ovulation (Murdoch *et al.*, 2001). La Vecchia *et al* further reported a reduced risk of ovarian cancer among women who use oral contraceptives to suppress ovulation (La Vecchia *et al.*, 1987). In 2007, Piek *et al* suggested a revision to the hypothesis by proposing that ovulation increases the risk of ovarian cancer by elevating the risk of inclusion of exfoliated tubal epithelial cells into the ovarian stroma and the subsequent enhanced mitotic activity within tubal epithelium (Piek *et al.*, 2007).

The pituitory/gonadotropin hypothesis suggests that under normal circumstances, the ovarian epithelium invaginates and forms inclusion cysts and clefts. Overstimulation by gonadotropins may trigger a cascade of events resulting in the entrapment of ovarian surface epithelium within the ovarian stroma and the subsequent malignant transformation (Cramer & Welch, 1983). However, recent experimental findings failed to support any link between hormone replacement theory and ovarian cancer (Rodriguez *et al.*, 2001), suggesting an inadequacy of this hypothesis alone to account for the process of ovarian carcinogenesis.

Alternative hypotheses have been proposed including the androgen/progesterone hypothesis which suggests an increased risk of ovarian cancer may result from

elevated levels of androgens produced by the ovarian thecal cells (Risch, 1998). Similar to the gonadotropin hypothesis, this hypothesis lacks extensive experimental support.

1.6.2 Dualistic model of ovarian carcinogenesis

Based on a review of clinicopathological and molecular studies, a dualistic model has been proposed to account for ovarian tumour progression. Briefly, ovarian cancer could be divided into two groups, namely type I and type II that correspond to two main pathways of tumorigenesis (Shih Ie & Kurman, 2004). Type I tumours are low-grade neoplasms which develop in a stepwise manner from well established precursor lesions called borderline tumours to invasive tumours (Shih Ie & Kurman, 2004; Singer *et al.*, 2005). In addition to low-grade serous carcinomas, type I tumours are composed of mucinous, endometrioid and clear cell carcinomas. Type I tumours are genetically stable and characterized by mutations in an array of genes such as *KRAS, BRAF, PTEN* and *β-catenin* (Shih Ie & Kurman, 2004; Singer *et al.*, 2005). In contrast, type II tumours include high-grade serous carcinoma, malignant mixed mesodermal tumours and undifferentiated carcinoma and are characterized by *de novo* development from *in situ* changes originated from ovarian surface epithelium or inclusion cysts (Shih Ie & Kurman, 2004). Unlike type I tumours, type II tumours tend to be highly genetically unstable and harbour frequent mutations of *TP53* (Shih Ie & Kurman, 2004).

1.6.3 Epithelial to mesenchymal transition

Epithelial to mesenchymal transition (EMT) is a process by which epithelial cells lose cell-cell adhesion and gain migratory and invasive properties necessary to become mesenchymal cells. EMT is crucial for numerous developmental processes and accumulating findings suggest EMT participates in the initiation of cancer metastasis (Thiery, 2003). Recently, Ahmed *et al* described a model based on the development of EMT to elucidate the progression of ovarian cancer (Ahmed *et al.*, 2007). According to this model, EMT is crucial for enhanced motility of epithelial ovarian cancer cells. Rupture of ovarian tumours leads to shedding of cancer cells into the surrounding peritoneum. Cytokines and growth factors secreted by the tumour microenvironment are indispensable for facilitating the invasiveness of cancer cells until reaching a secondary site of attachment (Ahmed *et al.*, 2007). On the other hand, mesenchymal to epithelial transition (MET) is required for tumour growth on the omentum (Ahmed *et al.*, 2007).

1.7 Conventional treatments of ovarian cancer

Optimal debulking surgery remains the major treatment for stage I ovarian cancer which involves total abdominal hysterectomy and bilateral salpingo-oophorectomy with careful surgical staging. Lymphadenectomy is also considered an important diagnostic and therapeutic procedure (Colombo *et al.*, 2003). However, given the non-specific symptoms of ovarian cancer and the lack of effective screening tests, majority of patients are diagnosed at an advanced stage. Treatment for advanced

ovarian cancer focuses on cytoreductive surgery and chemotherapy. Radiotherapy is mainly used as a palliative treatment to alleviate pain. The combination of carboplatin and paclitaxel has represented the first-line standard treatment.

Unfortunately, the effectiveness of chemotherapy is limited by drug resistance. Resistance to chemotherapeutic agents can be classified into two categories: intrinsic or acquired. For patients with intrinsic resistance, they tend to display resistance before receiving chemotherapy since the tumour cells already harbour resistance-mediating factors (Holohan *et al.*, 2013). On the other hand, acquired drug resistance can develop during treatment of tumours that were initially sensitive. This may be attributable to various mechanisms including mutations arising during treatment (Holohan *et al.*, 2013). The mode of action and mechanisms of resistance of several commonly adopted chemotherapeutic drugs will be discussed.

1.7.1 Paclitaxel

Paclitaxel, a member of taxanes, was first isolated in 1971 as the active ingredient of the crude bark extract of the Pacific yew, *Taxus brevifolia* (Wani *et al.*, 1971). Although paclitaxel showed anti-tumour activity shortly after its discovery, it was not until the mid-1980s that the impressive tumour suppressing activity of paclitaxel in human tumour xenografts in nude mice had been appreciated and applied to clinical trials (Oberlies & Kroll, 2004).

The chemical name of paclitaxel is 5β, 20-epoxy-1, 2α, 4, 7β, 10β, 13α-hexahydroxytax-11-en-9-one4, 10-diacetate2-benzoate 13 ester with (2R, 3S)-N-benzoyl-3-phenyliseseine. The chemical formula is $C_{47}H_{51}O_{14}$ with a molecular weight of 853.9 (Kumar *et al.*, 2010). The principal mechanism of action involves disruption of microtubule dynamics.

Microtubules play a pivotal role in a broad spectrum of biological processes including the initiation of DNA synthesis, mitosis, meiosis, motility as well as intracellular trafficking of macromolecules and organelles. Paclitaxel acts by binding selectively and reversibly to the B subunit of tubulin, thus promoting tubulin polymerization and the formation of stable microtubules (Kumar *et al.*, 2010). Exposed cells generally exhibit an accumulation of arrays of disorganized microtubules which eventually lead to profound cell cycle arrest at the G2/M phase and the subsequent apoptotic cell death (Downing, 2000). It has been demonstrated that paclitaxel-induced apoptosis and drug resistance is mediated at molecular level by altering the functions of p53, p21, Bcl-2 and Bcl-xL (Giannakakou *et al.*, 2000; Schmidt *et al.*, 2000). Although approximately two-thirds of ovarian cancer patients respond to the combinatorial chemotherapy involving taxanes and platinum compounds, the majority of them will have disease recurrence due in large to the acquisition of drug resistance (Kumar *et al.*, 2010).

Several mechanisms have been proposed to explain resistance to paclitaxel. It has

been suggested elevated expression of the efflux transporter Pgp, multidrug resistance-associated protein 2, and reduced expression of the influx transporter, such as the organic anion transporting polypeptide 1 B3 (OATP1B3/SLCO1B3) may account for the development of chemoresistance (Walle *et al.*, 1995). Other plausible mechanisms include overexpression of the paclitaxel-metabolizing enzyme CYP2C8, mutations in tubulin that alter binding capacity of paclitaxel as well as changes in signaling pathways associated with microtubule function (Bolis *et al.*, 2004; Kumar *et al.*, 2010; Walle *et al.*, 1995). Given the efficacy of paclitaxel as front-line chemotherapeutic agent for ovarian cancer, it is of utmost importance to elucidate detailed mechanisms underlying the acquisition of drug resistance while exploring novel agents.

1.7.2 Cisplatin

cis-diamminedichloroplatinum (II) (commonly known as cisplatin or CDDP) was first adopted for the treatment of testicular and bladder cancer in 1978. It has now become a widely employed platinum-based therapeutic compound for a broad spectrum of solid neoplasms including ovarian, colorectal and lung cancers (Galanski, 2006). Given the cytotoxicity of cisplatin affecting kidneys and peripheral nerves, *cis*-diammine (cyclobutane-1, 1-dicarboxylate-*O*, *O*') platinum (II) (carboplatin) was later developed as a second-generation platinum compound that functions similarly to cisplatin yet possesses reduced side effects (Harrap, 1985).

The most prominent mode of action of cisplatin involves the generation of DNA lesions followed by the activation of DNA damage response and apoptotic cell death (Galluzzi *et al.*, 2012). The major signaling pathway leading to apoptosis is the sequential activation of ataxia telangiectasia mutated (ATM)- and RAD3-related protein (ATR) and checkpoint kinase I (CHEK1) which in turn phosphorylates the tumour suppressing TP53 triggering apoptosis (Galluzzi *et al.*, 2012). Unlike most patients of lung, prostate and colorectal cancers that demonstrate intrinsic resistance to cisplatin-based therapies, acquired chemoresistance is frequently observed in ovarian cancer patients (Koberle *et al.*, 2010).

Resistance to cisplatin often presents a multifactorial nature. For example, down-regulation of copper transporter 1 (CTR1) has been documented in cisplatin-resistant cancer cell lines which might result in reduced cisplatin uptake (Ishida *et al.*, 2002). Overexpression of multidrug resistant protein 2 (MRP2) has also been shown to account for an increased efflux of cisplatin in resistant cells (Liedert *et al.*, 2003). Furthermore, mutations or down-regulations of MLH1 and MSH2, which constitute part of the mismatch repair system, have also been implicated in the context of acquired cisplatin resistance (Aebi *et al.*, 1996; Gifford *et al.*, 2004).

1.7.3 Tamoxifen

The anti-oestrogen tamoxifen is the most commonly used treatment for patients with oestrogen-receptor alpha (ER)-positive breast cancer. Although there has been some

evidence from observational studies suggesting that tamoxifen may generate a response in women with relapsed ovarian cancer, no conclusive findings from randomized controlled trials are available (Williams, 2001).

1.8 Survival and prognosis

As mentioned in previous sections, only approximately 25% of ovarian cancers are diagnosed at an early stage (I and II FIGO stages), while for 70% of cases the diagnosis is poised at late stages. The five-year survival of these patients is 70-90% in stage I, 50-60% in stage II, 20-40% in stage III and 10% in stage IV (Rescigno *et al.*, 2013). Tumour cell type was further reported to be the most relevant histopathological prognostic factor in patients treated with surgery and chemotherapy. Mucinous and clear cell carcinomas are considered highly lethal (Alexandre *et al.*, 2010; Zaino *et al.*, 2011).

1.9 Scope of Study

Hypothesis

FOXM1 is dysregulated in ovarian cancer and contributes to tumorigenesis and acquisition of paclitaxel resistance

Objectives

-To characterize the expression pattern of FOXM1 in ovarian cancer patient samples and determine potential correlation with clinicopathological parameters

-To study the roles of FOXM1 in ovarian cancer metastasis and acquisition of paclitaxel resistance

-To identify novel FOXM1 transcriptional targets that might be implicated in chemoresistance

Chapter 2

Forkhead box protein M1 (FOXM1) expression and clinicopathological parameters in ovarian cancer

2.1 Introduction

2.1.1 Forkhead box (FOX) proteins

Forkhead box (FOX) proteins constitute an extensive family of transcriptional regulators that are characterized by an evolutionarily conserved "forkhead" or "winged-helix" DNA-binding domain (DBD) (Myatt & Lam, 2007). With the first member being identified more than two decades ago, fifty FOX proteins have so far been identified and they are categorized into 19 subgroups (FOXA to FOXS) according to sequence homology (Kaestner *et al.*, 2000). The FOX superfamily of proteins are multifunctional transcription factors responsible for the spatio-temporal fine tuning of a broad repertoire of transcriptional programs that are involved in cell cycle progression, proliferation, differentiation, metabolism, senescence, survival and apoptosis (Myatt & Lam, 2007). In addition to serving as transcription activators and repressors, FOX proteins also act as pioneer factors capable of unwinding compacted chromatin rendering it accessible to other factors to bind (Lam *et al.*, 2013). Given the utmost importance of FOX proteins, it comes with no surprise that deregulation of their modes of transcriptional regulation and action will lead to pathological conditions including cancer. Key members of five subfamilies, FOXA, FOXC, FOXP, FOXO and FOXM1 will be discussed below with an emphasis on their emerging roles

in carcinogenesis.

2.1.2 FOXA

FOXA family members include FOXA1, FOXA2 and FOXA3. FOXA proteins are characterized by an N-terminal transactivation domain (TAD), followed by forkhead DNA-binding domain (FHD) and a C-terminal TAD (Lam *et al.*, 2013) (Figure 2.1). FOXA1 and FOXA2 have been demonstrated to be necessary in concert for hepatic specification in mice, suggesting a role in liver development (Lee *et al.*, 2005). FOXA2 is also required for normal bile acid homeostasis as the liver samples of individuals with different cholestatic syndromes harbour markedly reduced FOXA2 expression (Bochkis *et al.*, 2008). A recent study reported FOXA3 regulates adipocyte differentiation and depot-selective fat tissue expansion (Xu *et al.*, 2013). FOXA1 is also capable of regulating steroid hormone signaling as a pioneer factor by inducing chromatin modifications and the subsequent recruitment of other transcription factors (Gao *et al.*, 2003; Lupien *et al.*, 2008).

Intriguingly, FOXA1 and FOXA2 play differential roles in males and females during hepatocarcinogenesis in that they cooperate with estrogen receptor (ERα) to suppress and androgen receptor (AR) to promote liver cancer in females and males respectively (Z. Li *et al.*, 2012). The seemingly paradoxical role of FOXA proteins to serve as both oncoproteins and tumour suppressors has also been documented in other cancer types. For example, although amplification and overexpression of *FOXA1* has been reported

in cancers of lung, prostate, breast and oesophagus (Cancer Genome Atlas Network, 2012; Lin *et al.*, 2002; Robbins *et al.*, 2011), loss of FOXA1 is associated with high grade, late stage bladder cancer and enhanced tumour proliferation (DeGraff *et al.*, 2012).

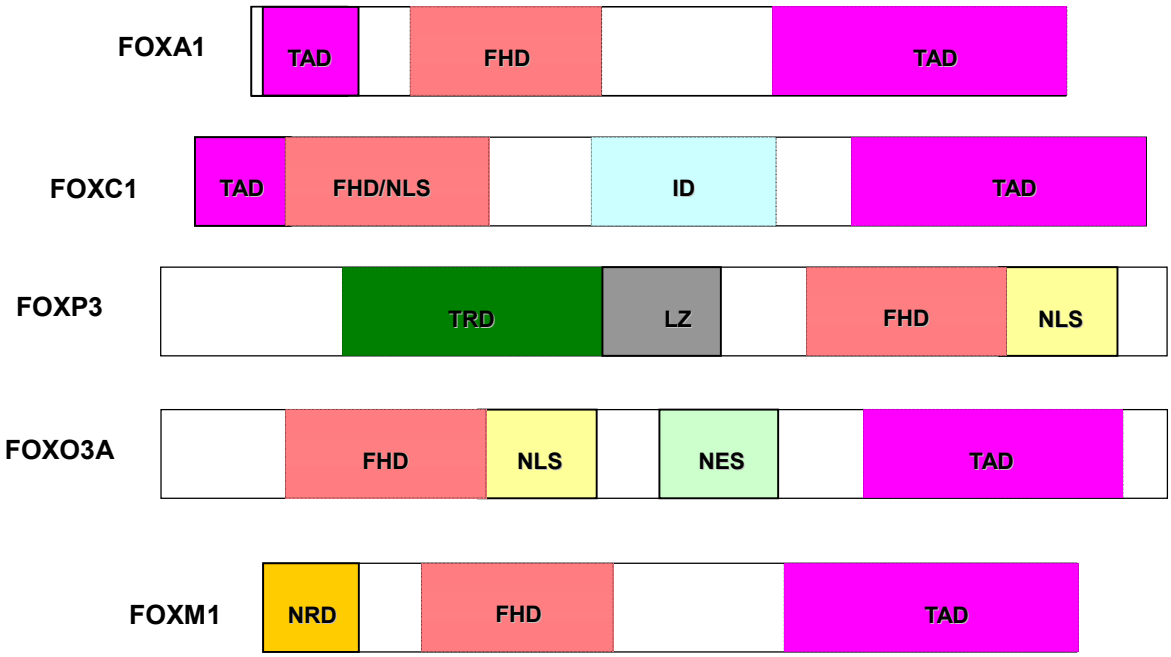


Figure 2.1. Schematic diagram depicting structural organization of FOXA1, FOXC1, FOXP3, FOXO3A and FOXM1 which are typical members of the five subfamilies. FHD: Forkhead binding domain; ID: Inhibitory domain; LZ: Leucine zipper; NES: Nuclear export signal; NLS: Nuclear localization signal; NRD: N-terminal repressor domain; TAD: Transactivation domain; TRD: Transcriptional repressor domain. Modified from (Lam *et al.*, 2013).

2.1.3 FOXC

FOXC family includes FOXC1 and FOXC2 that contain an N-terminal transactivation domain, forkhead binding domain followed by inhibitory domain and a C-terminal transactivation domain (Figure 2.1). FOXC1 and FOXC2 play interactive pivotal roles in the early processes of heart development by acting upstream of the Tbx1-FGF signaling cascade during the morphogenesis of the outflow tract (Seo & Kume, 2006). FOXC1 is also expressed by brain pericytes during development and is crucial for pericyte regulation of vascular development in the fetal brain (Siegenthaler *et al.*, 2013). Overexpression of FOXC2 has been shown to stimulate osteogenic differentiation and inhibit aipogenic differentiation in bone marrow mesenchymal stem cells (You *et al.*, 2014).

FOXC proteins are also implicated in promoting EMT and carcinogenesis. EMT is a key developmental program that is often activated during cancer invasion and metastasis. Mani *et al* reported the involvement of FOXC members in EMT and maintenance of stem cell properties, suggesting FOXC proteins participate in the development of cancer (Mani *et al.*, 2008). This is consistent with a recent finding establishing FOXC2 expression as a link between EMT and stem cell properties in breast cancer (Hollier *et al.*, 2013). FOXC1 expression predicts poor survival of breast cancer patients and may serve as a potential molecular therapeutic target in basal-like breast cancer (Ray *et al.*, 2010). Elevated expression of FOXC1 has been demonstrated to be associated with poor clinical outcome for non-small cell lung

cancer patients and pancreatic ductal adenocarcinoma patients (Wang *et al.*, 2013; Wei *et al.*, 2013). FOXC2 has recently been shown to be critical for resveratrol-mediated suppression of lung cancer progression (Yu *et al.*, 2013).

2.1.4 FOXP

FOXP proteins consist of FOXP1, FOXP2, FOXP3 and FOXP4. FOXP proteins are characterized by transcription repressor domain, leucine zipper followed by forkhead binding domain and nuclear localization signal (Figure 2.1). Similar to FOXA proteins, FOXP proteins have also been shown to possess both oncogenic and tumour suppressing properties. For example, although elevated expression of FOXP1 is associated with improved survival in patients with non-small cell lung cancer (Feng *et al.*, 2012), down-regulation of FOXP1 has been reported in endometrial and prostate cancer (Giatromanolaki *et al.*, 2006; Takayama *et al.*, 2008).

Mutations in *FOXP2* are the only known cause of developmental speech and language disorders in humans. Using chromatin immunoprecipitation coupled with microarray analysis, Spiteri *et al* provided the first insight into the functional network of genes directly regulated by FOXP2 in human brain (Spiteri *et al.*, 2007). The presence of leuzine zippers enables FOXP2 to heterodimerize and cooperate with FOXP1 in the modulation of lung and oesophageal development (Shu *et al.*, 2007). Elevated FOXP2 mRNA and protein expressions have been detected in lymphoma and multiple myeloma-derived cell lines (Campbell *et al.*, 2010). Recently, overexpression of

FOXP2 has been associated with advanced tumour stage and lymph node metastasis only in ERG fusion-negative prostate cancer, but not in the ERG fusion-positive cancer, implicating the paradoxical role of FOXP2 (Stumm *et al.*, 2013).

FOXP3 has long been established to play a pivotal role in the normal functioning of CD4+CD25+ regulatory T (T_{reg}) cells and mutations in *FOXP3* are associated with inherited autoimmune diseases (Ouyang *et al.*, 2010). FOXP3 also participates in normal reproductive function and it has been reported *FOXP3* mutant mice harbour significantly reduced expression of pituitary gonadotropins (Jasurda *et al.*, 2013). Zuo *et al* reported in a recent intriguing study that FOXP3 serves as an X-linked breast cancer suppressor gene (Zuo *et al.*, 2007a). The authors further established FOXP3 as a novel transcriptional repressor for the breast cancer oncogene *SKP2* (Zuo *et al.*, 2007b). Subsequent investigations revealed up-regulation of FOXP3 is capable of inhibiting cell proliferation, migration and invasion of epithelial ovarian cancer cells (Zhang & Sun, 2010).

FOXP4 is necessary for normal T cell cytokine recall responses to antigen following pathogenic infection (Wiehagen *et al.*, 2012). FOXP4 and FOXP2 are also progressively expressed upon neural differentiation in the spinal cord thus essential for the regulation of neuroepithelial character as well as progenitor maintenance in the central nervous system (Rousso *et al.*, 2012). Dysregulation of FOXP4 has been documented in various cancer types including prostate cancer (Takata *et al.*, 2010).

2.1.5 FOXO

FOXO family members include FOXO1 (FKHR), FOXO3A (FKHRL1), FOXO4 (AFX) and FOXO6. FOXO proteins contain the forkhead binding domain, nuclear localization signal, followed by nuclear export signal and a C-terminal transactivation domain (Figure 2.1). FOXO transcription factors are responsible for the regulation of a broad spectrum of biological functions, including cell proliferation, apoptosis, differentiation and metabolism. Dysregulations of FOXOs have been implicated in multiple bodily disorders such as diabetes mellitus, neurodegeneration and cancer (Monsalve & Olmos, 2011).

FOXO1 plays a critical role in normal vascular development by enabling endothelial cells to respond properly to a high dose of vascular endothelial growth factor (VEGF) (Furuyama *et al.*, 2004). FOXO1 also cooperates with other transcription factors in the regulation of hepatic gluconeogenesis (Oh *et al.*, 2013). FOXO4 has been shown to be necessary for neural differentiation of human embryonic stem cells (Vilchez *et al.*, 2013). In addition to promoting memory consolidation by regulating a program coordinating neuronal connectivity in the hippocampus (Salih *et al.*, 2012), FOXO6 is also an important regulator of hepatic glucose metabolism in response to insulin or physiological cues (Kim *et al.*, 2013). Not surprisingly, FOXO1, FOXO4 and FOXO6 have all been involved in the development of cancer. For example, the anti-apoptotic function of miR-96 in prostate cancer is mediated by inhibiting FOXO1 (Fendler *et al.*, 2013). FOXO1 is also able to mediate cisplatin resistance in gastric cancer cells by

activating the PI3K/Akt pathway (Park *et al.*, 2013). FOXO4 has been documented to mediate the oncogenic functions of miR-421 in human nasopharyngeal carcinoma (Chen *et al.*, 2013). Although FOXO6 has garnered relatively less attention as compared to other FOXO members, recent evidence suggests an oncogenic role of FOXO6 in promoting gastric cancer cell tumorigenicity through up-regulation of c-Myc (Qinyu *et al.*, 2013).

FOXO3A is an important member of the FOXO family of transcription factors. It is an established *bona fide* tumour suppressor involved in a variety of anti-tumour functions such as cell cycle arrest, DNA damage repair and apoptotic cell death. In particular, $p27^{Kip1}$, Gadd45α and Bim are direct gene targets of FOXO3A involved in these processes. It has been documented that FOXO3A is a regulator of ERα and its overexpression results in a reduction of ER-mediated tumour growth in ER-positive breast cancers (Zou *et al.*, 2008). However, emerging evidence has uncovered a flip side of FOXO3A (Lam *et al.*, 2012). Persistent activation of FOXO3A by doxorubicin in multi-drug resistant leukemia cells is capable of promoting survival and drug resistance through the hyperactivation of PI3K/Akt signaling pathway (Hui *et al.*, 2008). Previous finding on the exclusive nuclear accumulation of FOXO3A in doxorubicin resistant breast cancer cells is supported by a recent study in thyroid cancer thereby providing further evidence on the uncoupling of FOXO3A from the PKB/Akt signaling cascade (Chen *et al.*, 2010; Marlow *et al.*, 2012).

2.1.6 FOXM1

FOXM1 is one of the best characterized member of the FOX proteins and contains an N-terminal repressor domain, forkhead binding domain and a C-terminal transactivation domain (Figure 2.1). While the FOXO family of transcription factors behave as tumour suppressors, FOXM1 functions like a classic oncogene. FOXM1 is critical to a broad spectrum of biological functions including cell differentiation, apoptosis, angiogenesis, senescence, tissue homeostasis, cell cycle progression and DNA damage repair (Myatt & Lam, 2007). Overexpression of FOXM1 has been implicated in cancers of the liver, prostate, breast, lung and colon etc. (Myatt & Lam, 2007; Pilarsky *et al.*, 2004). Several independent gene expression profiling studies have consistently identified FOXM1 as one of the most commonly up-regulated genes in human solid tumours (Okabe *et al.*, 2001; Pilarsky *et al.*, 2004; Uddin *et al.*, 2011), suggesting a pivotal role of FOXM1 in carcinogenesis. Consistent with this notion, FOXM1 is capable of inducing the expansion of stem cell compartments, leading to the initiation of hyperplasia during tumorigenesis (Zhao & Lam, 2012).

In addition to initiating the development of cancer, FOXM1 also plays a central role in cancer progression by promoting the acquisition of stem cell (SC) and EMT phenotypes. Accordingly, FOXM1 enhances EMT and SC compartment expansion through activating the expression of mesenchymal/stem cell markers such as Snail2, E-cadherin and vimentin which eventually give rise to enhanced cell proliferation, self-renewal, cell migration, angiogenesis as well as drug resistance (Bao *et al.*, 2011).

Furthermore, it has been demonstrated FOXM1-deficient cells generally display polyploidy, aneuploidy and chromosome missegregation and an increase in the number of DNA breaks, highlighting FOXM1 in the maintenance of genomic integrity (Laoukili *et al.*, 2005; Laoukili *et al.*, 2007).

Accumulating evidence suggests FOXM1 serves a crucial role in drug responsiveness and resistance (Chen *et al.*, 2010; Francis *et al.*, 2009; Myatt & Lam, 2007). For instance, deregulated FOXM1 expression contributes to resistance of chemotherapeutic agents, such as cisplatin and epirubicin, and safeguards cells against DNA-damage induced cell death (Francis *et al.*, 2009; Kwok *et al.*, 2010; Millour *et al.*, 2011). The checkpoint kinases Chk1 and Chk2 phosphorylate and activate FOXM1 upon DNA damage, which in turn induces the expression of genes crucial for homologous recombination such as BRCA2, XRCC1 and PLK4 (Park *et al.*, 2012; Tan *et al.*, 2007). As a consequence, cancer cells with deregulated FOXM1 expression become more proficient in DNA-damage repair, resulting in resistance to genotoxic therapeutics. FOXM1 also participates in the acquisition of endocrine resistance in breast cancer (Millour *et al.*, 2010). This is attributable to the fact that FOXM1 is subjected to transcriptional regulation of ERα, and that FOXM1 also regulates ERα expression in breast cancer cells (Madureira *et al.*, 2006). The mutual regulation culminates in a positive forward feeding loop that contributes to tumorigenesis and hormone-insensitivity in endocrine-related malignancies (Madureira *et al.*, 2006).

2.1.7 FOXO-FOXM1 axis in tumorigenesis and drug resistance

Intriguingly, FOXM1 and FOXO3A possess antagonistic functions in the regulation of target genes in that genes activated by FOXM1 are often suppressed by FOXO3A (Koo *et al.*, 2012). FOXM1 is a direct transcriptional target inhibited by FOXO proteins and a pivotal effector of the PI3K-AKT-FOXO signaling axis in the mediation of multiple biological processes including cell migration and differentiation (Francis *et al.*, 2009; McGovern *et al.*, 2009; Wilson *et al.*, 2011) (Figure 2.2). FOXO and FOXM1 are also indirect targets of several widely adopted chemotherapeutic agents. For example, cytotoxic drugs such as doxorubicin and cisplatin function partly by targeting the PI3K-AKT-FOXO-FOXM1 axis (Lam *et al.*, 2013) (Figure 2.2). Given the vital roles of FOXO and FOXM1 in the normal functioning of anti-cancer drugs, it is not surprising that deregulation of this functional axis results in the acquisition of drug resistance.

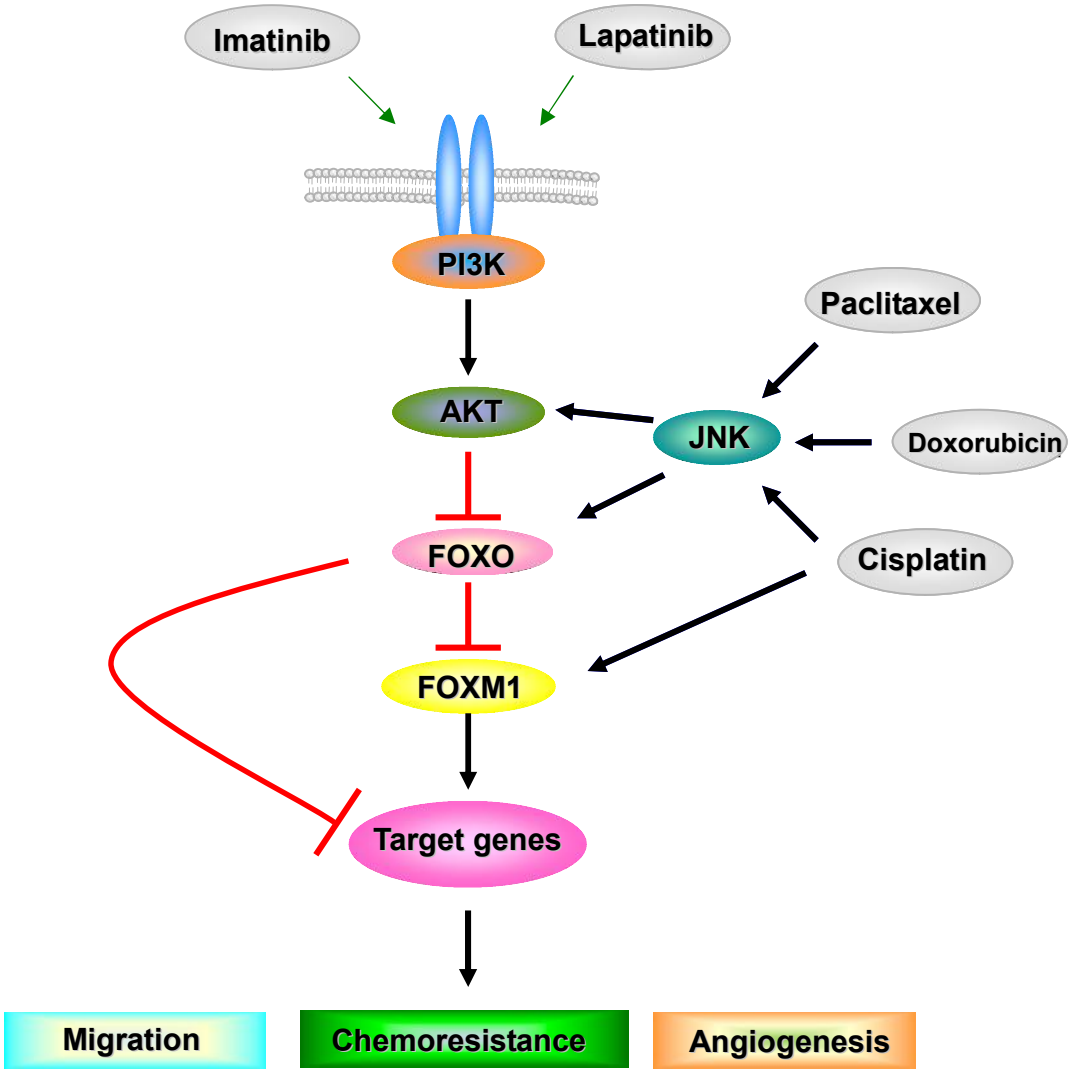


Figure 2.2. Targeting the FOXO-FOXM1 axis in cancer therapeutics. Chemotherapeutic drugs have various modes of action but ultimately integrate signals with the PI3K-AKT-FOXO-FOXM1 signaling cascade. FOXO and FOXM1 then exert antagonistic functions in the regulation of target genes, which in turn control cancer-related processes including drug resistance, angiogenesis and migration. Modified from (Zhao & Lam, 2012).

2.1.8 Aim:

1. To characterize the expression profile of FOXM1 in ovarian cancer tissue samples

2. To determine if FOXM1 expression correlates with clinicopathological parameters

3. To study if FOXM1 affects migration and invasion of ovarian cancer cells

4. To elucidate the role of FOXM1 in the acquisition of paclitaxel resistance in ovarian cancer

2.2 Materials and methods

2.2.1 Clinical samples and cell lines

Archival paraffin embedded tissue blocks from year 1987 to 2002 were retrieved from the Department of Pathology, Queen Mary Hospital, the University of Hong Kong. The samples included 2 benign cystadenomas, 2 borderline tumours, 94 primary carcinomas and 21 metastatic foci of cancer (at ligament, gut, lymph node and uterine serosa). All patients underwent surgery followed by the standard first-line chemotherapy including platinum/paclitaxel. The follow-up period ranged from 5 to 209 months (median 63 months). The use of these samples was approved by the Institutional Ethical Review Board. Each patient sample was assessed by pathologists and ensured to contain more than 70% tumour cells.

Ovarian cancer cell lines SKOV-3 and OVCAR-3 were purchased from American Type Culture Collection (Manassas, VA, USA). SKOV3-TR cells were a generous gift from Dr Lawrence XF Le (Division of Cancer Medicine, University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA) (Siu, *et al.*, 2010a). OVCAR-3 was cultured in 1:1 Medium 199 (Invitrogen, CA, USA): MCDB105 (Sigma, MO, USA) supplemented with 10% foetal bovine serum (FBS) and 100 units/ml penicillin-streptomycin (Invitrogen). SKOV-3 and SKOV3-TR were cultured in RPMI1640 (Sigma) supplemented with 10% foetal bovine serum (FBS) and 100 units/ml penicillin-streptomycin (Invitrogen). All cell lines were maintained at 37°C in humidified incubator with 5% CO_2. Cell culture medium was changed every 3 to 5

days depending on cell density. For routine passage, when cells reached 85% to 90% confluency, they were split at a ratio of 1:4.

2.2.2 Immunohistochemistry

Immunohistochemistry was performed as described previously (F. Zhao *et al.*, 2011). Briefly, formalin-fixed paraffin sections were stained with anti-FOXM1 antibody (NBP1-30961, 1:40, Novus Biologicals, CO, USA) using EnVision+ Dual Link System (K4061; Dako, CA, USA). Antigen retrieval was performed using EDTA buffer, pH8.0, in a pressure cooker for 30 min. Benign cystadenomas with no FOXM1 expression were included as negative tissue control. All sections were assessed by two independent investigators. The immunoreactivity of FOXM1 antibody, the intensity of stained cells and their percentages were measured in terms of intensity and percentage scores respectively. The percentage score ranged from 0 to 4: 0 = <5% of positively stained cells, 1 = 5-25% of positively stained cells, 2 = 26-60% of positively stained cells, 3 = 61-85% of positively stained cells, and 4 = 86-100% of positively stained cells. Immunohistochemical (IHC) score (from 0 to 16) was calculated by multiplying the intensity score (0-4) and the percentage score (0-4), with a maximum score of 16 (Siu *et al.*, 2010a; F. Zhao *et al.*, 2011). FOXM1 nuclear and cytoplasmic immunoreactivities were scored separately.

2.2.3 Western blot

Cells were harvested with lysis buffer [0.125 m Tris, pH 6.8 at 22°C containing 1%

NP-40 (v/v), 2 mM ethylenediamine tetraacetic acid (EDTA), 2 mM N-ethylmaleimide, 2 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM sodium orthovanadate and 0.1 μm sodium okadate] and centrifuged at 4°C for 10 min. Protein concentration was determined by detergent-compatible (DC) protein assay (Bio-Rad). Twenty micrograms of protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane and hybridized with the following anti-bodies: anti-FOXM1 (sc-502, Santa Cruz Biotechnology, CA, USA), anti-Caspase 9 (9502, Cell Signaling Technology, MA, USA), anti-Caspase 7 (9492, Cell Signaling) and anti-β-tubulin (sc-9104, Santa Cruz Biotechnology).

2.2.4 Transient knockdown of FOXM1

ON-TARGET Plus Human FOXM1 siRNA and Non-targeting Control siRNAs (Thermo Scientific, CO, USA) were employed for transient silencing of FOXM1 using Oligofectamine transfection reagent (Invitrogen). Briefly, for each well of a 6-well plate of cells at 80-90% confluency, two reaction mixes were prepared. Mix 1 contains 60μl of Opti-MEM Reduced Serum Medium (Gibco) and 5μl of Oligofectamine transfection reagent, whereas mix 2 contains 250μl of Opti-MEM Reduced Serum Medium, 7.5μl of 1X siRNA buffer (Dharmacon) and 7.5μl of 20μM non-targeting control or FOXM1 siRNA. Mix 2 was added to mix 1 and the resulting mixture was left at RT for 25 min, before being diluted with 170μl of Opti-MEM Reduced Serum Medium. 500μl of the reaction mixture was then added to each well

and 2ml of RPMI1640 medium was added after an incubation of 4 h.

2.2.5 *In Vitro* migration and invasion assays

In Vitro migration and invasion assays were performed as described previously (Liao *et al.*, 2009). Briefly, $1.25x10^5$ cells were plated on the upper compartment of a Transwell chamber (Corning Life Sciences, MA, USA). For migration assays, cells were allowed to migrate through a gelatin-coated membrane. For invasion assays, cells were allowed to invade through a matrigel-coated membrane. After 24 h, cells on the upper side of the membrane were removed and the migrated or invaded cells were fixed, stained and counted.

2.2.6 TdT-mediated dUTP nick end labeling (TUNEL) assay and evaluation of mitotic catastrophe index

Following FOXM1 knockdown for 48h and paclitaxel treatment (50nM) for 24h, TUNEL assay was performed using In Situ Death Detection Kit (Roche Biochemical, IN, USA) following the manufacturer's protocol (Liao *et al.*, 2009). Apoptotic and mitotic catastrophe figures were assessed under fluorescence microscopy. Mitotic catastrophe figures were observed by morphological changes in nuclei (DAPI staining) (Jiang *et al.*, 2011). More than 1000 viable cells in each experiment were examined and the mitotic catastrophe index was evaluated as percentages of the cells counted. Every assay was run in triplicate.

2.2.7 Cell cycle analysis

Cell cycle analysis was performed by propidium iodide staining as described previously (Kwok *et al.*, 2008). Briefly, both adherent and suspension cells were harvested and stained with propidium iodide (1mg/mL) in the presence of DNase-free RNase for flow cytometric analysis. Cell cycle profile was analyzed by using the Cell Diva software (Becton Dickinson UK Ltd.).

2.2.8 Statistical analysis

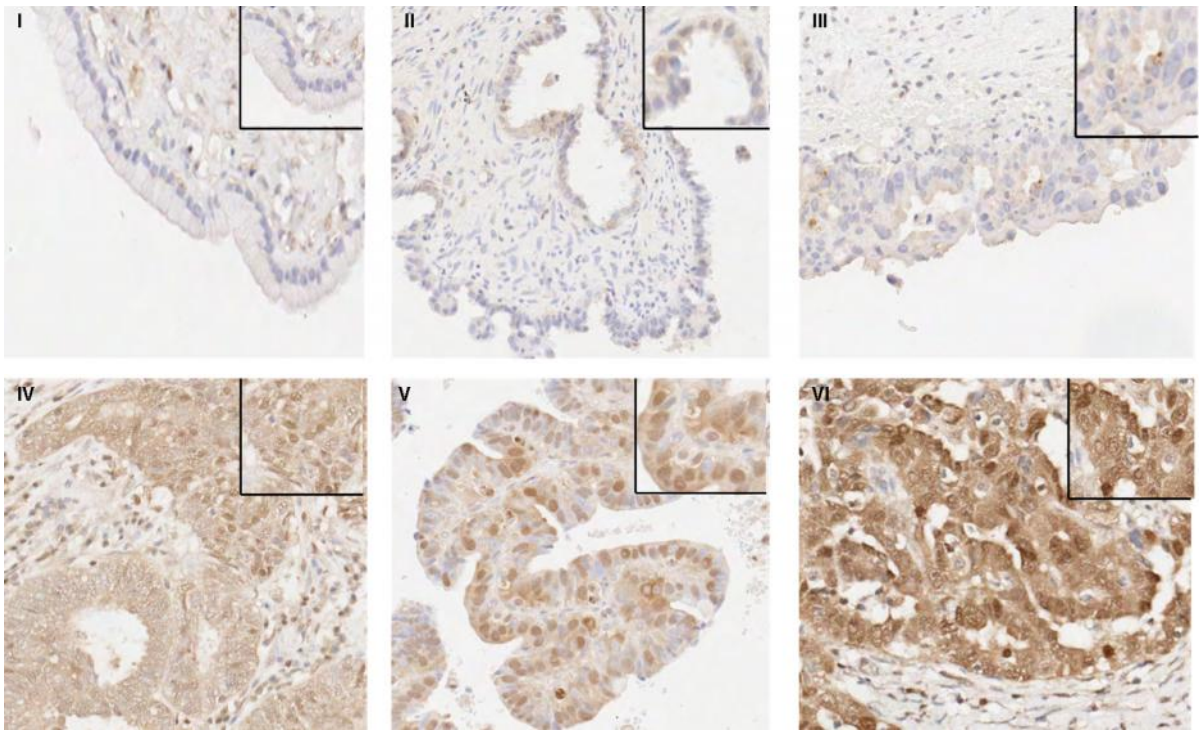
Statistical analysis was performed by the Statistical Package for Social Science version 19.0 for Windows (IBM). Comparison between two groups of non-parametric data was performed by Mann-Whitney *U*-test. Probability of survival was analyzed using the Kaplan-Meier approach without stratification to other clinical and pathological parameters such as stage and grade. Multivariate analysis taking other parameters into consideration was performed using Cox's regression model. *P*-values of <0.05 were considered to be statistically significant.

2.3 Results

2.3.1 FOXM1 overexpression correlates with poor survival

The expression of FOXM1 in ovarian benign and borderline tumours as well as invasive cancers was evaluated by immunohistochemistry. Ovarian cancers displayed stronger nuclear FOXM1 staining than benign and borderline tumours. However, there was no significant difference in FOXM1 expression between primary carcinomas and their metastatic foci. Higher nuclear FOXM1 expression was significantly associated with advanced stages of ovarian cancer (P=0.035) (Figure 2.3 Upper panel). Although not reaching statistical significance, FOXM1 overexpression displayed a trend related to serous histological type (P=0.142), high grade cancers (poor differentiation) (P=0.235) and chemoresistance (P=0.282) (Table 2.1). In order to study the association of FOXM1 expression with patients' outcome, patients were categorized into two groups using the cutoff point of IHC score >0. As shown in the Kaplan-Meier overall survival plot (Figure 2.3 Lower panel), patients with a low FOXM1 level had a significantly longer overall (P=0.019) and disease-free survival (P=0.014) than those with high FOXM1 expression. Multivariate progression analysis showed high expression of FOXM1, advanced cancer stages and poor histological differentiation (high grade) were found to be independent prognostic factors for short overall survival (95% CI, 0.906-5.754, P=0.08; 95% CI, 0.953-5.963, P=0.06; 95% CI, 2.137-40.638, P=0.003, respectively) and disease-free survival (95% CI, 1.025-6.631, P=0.04; 95% CI, 1.039-6.555, P=0.04; 95% CI, 1.821-35.091, P=0.006, respectively). Interestingly, cytoplasmic staining of FOXM1 was also detected in

addition to nuclear expression, but no significant correlation with clinicopathological parameters was observed.



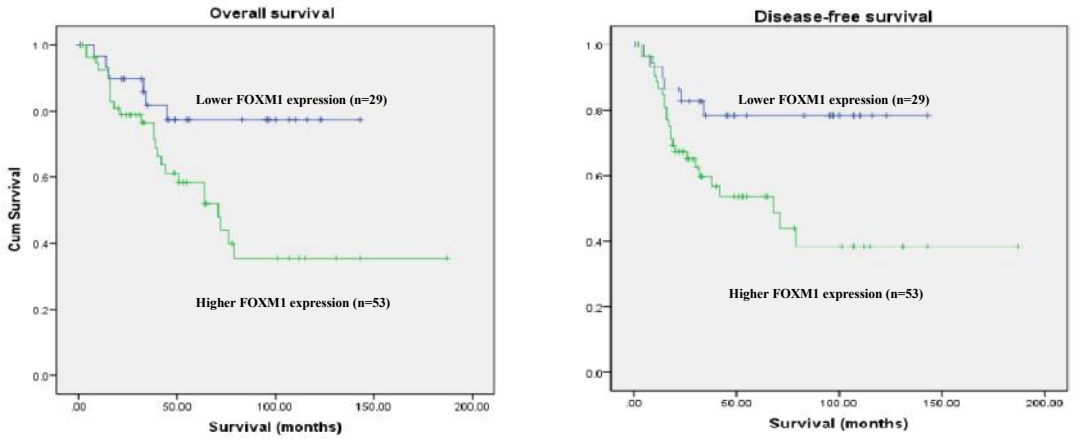


Figure 2.3. Elevated nuclear FOXM1 expression was associated with advanced stages and poor patient survival of ovarian cancer. Upper panel: Representative images of Immunoreactivity of nuclear FOXM1 in (I) benign cystadenoma (staining intensity 0, staining percentage 0, IHC score 0), (II) borderline cystadenoma (staining

intensity 2, staining percentage 1, IHC score 2), (III) stage I invasive cancer (staining intensity 0, staining percentage 0, IHC score 0), (IV) stage II invasive cancer (staining intensity 4, staining percentage 1, IHC score 4), (V) stage III invasive cancer (staining intensity 4, staining percentage 2, IHC score 8) and (VI) stage IV invasive cancer (staining intensity 4, staining percentage 3, IHC score 12). Magnifications X400. Insets: Regions with higher magnifications of nuclear FOXM1 staining. **Lower panel**: Cumulative overall and disease-free survival plots using the Kaplan-Meier approach.

Table 2.1. Summary of nuclear FOXM1 immunohistochemical staining results in various types of ovarian tumours.

		Nuclear expression of FOXM1					
		Absent (IHC=0)	Weak (IHC=1-3)	Moderate (IHC=4-6)	Strong (IHC=7-12)	Total	p-value
Diagnostic Categories	Benign	1 (50%)	1 (50%)	0 (0%)	0 (0%)	2	
	Borderline	0 (0%)	2 (100%)	0 (0%)	0 (0%)	2	
	Invasive cancer	31 (33%)	28 (29.8%)	25 (26.6%)	10 (10.6%)	94	
	Metastatic foci	3 (14.3%)	8 (38.1%)	6 (28.6%)	4 (19%)	21	
Histological Types	Mucinous	3 (33.3%)	4 (44.4%)	1 (11.1%)	1 (11.1%)	9	
	Serous	11 (31.4%)	7 (20%)	11 (31.4%)	6 (17.1%)	35	0.142*
	Endometrioid	14 (45.2%)	10 (32.3%)	7 (22.6%)	0 (0%)	31	
	Clear cell	4 (21.1%)	7 (36.8%)	5 (26.3%)	3 (15.8%)	19	
Stages	Stage I	15 (41.7%)	11 (30.6%)	9 (25%)	1 (2.78%)	36	
	Stage II-IV	12 (24%)	13 (26%)	16 (32%)	9 (18%)	50	0.035†
Grades	Grade I	10 (41.7%)	8 (33.3%)	5 (20.8%)	1 (4.17%)	24	
	Grades II-III	22 (31.9%)	19 (27.5%)	19 (27.5%)	9 (13%)	69	0.235‡

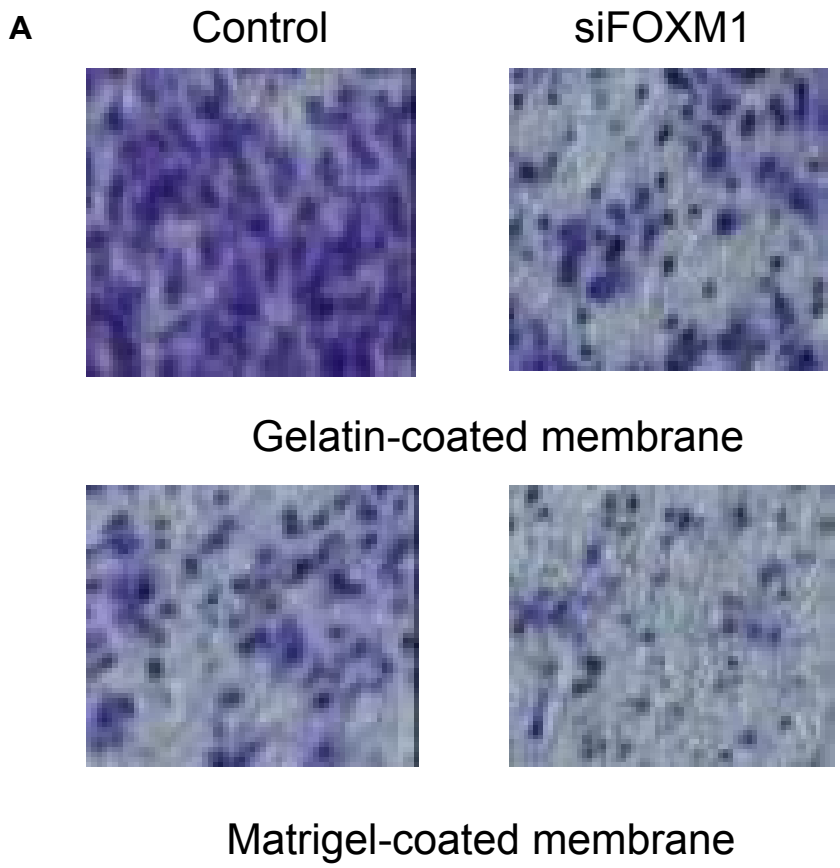
* p value reflects the comparison of serous ovarian cancer vs. all the other histological types combined

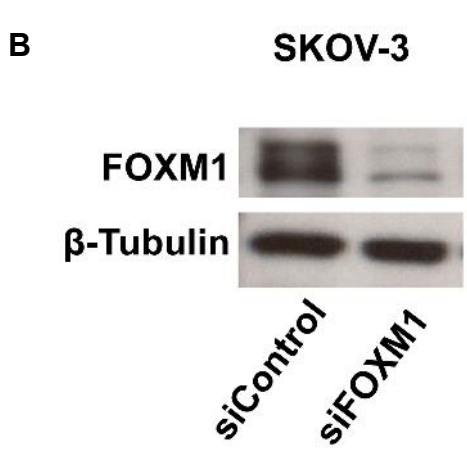
† p value reflects the comparison of stage I vs. stage II-IV

‡ p value reflects the comparison of low grade (grade I) vs. high grade (grade II and III)

2.3.2 Transient knockdown of FOXM1 inhibits SKOV-3 cell migration and invasion

We next studied the role of FOXM1 in ovarian cancer progression. *In Vitro* Transwell assays were employed to study the effects of transient silencing of FOXM1 on ovarian cancer cell motility and invasion. Significantly decreased migration and invasion ($P<0.05$) was observed in SKOV-3 cells transfected with ON-TARGET Plus Human FOXM1 siRNA (siFOXM1) as compared to cells transfected with non-targeting controls, indicating that knockdown of FOXM1 was capable of inhibiting migration and invasion of SKOV-3 cells (Figure 2.4). No changes in cell proliferation were observed after FOXM1 knockdown.


A
Control
siFOXM1
Gelatin-coated membrane
Matrigel-coated membrane


B
SKOV-3
FOXM1
β-Tubulin
siControl
siFOXM1

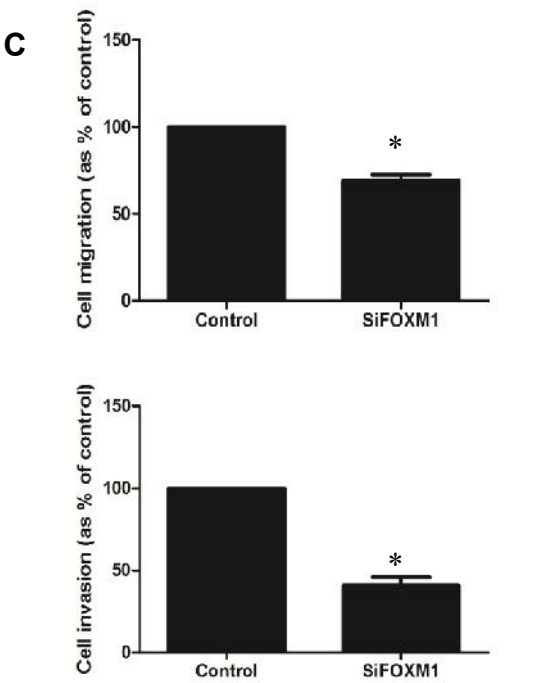

C
Cell migration (as % of control)
150
100
50
0
Control
SiFOXM1
*
Cell invasion (as % of control)
150
100
50
0
Control
SiFOXM1
*

Figure 2.4. Silencing of FOXM1 reduced migration and invasion of SKOV-3. A: Representative images showing cells migrated (gelatin-coated membrane) or invaded (matrigel-coated membrane) after 24 h. **B.** Representative Western blot analysis demonstrating the effectiveness of FOXM1 transient knock-down in SKOV-3. **C.** Graphic representation of migration (left panel) and invasion (right panel) results as fold change of migrated and invaded cells relative to the control, respectively, in five fields of triplicate wells from three independent experiments; * $P<0.05$, significant; Mann-Whitney *U*-test.

2.3.3 Paclitaxel treatment downregulates the expression of FOXM1 in SKOV-3 but not in the paclitaxel-resistant SKOV3-TR

In view of the IHC staining showing that elevated FOXM1 expression is associated with poor prognosis and thus chemoresistance, a pair of established ovarian cancer cell line sensitive and resistant to paclitaxel, namely SKOV-3 and SKOV3-TR respectively, were used to study the effect of paclitaxel treatment on the expression of FOXM1. Cells were treated with paclitaxel (100nM) (Ofir *et al.*, 2002) and harvested at various time points 0, 8, 16, 24, 48 and 72 h. Intriguingly, immunoblotting showed FOXM1 expression to be up-regulated transiently at 8 h and decreased at 48 h and 72 h in SKOV-3. However, FOXM1 expression remained relatively constant at high levels in SKOV3-TR upon paclitaxel treatment (Figure 2.5), suggesting a role of FOXM1 in mediating paclitaxel resistance in ovarian cancer cells. Notably, there also appeared to be only marginal increases in the expression of cleaved Caspase-9 and Caspase-7 in both SKOV-3 and SKOV3-TR upon paclitaxel treatment (Figure 2.5), suggesting that apoptosis may not be the predominant mechanism of inducing cell death in these ovarian carcinoma cells.

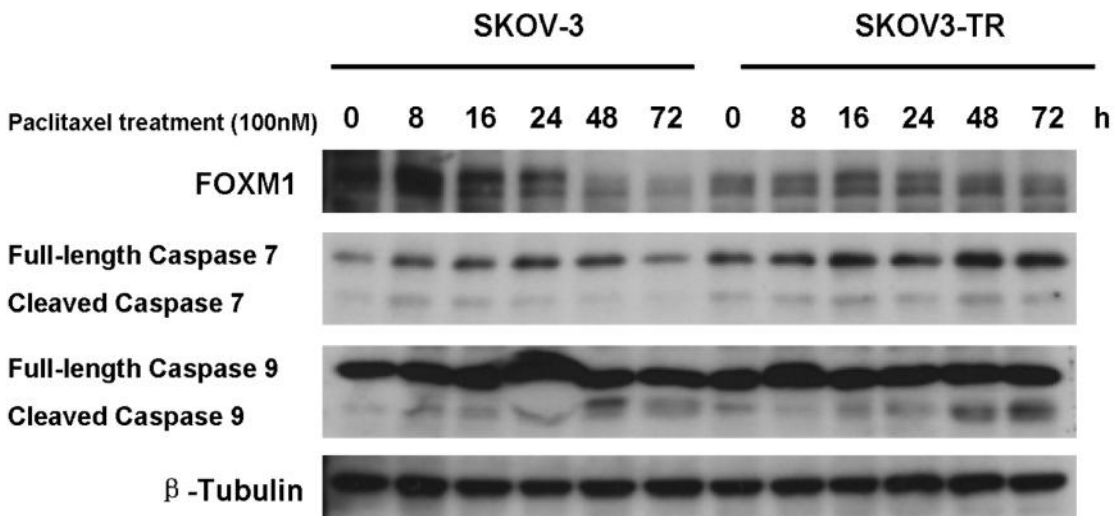
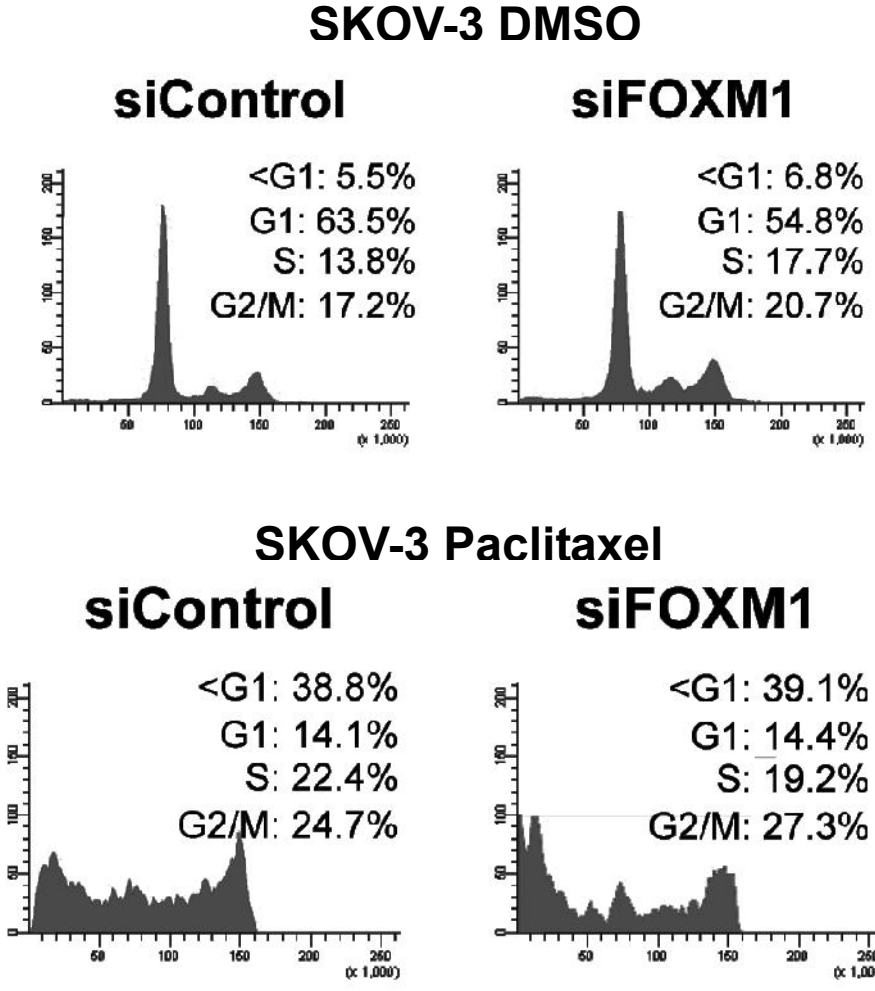


Figure 2.5. Paclitaxel treatment down-regulated FOXM1 expression in SKOV-3 but not in SKOV3-TR cells.

The paclitaxel sensitive SKOV-3 and resistant SKOV3-TR ovarian cancer cells were treated with 100nM paclitaxel and harvested at times indicated for Western blot analysis. Paclitaxel treatment down-regulated FOXM1 expression at time points 48h and 72h in SKOV-3 but not in SKOV3-TR as shown by immunoblotting. There were only marginal increases in the expression of cleaved Caspase-9 and Caspase-7. Representative images were shown from three independent experiments. .

2.3.4 FOXM1 knockdown increases the accumulation of G2/M and dead cells upon paclitaxel treatment in the resistant SKOV3-TR cell lines.

Cell cycle analysis was then performed to investigate the role of FOXM1 in paclitaxel-mediated cell death. To this end, SKOV-3 and SKOV3-TR cells were transiently transfected with control and FOXM1 siRNA for 48 h and cultured in the presence or absence of paclitaxel treatment (100nM) for 48 h. Cells were then harvested, stained with propidium iodide and subjected to flow cytometric analysis. The results showed that paclitaxel induced significant levels of cell death (sub-G1 population) in SKOV-3 cells transfected with either FOXM1 or control siRNA pool (Figure 2.6). Increased number of cells accumulated with sub-G1 DNA contents in SKOV3-TR with FOXM1 knockdown (8.1%) (Figure 2.7) compared to SKOV3-TR transfected with control siRNA (4.2%) (Figure 2.7), suggesting FOXM1 contributes to paclitaxel resistance in ovarian cancer cells and that FOXM1 silencing can induce paclitaxel-mediated cell death. Upon paclitaxel treatment, though not reaching statistical significance, a modest increase in cells blocked at G2/M phase was also observed in SKOV3-TR treated with siRNA against FOXM1 as compared to control, suggesting FOXM1 silencing might enhance paclitaxel-mediated cell death via mitotic catastrophe (Figure 2.7, lower panel). Depletion of FOXM1 in the sensitive SKOV-3 cells has no additive effect to paclitaxel treatment which is likely to be due to the fact that paclitaxel functions through downregulating FOXM1 expression as revealed by the Western blot analysis (Figure 2.5).


SKOV-3 DMSO
siControl
siFOXM1
<G1: 5.5%
G1: 63.5%
S: 13.8%
G2/M: 17.2%
<G1: 6.8%
G1: 54.8%
S: 17.7%
G2/M: 20.7%
SKOV-3 Paclitaxel
siControl
siFOXM1
<G1: 38.8%
G1: 14.1%
S: 22.4%
G2/M: 24.7%
<G1: 39.1%
G1: 14.4%
S: 19.2%
G2/M: 27.3%

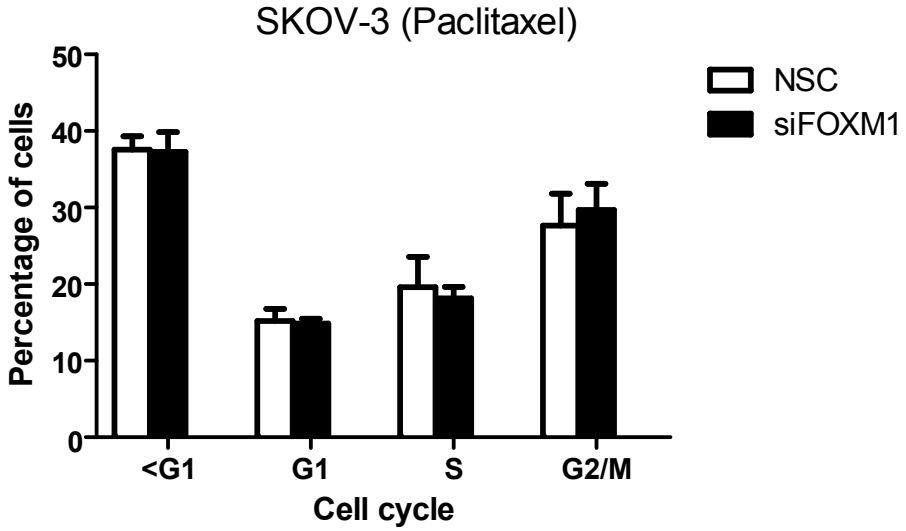
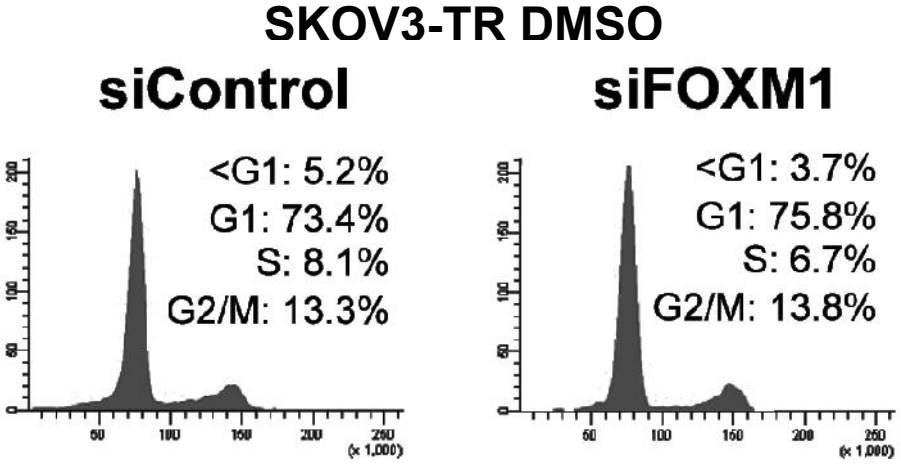
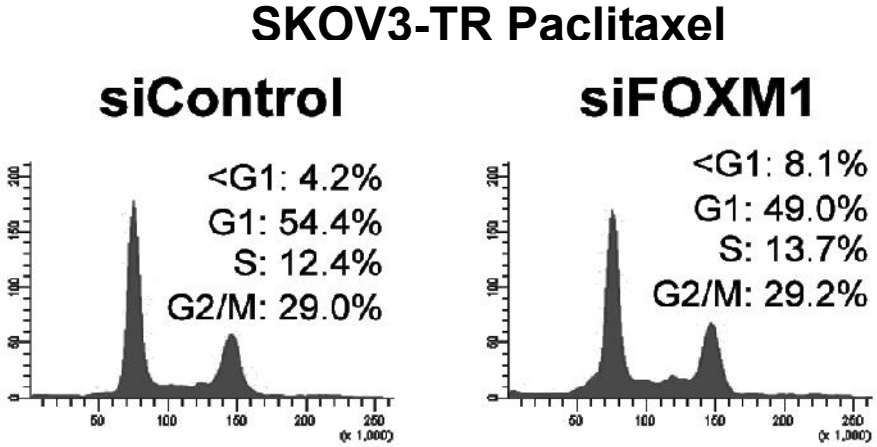

SKOV-3 (Paclitaxel)
NSC
siFOXM1
Percentage of cells
50
40
30
20
10
0
<G1
G1
S
G2/M
Cell cycle

Figure 2.6. Flow cytometric analysis of SKOV-3 cells with and without FOXM1 depletion in the presence or absence of paclitaxel treatment.

Upper panel: Flow cytometric analysis was performed following propidium iodide staining on SKOV-3 cells treated with paclitaxel (100nM) or remained untreated after transfection with siRNA pools against FOXM1 or control siRNA pools. Representative histogram was shown. Lower panel: Bar chart of different phases of cell cycle in SKOV-3 treated with paclitaxel after transfection with control or siRNA against FOXM1. Results represent data from two independent experiments.


SKOV3-TR DMSO
siControl
siFOXM1
<G1: 5.2%
G1: 73.4%
S: 8.1%
G2/M: 13.3%
<G1: 3.7%
G1: 75.8%
S: 6.7%
G2/M: 13.8%
(x 1,000)


SKOV3-TR Paclitaxel
siControl
siFOXM1
<G1: 4.2%
G1: 54.4%
S: 12.4%
G2/M: 29.0%
<G1: 8.1%
G1: 49.0%
S: 13.7%
G2/M: 29.2%
(x 1,000)

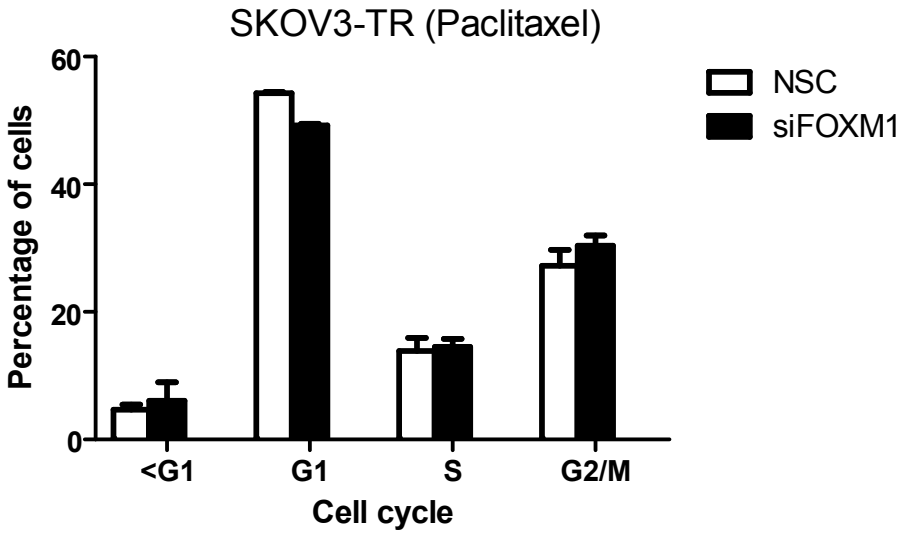

SKOV3-TR (Paclitaxel)
NSC
siFOXM1
Percentage of cells
60
40
20
0
<G1
G1
S
G2/M
Cell cycle

Figure 2.7. Flow cytometric analysis of SKOV3-TR cells with and without FOXM1 depletion in the presence or absence of paclitaxel treatment.

Upper panel: Flow cytometric analysis was performed following propidium iodide staining on SKOV3-TR cells treated with paclitaxel (100nM) or remained untreated after transfection with siRNA pools against FOXM1 or control siRNA pools. FOXM1 silencing is capable of inducing a modest increase in the number of dead cells in sub-G1 population and cells blocked at G2/M cell cycle phase in SKOV3-TR as compared to cells treated with control siRNA. Representative histogram was shown. Lower panel: Bar chart of different phases of cell cycle in SKOV3-TR treated with paclitaxel after transfection with control or siRNA against FOXM1. Results represent data from two independent experiments.

2.3.5 Transient silencing of FOXM1 significantly enhances paclitaxel-mediated mitotic catastrophe

To elucidate the potential role of FOXM1 in ovarian cancer chemoresistance, the SKOV-3 and OVCAR3 ovarian cancer cell lines were treated with paclitaxel for 24 h following FOXM1 depletion by siRNA, subjected to TUNEL assay and examined by fluorescent microscopy. These two cell lines were chosen to study the role of FOXM1 on paclitaxel-mediated mitotic catastrophe (Jiang *et al.*, 2011). The result showed there was an increased number of multi-nucleated cells (arrow) in ovarian cancer cells with FOXM1 knockdown (P=0.03 and 0.01, respectively) as revealed by DAPI staining (Figure 2.8), suggesting FOXM1 depletion significantly enhanced paclitaxel-mediated mitotic catastrophe in both SKOV-3 and OVCAR3. It is notable that apoptosis was barely detectable in these paclitaxel-treated cells as shown by the insignificant positive green staining of TUNEL assay (data not shown) This was probably due to the fact that both SKOV-3 and OVCAR3 harbour dysfunctional p53, and functional p53 is required for paclitaxel-induced apoptosis in ovarian cancer cells.

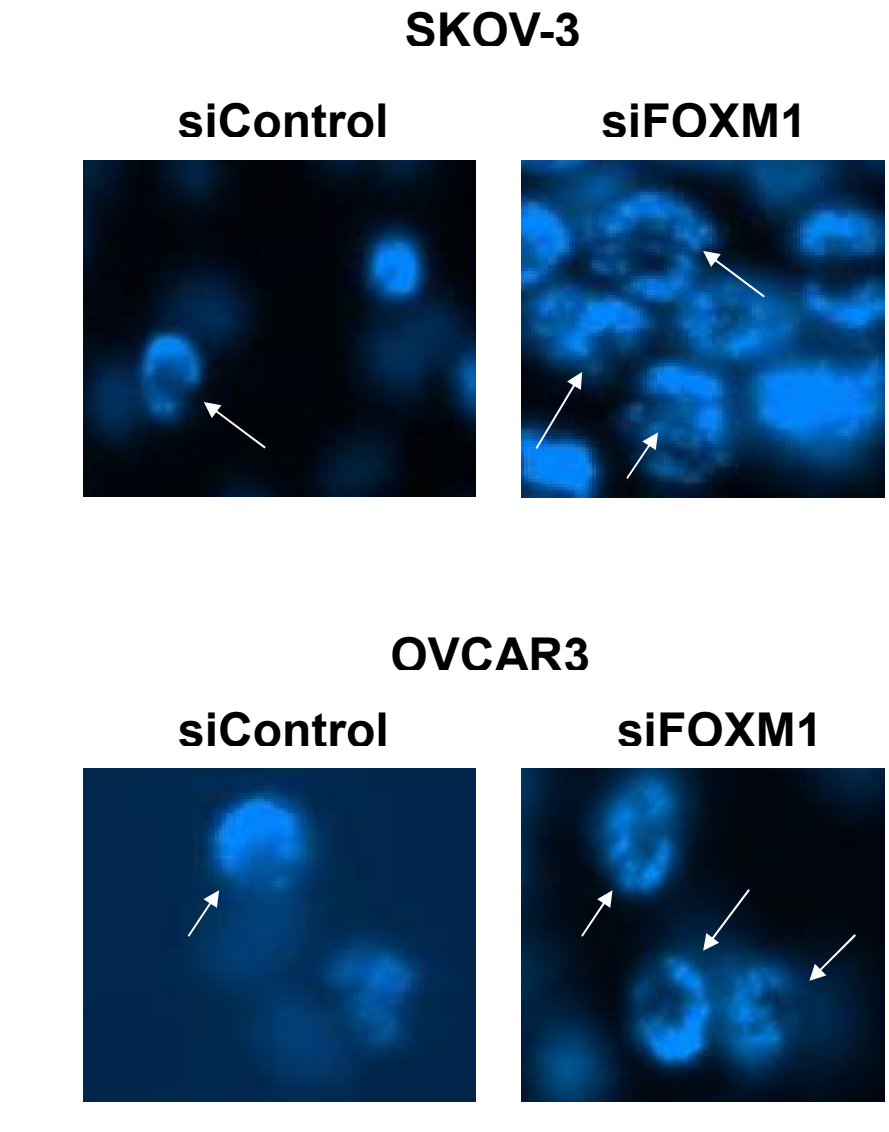

SKOV-3
siControl
siFOXM1
OVCAR3
siControl
siFOXM1
SKOV-3
OVCAR3
% of cells in mitotic catastrophe
45
40
35
30
25
20
15
10
5
0
*
siControl
siFOXM1

Figure 2.8. Transient FOXM1 knockdown significantly enhanced paclitaxel-induced mitotic catastrophe in SKOV-3 and OVCAR3 cells. SKOV-3 and OVCAR3 cells transfected with either siRNA pools against FOXM1 or control siRNA pools were treated with paclitaxel (100nM) and stained with DAPI. **Upper panel:** Representative staining results were shown showing that transient FOXM1 knockdown significantly enhanced paclitaxel-induced mitotic catastrophe (arrow) in SKOV-3 and OVCAR3 cells respectively (Upper panel). Magnifications X400. **Lower panel:** Graphs represent the results of 3 independent experiments, showing the percentage of cells undergoing mitotic catastrophe, * $P<0.05$, significant; Mann-Whitney *U*-test.

2.4 Discussion

This is the first study demonstrating that high nuclear FOXM1 expression is significantly correlated with stage, shorter overall survival and disease-free survival of ovarian cancer patients. Multivariate analysis indicates that FOXM1 expression could serve as an independent prognostic factor. Furthermore, transient FOXM1 depletion is capable of inhibiting ovarian cancer cell migration and invasion. These findings suggest that FOXM1 can have a crucial role in ovarian carcinogenesis and progression and may serve as a prognostic factor to predict patients' outcome.

Although FOXM1's association with high grade ovarian cancer tumours has been reported (Chan *et al.*, 2012), whether FOXM1 contributes to the acquisition of paclitaxel resistance remains undefined. Indeed, deregulated FOXM1 expression has been shown to confer resistance to chemotherapeutic drugs such as cisplatin and epirubicin (Kwok *et al.*, 2010; Millour *et al.*, 2011). This is attributable in part to the regulation of DNA damage repair genes, particularly those involved in homologous recombination, such as *XRCC1*, *BRCA2*, *RAD51* and *BRIP1* (Lam *et al.*, 2013; Monteiro *et al.*, 2013; N. Zhang *et al.*, 2012). Knockdown of FOXM1 is able to re-sensitize drug resistance cancer cells to chemotherapy partly by suppressing the activation of DNA damage repair genes (Lam *et al.*, 2013; N. Zhang *et al.*, 2012). In view of the immunohistochemical finding suggesting association between FOXM1 and chemoresistance, a pair of established paclitaxel-sensitive and -resistant cell lines, SKOV-3 and SKOV3-TR (Jiang *et al.*, 2011), were recruited to study the effect of

paclitaxel on FOXM1. Interestingly, paclitaxel treatment resulted in down-regulation of FOXM1 in SKOV-3 but not in the resistant cell line SKOV3-TR, implying a role of FOXM1 in mediating paclitaxel resistance in ovarian cancer cells. Immunofluorescence study further showed transient FOXM1 knockdown could enhance paclitaxel-mediated cell death in two ovarian cancer cell lines, SKOV-3 (deleted p53) (Hamroun *et al.*, 2006) and OVCAR3 (mutant p53) (Wolf *et al.*, 1999). It is not surprising that apoptotic cells were barely detectable as both cell lines harbour dysfunctional p53 while functional p53 is required for paclitaxel-induced apoptosis in ovarian cancer cells. Recently, it has been suggested that the induction of p53-independent apoptosis takes place through the activation of Caspase-9 (Yamakawa *et al.*, 2008). However, immunoblotting revealed Caspase-9 was only marginally activated upon paclitaxel treatment in SKOV-3 and SKOV-3-TR cells, indicating that both paclitaxel and FOXM1 silencing induce cell death primarily through enhancing mitotic catastrophe rather than apoptosis in ovarian cancer cells, which commonly have dysfunctional p53 pathway. This is in line with previous findings on paclitaxel in breast cancer (Wonsey & Follettie, 2005). Immunofluorescence staining will also be performed in SKOV3-TR in future studies.

Mitotic catastrophe can be considered as a type of cell death occurring during mitosis or resulting from mitotic failure (Castedo *et al.*, 2004). Two mechanisms have been proposed as crucial for mitotic catastrophe, namely the G2/M and mitotic spindle checkpoints (Cahill *et al.*, 1998). For the G2/M checkpoint, the inactivation of genes

such as *p53*, $p21^{Cip1}$ and *14-3-3Sigma* have been reported to induce DNA damage-induced mitotic catastrophe (Bunz *et al.*, 1998; Chan *et al.*, 1999). Flow cytometric analysis performed in our study suggested FOXM1 knockdown in the chemoresistant ovarian cancer cell line SKOV3-TR could induce G2/M arrest and enhance paclitaxel-mediated cell death via mitotic catastrophe in a p53-independent and Caspase-9-independent manner. Delineation of the underlying mechanism would be informative and will be pursued in further studies.

In conclusion, overexpression of FOXM1 was found to be correlated with poor patients' survival and to paclitaxel-mediated mitotic catastrophe in ovarian cancer cells. Our findings help to define FOXM1 as a potential prognostic marker as well as a therapeutic target in ovarian cancer.

Chapter 3

Identification and characterization of KIF2C and KIF20A as novel FOXM1 transcriptional targets

3.1 Introduction

3.1.1 Kinesin superfamily proteins (KIFs)

Intracellular trafficking is of utmost importance to normal cellular morphology and function. After synthesis, lipids and proteins are sorted and transported to designated locations within the cell as membranous organelles or protein complexes, whereas mRNAs are carried in large protein complexes (Hirokawa, 1998). Three superfamilies of molecular motors-kinesins, dyneins and myosins- have so far been identified (Vale, 2003).

First being isolated from squid nervous tissue in 1985 (Vale *et al.*, 1985), kinesin superfamily proteins (KIFs) are ubiquitously present in all eukaryotes. More than 40 KIFs have been identified to date and are classified into 15 families, which are named kinesin 1 to kinesin 14B based on findings of phylogenetic analyses (Miki *et al.*, 2005). These families can be further grouped into three subtypes depending on the location of the motor domain in the molecule. Motor domains are located in N-kinesins, M-kinesins and C-kinesins in the amino-terminal region, the middle region and the carboxy-terminal region respectively (Liu *et al.*, 2013). Many motor kinesins possess adenosine triphosphatase activity and microtubule-dependent

plus-end motion ability. The highly conserved motor domain is responsible for motor binding and stepping across microtubules by converting chemical energy generated from ATP hydrolysis into mechanical force (Liu *et al.*, 2013).

KIFs are not only involved in the intracellular transport of organelles, protein complexes and mRNAs, but also play a crucial role in chromosomal and spindle movements during mitosis and meiosis (Sharp *et al.*, 2000). For instance, the activities of microtubule motors on spindle microtubule are under precise regulation in order to ensure that mitotic events are adequately orchestrated throughout the progression of mitosis. Given the importance of proper sister chromatid separation during mitosis, it comes with no surprise that dysregulated KIF proteins could result in aneuploid daughter cells, eventually leading to the aggressive progression of cancer (Oki *et al.*, 2012). Indeed, accumulating evidence has been reported suggesting many KIF members play critical roles in the genesis and development of various types of cancers.

3.1.2 KIF2A

KIF2A belongs to the kinesin-13 family and is a microtubule depolymerase responsible for ensuring proper bipolar spindle assembly during mitosis (Uehara *et al.*, 2013). KIF2A has been shown to control the length and alignment of central spindle microtubules through depolymerization at their minus ends, and the distribution of KIF2A is regulated by Aurora B activity gradient (Uehara *et al.*, 2013). Recently,

overexpression of KIF2A has been documented in squamous cell carcinoma of the oral tongue (C. Q. Wang *et al.*, 2010). Higher expression of KIF2A is also significantly associated with lymph node metastasis and tumour stage. *In Vitro* Transwell assay further demonstrated KIF2A silencing is capable of retarding the migratory ability of cancer cells (C. Q. Wang *et al.*, 2010). In another study, Li *et al* reported that microRNA-183 (miR-183) directly inhibits the expression of KIF2A and that ectopic expression of miR-183 could lead to significantly reduced cancer cell migration and invasion, suggesting KIF2A plays a pivotal role in cancer metastasis (Li *et al.*, 2010).

3.1.3 KIF4A

KIF4A, a member of the kinesin-4 family, has also been widely reported to be dysregulated in various cancer types. KIF4A expression is associated with shorter survival for patients with non-small cell lung cancer, and KIF4A is capable of regulating the growth of cancer cells as revealed by transient knockdown assays (Taniwaki *et al.*, 2007). However, contradictory findings regarding the role of KIF4A in carcinogenesis have also been documented. For instance, Gao *et al* found overexpression of KIF4A inhibits proliferation of human gastric carcinoma cells both *in vitro* and *in vivo* (Gao *et al.*, 2011). Thus, whether KIF4A possesses oncogenic or tumour suppressing characteristics awaits further investigation.

3.1.4 KIF11

KIF11 (or Eg5) is a member of the most conserved kinesin-5 family. With no exception, KIF11 has also been suggested to contribute to the development of cancer. KIF11 is found to be significantly associated with tumour stage, grade and poor survival for patients with renal cell carcinoma (Sun *et al.*, 2013). Up-regulation of KIF11 has also been reported in pancreatic cancer cells (Sun *et al.*, 2011). Furthermore, overexpression of KIF11 could predict unfavorable prognosis in non-muscle invasive bladder urothelial carcinoma (Ding *et al.*, 2011).

3.1.5 KIF14

KIF14, a member of the kinesin-3 family, plays a crucial role in the cytokinesis of eukaryotic cells. KIF14 is dysregulated in primary cancers including lung, breast, hepatocellular carcinoma and retinoblastoma cancer (Basavarajappa & Corson, 2012). In lung cancer, KIF14 overexpression is associated with shorter overall- and disease-free survival, suggesting KIF14 could serve as an independent prognostic marker (Corson *et al.*, 2007). Ahmed *et al* reported that KIF14 negatively regulates Rap1a-Radil signaling during breast cancer progression and that depletion of KIF14 could lead to enhanced cell spreading, altered focal adhesion dynamics as well as inhibition of cell migration and invasion (Ahmed *et al.*, 2012). Intriguingly, KIF14 mRNA has been reported to be an independent prognostic marker in serous ovarian carcinoma (Theriault *et al.*, 2012). In the same study, ectopic expression of KIF14 could increase proliferation and colony formation of ovarian cancer cells, suggesting a

role of KIF14 in promoting tumorigenic phenotype (Theriault *et al.*, 2012).

3.1.6 KIF15

KIF15 belongs to the kinesin-12 family. It has been reported KIF15 bound to kinetochore fibers antagonizes centrosome separation while KIF15 bound to non-kinetochore microtubules mediates centrosome separation (Sturgill & Ohi, 2013). Whether KIF15 contributes to the genesis and development of cancer remains to be answered.

3.1.7 KIFC3

KIFC3, a member of the kinesin-14B family, participates in the apical transport of annexin XIIIb-associated Triton-insoluble membranes and plays a complementary role in Golgi positioning and integration with cytoplasmic dynein (Noda *et al.*, 2001; Xu *et al.*, 2002). A recent study showed ectopic expression of KIFC3 in breast cancer cell lines could make the cells more resistant to docetaxel, indicating a role of KIFC3 in mediating docetaxel resistance in breast cancer cells (De *et al.*, 2009).

3.1.8 KIF20A

KIF20A (or Rab6Kinesin) belongs to kinesin-6 family and is the first KIF to be reported to bind to a Rab GTPase (Liu *et al.*, 2013). Similar to other members of the family, KIF20A is involved in cytokinesis and organelle transport (Liu *et al.*, 2013). Recent findings have been suggesting a pivotal role of KIF20A in the development of

cancer. Transient knockdown of endogenous KIF20A expression in pancreatic ductal adenocarcinoma cell lines has been demonstrated to be capable of drastically attenuating growth of cancer cells, implying a role of KIF20A in pancreatic carcinogenesis (Taniuchi *et al.*, 2005). KIF20A has also been suggested as a novel promising candidate for anticancer immunotherapeutic target for pancreatic cancers (Imai *et al.*, 2011). Furthermore, Gasnereau *et al* reported a strong accumulation of KIF20A mRNA in a large series of human hepatocellular carcinomas, with the highest expression observed in tumours harboring genomic instability (Gasnereau *et al.*, 2012). KIF20A has also been suggested to play a crucial role in mediating the anti-cancer actions of genistein, and may thus be treated as a potential molecular target for drug intervention of gastric cancer (Yan *et al.*, 2012).

3.1.9 KIF20B

KIF20B, also known as M-phase phosphoprotein 1, acts as a mitotic molecular motor crucial for faithful completion of cytokinesis (Abaza *et al.*, 2003). Using a genome-wide expression profile analysis, KIF20B has been demonstrated to be significantly up-regulated in bladder cancer samples. In the same study, transient knockdown of KIF20B resulted in a drastic increase in multinucleated cells and cancer cell death, suggesting a role of KIF20B in the tumorigenesis of bladder cancer (Kanehira *et al.*, 2007). Recently, KIF20B has also been reported to contribute to tumour cell growth and failed mitotic arrest (Liu *et al.*, 2013).

3.1.10 KIF2C

Being the founding and best-characterized member of the kinesin-13 family, KIF2C/MCAK harbours an N-terminal domain, a positively charged neck followed by the conserved kinesin motor domain and a C-terminal dimerization domain (Sanhaji *et al.*, 2011). Unlike other kinesin proteins, KIF2C depolymerizes microtubules by disassembling tubulin units from the polymer end using energy harnessed from ATP hydrolysis instead of moving directionally along microtubules (Liu *et al.*, 2013). This depolymerizing activity is of utmost importance in spindle formation, correcting erroneous attachments of microtubule-kinetochore and in chromosome movement. Therefore, an adequately orchestrated regulation of KIF2C is crucial for ensuring the faithful segregation of chromosomes in mitosis and for safeguarding chromosomal stability (Maney *et al.*, 1998). Recent studies indicated KIF2C is subjected to a complex spatio-temporal regulation during mitosis by Aurora B, Aurora A, PLK1 and CDK1/Cyclin B1 (Sanhaji *et al.*, 2011).

Given the pivotal roles of KIF2C in ensuring faithful progression of mitotic events, it comes with no surprise that deregulation of KIF2C can result in aberrant mitosis, chromosomal instability and ultimately cancer development. Indeed, KIF2C has been established as one of the up-regulated genes in a genome-wide expression analysis of 81 breast cancer tissues using cDNA microarray laser capture microdissection (Nishidate *et al.*, 2004). Shimo *et al* further demonstrated KIF2C overexpression in breast cancer cell lines as well as cancer tissues and that KIF2C expression can be

suppressed by ectopic expression of p53, suggesting a role of KIF2C in breast carcinogenesis (Shimo *et al.*, 2008). Elevated expression of KIF2C has also been documented in gastric cancer (Nakamura *et al.*, 2007). In the same study, elevated KIF2C expression was also shown to be significantly associated with lymphatic invasion, lymph node metastasis and poor survival of gastric cancer patients (Nakamura *et al.*, 2007). Recently, KIF2C has been reported to be up-regulated at both mRNA and protein levels in colorectal cancer samples (Ishikawa *et al.*, 2008). Enhanced expression of KIF2C is markedly associated with lymph node metastasis, venous invasion, peritoneal dissemination, Dukes' classification and poor survival rate of colorectal cancer patients (Ishikawa *et al.*, 2008). Taken together, KIF2C is dysregulated in multiple cancer types and elevated KIF2C expression is correlated with cancer invasiveness, metastasis and poor prognosis.

Intriguingly, KIF2C not only participates in the progression of cancer, but also has a role in the development of taxane resistance. It has been reported that overexpression of KIF2C confers resistance to paclitaxel (Ganguly *et al.*, 2011). Using Chinese hamster ovary (CHO) cells, it was further demonstrated that paclitaxel resistant cells resulting from KIF2C overexpression shows a reduction in microtubule polymer and an increase in the frequency of microtubule detachment from centrosomes. Conversely, loss of KIF2C reverses the high frequency of microtubule detachment and increases sensitivity of cells to paclitaxel (Ganguly *et al.*, 2011).

In view of the emerging role of KIFs in the acquisition of drug resistance, a correlation analysis was performed to determine if KIFs could serve as potential downstream targets of FOXM1 implicated in paclitaxel resistance of ovarian cancer cells. Interestingly, KIF2C, KIF4A, KIF11, KIF15 and KIF20A appeared to have high correlation scores with FOXM1 and are among the most probable target genes of FOXM1. It is thus tempting to investigate if the KIFs (focusing on KIF2C) could be established as novel FOXM1 transcriptional targets implicated in the acquisition of paclitaxel resistance in ovarian cancer cells.

3.1.11 Aim:

1. To investigate the roles of KIF2C & KIF20A in the acquisition of paclitaxel resistance in ovarian cancer cells

2. To establish KIF2C & KIF20A as candidate FOXM1 transcriptional targets in mediating chemoresistance

3.2 Materials and methods

3.2.1 Cell lines

A pair of paclitaxel-sensitive (PEO1) and -resistant (PEO1-TaxR) ovarian cancer cell lines were used in the study (Jiang *et al.*, 2011) instead of SKOV-3 and SKOV3-TR. PEO1 and PEO1-TaxR are recently developed cell lines and have been authenticated at Cancer Research UK facility. The use of PEO1 and PEO1-TaxR offers extra means to ensure that resistant mechanisms identified in SKOV-3 and SKOV3-TR are common to all paclitaxel resistant ovarian cancer cells and not unique to SKOV-3 and SKOV3-TR. Importantly, both PEO1 and PEO1-TaxR are kept to lower passages to avoid drifts in resistance and the acquisition of secondary mutations. PEO1 and PEO1-TaxR were cultured in RPMI1640 (Sigma) supplemented with 10% foetal bovine serum (FBS) and 100 units/ml penicillin-streptomycin (Invitrogen). PEO1-TaxR is supplemented with 50nM of paclitaxel. All cell lines were maintained at 37°C in humidified incubator with 5% CO_2. Cell culture medium was changed every 3 to 5 days depending on cell density. For routine passage, when cells reached 85% to 90% confluency, they were split at a ratio of 1:4.

3.2.2 Western blot

Cells were harvested with lysis buffer [0.125 m Tris, pH 6.8 at 22°C containing 1% NP-40 (v/v), 2 mM ethylenediamine tetraacetic acid (EDTA), 2 mM N-ethylmaleimide, 2 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM sodium orthovanadate and 0.1 μm sodium okadate] and centrifuged at 4°C for 10 min. Protein

concentration was determined by detergent-compatible (DC) protein assay (Bio-Rad). Twenty micrograms of protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane and hybridized with the following antibodies: anti-FOXM1 (sc-502, Santa Cruz Biotechnology, CA, USA), anti-RAD51 (sc-8349. Santa Cruz), anti-KIF2C (WH0011004M1, Sigma) and anti-β-tubulin (sc-9104, Santa Cruz Biotechnology).

3.2.3 Transient overexpression of FOXM1 and transient knockdown of FOXM1 and KIF2C

For transient overexpression, cells were transfected with pcDNA3-FOXM1 and control vector using Fugene 6 Transfection Reagent as per the manufacturer's instructions (Roche Applied Science, IN, USA). ON-TARGET Plus Human FOXM1 siRNA, KIF2C siRNA and Non-targeting Control siRNAs (Thermo Scientific, CO, USA) were employed for transient silencing using Oligofectamine transfection reagent (Invitrogen) as previously described. Cells were harvested 72 h after transfection.

3.2.4 RNA extraction

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, CA, USA). Briefly, cells were lysed by continuous pipetting with 350µl of buffer RLT followed by addition of equal volume of 70% ethanol. After binding to RNeasy Mini spin

column, samples were washed sequentially with buffer RW1 and buffer RPE. RNA was eluted with 50µl of RNase-free water.

3.2.5 Complementary DNA synthesis

Complementary DNA (cDNA) was synthesized by Superscript™ III First-Strand synthesis System (Invitrogen). First, 1µl of 50µM oligo-$(dT)_{20}$ and 1µl of 10mM dNTP mix were added to 1µg of RNA and the final volume was made up to 13µl with DEPC-treated water. The mixture was heated at 65°C for 5 minutes and chilled on ice for 1 minute. Afterwards, 7µl of a synthesis mix containing 4µl of 5X first-strand buffer, 1µl of 0.1M DTT, 1µl of RNaseOUT™ (40U/µl) and 1µl of Superscript III RT (200U/µl) was added to each primer/RNA mixture. The sample mixtures were then applied to the synthesizing reaction which was carried out at 50°C for 50 minutes and terminated at 85°C for 5 minutes followed by brief chilling on ice.

3.2.6 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and analysed by ABI7900 Sequence Detection System (Applied Biosystems). Housekeeping gene L19 was used as internal control. The following primers were used:

Primer	Forward 5' to 3'	Reverse 5' to 3'
KIF2C	CATGATTGCCACGATCTCAC	CGTTAGAGCAGGCTTCCATC
KIF20A	GCCAACTTCATCCAACACCT	GTGGACAGCTCCTCCTCTTG
KIF4A	ACCTCGCTGGATCAGAAAGA	CACAAAGCCACCCTTTTTGT
KIF11	GGCAGTTGACCAACACAATG	TCTAGCATGGCCTTTTGCTT
KIF15	AGGTGTTGTCTGGAGGATGG	TTTGCCTTTCAGATCCTGTC
L19	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA

Table 3.1. Primers used in the analysis of expressions of KIFs upon FOXM1 knockdown

3.2.7 Cloning of pGL3-KIF2C and pGL3-KIF20A

Total DNA was purified from PEO1 cells using DNeasy Blood & Tissue Kit (Qiagen). Two pairs of primers were designed to amplify a region 1.2kb upstream of the transcription start sites of KIF2C and KIF20A respectively. Restriction digestion sites for KpnI and HindIII were included in the primers. *Pfu* DNA polymerase (Promega) was used in PCR to amplify the desired band, followed by agarose gel electrophoresis and band purification using Wizard SV Gel and PCR Clean-Up System (Promega). Restriction digestion was performed at 37°C for 4 h and ligation with pGL3 vector was achieved at 16°C O/N using T4 DNA Ligase (New England Biolabs, UK). Competent *E.coli* cells were then transformed with the ligated products and positive

colonies were selected by restriction digestion to confirm the insert. Plasmid Maxiprep was used to generate ample amounts of pGL3-KIF2C and pGL3-KIF20A.

Primer	Forward 5' to 3'	Reverse 5' to 3'
KIF2C-Promoter	GGGGTACCCCCGTCCGGGAGATGACGAG	CCCAAGCTTGGGTTAAAACGTCATTCCGCT
KIF20A-Promoter	GGGGTACCCCGACCGGGGTACCTATTCGTT	CCCAAGCTTGGGCTGTCGTGGATTCGCACT

Table 3.2. Primers used in the cloning of pGL3-KIF2C and pGL3-KIF20A

3.2.8 Luciferase reporter assay

PEO1 cells seeded in 96-well plate were transfected FuGENE 6 Transfection Reagent (Roche) with a mixture of pGL3-KIF2C/pGL3-KIF20A, renilla and an increasing amount (0, 5, 10, 25, 50, 100 ng) of pcDNA3-FOXM1. Luciferase activity was detected by steadylite plus system (PerkinElmer, MA, USA) after 24 h. Briefly, one pot of luclite luciferase reagent was mixed with 10ml of luclite buffer, 10µl of 1M $MgCl_2$ and 5µl of 2M $CaCl_2$. The reagent mixture was diluted with equal volume of PBS and 100µl was applied to each well. After incubating at RT for 15 min in darkness, cells were transferred to opaque plate for measuring fluorescence activity. Afterwards, 25µl of renlite reagent (0.5M HEPES pH7.8, 40mM EDTA, coelenterazine at 1mg/ml) was added to each well and the plate was incubated at RT for 20 min in darkness followed by measurement. Relative reading was obtained by dividing luciferase activity over renilla activity.

3.2.9 Chromatin immunoprecipitation (ChIP)

40µl of Dynabeads Protein A (Invitrogen) was washed with 200µl of TSE I buffer for three times and diluted with 40µl of TSE I buffer. Anti-FOXM1 (sc502, Santa Cruz Biotechnology) (4µg) and rabbit IgG control (DAKO) (4µg) were first separately diluted in Buffer D, mixed with diluted Dynabeads and then rotated O/N at 4°C. PEO1 and PEO1-TaxR cells at 90% confluency in 100mm culture dish were crosslinked with 1% formaldehyde for 10 min, rinsed with ice-cold PBS and incubated with 2.5M glycine for 5 min. Cells were then harvested with 2ml of scrapping buffer. After a sequential wash with PBS, Buffer I and Buffer II, cell pellet was resuspended in 300µl of Lysis buffer and subjected to sonication under optimized condition (20 min with 30s on and 30s off). Supernatant was then diluted in 300µl of Buffer D from which 100µl was taken as INPUT control. 200µl of cell lysate was mixed with prepared Dynabeads and rotated O/N at 4°C. After a sequential wash with TSE I, TSE II, Buffer III and TE buffer, 100µl of elution buffer was added to the Dynabeads and the mixture was rotated at RT for 1 h. Eluted sample was collected in eppendorf and the Dynabeads was re-eluted with another 100µl of elution buffer. 200µl of sample was de-crosslinked by incubating at 65°C O/N. PCR Purification Kit (Qiagen) was then used to purify DNA. Quantitative real-time PCR was performed with the following primers: KIF2C (Forward 5' to 3': GCCAAGTCTCCAACTTGCTC; Reverse 5' to 3': TTCCCAACCATCTTCCTACG).

Buffer	Composition
Buffer I	0.25% Triton X-100, 10mM EDTA, 0.5nM EGTA, 10mM HEPES pH6.5
Buffer II	200mM NaCl, 10mM EDTA, 0.5mM EGTA, 10mM HEPES pH6.5
Buffer III	0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCL pH8.1
Lysis buffer	1% SDS, 10mM EDTA, 50mM Tris-HCL pH8.1
Buffer D	1% Triton X-100, 2mM EDTA, 20mM Tris-HCL pH8.1, 150mM NaCl
TSE I	0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCL pH8.1, 150mM NaCl
TSE II	0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCL pH8.1, 500mM NaCl
TE buffer	10mM Tris-HCL pH8.0, 1mM EDTA
Scrapping buffer	100mM Tris-HCL pH9.4, 0.1% SDS
Elution buffer	0.1M $NaHCO_3$, 1% SDS

Table 3.3. Composition of buffers used in ChIP

3.2.10 Clonogenic assay

Transient knockdown of KIF2C was performed on PEO1 and PEO1-TaxR cells seeded in 6-well plate. After 24 h, cells were re-seeded at a density of 1000 cells/well and medium was changed after another 24 h supplemented with different concentrations (0nM, 5nM, 10nM, 20nM) of paclitaxel. Fresh medium with drug was changed every 3 days. Cells were fixed and stained with crystal violet after an incubation of 14 days. Colonies were dissolved with 33% acetic acid and survival rates were expressed as absorbance at 592nm.

3.2.11 Cell cycle analysis

Cell cycle analysis was performed by propidium iodide staining as described previously (Kwok *et al.*, 2008). Briefly, both adherent and suspension cells were harvested and stained with propidium iodide (1mg/mL) in the presence of DNase-free RNase for flow cytometric analysis. Cell cycle profile was analyzed by using the Cell Diva software (Becton Dickinson UK Ltd.).

3.2.12 Statistical analysis

Statistical analysis was performed by the Statistical Package for Social Science version 19.0 for Windows (IBM). Comparison between two groups of non-parametric data was performed by Mann-Whitney *U*-test. *P*-values of <0.05 were considered to be statistically significant.

3.3 Results

3.3.1 Paclitaxel treatment downregulates the expression of FOXM1 in PEO1 but not in the paclitaxel-resistant PEO1-TaxR

In view of the immunohistochemical data showing elevated FOXM1 expression was associated with chemoresistance, a pair of established ovarian cancer cell line sensitive and resistant to paclitaxel, namely PEO1 and PEO1-TaxR respectively, was used to study the effect of drug treatment on the expression of FOXM1 as well as some potential downstream targets. Cells were treated with paclitaxel (50nM) (the optimum concentration for PEO1 and PEO1-TaxR which is different from SKOV-3 and SKOV3-TR) and harvested at various time points 0, 8, 16, 24, 48 and 72 h. Immunoblotting showed FOXM1 expression to be up-regulated transiently at 8 h and decreased at 48 h and 72 h in PEO1. However, FOXM1 expression remained relatively constant at high levels in PEO1-TaxR upon paclitaxel treatment (Figure 3.1), suggesting a role of FOXM1 in mediating paclitaxel resistance in ovarian cancer cells. The expression of RAD51, an established downstream mediator of FOXM1 (N. Zhang *et al.*, 2012), also appeared to be down-regulated upon prolonged paclitaxel treatment in the sensitive cell line. The reduced expression was not observed in the resistant cell line. Intriguingly, protein expression of KIF2C showed a similar pattern as that of FOXM1 with a transient up-regulation at 8 h and decrease at 48 h and 72 h in PEO1. KIF2C level remained fairly constant in PEO1-TaxR.

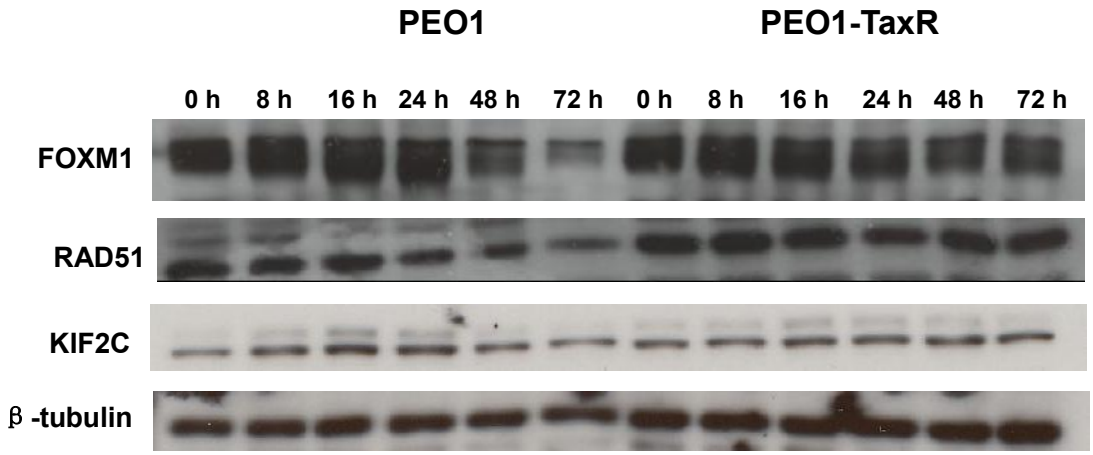


Figure 3.1. Representative immunoblots of FOXM1, RAD51 and KIF2C upon paclitaxel treatment (50nM) at different time points (0h, 8h, 16h, 24h, 48h, 72h) in sensitive ovarian cancer cell line PEO1 and paclitaxel-resistant cell line PEO1-TaxR. Paclitaxel treatment down-regulated FOXM1 expression at time points 48h and 72h in PEO1 but not in PEO1-TaxR. Similar expression patterns were also observed for RAD51 and KIF2C. Representative blot from three experiments.

3.3.2 Transient knockdown of FOXM1 leads to down-regulations of KIF2C and KIF20A

Since *In silico* analysis revealed correlation between FOXM1 and KIF superfamily of proteins, quantitative real-time PCR (qPCR) was used to study the effects of transient silencing and overexpression of FOXM1 on the transcript levels of selected KIF genes including KIF2C, KIF4A, KIF11, KIF15 and KIF20A. Though ectopic expression of FOXM1 did not lead to changes in expression of the five selected KIF genes, FOXM1 knockdown resulted in significantly down-regulated mRNA expressions of KIF2C and KIF20A ($P<0.05$) (Figure 3.2). However, the transcript levels of KIF4A, KIF11 and KIF15 remained the same (Figure 3.3). Transient silencing of FOXM1 also resulted in reduced protein expression of KIF2C in PEO1-TaxR (Figure 3.4).

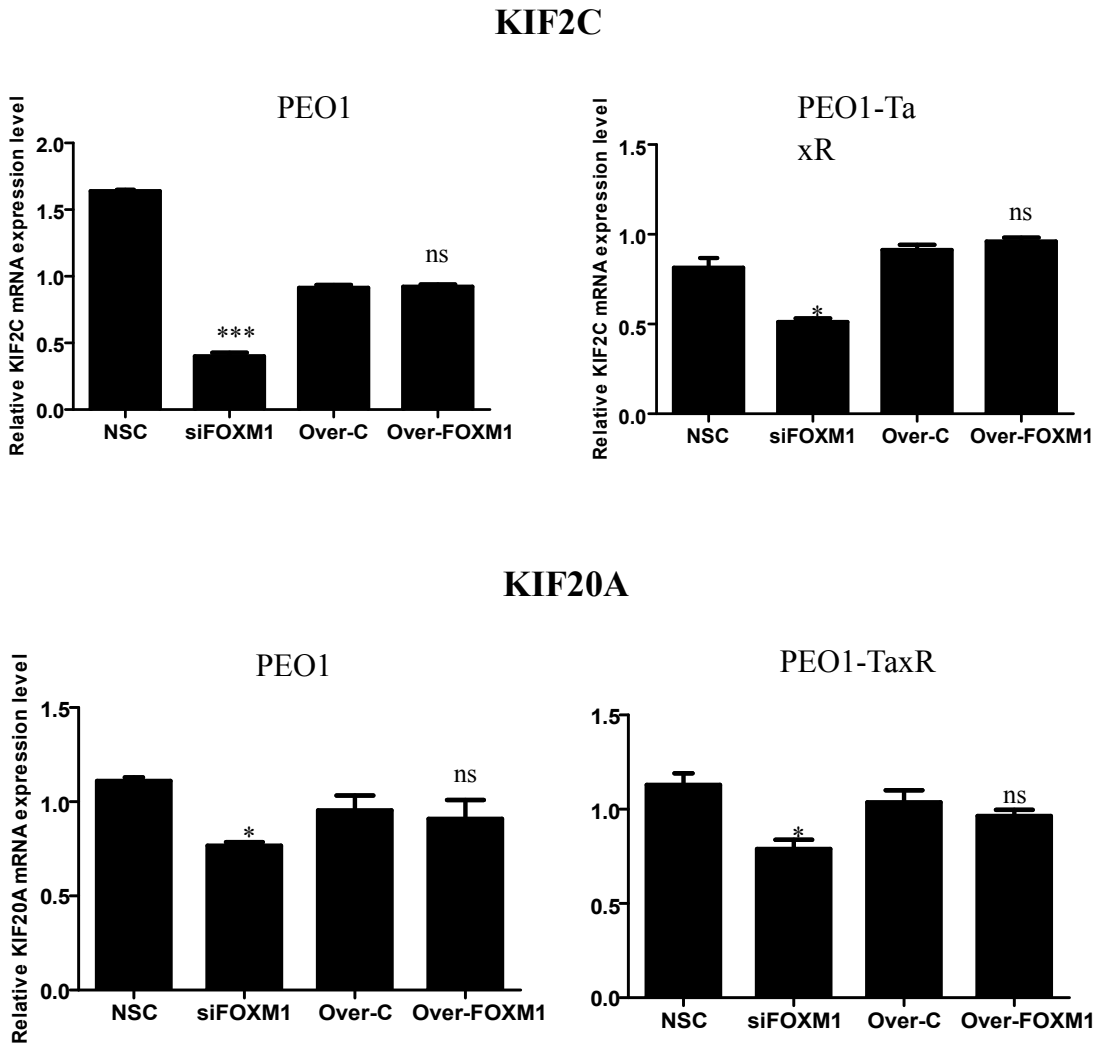


Figure 3.2. Quantitative real-time PCR analysis of the transcript levels of KIF2C and KIF20A after transient silencing (NSC: Non-specific control) and overexpression of FOXM1 (Over-C: control vector pcDNA3, Over-FOXM1: pcDNA3-FOXM1) in ovarian cancer cell lines PEO1 and PEO1-TaxR. Housekeeping gene L19 was used for normalization. Data represent triplicates from three experiments. *P<0.05, ***P=0.0003. ns: not significant. Transient knockdown of FOXM1 was capable of significantly down-regulating the expressions of KIF2C and KIF20A.

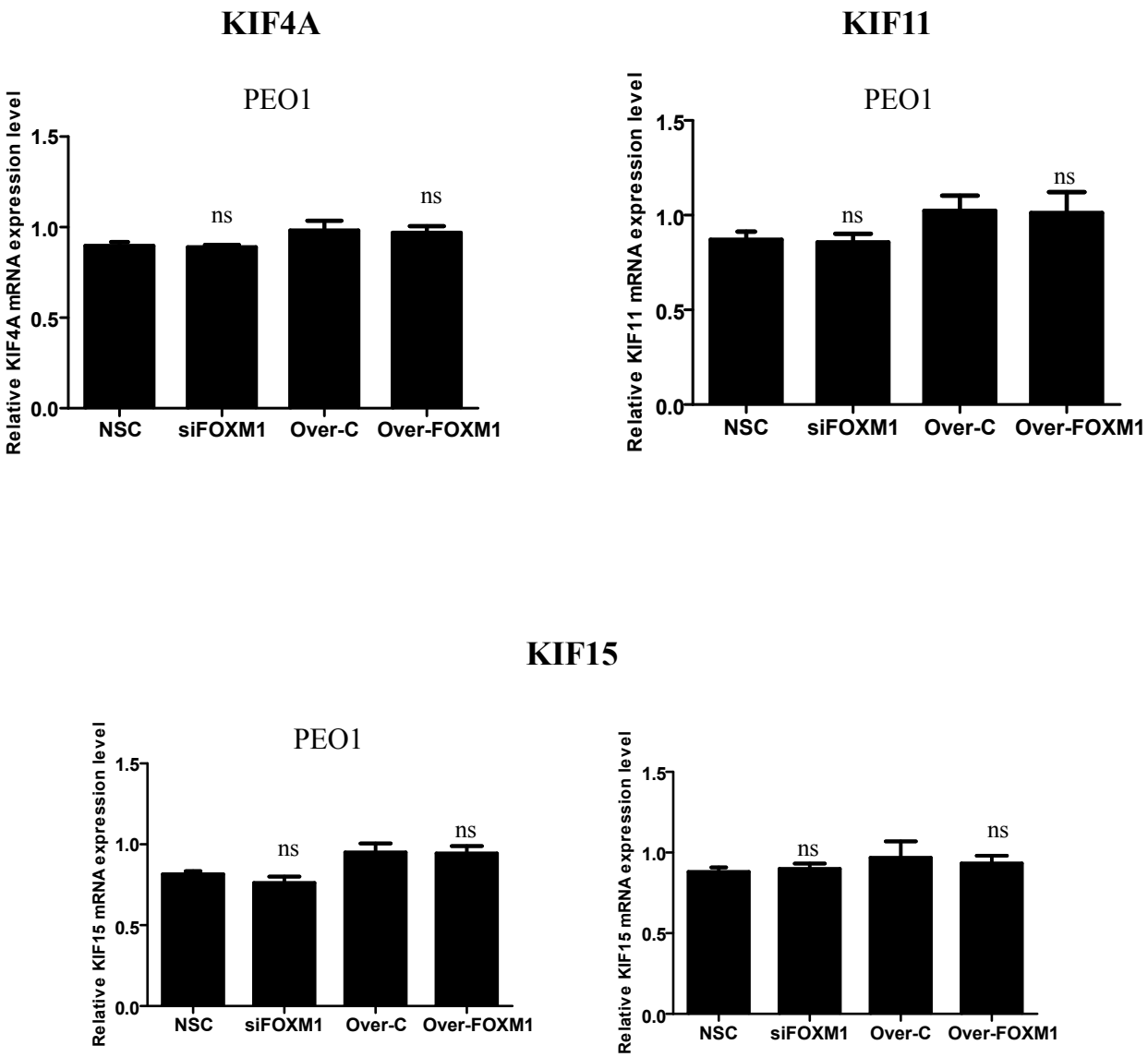


Figure 3.3. Quantitative real-time PCR analysis of the transcript levels of KIF4A, KIF11 and KIF15 after transient silencing (NSC: Non-specific control) and overexpression of FOXM1 (Over-C: control vector pcDNA3, Over-FOXM1: pcDNA3-FOXM1). Housekeeping gene L19 was used for normalization. Data represent triplicates from three experiments. ns: not significant. Transient overexpression or silencing of FOXM1 appeared to have no effects on the expressions of KIF4A, KIF11 and KIF15.

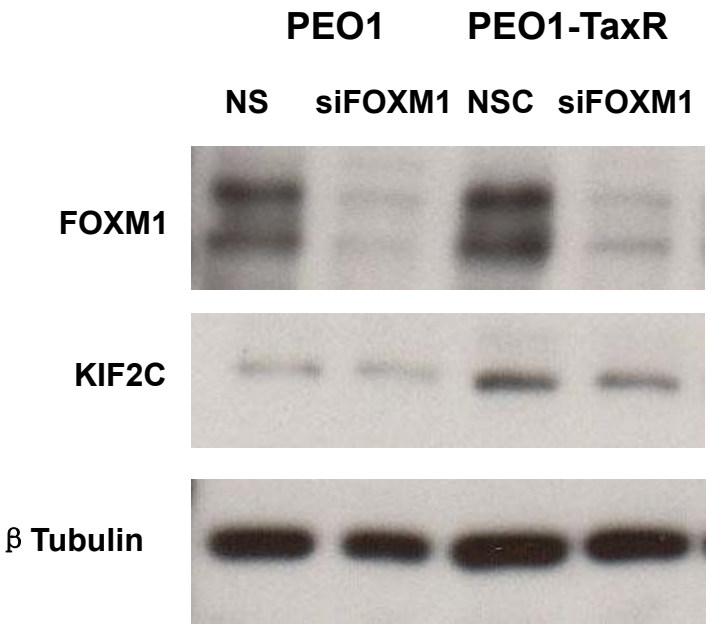


Figure 3.4. Immunoblotting analysis demonstrating reduced expression of KIF2C in PEO1-TaxR after FOXM1 knockdown. (NSC: Non-specific control; siFOXM1: transient silencing of FOXM1). Representative blot from three experiments.

3.3.3 FOXM1 could activate the promoter of KIF2C but not KIF20A

Several FOXM1 consensus binding sites were identified in the promoter region of KIF2C and KIF20A. In order to investigate if FOXM1 could transcriptionally activate KIF2C and KIF20A, around 1.2kb region upstream of the transcription start sites of KIF2C and KIF20A were separately cloned into pGL3 vector. The resulting pGL3-KIF2C and pGL3-KIF20A were subjected to restriction enzyme digestion and agarose gel electrophoresis to confirm the insert (Figure 3.5). Luciferase reporter assay was then performed by titrating pGL3-KIF2C and pGL3-KIF20A with increasing amounts (ng) of pcDNA3-FOXM1 at 0, 5, 10, 25, 50, 100. Empty pcDNA3 was used as control. While pGL3-KIF2C appeared to be gradually activated with increasing amounts of pcDNA3-FOXM1 ($P<0.05$) as revealed by rising luciferase reading, the activation of pGL3-KIF20A was not significant when compared to control (Figure 3.6).

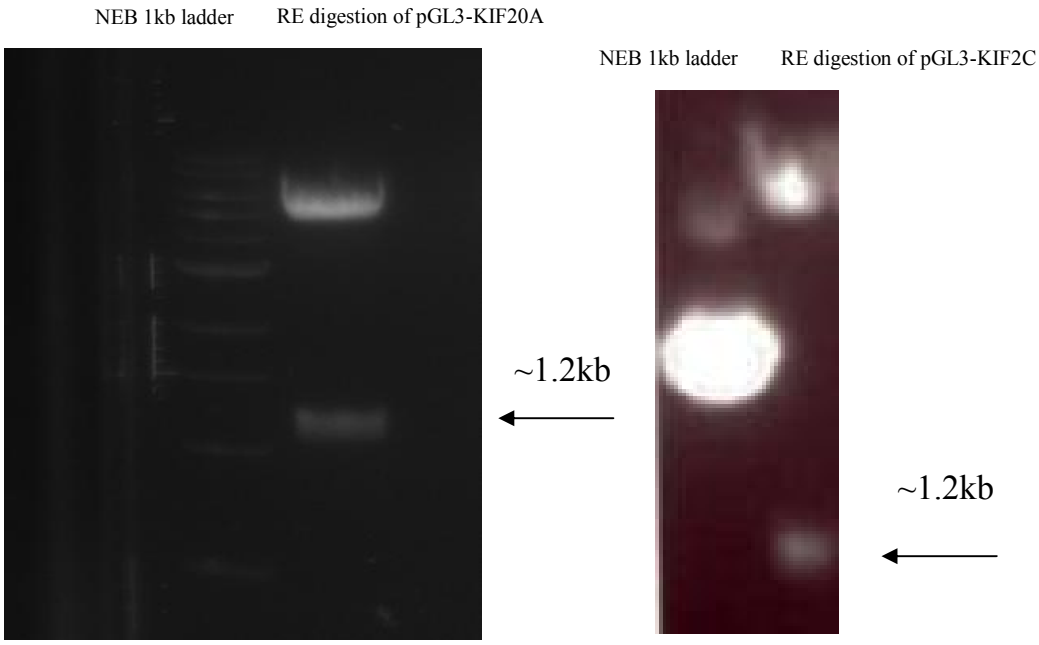


Figure 3.5. Restriction digestion (XbaI and NotI) and agarose gel electrophoresis of plasmids with inserts. Left: pGL3-KIF20A, Right: pGL3-KIF2C. 1.2kb region upstream of the transcription start sites of KIF2C and KIF20A were separately amplified and cloned into pGL3 vector. Positive clones containing pGL3-KIF2C and pGL3-KIF20A were selected by restriction digestion and electrophoresis.

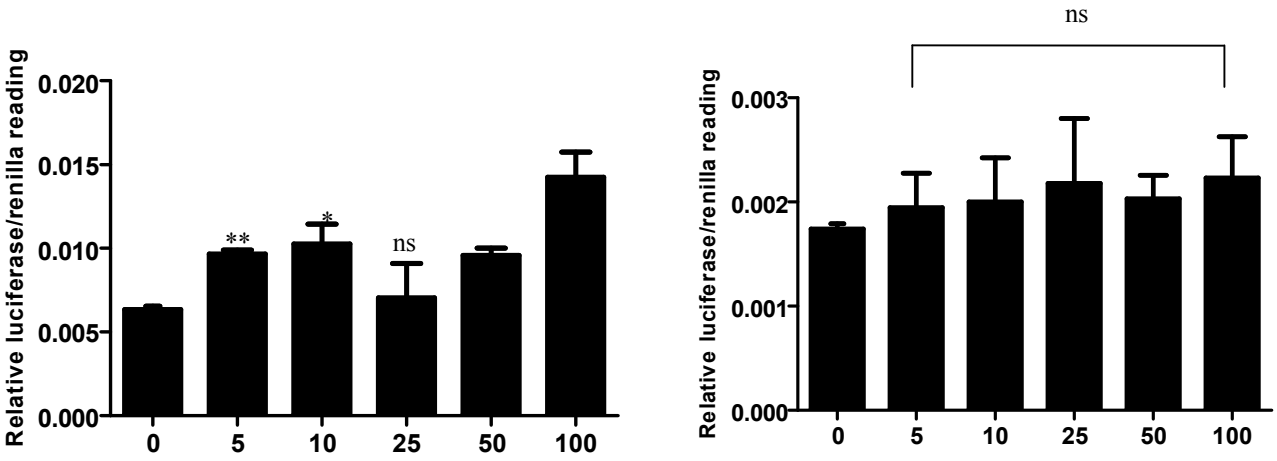


Figure 3.6. Titration of pGL3-KIF2C with increasing amount (ng: 0, 5, 10, 25, 50, 100) of pcDNA3-FOXM1 (left) and titration of pGL3-KIF20A with increasing amount (ng) of pcDNA3-FOXM1 (right). Data represent triplicates from three experiments. *P<0.05, **P=0.006. ns: not significant. FOXM1 could activate the promoter of KIF2C but not that of KIF20A. Treatments with different amount of pcDNA3-FOXM1 (5-100ng) were compared individually to the treatment without pcDNA3-FOXM1 (0ng). Comparison among different concentrations showed no significant difference.

3.3.4 FOXM1 is capable of binding to the promoter region of KIF2C

Given the findings that knockdown of FOXM1 resulted in down-regulation of KIF2C and that FOXM1 could activate the promoter of KIF2C as revealed by Luciferase reported assay, chromatin immunoprecipitation (ChIP) was performed in an attempt to evaluate if FOXM1 can bind to the promoter region of KIF2C. ChIP-qPCR analysis showed significantly enhanced pull-down of KIF2C by anti-FOXM1 antibody when compared to the IgG control in both PEO1 (51 folds enrichment, $P=0.04$) and PEO1-TaxR (31 folds enrichment, $P<0.0001$) (Figure 3.7), indicating FOXM1 is capable of binding to the promoter region of KIF2C.

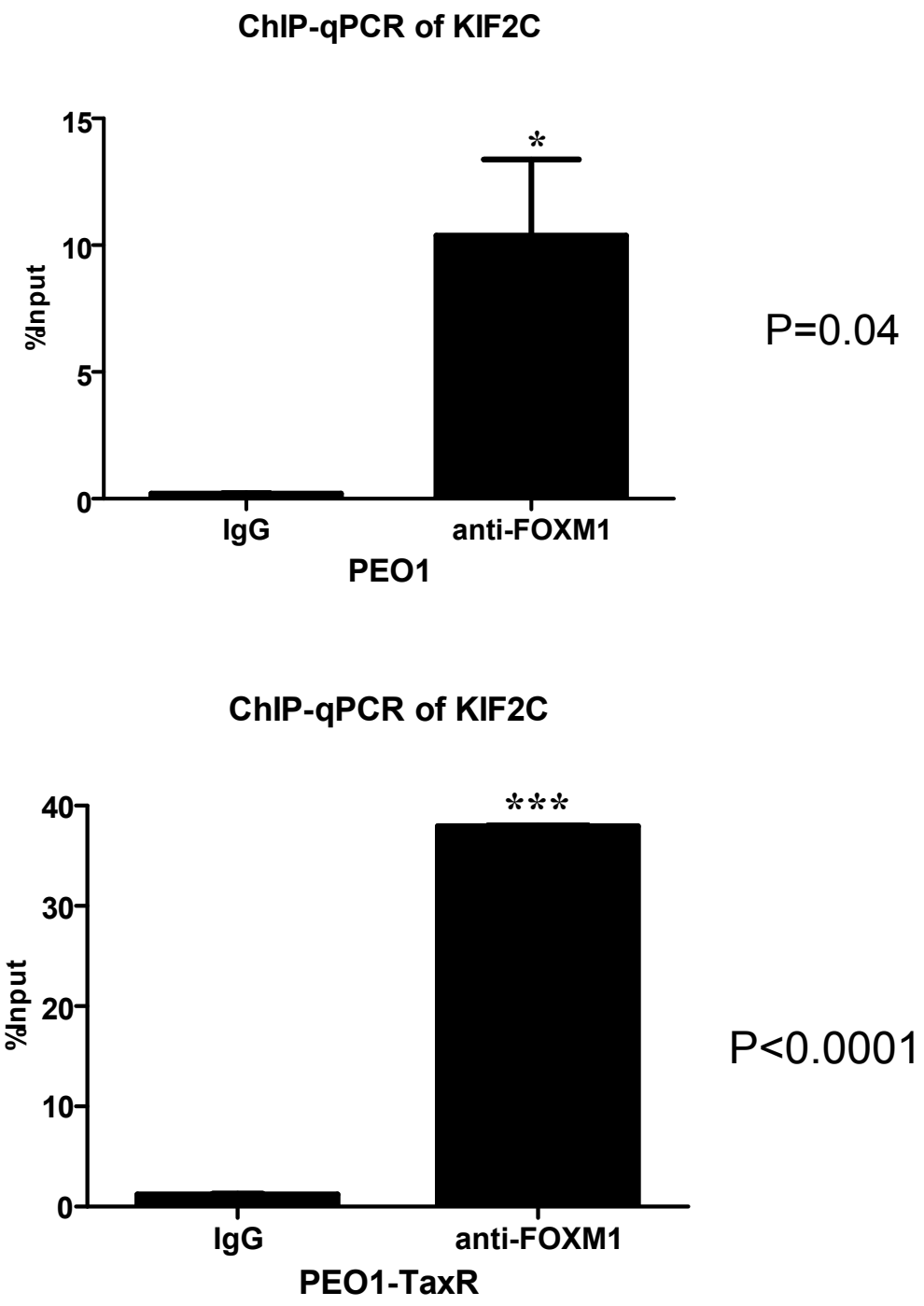


Figure 3.7. Chromatin immunoprecipitation-qPCR (ChIP-qPCR) revealing significantly enhanced pull-down of KIF2C by anti-FOXM1 antibody as compared to the negative IgG control.

3.3.5 KIF2C knockdown appeares to re-sensitize PEO1-TaxR to paclitaxel

Clonogenic assay was used to investigate the role of KIF2C in the acquisition of paclitaxel resistance in ovarian cancer cells. Following KIF2C knockdown and paclitaxel treatment at various concentrations (0, 5, 10, 20nM) for a period of 14 days, PEO1 and PEO1-TaxR cells were harvested and survival rates were measured in terms of absorbance. Although no difference in survival was observed between non-specific control (NSC) and KIF2C knockdown (siKIF2C) in PEO1 upon paclitaxel treatment, there appeared to be lower survival rates in PEO1-TaxR transfected with siKIF2C than PEO1-TaxR transfected with control upon paclitaxel treatment (Figure 3.8), suggesting KIF2C might contribute to the development of paclitaxel resistance in ovarian cancer cells. Efficacy of KIF2C knockdown was confirmed by Western blot analysis (Figure 3.9).

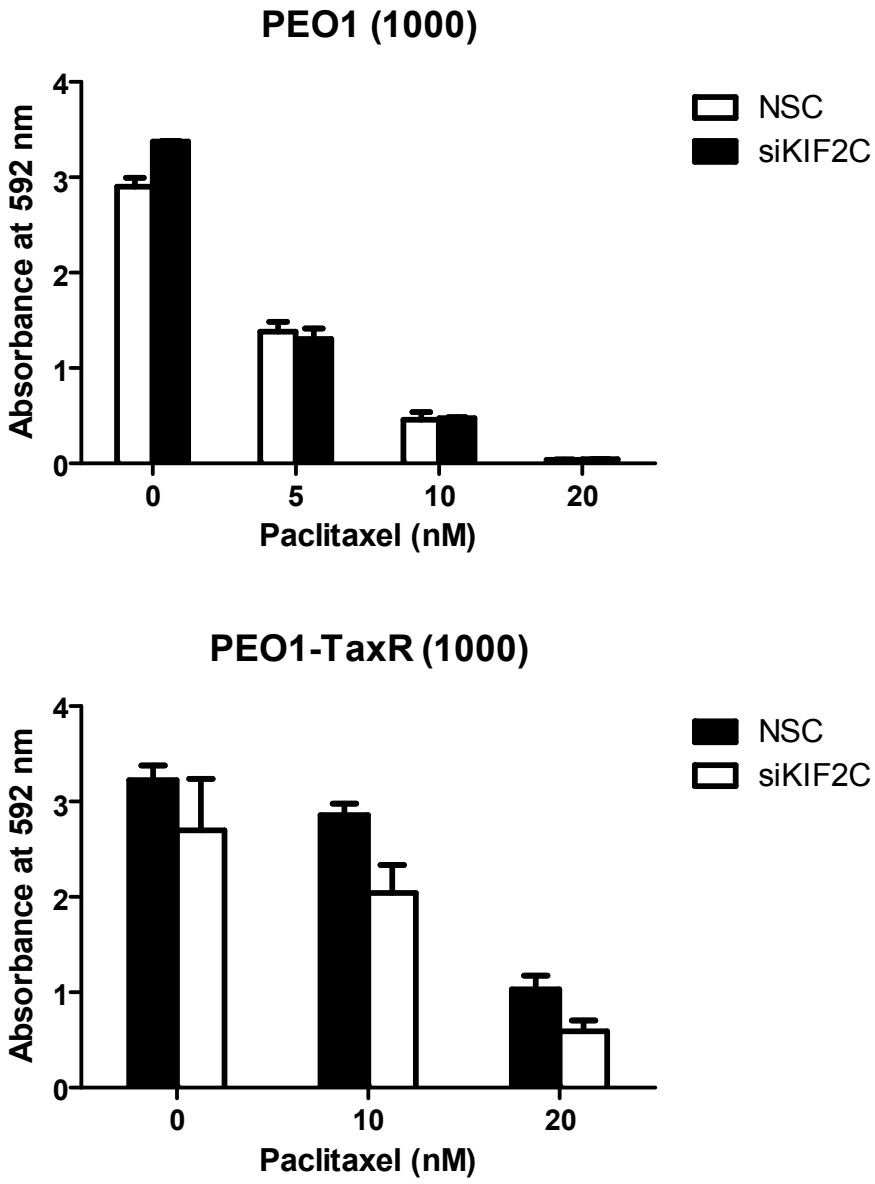


Figure 3.8. Clonogenic assay showing KIF2C knockdown appeared to re-sensitize the PEO1-TaxR cells to paclitaxel treatment. NSC: non-specific control; siKIF2C: KIF2C knockdown. Data represent triplicates from three experiments.

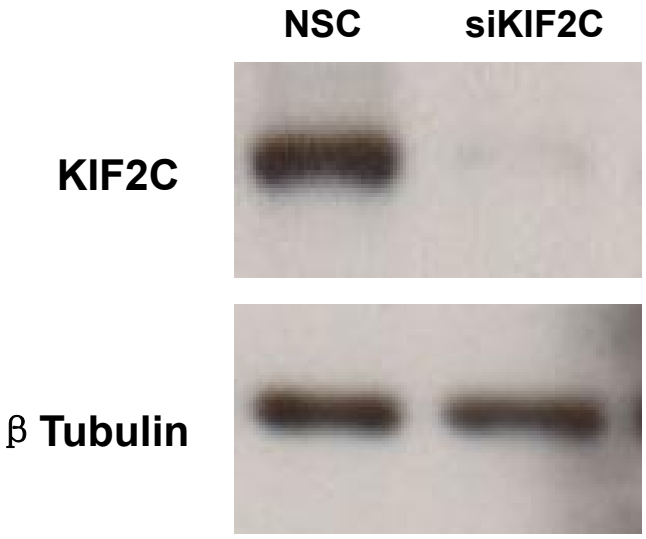
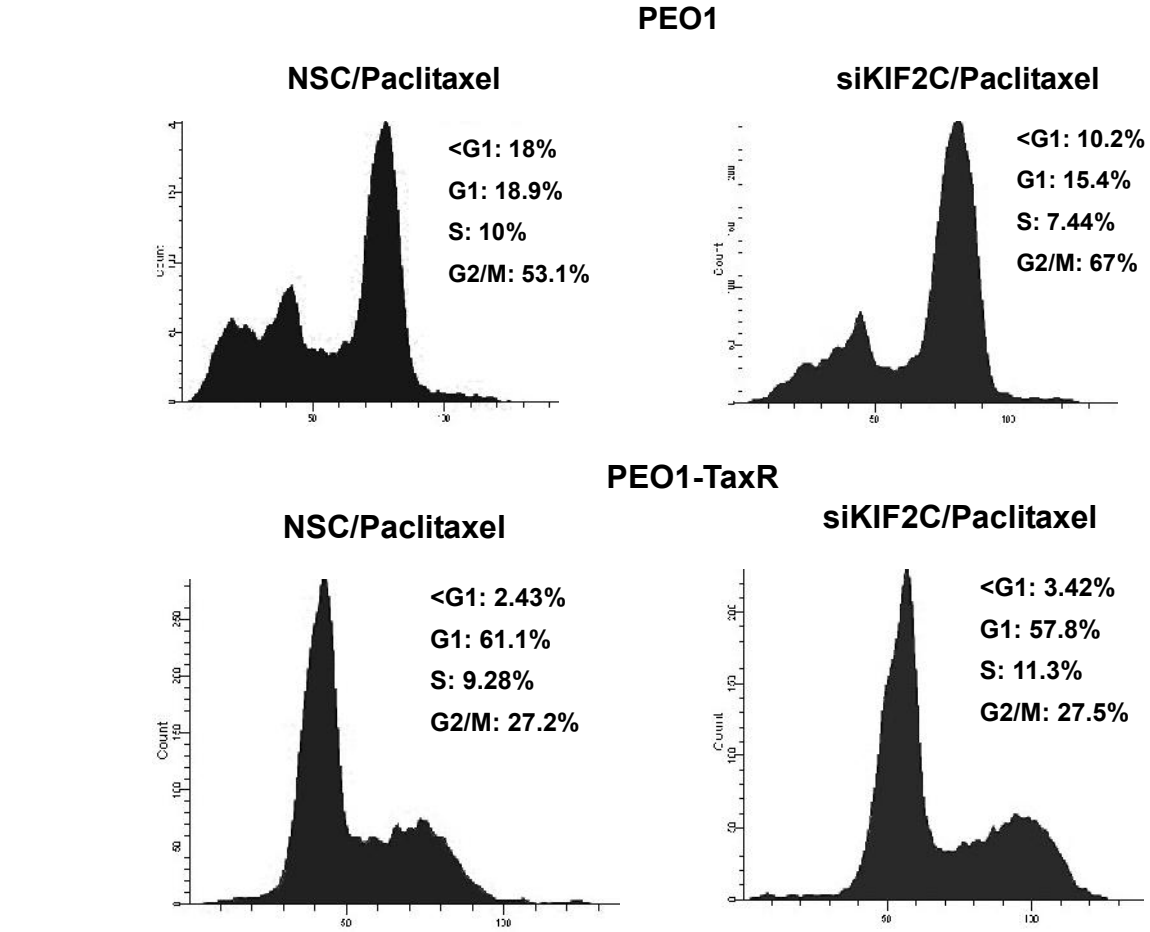


Figure 3.9. Western blot confirming the efficacy of KIF2C knockdown. NSC: non-specific control; siKIF2C: KIF2C knockdown. Representative blot from three experiments.

3.3.6 KIF2C knockdown increases accumulation of cells at G2/M in PEO1 and dead cells in PEO1-TaxR upon paclitaxel treatment

Cell cycle analysis was then performed to investigate the role of KIF2C in paclitaxel-mediated cell death. To this end, PEO1 and PEO1-TaxR cells were transiently transfected with non-specific control (NSC) and KIF2C siRNA (siKIF2C) for 48 h and cultured in the presence of paclitaxel treatment (50nM) for 48 h. Cells were then harvested, stained with propidium iodide and subjected to flow cytometric analysis. The results showed that though not significant, KIF2C depletion by siRNA decreased the number of dead cells in the sub-G1 population (18% vs 10.2%) and increased the levels of G2/M cells in the PEO1 cells treated with paclitaxel as compared to control (67% vs 53.1%) (Figure 3.10). Paclitaxel also induced a minor increase in cell death (sub-G1 population) in PEO1-TaxR cells transfected with KIF2C siRNA (3.42% vs 2.43%) (Figure 3.10).


PEO1
NSC/Paclitaxel
<G1: 18%
G1: 18.9%
S: 10%
G2/M: 53.1%
siKIF2C/Paclitaxel
<G1: 10.2%
G1: 15.4%
S: 7.44%
G2/M: 67%
PEO1-TaxR
NSC/Paclitaxel
<G1: 2.43%
G1: 61.1%
S: 9.28%
G2/M: 27.2%
siKIF2C/Paclitaxel
<G1: 3.42%
G1: 57.8%
S: 11.3%
G2/M: 27.5%

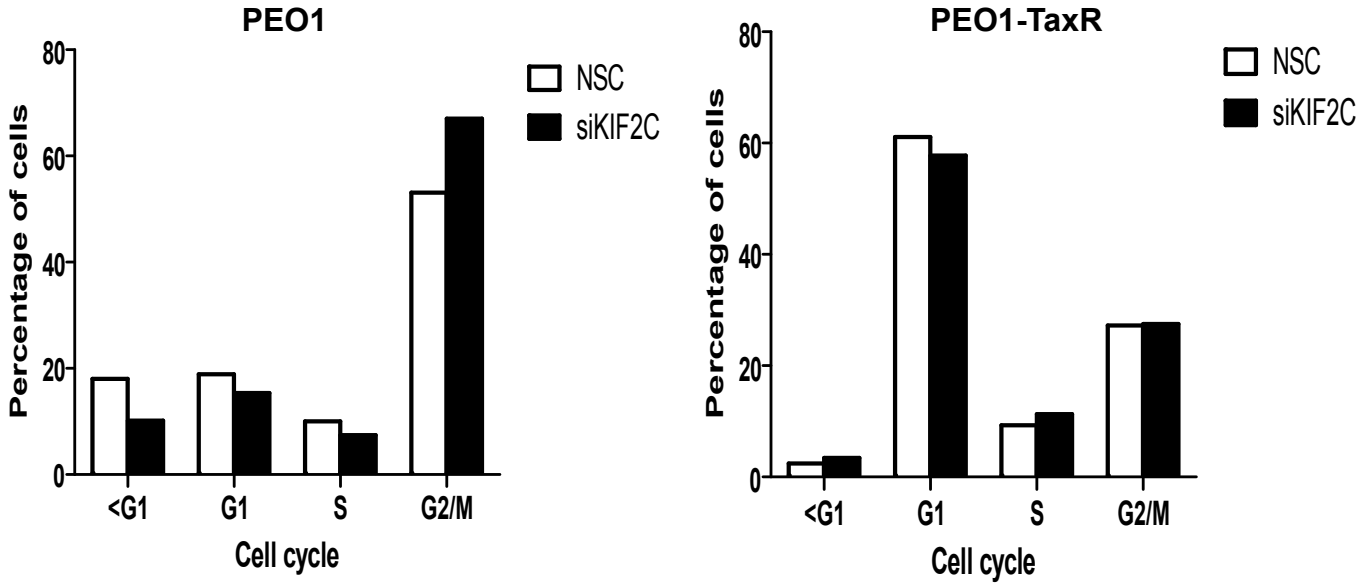

PEO1
Percentage of cells
80
60
40
20
0
NSC
siKIF2C
<G1
G1
S
G2/M
Cell cycle
PEO1-TaxR
Percentage of cells
80
60
40
20
0
NSC
siKIF2C
<G1
G1
S
G2/M
Cell cycle

Figure 3.10. Flow cytometric analysis of PEO1 and PEO1-TaxR cells with and without KIF2C depletion in the presence of paclitaxel treatment.

Upper panel: Flow cytometric analysis was performed following propidium iodide staining on PEO1 and PEO1-TaxR cells treated with paclitaxel (50nM) after transfection with siRNA pools against KIF2C. Data indicated that KIF2C silencing (siKIF2C) is capable of decreasing the number of dead cells in the sub-G1 population and increasing the number cells blocked at G2/M cell cycle phase in PEO1 and raising the number of dead cells in PEO1-TaxR as compared to cells treated with control (NSC). **Lower panel**: Bar charts of the cell cycle distribution of PEO1 and PEO1-TaxR upon paclitaxel treatment after transfection with NSC or siKIF2C of an independent experiment.

3.4 Discussion

Kinesin superfamily proteins (KIFs) play pivotal roles in intracellular transport of organelles and maintenance of spindle assembly during mitosis and meiosis. Emerging evidence suggests dysregulations of KIF members contribute to tumorigenesis of multiple types of human cancer. In this study, KIF2C (MCAK) was identified as a novel transcriptional target of FOXM1 and KIF2C participates in the acquisition of paclitaxel resistance in ovarian cancer.

In view of the bioinformatics analysis revealing a positive correlation between FOXM1 and KIF proteins, several pairs of primers were designed and qPCR was used to study whether FOXM1 affects the expressions of KIF proteins. Transient knockdown of FOXM1 resulted in reduced mRNA expressions of KIF2C and KIF20A but not of KIF4A, KIF11 and KIF15. The down-regulation at protein level was also observed for KIF2C. However, ectopic expression of FOXM1 did not alter the expressions of KIFs which might be attributable to the fact that ovarian cancer cells already harbour high FOXM1 expression. This finding is in line with a microarray analysis implying KIF20A to be a target gene of FOXM1 (Wonsey & Follettie, 2005). Reporter plasmids containing the promoter regions of KIF2C and KIF20A were subsequently cloned and Luciferase reporter assay was employed to study if FOXM1 can induce activation of the promoters. Upon titration of increasing amounts of FOXM1, it was observed that only pGL3-KIF2C was activated but not pGL3-KIF20A, indicating KIF2C may be a direct transcriptional target of FOXM1. Chromatin

immunoprecipitation-qPCR (ChIP-qPCR) further demonstrated that FOXM1 could bind to and pull-down KIF2C in ovarian cancer cell lines, indicating KIF2C may be a novel transcriptional target of FOXM1.

Overexpression of KIF2C is not only associated with malignant progression, but also with drug resistance (Sanhaji *et al.*, 2011). It has been reported in a recent study recruiting Chinese hamster ovary (CHO) cells that paclitaxel resistant cells resulting from KIF2C overexpression displays a reduction in microtubule polymer and an increase in the frequency of microtubule detachment from centrosomes. Conversely, loss of KIF2C reverses the high frequency of microtubule detachment and increases sensitivity of cells to paclitaxel (Ganguly *et al.*, 2011). In the current study, immunoblotting analysis showed KIF2C expression in PEO1 altered in a similar pattern as FOXM1 expression by displaying an initial induction at 8 h followed by a subsequent decrease at 48 h and 72 h upon paclitaxel treatment. In contrast, KIF2C expression remained relatively constant in PEO1-TaxR, implicating KIF2C might be involved in the development of paclitaxel resistance in ovarian cancer. Furthermore, KIF2C knockdown is capable of re-sensitizing PEO1-TaxR to paclitaxel treatment as revealed by clonogenic assay. Flow cytometric analysis showed KIF2C silencing is able to induce cell cycle arrest at G2/M in PEO1 and increased cell death in PEO1-TaxR. Further experiments are required to confirm the finding.

Taken together, this study identified KIF2C as a novel FOXM1 transcriptional target

and KIF2C contributes to the acquisition of paclitaxel resistance in ovarian cancer cells by inducing enhanced G2/M arrest and cell death. Site-directed mutagenesis coupled with Luciferase reporter assay will be performed in future studies in order to consolidate the interaction between FOXM1 and KIF2C. Functional experiments will also be pursued to further understand the role of KIF2C in drug resistance.

Chapter 4

Identification of PAK4 as a novel transcriptional target of FOXM1

4.1 Introduction

4.1.1 p21-activated kinases (PAKs)

p21-activated serine/threonine kinases (PAKs) are identified as major effectors of the small Rho GTPases Rac1 and Cdc42. Based on structural and functional similarities, the six members of PAK family are classified into group I (PAK1, PAK2, PAK3) and group II (PAK4, PAK5, PAK6) (Kumar *et al.*, 2006). PAKs are evolutionarily conserved and generally expressed in a variety of tissues playing pivotal roles in morphogenesis, cytoskeletal reorganization, apoptosis, survival and angiogenesis (Kumar *et al.*, 2006). Over the years, accumulating evidence has suggested PAK family members contribute to the processes of tumour formation and cell invasiveness.

4.1.2 Group I PAKs

4.1.2.1 PAK1

Similar to other members of the PAK family, PAK1 consists of a C-terminal kinase domain and an N-terminal regulatory domain containing a GTPase binding domain and an inhibitory domain. When inactive as homodimers, the kinase inhibitory domain of PAK1 binds to the other kinase domain keeping it in an inactive state.

When bound by GTP-bound forms of Cdc42 and Rac, the inhibition is released thereby allowing phosphorylation of the kinase domain and activation of PAK1 (Pirruccello *et al.*, 2006). However, group I PAKs can also be activated in a GTPase-independent manner. Regardless of the mode of activation, activated PAKs phosphorylate their substrate proteins and in turn activate a myriad of biological functions (Molli *et al.*, 2009).

PAKs participate in cytoskeleton remodeling by phosphorylating a wide array of downstream effectors involved in regulation of cytoskeletal structure including LIM-kinases (LIMK), paxillin, filamin A, cortactin, myosin light-chain kinase (MLCK), PIX/COOL guanine nucleotide exchange factors, stathmin and tubulin cofactor B (Kumar *et al.*, 2006). The forkhead box transcription factors (FOXs), estrogen receptor α (ERα), Snail homologue 1 and C-terminal binding protein 1 (CtBP1) have also been identified as PAK1-interacting substrates involved in crucial biological processes such as epithelial-mesenchymal transition (EMT) (Come *et al.*, 2004; Grooteclaes & Frisch, 2000).

In addition to regulating cytoskeletal reorganization, PAK1 also plays an important role in the faithful progression of cell cycle. For instance, it has been demonstrated cyclin B1/Cdc2 mediated phosphorylation of PAK1 during mitosis is crucial for cell division (Thiel *et al.*, 2002). Li *et al* reported that activated PAK1 is specifically localized within chromosomes during prophase and on centrosomes during metaphase

before moving to the contraction ring during cytokinesis (Li *et al.*, 2002). An interaction between PAK1 and histone H3 has also been shown in the same study (Li *et al.*, 2002). Furthermore, PAK1 has been documented to bind to and phosphorylate Aurora-kinase A and polo-like kinase 1 (PLK1), suggesting the importance of PAK1 in cell-cycle checkpoints and adequately orchestrated cell-cycle progression (Maroto *et al.*, 2008; Zhao *et al.*, 2005).

PAK1 is responsible for modulating cell survival via multiple mechanisms. Schurmann *et al* demonstrated PAK1 phosphorylates Bad *in vitro* and *in vivo* on Ser-112 and Ser-136, resulting in a markedly reduced interaction between Bad with Bcl2 family members and thus an anti-apoptotic action (Schurmann *et al.*, 2000). Under apoptotic stimuli, however, PAK1 can phosphorylate dynein light chain 1 (DLC1) and BimL leading to inactivation of the anti-apoptotic function of Bcl2 (Vadlamudi *et al.*, 2004). Furthermore, PAK1 has been reported to be an important mediator of estrogen's cell survival functions by phosphorylating and inactivating the forkhead transcription factors, thereby exerting an anti-apoptotic function (Mazumdar & Kumar, 2003).

In recent years, an emerging body of evidence has indicated a crucial role of PAK1 in the carcinogenesis of a broad spectrum of cancers. PAK1 amplification is prevalent in luminal breast cancer, and PAK1 protein expression is significantly associated with lymph node metastasis (Ong *et al.*, 2011). PAK1 hyperactivation has also been shown

to enhance the stimulation of downstream effectors MEK1/2 and p38-MAPK in mammary tumour epithelial cells (Wang *et al.*, 2006). Overexpression of PAK1 is also detected in 70% of colon cancer samples and is associated with multiple signaling pathways including Wnt, Erk and Akt pathways (Ye & Field, 2012). Down-regulation of PAK1 has been reported to be able to reduce β-catenin accumulation and proliferation of colon cancer cells (Zhu *et al.*, 2012). Strong nuclear and cytoplasmic PAK1 expression is observed in squamous non-small cell lung carcinomas (NSCLCs). Selective PAK1 inhibition could postpone cell-cycle progression both *in vitro* and *in vivo* (Molli *et al.*, 2009). Siu *et al* further identified PAK1 as an independent prognostic factor in ovarian cancer, affecting cell migration and invasion via p38 pathway (Siu *et al.*, 2010a).

4.1.2.2 PAK2

PAK2 can affect both cell survival and cell death pathways depending on cellular contexts. Cellular stresses such as DNA damage and serum starvation activate PAK2 to generate a proteolytic fragment, PAK-2p34, and lead to apoptosis (Roig & Traugh, 1999). In contrast, Activation of full-length PAK2 is capable of promoting cell survival by phosphorylating Bad and enhancing the interaction between Bad and 14-3-3tau, thereby promoting cell survival (Jakobi *et al.*, 2001).

Although less well characterized than PAK1, increasing attention has been drawn to the oncogenic role of PAK2. Up-regulation of phosphorylated PAK2 (pPAK2) has

been documented in ovarian cancer cell lines, and transient knockdown of PAK2 could retard cancer cell migration and invasion (Siu *et al.*, 2010a). Recently, clinicopathological analyses indicated that the phosphorylation level of PAK2 is closely correlated with tumour progression, metastasis and early recurrence of hepatocellular carcinoma (Sato *et al.*, 2013). The same study also suggested PAK2 may serve as a critical mediator of TGF-β-mediated hepatoma cell migration, thus establishing PAK2 as a promising target for the treatment of hepatocellular carcinoma (Sato *et al.*, 2013).

4.1.2.3 PAK3

With no exception, PAK3 is also involved in the regulation of cytoskeleton dynamics. Hashimoto *et al* reported paxillin is the sole member among several representative focal adhesion proteins that associates with PAK3. paxillin alpha was further shown to be capable of linking both the kinase-inactive and activated forms of PAK3 to integrins (Hashimoto *et al.*, 2001). It has been documented that PAK3 positively regulates Raf-1 activity through phosphorylation of Ser-338 (King *et al.*, 1998).

NADPH oxidase is an enzyme responsible for regulating the intracellular context of reactive oxygen species (ROS). PAK3 has been shown to confer survival advantage to cancer cells by activating NADPH oxidase and thus altering the redox potential of cells (Molli *et al.*, 2009). There has been little data regarding whether PAK3 participates in the development of cancer, though overexpression of PAK3 has been

found in thymic neuroendocrine tumours (Liu *et al.*, 2010). Intriguingly, PAK3 is the sole member of the group I PAKs known to be associated with a human genetic disease. Mutations in the *PAK3* gene have been reported in X-linked non-syndromic mental retardation syndromes in which an A365E mutation adversely affects a highly conserved region of PAK3 (Gedeon *et al.*, 2003).

4.1.3 Group II PAKs

Group II PAKs consists of PAK4, PAK5, PAK6 and are structurally distinct from group I PAKs. They merely contain an N-terminal p21-binding domain and a C-terminal kinase domain but lack other motifs generally found in group I PAKs (Molli *et al.*, 2009). Although interaction of group II PAKs with GTPases barely has an effect on kinase activity, PAK4-6 do play vital roles in a broad spectrum of biological processes including cell motility and survival (Molli *et al.*, 2009).

4.1.3.1 PAK5

The constitutively active PAK5 (also known as PAK7) binds to the GTPases Cdc42 and Rac and antagonizes Rho in the pathway, resulting in filopodia formation and neurite development (Dan *et al.*, 2002). Timm *et al* demonstrated PAK5 contributes to microtubule stability by preventing MARK (a kinase promoting microtubule disruption)-induced phosphorylation of tau (Timm *et al.*, 2006). PAK5 has also been reported to be constitutively localized to mitochondria and inhibit apoptosis by phosphorylating Bad (Cotteret *et al.*, 2003).

Studies focusing on the role of PAK5 in carcinogenesis are on the rise. Overexpression of PAK5 has been shown in a range of colorectal carcinoma cell lines and the elevated expression is significantly associated with metastatic foci, high cancer grades and poor differentiation of colorectal cancer (Gong *et al.*, 2009). The same investigators then documented elevated expression of PAK5 is capable of antagonizing camptothecin-induced apoptosis by inhibiting the activity of caspase-8 in colorectal carcinoma cell lines (X. Wang *et al.*, 2010). Transient silencing of PAK5 has been reported to inhibit human gastric cancer cell proliferation by inducing cell cycle arrest in G(0)/G(1) phase (Gu *et al.*, 2013). An oncogenic role of PAK5 has also been observed in glioma cells (Han *et al.*, 2013). Interestingly, PAK5 expression was found to be correlated with advanced stages and high grades of epithelial ovarian cancer and contribute to the acquisition of paclitaxel resistance of ovarian cancer cells (Li *et al.*, 2013).

4.1.3.2 PAK6

PAK6 is a 75-kDa protein harbouring an N-terminal Cdc42/Rac binding motif and a C-terminal kinase domain (Yang *et al.*, 2001). PAK6 is highly expressed in the brain and although PAK6 double knockout mice remained viable and fertile, they displayed several locomotor and behavioral deficits, suggesting PAK6 is important for certain neuronal functions (Minden, 2012). Zhao *et al* further demonstrated PAK6 is involved in central nervous system pathophysiology after traumatic brain injury (W. Zhao *et al.*, 2011).

Similar to other PAKs, PAK6 also participates in the development of multiple types of cancer. Overexpression of PAK6 has been observed in both primary and metastatic prostate cancer cells and contributes to prostate cancer progression post androgen deprivation therapy (Kaur *et al.*, 2008). Recently, it has been shown knockdown of PAK6 inhibits prostate cancer growth and enhances chemosensitivity to docetaxel (Wen *et al.*, 2009). Whether PAK6 contributes to the carcinogenesis of other cancer types awaits further investigation.

4.1.3.3 PAK4

PAK4 is the first identified and most intensively studied member of group II PAKs, serving a pivotal role in a wide array of cellular functions. PAK4 is an effector of Cdc42 crucial for regulating cytoskeleton reorganization (Kumar *et al.*, 2006). PAK4 also phosphorylates LIMK1 and enhances its ability to phosphorylate cofilin thereby modulating cytoskeletal changes (Dan *et al.*, 2001). Activated PAK4 is capable of protecting cells from apoptosis by phosphorylating Bad and by inhibiting caspase activation (Gnesutta *et al.*, 2001). Qu *et al* reported activated PAK4 regulates cell adhesion and anchorage-independent growth (Qu *et al.*, 2001).

Though PAK4 is highly expressed in embryos, its protein levels are generally low in adult tissues. Taken into account the finding that PAK4 null mice are embryonic lethal, PAK4 is indispensible for embryonic development (Qu *et al.*, 2003). Early death of PAK4 null embryos is due in part to abnormal growth and branching of blood vessels,

suggesting a role of PAK4 in angiogenesis (Qu *et al.*, 2003). PAK4 also participates in the normal functioning of heart. For instance, it has been documented PAK4 null embryos harbour a thinning of the myocardial walls of the bulbus cordis and the ventricle, and dilation as well as distortion of the sinus venosus region of the heart has been observed (Qu *et al.*, 2003). Nekrasova *et al* further demonstrated knockdown of PAK4 in cultured cardiomyocytes could lead to severely compromised sarcomeric structure and deficits in contraction, emphasizing PAK4 is crucial for normal development of the heart (Nekrasova & Minden, 2012).

Given the pivotal roles of PAK4 in a broad spectrum of biological processes, it comes with no surprise that dysregulation of PAK4 could result in oncogenic transformation. In athymic mice, overexpression of PAK4 results in tumour formation and depletion of PAK4 inhibits tumorigenesis (Liu *et al.*, 2008). In fact, PAK4 is up-regulated in most cancer cell lines (Callow *et al.*, 2002). This is in contrast to a generally low PAK4 expression in most normal tissues. In addition to cell lines, PAK4 has been reported to be elevated in many primary tumours. PAK4 overexpression has been documented in a subset of gastric cancer cell lines, and patients with high PAK4 expression tend to have poor survival rates (Ahn *et al.*, 2011). Increased level of PAK4 was also detected in hepatocellular carcinoma samples (Minden, 2012). Mak *et al* showed overexpression of CDK5 kinase regulatory subunit-associated protein 3 (CDK5RAP3) promotes hepatocellular carcinoma metastasis through PAK4 activation (Mak *et al.*, 2011). Recently, the microRNA miR-199a/b-3p was found to be

consistently down-regulated in hepatocellular carcinoma and miR-199a/b-3p can target PAK4 to suppress growth of cancer cells (Hou *et al.*, 2011). An oncogenic role of PAK4 has also been reported in breast and endometrial cancer (Lu *et al.*, 2013; Wong *et al.*, 2013).

Ovarian cancer cell lines have also been reported to have high levels of PAK4. Elevated expressions of PAK4 and phospho-PAK4 are significantly associated with metastasis of ovarian cancer, shorter survival, advanced stage as well as reduced chemosensitivity (Siu *et al.*, 2010b). Knockdown of PAK4 in ovarian cancer cell lines retards cell migration, invasion and proliferation whereas ectopic expression of PAK4 has the opposite effect (Siu *et al.*, 2010b).

Since PAK4 plays an important role in the acquisition of drug resistance, a correlation analysis was performed to determine if FOXM1 could regulate PAK4 which might contribute to paclitaxel resistance of ovarian cancer cells. Interestingly, PAK4 appeared to have high correlation score with FOXM1 and is a potential target gene of FOXM1. It is thus tempting to investigate if the PAK4 could be established as novel FOXM1 transcriptional targets that might be implicated in the acquisition of paclitaxel resistance in ovarian cancer cells.

4.1.4 Aim:

1. To elucidate the role of PAK4 in acquisition of paclitaxel resistance in ovarian cancer

2. To study if PAK4 expression correlates with FOXM1 expression in ovarian cancer tissue samples

3. To investigate if FOXM1 is able to transcriptionally regulate PAK4

4.2 Materials and methods

4.2.1 Cell lines

The paclitaxel-sensitive ovarian cancer cell line PEO1 and -resistant cell line PEO1-TaxR were used in the study. Cell culture conditions were the same as mentioned in chapter 3.

4.2.2 Immunohistochemistry (IHC)

Immunohistochemical staining of FOXM1 was performed in chapter 3. Immunohistochemical staining of PAK4 was done independently in a separate project and the data has been published (Siu *et al.*, 2010b). New PAK4 staining images were captured with agreement of the authors.

4.2.3 Transient knockdown of FOXM1

ON-TARGET Plus Human FOXM1 siRNA and Non-targeting Control siRNAs (Thermo Scientific, CO, USA) were employed for transient silencing of FOXM1 and control using Oligofectamine transfection reagent (Invitrogen).

4.2.4 RNA extraction

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, CA, USA) as described above.

4.2.5 Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was synthesized by Superscript™ III First-Strand synthesis System (Invitrogen) as described above.

4.2.6 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and analysed by ABI7900 Sequence Detection System (Applied Biosystems). Housekeeping gene L19 was used as internal control. The following primers were used:

Primer	Forward 5' to 3'	Reverse 5' to 3'
PAK4	ATGTGGTGGAGATGTACAACAGCTA	GTTCATCCTGGTGTGGGTGAC
L19	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA

Table 4.1. Primers used in qPCR analysis of PAK4

4.2.7 Western blot

Immunoblotting was performed as described above. The following antibodies were used: anti-PAK4 (#3242, Cell Signaling), anti-β-tubulin (sc-9104, Santa Cruz Biotechnology).

4.2.8 Cloning of pGL3-PAK4

Generation of reporter plasmid pGL3-PAK4 was performed similarly to the cloning of pGL3-KIF2C and pGL3-KIF20A. Briefly, a pair of primer with restriction sites (KpnI and HindIII) was designed to amplify a region 1.2kb upstream of the transcription start sites of PAK4. The resulting product was purified and ligated to pGL3 vector. Following transformation, colonies were picked and restriction digestion was used to confirm positive clones harbouring pGL3-PAK4. The following primers were used:

Primer	Forward 5' to 3'	Reverse 5' to 3'
PAK4-Promoter	GGGGTACCGGAATGCAGCAGTGAACAAA	CCCAAGCTTAGTTAGGGGCTGGGAGAAAG

Table 4.2. Primers used in the cloning of pGL3-PAK4

4.2.9 Luciferase reporter assay

Luciferase reporter assay was done as described above.

4.2.10 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was done as described above. qPCR was performed using the following primer: PAK4 ChIP Forward 5' to 3': CCGACCTCCACTACAACTCC; PAK4 ChIP Reverse 5' to 3': CAGACAGACCAGCGTTCAAA.

4.2.11 Statistical analysis

Statistical analysis was performed by the Statistical Package for Social Science version 19.0 for Windows (IBM). Comparison between two groups of non-parametric data was performed by Mann-Whitney *U*-test. Correlation analysis was achieved by Pearson's correlation test. Probability of survival was analyzed using the Kaplan-Meier approach. *P*-values of <0.05 were considered to be statistically significant.

4.3 Results

4.3.1 Nuclear FOXM1 immunohistochemical staining correlates with total PAK4 expression and the co-expression of FOXM1 and PAK4 is a poor prognostic factor for ovarian cancer patients

In order to evaluate if FOXM1 expression correlates with PAK4 expression in ovarian cancer tissue samples, previous PAK4 immunohistochemical stainings and scores were retrieved (Siu *et al.*, 2010b). Although no correlation was observed between nuclear FOXM1 and nuclear PAK4 expressions, using cutoffs of 3 and 4.8 for nuclear FOXM1 IHC score and total PAK4 scores (a sum of PAK4 nuclear and cytoplasmic scores) respectively, nuclear FOXM1 expression was found to be significantly correlated with total PAK expression (P=0.021) (Figure 4.1). Representative images were re-captured from archival PAK4 tissue slides and a similar staining pattern was observed between nuclear FOXM1 and total PAK4 (Figure 4.2). Furthermore, the high co-expression of nuclear FOXM1 and total PAK4 is significantly associated with shorter overall survival (P=0.028) and disease-free survival (P=0.026) of ovarian cancer patients (Figure 4.3).

Count

		FOXM1_nucl_cutoff_3		
		.00	1.00	Total
PaK4_total_cutoff_4.8	.00	13	11	24
	1.00	9	27	36
Total		22	38	60

Correlations

		PaK4_total_cutoff_4.8	FOXM1_nucl_cutoff_3
PaK4_total_cutoff_4.8	Pearson Correlation	1	.297*
	Sig. (2-tailed)		.021
	N	66	60
FOXM1_nucl_cutoff_3	Pearson Correlation	.297*	1
	Sig. (2-tailed)	.021	
	N	60	60

Figure 4.1. Pearson's correlation analysis demonstrating a significant correlation between nuclear FOXM1 expression and total PAK4 expression. Nuclear FOXM1 score was found to be significantly correlated with total PAK4 score (a sum of PAK4 nuclear and cytoplasmic scores) in ovarian cancer patient samples.

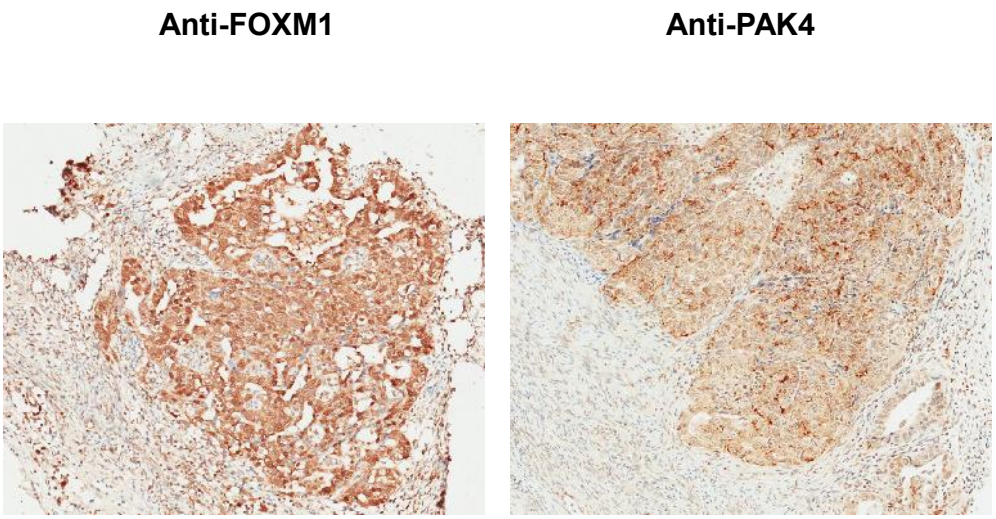


Figure 4.2. Representative IHC images showing a similar staining pattern between nuclear FOXM1 and total PAK4 in ovarian cancer tissue slides. Magnifications X400. Close correlation between FOXM1 and PAK4 stainings in selected sample tissues confirmed the correlation between IHC scores.

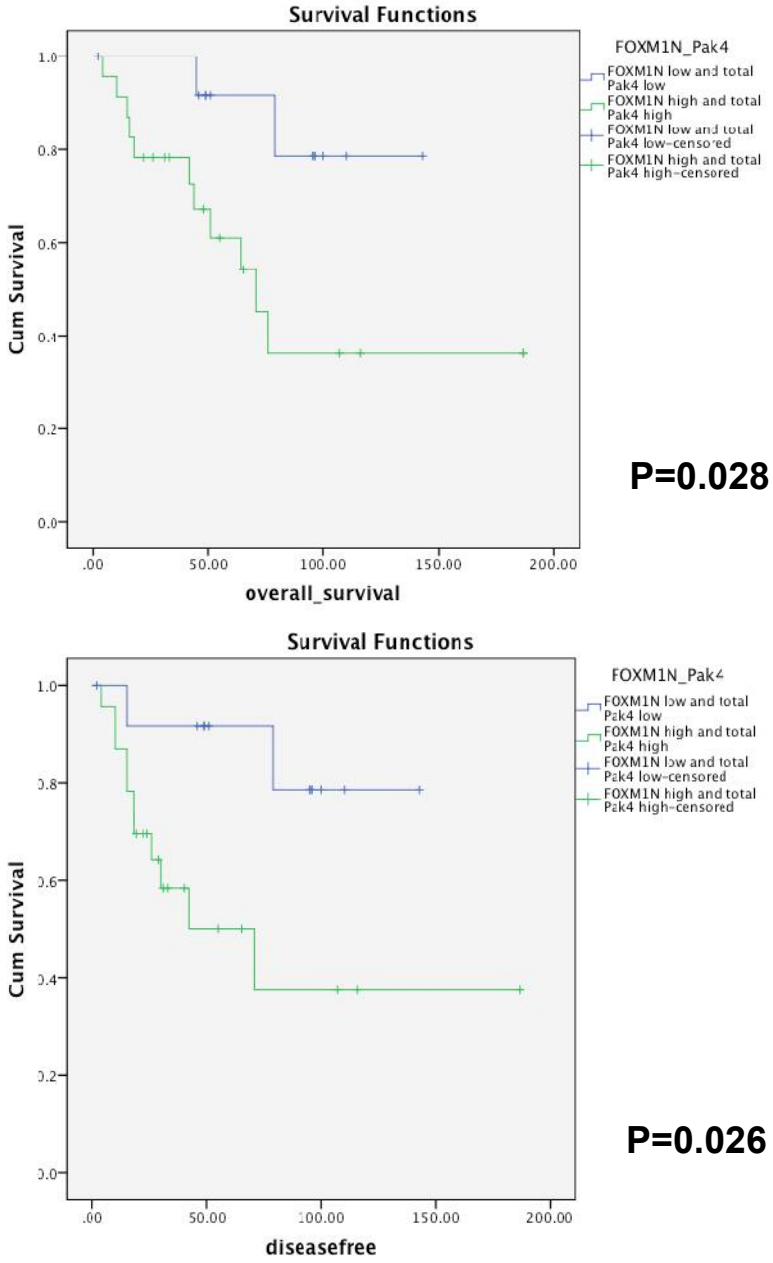


Figure 4.3. Kaplan-Meier analysis showing high co-expression of high nuclear FOXM1 and total PAK4 significantly correlates with poor overall and disease-free survival of ovarian cancer patients.

4.3.2 Paclitaxel treatment down-regulates PAK4 expression at 48 and 72 h in PEO1 but not in PEO1-TaxR

PEO1 and PEO1-TaxR were treated with paclitaxel (50nM) and cells were harvested at various time points (0, 8, 16, 24, 48, 72 h). Immunoblotting analysis showed PAK4 expression is reduced at 48 and 72 h in PEO1 while remaining fairly constant in PEO1-TaxR (Figure 4.4).

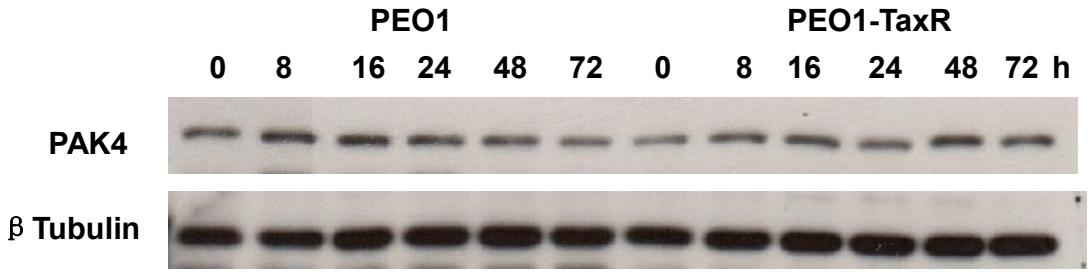


Figure 4.4. Western blot analysis showing PAK4 expression is reduced at 48 and 72 h only in PEO1 but not in PEO1-TaxR. Representative blot was shown from three experiments.

4.3.3 FOXM1 knockdown results in mildly reduced PAK4 expression

Transient FOXM1 knockdown resulted in a mild reduction in PAK4 protein expression as shown by Western blot analysis (Figure 4.5). However, Transcript level of PAK4 remained unchanged upon FOXM1 silencing as shown by qPCR (Figure 4.5).

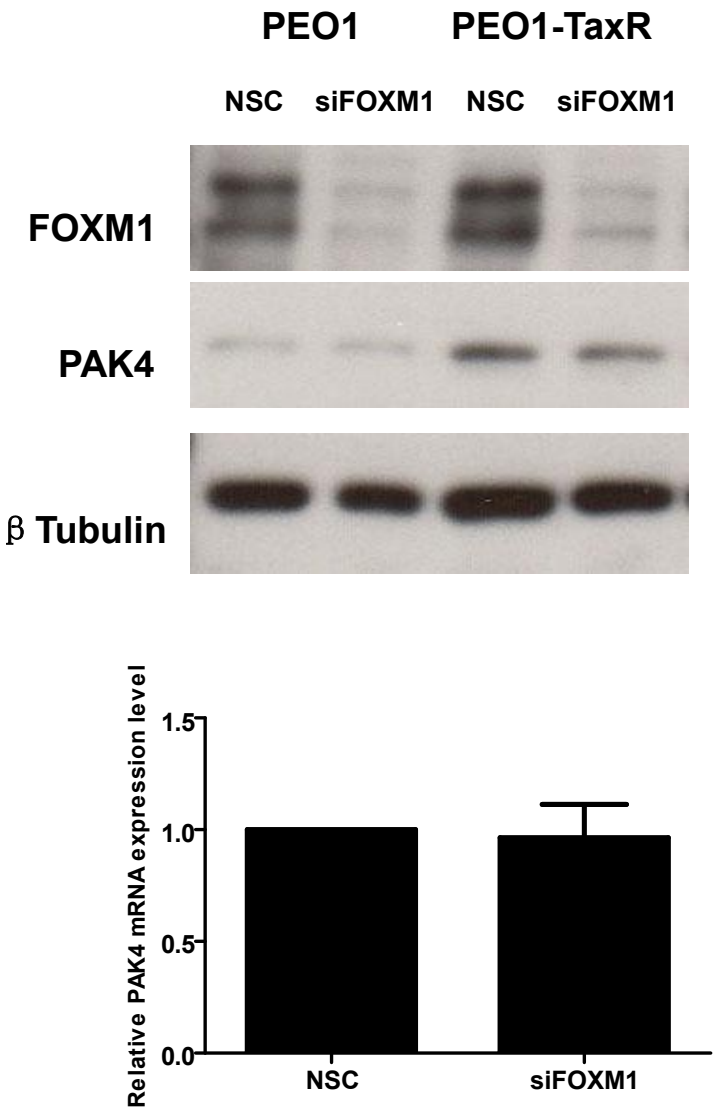


Figure 4.5. Immunoblotting (upper panel) and qPCR (lower panel) showing the effects of FOXM1 knockdown on protein and mRNA expressions of PAK4 respectively. Western blot analysis showed FOXM1 knockdown reduced PAK4 protein expression in PEO1-TaxR. PAK4 transcript level was unaffected upon FOXM1 silencing. Data represent triplicates from three experiments. ns: not significant.

4.3.4 FOXM1 can activate the promoter of PAK4

Several FOXM1 consensus binding sites were identified in the promoter region of PAK4. In order to investigate if FOXM1 could transcriptionally activate PAK4, around 1.2kb region upstream of the transcription start site of PAK4 was cloned into pGL3 vector. The resulting pGL3-PAK4 was subjected to restriction enzyme digestion and agarose gel electrophoresis to confirm the insert (Figure 4.6). Luciferase reporter assay was then performed by titrating pGL3-PAK4 with increasing amounts of pcDNA3-FOXM1 (0, 5, 10, 15, 20 ,25 ng). Empty pcDNA3 was used as control. pGL3-PAK4 appeared to be gradually activated in PEO1 cells with increasing amounts of pcDNA3-FOXM1 as revealed by rising luciferase reading (Figure 4.7).

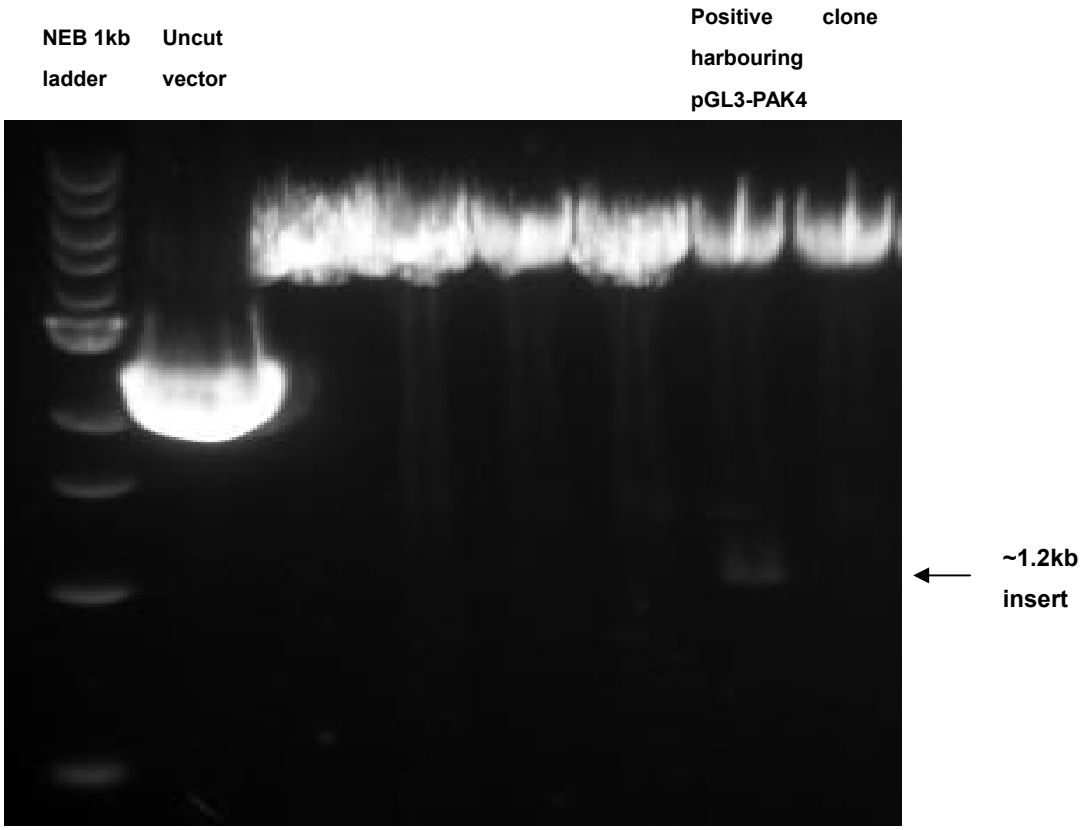


Figure 4.6. Restriction digestion and agarose gel electrophoresis to select positive clones containing pGL3-PAK4 reporter plasmid. 1.2kb region upstream of the transcription start site of PAK4 was amplified and cloned into pGL3 vector. Positive clone containing pGL3-PAK4 was selected by restriction digestion and electrophoresis.

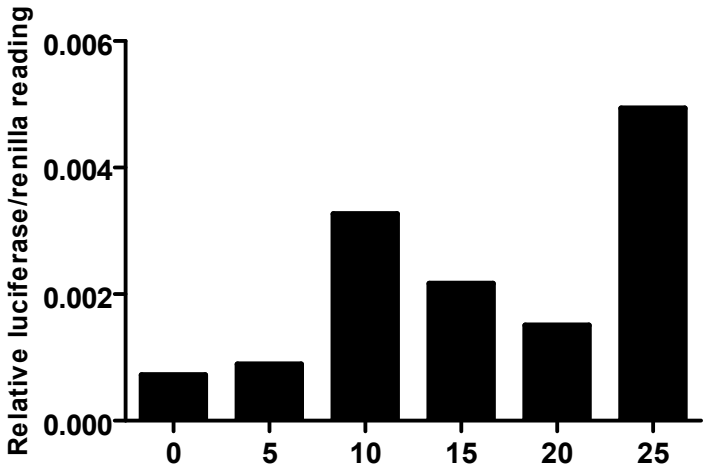


Figure 4.7. pGL3-PAK4 appeared to be gradually activated upon titration with increasing amount (0, 5, 10, 15, 20, 25 ng) of pcDNA3-FOXM1 in PEO1 cells. Luciferase reporter assay demonstrated FOXM1 could activate the promoter region of PAK4.

4.3.5 FOXM1 is capable of binding to the promoter region of PAK4

Given the finding that FOXM1 could activate the promoter of PAK4 as revealed by Luciferase reported assay, chromatin immunoprecipitation (ChIP) was performed in an attempt to evaluate if FOXM1 can bind to the promoter region of PAK4. ChIP-qPCR analysis showed significantly enhanced pull-down of PAK4 by anti-FOXM1 antibody when compared to the IgG control in both PEO1 and PEO1-TaxR (Figure 4.8), indicating FOXM1 is capable of binding to the promoter region of PAK4.

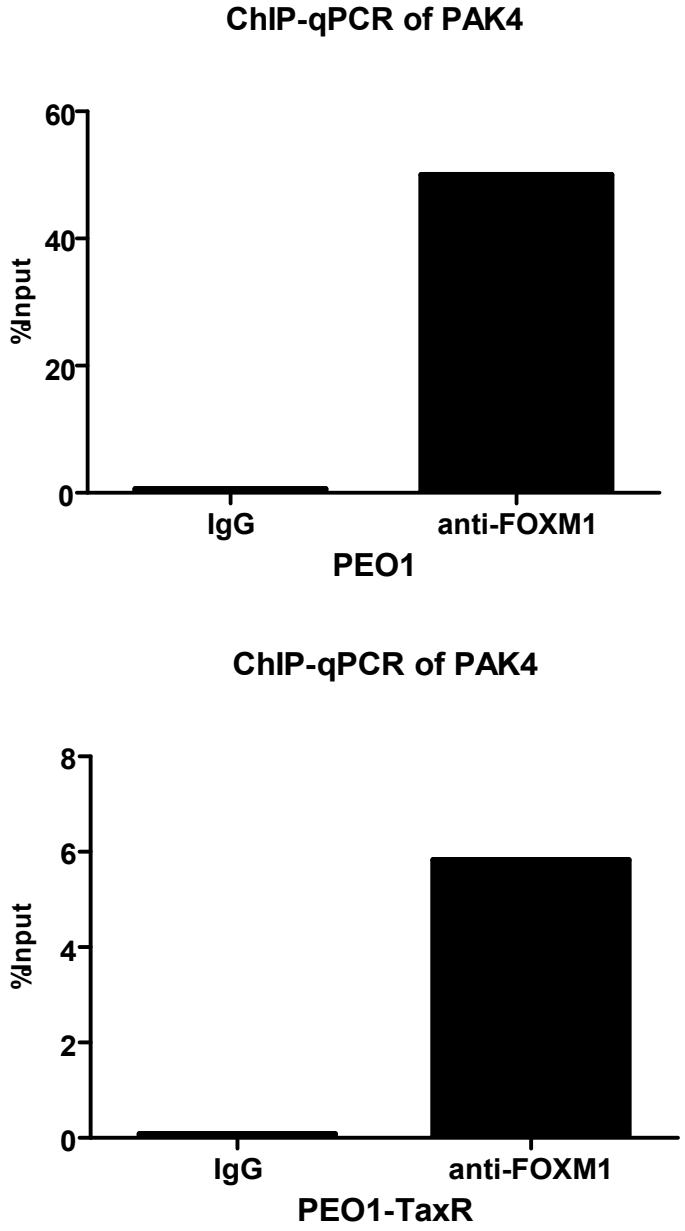


Figure 4.8. Chromatin immunoprecipitation (ChIP)-qPCR showing FOXM1 is able to bind to the promoter region of PAK4 in PEO1 and PEO1-TaxR. ChIP-qPCR analysis demonstrated anti-FOXM1 enhanced the pull-down of PAK4 as compared to the negative IgG control.

4.4 Discussion

Although no apparent association was observed between nuclear FOXM1 expression and nuclear PAK4 expression in this study, nuclear FOXM1 expression displays a significant positive correlation with total PAK4 level as revealed in the immunohistochemical study. Indeed, elevated nuclear and cytoplasmic PAK4 expression has been shown to be associated with shorter overall and disease-free survival of ovarian cancer patients (Siu *et al.*, 2010b). In addition to playing an important role in the regulation of actin cytoskeleton (Callow *et al.*, 2005), nuclear PAK4 has also been documented to promote gene transcription by modulating β-catenin signaling (Y. Li *et al.*, 2012). FOXM1 may thus be implicated in the regulation of both the nuclear as well as cytoplasmic functions of PAK4. Transient silencing of FOXM1 resulted in reduced PAK4 protein expression in PEO1-TaxR. Surprisingly, no change in PAK4 transcript level was observed upon FOXM1 knockdown. This could be attributed to the fact that PAK4 has stable mRNA and unstable protein. Indeed, it has been documented that mRNA correlates poorly with protein levels and only about 40% of protein levels in cultured cells could be explained by mRNA levels (Schwanhausser *et al.*, 2011).

In an attempt to elucidate whether FOXM1 is able to transcriptionally activate PAK4, luciferase reporter assay and chromatin immunoprecipitation studies were performed. Intriguingly, FOXM1 is capable of pulling down PAK4 and inducing activation of the promoter region of PAK4, indicating PAK4 might serve as a novel transcriptional

target of FOXM1. Furthermore, paclitaxel treatment and immunoblotting showed PAK4 expression follows a similar pattern to FOXM1 expression by displaying a reduction at 48 and 72 h only in the sensitive PEO1 cells, but not in the resistant PEO1-TaxR cells, suggesting a role of PAK4 in the development of paclitaxel resistance in ovarian cancer cells. This is in line with a recent study demonstrating overexpression of PAK4 is significantly associated with resistance to chemotherapy in ovarian cancer (Siu *et al.*, 2010b). In figure 4.4, the level of PAK4 expression in PEO1-TaxR at time point 0 is comparable to that in PEO1 at time point 0. On the other hand, a higher expression of PAK4 in non-specific control (NSC) of PEO1-TaxR than that of PEO1 was shown in figure 4.5. Possible reasons may include use of scrambled control in experiments of figure 4.5 but not in that of figure 4.4.

Recently, PAK4 was found to be involved in the regulation of the G1 phase and the G2/M transition of the cell cycle. PAK4 is also crucial for metaphase spindle positioning and anchoring (Bompard *et al.*, 2013). Future experiments will focus on how FOXM1 contributes to cell cycle progression through regulation of PAK4 transcription.

Chapter 5

Up-regulation of hsa-miR-590 and hsa-miR-370 in chemoresistant breast and ovarian cancer cells

5.1 Introduction

5.1.1 MicroRNA

Ever since the discovery of *lin*-4 in the nematode *Caenorhabditis elegans* (*C.elegans*) in 1993, members of the novel class of small non-coding single strand regulatory RNAs , the microRNA (miRNA) family, have been expanding (Chaudhuri & Chatterjee, 2007). miRNAs are comprised of approximately 22 nucleotides and are found in a diverse array of organisms ranging from prokaryotes, eukaryotes to viruses. miRNAs could be either encoded by specific genes or located in the introns or exons of protein-coding genes and expressed as a by-product (Chaudhuri & Chatterjee, 2007). They play crucial roles in a wide spectrum of cellular and physiological functions, including cell proliferation, cell death, metabolism, haematopoiesis, chromatin modification by modulating the expression of target genes (Alvarez-Garcia & Miska, 2005).

5.1.2 Biogenesis and mechanism of action of miRNAs

Biogenesis of miRNAs in vertebrates initiates with the generation of a long primary miRNA (pri-miRNA) which is transcribed mostly by RNA polymerases type II (Pol-II). Each pri-miRNA is then processed into hairpin-shaped precursor miRNA

(pre-miRNA) of approximately 60-70 nucleotidxes by Drosha-like RNase III endonucleases (Borchert *et al.*, 2006). The pre-miRNA is subsequently transported out of nucleus into cytoplasm by Exportin-5 and Ran-GTP, and is then cleaved by Dicer-like RNase III endonuclease to form the mature miRNA duplex. Afterwards, one strand is usually incorporated into the RNA-induced silencing complex (RISC) whereas the other strand is degraded (Borchert *et al.*, 2006). Regulation of gene expression is mediated through the canonical base pairing of miRNA seed sequence and the complementary sequence of target mRNAs followed by silencing or degradation of target mRNAs (Inui *et al.*, 2010). It has been reported an average miRNA has approximately 100 target sites, indicating that miRNAs are capable of regulating a large fraction of protein-coding genes (Brennecke *et al.*, 2005).

5.1.3 miRNAs and cancer

Since a large proportion of the transcriptome is subjected to miRNA regulation, It comes with no surprise that alterations in miRNA expressions contribute to the pathogenesis of many cancer types. Indeed, dysregulated miRNAs have been documented in chronic lymphocytic leukemia (Mraz & Pospisilova, 2012), breast cancer (F. Wang *et al.*, 2010), lung cancer (Sotiropoulou *et al.*, 2009), colorectal cancer (Yang *et al.*, 2009), prostate cancer (Walter *et al.*, 2013), ovarian cancer (Iorio *et al.*, 2007), etc. Recently, accumulating evidence suggests that dysregulations of miRNAs might play an important role in tumorigenesis, metastasis and chemoresistance (Sotiropoulou *et al.*, 2009). For example, overexpression of miR-451

has been reported to increase the sensitivity of MCF-7 and resistant breast cancer cells to doxorubicin (Kovalchuk *et al.*, 2008). Distinct miRNA fingerprint has also been associated with ovarian cancer drug resistance as revealed by high-throughput profiling analysis (Sorrentino *et al.*, 2008).

5.1.4 miR-590

Located on chromosome 7q11.23, hsa-miR-590 is but one member of the microRNA family whose roles in orchestrating normal cellular functions as well as cancer development have been drawing accumulating research focus. For instance, Eulalio *et al.* recently reported miR-590 could promote cardiomyocyte proliferation in both neonatal and adult animals, thus establishing miR-590 as a target for treatment of cardiac pathologies consequent to cardiomyocyte loss (Eulalio *et al.*, 2012). Dysregulations of miR-590-3p and miR-590-5p have also been reported in bladder cancer (Mo *et al.*, 2013) and renal cell carcinoma (Xiao *et al.*, 2013). However, the expression profile and target genes of miR-590 in breast cancer and ovarian cancer tumorigenesis and development of drug resistance have not been studied.

5.1.5 miR-370

miR-370 is located on chromosome 14q32.2 and participates in a myriad of biological processes including regulation of lipid metabolism (Iliopoulos *et al.*, 2010) and development of coronary artery disease (Gao *et al.*, 2012). Accumulating investigations suggest a crucial role of miR-370 in the development of cancer. For

example, overexpression of miR-370 has been reported in gastric carcinoma and miR-370 contributes to carcinogenesis by down-regulating TGFβ-RII (Lo *et al.*, 2012). On the other hand, it has also been suggested that miR-370 could function as a tumour suppressor by down-regulating FOXM1 (Feng *et al.*, 2013; X. Zhang *et al.*, 2012).

In view of the emerging roles of miR-590 and miR-370 in tumorigenesis, a correlation analysis was performed to determine if these miRNAs could have potential interactions with FOXM1 implicated in the development of drug resistance in breast and ovarian cancer cells. Interestingly, both miR-590 and miR-370 appeared to have high correlation scores with FOXM1. It is thus tempting to investigate how miR-590 and miR-370 would interact with FOXM1 that might be implicated in the acquisition of drug resistance in cancer cells.

5.1.6 Aim:

1. To delineate the expression profiles of miR-590-5p, miR-590-3p and miR-370 in a panel of chemosensitive and chemoresistant breast cancer and ovarian cancer cell lines.

2. To identify candidate target genes of miR-590-5p.

3. To study potential interaction between FOXM1 and miR-590

5.2 Materials and methods

5.2.1 Cell culture

Human breast cancer cell lines MCF-7 and MDA MB-231 were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, UK) supplemented with 10% foetal bovine serum (FBS) and 100 units/ml of penicillin-streptomycin (Invitrogen). Breast cancer cell lines LCC9 and TAMR 4/7 were cultured in DMEM and DMEM F-12 (Gibco) supplemented with 10% double charcoal stripped foetal bovine serum (Globepharm Ltd.) respectively. Breast cancer cell lines MLET 1/2/5 cells were cultures in DMEM lacking phenol red (DMEM-PR) (Gibco). Ovarian cancer cell lines PEO1 and PEO1-TaxR were cultured in RPMI1640 (Sigma) supplemented with 10% foetal bovine serum (FBS) and 100 units/ml penicillin-streptomycin (Invitrogen). PEO1-TaxR is supplemented with 50nM of paclitaxel. All cell lines were maintained at 37°C in humidified incubator with 5% CO_2. Cell culture medium was changed every 3 to 5 days depending on cell density. For routine passage, when cells reached 85% to 90% confluency, they were split at a ratio of 1:4.

5.2.2 RNA extraction and cDNA synthesis

Total RNA was extracted using miRNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Reverse transcription was carried out with miScript II RT Kit (Qiagen) using HiFlex buffer to generate cDNA from total RNA and HiSpec buffer to generate cDNA from miRNAs.

5.2.3 Quantitative real-time PCR

Quantitative real-time PCR was performed using miScript SYBR Green PCR Kit (Qiagen) for miRNA analysis and Power SYBR Green PCR Master Mix (Applied Biosystems) for mRNA analysis. Reactions were run on ABI7900 Sequence Detection System (Applied Biosystems). Housekeeping genes L19 and RNU6B were used for normalization for mRNA and miRNA levels respectively. Primers used were listed as follows:

Primer	Forward 5' to 3'	Reverse 5' to 3'
FOXM1	CGTTTCTGCTGTGATTCCAAG	GCCAACCGCTACTTGACATT
FOXO3a	TCTACGAGTGGATGGTGCGTT	CGACTATGCAGTGACAGGTTGTG
FOXA1	GAAGATGGAAGGGCATGAAA	GCCTGAGTTCATGTTGCAGA
FOXC1	AGTTCATCATGGACCGCTTC	AGCCTGTCCTTCTCCTCCTT
FOXC2	AGTTCATCATGGACCGCTTC	TCTCCTTGGACACGTCCTTC

Table 5.1. Primers used in the analysis of expressions of Forkhead box genes

5.2.4 Ectopic expression of hsa-miR-590-5p mimic

MCF-7 cells were transfected with hsa-miR-590-5P mimic (C-300914-01, Thermo Scientific, UK) and non-specific control (NSC) (Dharmacon) using Oligofectamine transfection reagent (Invitrogen) according to manufacturer's instructions.

5.2.5 Western blot

Cells were harvested with lysis buffer [0.125 m Tris, pH 6.8 at 22°C containing 1% NP-40 (v/v), 2 mM ethylenediamine tetraacetic acid (EDTA), 2 mM N-ethylmaleimide, 2 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM sodium orthovanadate and 0.1 μm sodium okadate] and centrifuged at 4°C for 10 min. Protein concentration was determined by detergent-compatible (DC) protein assay (Bio-Rad). Twenty micrograms of protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane and hybridized with the following anti-bodies: anti-FOXM1 (sc-502, Santa Cruz Biotechnology), anti-FOXO3a (sc-11351, Santa Cruz Biotechnology), anti-FOXA1 (ab-55178, Abcam, UK), anti-FOXC1 (ab-24067, Abcam), anti-FOXC2 (ab-ab65141, Abcam) and anti-β-tubulin (sc-9104, Santa Cruz Biotechnology).

5.2.6 Transient knockdown of FOXM1

ON-TARGET Plus Human FOXM1 siRNA and Non-targeting Control siRNAs (Thermo Scientific, CO, USA) were employed for transient silencing of FOXM1 using Oligofectamine transfection reagent (Invitrogen) as previously described.

5.2.7 Luciferase reporter assay

MCF-7 cells seeded in 96-well plate were transfected FuGENE 6 Transfection Reagent (Roche) with a mixture of pGL3-miR-590-3p (a kind gift of Dr Caroline, University Claude Bernard Lyon 1) renilla and an increasing amount (0, 5, 10, 25, 50, 100 ng) of pcDNA3-FOXM1. Luciferase activity was detected by steadylite plus system (PerkinElmer, MA, USA) after 24 h. Briefly, one pot of luclite luciferase reagent was mixed with 10ml of luclite buffer, 10µl of 1M $MgCl_2$ and 5µl of 2M $CaCl_2$. The reagent mixture was diluted with equal volume of PBS and 100µl was applied to each well. After incubating at RT for 15 min in darkness, cells were transferred to opaque plate for measuring fluorescence activity. Afterwards, 25µl of renlite reagent (0.5M HEPES pH7.8, 40mM EDTA, coelenterazine at 1mg/ml) was added to each well and the plate was incubated at RT for 20 min in darkness followed by measurement. Relative reading was obtained by dividing luciferase activity over renilla activity.

5.3 Results

5.3.1 General expression levels of hsa-miR-590-5p (miR-590-5p), hsa-miR-590-3p (miR-590-3p) and hsa-miR-370 (miR-370) in a panel of breast cancer cell lines and a pair of ovarian cancer cell line

Quantitative real-time PCR (qPCR) was employed to assess the endogenous expression profiles of miR-590-5p, miR-590-3p and miR-370 in breast cancer cell lines MCF7, MDA MB-231, LCC9 (steroidal antiestrogen ICI-resistant), TAMR4 and TAMR7 (tamoxifen-resistant), MLET1, MLET2 and MLET5 (estrogen-deprived) as well as ovarian cancer cell lines PEO1 and PEO1-TaxR (paclitaxel-resistant). MCF7 displayed low expression of miR-590-5p, miR-590-3p and miR-370 among the panel of breast cancer cell lines, whereas LCC9, TAMR4 and TAMR7, MLET1, MLET2 and MLET5 harbored elevated expression of the miRNAs ($P<0.05$) (Figures 5.1-5.3). Intriguingly, MDA MB-231 exhibited lowest expression of miR-590-5p, and the levels of miR-590-3p and miR-370 were slightly higher than that of MCF7. Though PEO1-TaxR showed reduced expression of miR-370 when compared to the sensitive counterpart PEO1, no observable differences in the levels of miR-590-5p and miR-590-3p were observed between PEO1 and PEO1-TaxR (Figures 5.1-5.3).

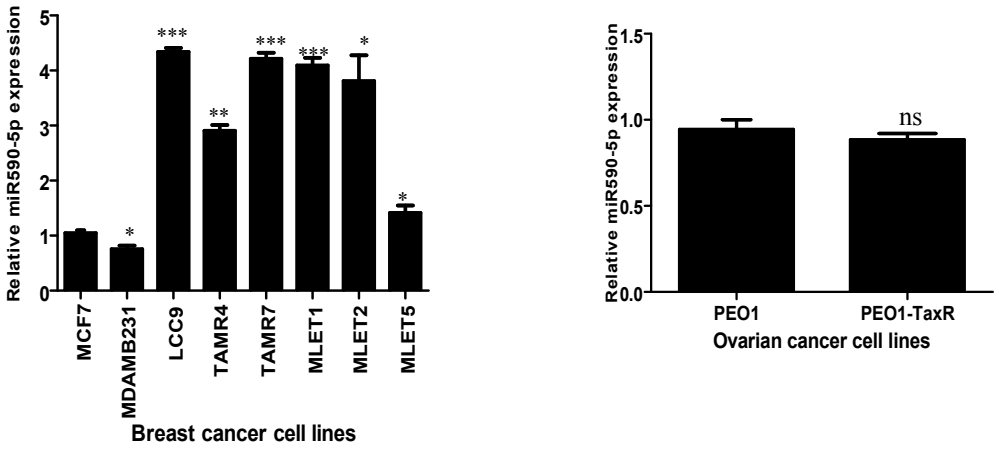


Figure 5.1. qPCR analysis of the general expression level of miR-590-5p in a panel of breast cancer and ovarian cancer cell lines. Housekeeping gene RNU6B was used for normalization. Data represent triplicates from three experiments. *P<0.05, **P=0.002, ***P<0.0005, ns: not significant.

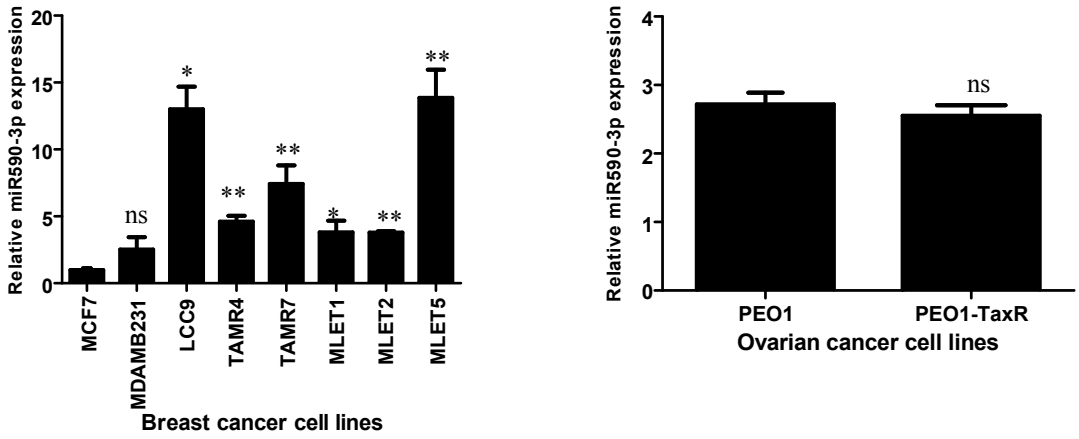


Figure 5.2. qPCR analysis of the general expression level of miR-590-3p in a panel of breast cancer and ovarian cancer cell lines. Housekeeping gene RNU6B was used for normalization. Data represent triplicates from three experiments. *P<0.05, **P<0.002, ns: not significant.

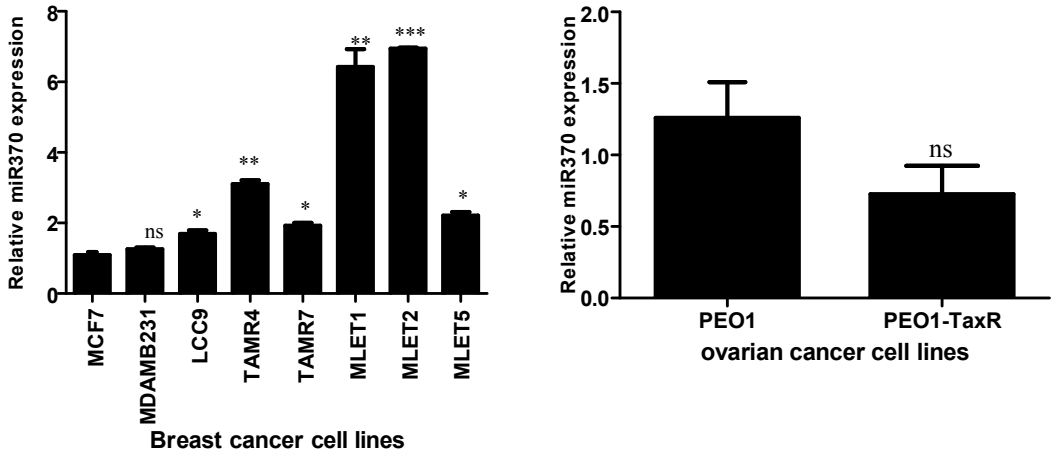


Figure 5.3. qPCR analysis of the general expression level of miR-370 in a panel of breast cancer and ovarian cancer cell lines. Housekeeping gene RNU6B was used for normalization. Data represent triplicates from three experiments. *P<0.05, **P<0.003, ***P<0.0005, ns: not significant.

5.3.2 Identification of candidate target Forkhead box genes of miR-590-5p by ectopic expression of hsa-miR-590-5p mimic

In order to identify candidate target forkhead box genes of miR-590-5p, transient transfection of hsa-miR-590-5p mimic was applied to MCF7 harboring lowest miRNA level. Interestingly, overexpression of miR-590-5p resulted in up-regulations of FOXO3a and FOXC2 at mRNA level, though the increase in protein level was not as obvious (Figure 5.4). On the other hand, both the transcript and protein levels of FOXM1 appeared to be mildly reduced. The protein expression of FOXA1 was down-regulated to a greater extent than that of the mRNA expression. There were no obvious changes in the expression of FOXC1 upon ectopic expression of miR-590-5p (Figure 5.4).

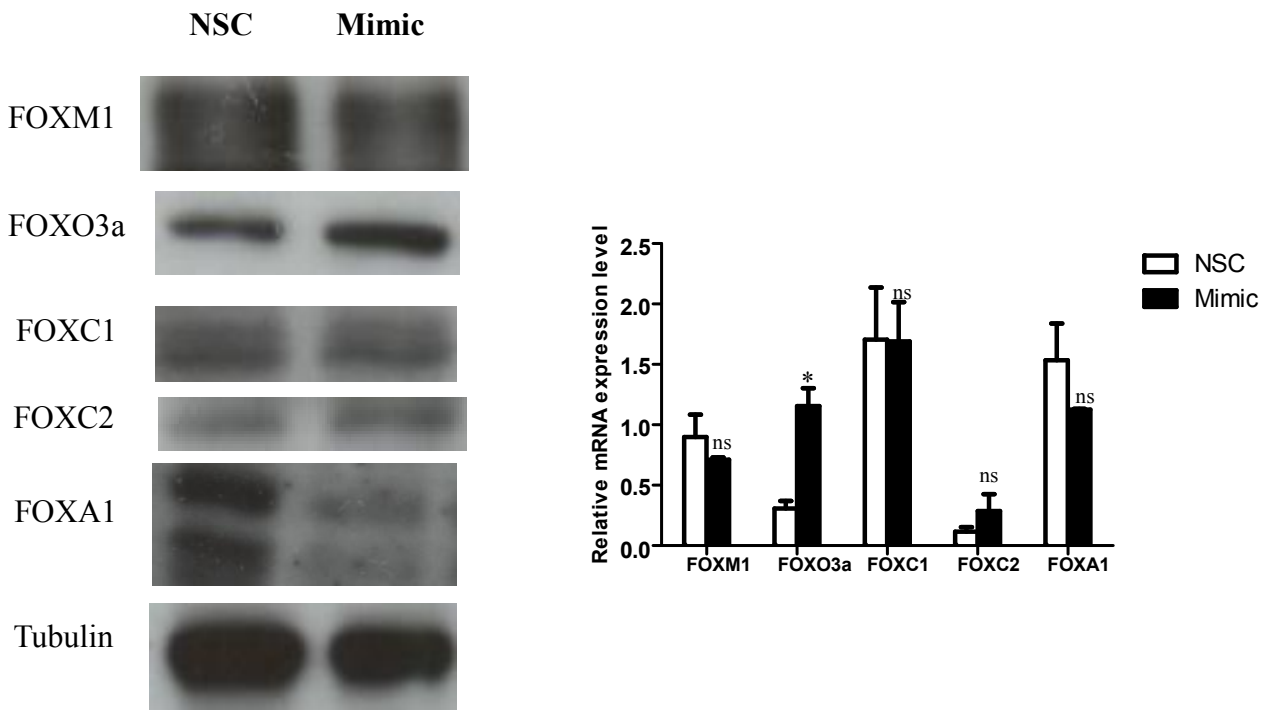


Figure 5.4. Left panel: Representative immunoblots of selected Forkhead box targets of miR-590-5p after treatment with hsa-miR-590-5p mimic. NSC: Non-specific control. Right panel: qPCR analysis of mRNA expressions of corresponding Forkhead box genes after treatment with hsa-miR-590-5p mimic. Housekeeping gene L19 was used for normalization. Data represent triplicates from three experiments. *P=0.02, ns: not significant.

5.3.3 Transient FOXM1 knockdown down-regulates miR-590-5p and miR-590-3p

FOXM1 silencing was used to determine if FOXM1 could regulate miR-590. Quantitative real-time PCR showed the expressions of miR-590-5p and miR-590-3p were down-regulated after transient FOXM1 knockdown (Figure 5.5), indicating FOXM1 is capable of regulating the expression of miR-590.

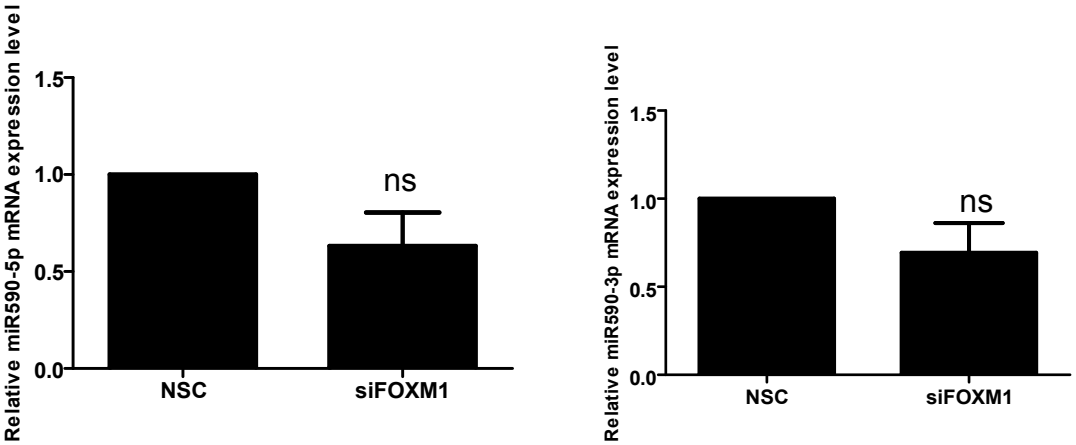


Figure 5.5. Quantitative real-time PCR showing down-regulation of miR-5905p and miR-590-3p after transient FOXM1 knockdown. Data represent triplicates from three experiments. ns: not significant.

5.3.4 FOXM1 is capable of activating the promoter of miR-590

Luciferase reporter assay was performed in an attempt to elucidate if FOXM1 could transcriptionally regulate the expression of miR-590. Titration of pGL3-miR-590-3p with increasing amounts of pcDNA-FOXM1 revealed a gradual rise in luciferase reading, with an abrupt increase at 25ng of pcDNA-FOXM1 (Figure 5.6), indicating FOXM1 could transcriptionally activate the expression of miR590.

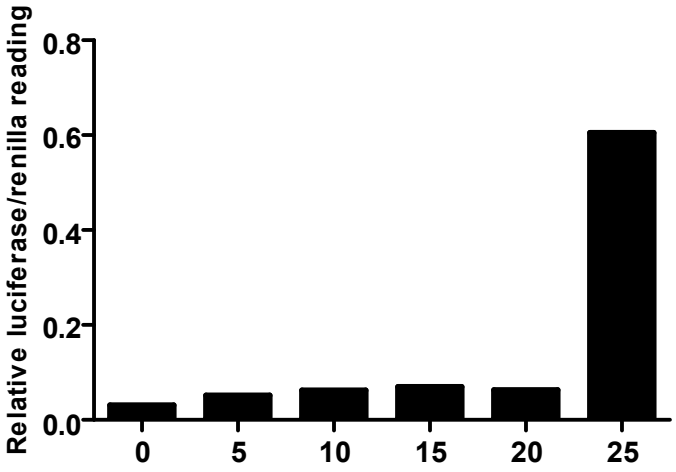


Figure 5.6. Luciferase reporter assay demonstrating FOXM1 could transcriptionally activate the expression of miR-590

5.4 Discussion

Breast cancer is the second leading cause of cancer death in women accounting for around 15% of all female cancer deaths. Although breast cancer mortality has been decreasing, the development of chemoresistance is a perennial problem. Accumulating evidence suggests that various cell types and human cancers display distinct miRNA expression profiles, and that miRNAs may be implicated in a variety of physiological and pathological processes including cell cycle progression, cell differentiation, apoptosis, inflammation and tumorigenesis.

In addition to regulating cardiomyocyte proliferation in both neonatal and adult animals (Eulalio *et al.*, 2012), dysregulations of miR-590 have been documented in bladder cancer and renal cell carcinoma (Mo *et al.*, 2013; Xiao *et al.*, 2013). Recently, down-regulation of miR-590-5p was shown to be involved in hepatocellular carcinoma and restoration of miR-590-5p could inhibit the growth of cancer cells (Shan *et al.*, 2013). In contrast, Chu *et al* demonstrated miR-590 possesses oncogenic properties in that miR-590 is capable of promoting cervical cancer cell growth and invasion by targeting Close Homologue of L1 (CHL1) (Chu *et al.*, 2014). In the current study, we found miR-590-5p and miR-590-3p may be involved in the development of drug resistance in breast cancer cells. Quantitative real-time PCR revealed overexpressions of miR-590-5p and miR-590-3p in ICI resistant cell line LCC9 as well as tamoxifen resistant cell lines TAMR4 and TAMR7 when compared to MCF7. Intriguingly, although up-regulation of miR-590-3p was observed in MDA

MB-231, reduced expression of miR-590-5p was detected in MDA MB-231 as compared with MCF7, implying differential roles of miR-590-5p and miR-590-3p in MDA MB-231 which harbours mutations in *TP53*. Furthermore, ectopic expression of miR-590-5p resulted in down-regulation of FOXM1 and FOXA1 at both transcript and protein levels in MCF7, suggesting FOXM1 and FOXA1 might be candidate downstream targets of miR-590. In view of the bioinformatics analysis revealing miR-590 to be a potential transcriptional target of FOXM1, functional *in vitro* experiments were performed. Transient knockdown of FOXM1 reduces the expressions of miR-590-5p and miR-590-3p. FOXM1 is able to induce the activation of promoter region of miR-590-3p as revealed by Luciferase reporter assay. Further investigations are necessary to elucidate the mechanism by which miR-590 contributes to the acquisition of drug resistance in breast cancer as well as the role of interaction between FOXM1 and miR-590.

Similarly, quantitative real-time PCR also showed up-regulation of miR-370 in LCC9, TAMR4 and TAMR7 when compared to MCF7, suggesting a role of miR-370 in the acquisition of chemoresistance in breast cancer. Interestingly, the expression of miR-370 is also lower in paclitaxel resistant ovarian cancer cell line PEO1-TaxR than the sensitive cell line PEO1. Given the recent finding that miR-370 may function as a tumour suppressor in laryngeal squamous cell carcinoma (LSCC) through down-regulation of FOXM1 (Yungang *et al.*, 2014), it is tempting to carry out further studies in order to delineate potential interactions between miR-370 with FOXM1 as

well as other FOX proteins with emphasis on the role of miR-370 in the development of breast and ovarian cancer. Potential interactions between miR-590 and miR-370 with KIF2C and PAK4 will also be pursued in further studies.

Chapter 6

Discussion

6.1 Summary of findings

Ovarian carcinoma is the most lethal gynecological malignancy. Identifying novel prognostic and therapeutic targets is therefore crucial for improving the long-term survival rate of patients. Forkhead box protein M1 (FOXM1), which is a widely studied member of the FOX superfamily of proteins, plays a vital role in a broad spectrum of biological processes. Recently, overexpression of FOXM1 has been documented in multiple human malignancies including cancers of lung, prostate and breast. In this study, elevated nuclear FOXM1 expression was found to be significantly associated with advanced stages of ovarian cancer. Though not reaching statistical significance, FOXM1 overexpression also showed correlation with serous histologic subtype, high grade cancers (poor differentiation) and chemoresistance. Patients with a high FOXM1 level had a significantly shorter overall and disease-free survival than those with low FOXM1 expression. Multivariate progression analysis further established high expression of FOXM1, advanced cancer stages and poor histological differentiation (high grade) as independent prognostic factors for short overall and disease-free survival. Consistently, Transwell assays demonstrated transient knockdown of FOXM1 was capable of reducing the migratory and invasive abilities of ovarian cancer cells.

Although FOXM1's association with high grade ovarian cancer tumours has been reported (Chan *et al.*, 2012), whether FOXM1 contributes to the acquisition of paclitaxel resistance in ovarian cancer remains unanswered. In view of the immunohistochemical finding suggesting an association between FOXM1 and chemoresistance, a pair of established paclitaxel-sensitive and -resistant ovarian cancer cell lines were employed to investigate the effect of paclitaxel on FOXM1. Intriguingly, paclitaxel treatment resulted in reduced expression of FOXM1 in SKOV-3 but not in the resistant cell line SKOV3-TR, implicating a role of FOXM1 in mediating paclitaxel resistance in ovarian cancer cells. Immunofluorescence study further showed FOXM1 knockdown could enhance paclitaxel-mediated cell death in two ovarian cancer cell lines. Western blot and flow cytometric analyses suggested FOXM1 knockdown in the chemoresistant ovarian cancer cell line SKOV3-TR could induce G2/M arrest and enhance paclitaxel-mediated mitotic catastrophe in a p53-independent and Caspase-9-independent manner.

During recent years increasing attention has been directed towards the oncogenic roles of KIF2C and PAK4. In this study, the expressions of KIF2C and PAK4 were observed to alter in a similar pattern to FOXM1 expression upon paclitaxel treatment by exhibiting reduced expression only in the paclitaxel sensitive cell line at later time points. An analysis of immunohistochemical scores revealed a significant positive correlation between nuclear FOXM1 expression and total PAK4 expression. FOXM1 silencing, qPCR, luciferase reporter assay and chromatin immunoprecipitation

analyses confirmed KIF2C and PAK4 to be novel transcriptional targets of FOXM1. Clonogenic assay and flow cytometry further demonstrated KIF2C contributes to the acquisition of paclitaxel resistance in ovarian cancer cells.

Given the emerging roles of miRNAs in tumorigenesis and chemoresistance, it is tempting to investigate if miRNAs contribute to the development of drug resistance in breast and ovarian cancer cells. qPCR analysis revealed up-regulations of miR-590 and miR-370 in a panel of drug resistant breast cancer cell lines and a paclitaxel resistant cell line. Overexpression of miR-590 resulted in reduced FOXM1 expression. Interestingly, FOXM1 could also regulate the expression of miR-590 at transcriptional level, suggesting a mutual regulation between FOXM1 and miR-590.

In summary, this study demonstrated overexpression of FOXM1 in ovarian cancer correlated with poor patients' survival and acquisition of paclitaxel resistance. KIF2C and PAK4 were identified as novel transcriptional targets of FOXM1 implicated in chemoresistance. Although therapeutics targeting PI3K are being developed at a rapid pace, the central role of PI3K in a large array of diverse biologic processes raises concerns about its use in therapeutics (Courtney *et al.*, 2010). Instead, FOXM1 may emerge as a potential prognostic marker as well as a therapeutic target in ovarian cancer. Thiazole antibiotics siomycin A and thiostrepton have been reported to act as potent inhibitors of FOXM1 (Alvarez-Fernandez & Medema, 2013).

6.2 Limitations

Although considerable evidence has been gathered regarding the association between FOXM1 and clinicopathological parameters of ovarian cancer, recruitment of a larger number of tissue samples for immunohistochemical study is necessary to determine if a significant correlation exists between FOXM1 expression and chemoresistance. *In vivo* studies involving nude mice could be performed to further validate the findings of *in vitro* functional assays.

6.3 Plan for future study

Site-directed mutagenesis, generation of mutant reporter plasmids and luciferase reporter assay will be pursued to validate if FOXM1 is able to activate the promoter regions of KIF2C and PAK4. Overexpressions of KIF2C and PAK4 in FOXM1 knockout ovarian cancer cell lines will further shed light on how the interaction would affect the migratory and invasive abilities as well as sensitivity to chemotherapeutic drugs of cancer cells. Ovarian cancer cell lines with stable overexpression or knockdown of FOXM1 and KIF2C will be generated and *in vivo* experiments involving nude mice will be performed. In situ hybridization (ISH) will be performed to determine the expression profiles of miR-590 and miR-370 in breast and ovarian cancer clinical samples. Inhibitors of the miR-590 and miR-370 will be employed to study the function of the miRNAs regarding chemoresistance and to identify novel regulatory targets.

References

Aarnio, M., Sankila, R., Pukkala, E., Salovaara, R., Aaltonen, L. A., de la Chapelle, A., Peltomaki, P., Mecklin, J. P., & Jarvinen, H. J. (1999). Cancer risk in mutation carriers of DNA-mismatch-repair genes. *Int J Cancer, 81*(2), 214-218.

Abaza, A., Soleilhac, J. M., Westendorf, J., Piel, M., Crevel, I., Roux, A., & Pirollet, F. (2003). M phase phosphoprotein 1 is a human plus-end-directed kinesin-related protein required for cytokinesis. *J Biol Chem, 278*(30), 27844-27852.

Aebi, S., Kurdi-Haidar, B., Gordon, R., Cenni, B., Zheng, H., Fink, D., Christen, R. D., Boland, C. R., Koi, M., Fishel, R., & Howell, S. B. (1996). Loss of DNA mismatch repair in acquired resistance to cisplatin. *Cancer Res, 56*(13), 3087-3090.

Ahmed, N., Thompson, E. W., & Quinn, M. A. (2007). Epithelial-mesenchymal interconversions in normal ovarian surface epithelium and ovarian carcinomas: an exception to the norm. *J Cell Physiol, 213*(3), 581-588.

Ahmed, S. M., Theriault, B. L., Uppalapati, M., Chiu, C. W., Gallie, B. L., Sidhu, S. S., & Angers, S. (2012). KIF14 negatively regulates Rap1a-Radil signaling during breast cancer progression. *J Cell Biol, 199*(6), 951-967.

Ahn, H. K., Jang, J., Lee, J., Se Hoon, P., Park, J. O., Park, Y. S., Lim, H. Y., Kim, K. M., & Kang, W. K. (2011). P21-activated kinase 4 overexpression in metastatic gastric cancer patients. *Transl Oncol, 4*(6), 345-349.

Akhmedkhanov, A., Toniolo, P., Zeleniuch-Jacquotte, A., Kato, I., Koenig, K. L., & Shore, R. E. (2001). Aspirin and epithelial ovarian cancer. *Prev Med, 33*(6), 682-687.

Alexandre, J., Ray-Coquard, I., Selle, F., Floquet, A., Cottu, P., Weber, B., Falandry, C., Lebrun, D., & Pujade-Lauraine, E. (2010). Mucinous advanced epithelial ovarian carcinoma: clinical presentation and sensitivity to platinum-paclitaxel-based chemotherapy, the GINECO experience. *Ann Oncol, 21*(12), 2377-2381.

Alvarez-Fernandez, M., & Medema, R. H. (2013). Novel functions of FoxM1: from molecular mechanisms to cancer therapy. *Front Oncol, 3*, 30.

Alvarez-Garcia, I., & Miska, E. A. (2005). MicroRNA functions in animal development and human disease. *Development, 132*(21), 4653-4662.

Au, C. W., Siu, M. K., Liao, X., Wong, E. S., Ngan, H. Y., Tam, K. F., Chan, D. C., Chan, Q. K., & Cheung, A. N. (2009). Tyrosine kinase B receptor and BDNF expression in ovarian cancers - Effect on cell migration, angiogenesis and clinical outcome. *Cancer Lett, 281*(2), 151-161.

Auersperg, N., Wong, A. S., Choi, K. C., Kang, S. K., & Leung, P. C. (2001). Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocr Rev, 22*(2), 255-288.

Baldwin, R. L., Nemeth, E., Tran, H., Shvartsman, H., Cass, I., Narod, S., & Karlan, B. Y. (2000). BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study. *Cancer Res, 60*(19), 5329-5333.

Bao, B., Wang, Z., Ali, S., Kong, D., Banerjee, S., Ahmad, A., Li, Y., Azmi, A. S., Miele, L., & Sarkar, F. H. (2011). Over-expression of FoxM1 leads to epithelial-mesenchymal transition and cancer stem cell phenotype in pancreatic cancer cells. *J Cell Biochem, 112*(9), 2296-2306.

Basavarajappa, H. D., & Corson, T. W. (2012). KIF14 as an oncogene in retinoblastoma: a target for novel therapeutics? *Future Med Chem, 4*(17), 2149-2152.

Bell, K. A., & Kurman, R. J. (2000). A clinicopathologic analysis of atypical proliferative (borderline) tumors and well-differentiated endometrioid adenocarcinomas of the ovary. *Am J Surg Pathol, 24*(11), 1465-1479.

Bertone, E. R., Newcomb, P. A., Willett, W. C., Stampfer, M. J., & Egan, K. M. (2002). Recreational physical activity and ovarian cancer in a population-based case-control study. *Int J Cancer, 99*(3), 431-436.

Bhatti, P., Cushing-Haugen, K. L., Wicklund, K. G., Doherty, J. A., & Rossing, M. A. (2013). Nightshift work and risk of ovarian cancer. *Occup Environ Med, 70*(4), 231-237.

Bochkis, I. M., Rubins, N. E., White, P., Furth, E. E., Friedman, J. R., & Kaestner, K. H. (2008). Hepatocyte-specific ablation of Foxa2 alters bile acid homeostasis and results in endoplasmic reticulum stress. *Nat Med, 14*(8), 828-836.

Bodelon, C., Wentzensen, N., Schonfeld, S. J., Visvanathan, K., Hartge, P., Park, Y., & Pfeiffer, R. M. (2013). Hormonal risk factors and invasive epithelial ovarian cancer risk by parity. *Br J Cancer, 109*(3), 769-776.

Bolis, G., Scarfone, G., Polverino, G., Raspagliesi, F., Tateo, S., Richiardi, G., Melpignano, M., Franchi, M., Mangili, G., Presti, M., Villa, A., Conta, E., Guarnerio, P., Cipriani, S., & Parazzini, F. (2004). Paclitaxel 175 or 225 mg per meters squared with carboplatin in advanced ovarian cancer: a randomized trial. *J Clin Oncol, 22*(4), 686-690.

Bompard, G., Rabeharivelo, G., Cau, J., Abrieu, A., Delsert, C., & Morin, N. (2013). P21-activated kinase 4 (PAK4) is required for metaphase spindle positioning and anchoring. *Oncogene, 32*(7), 910-919.

Borchert, G. M., Lanier, W., & Davidson, B. L. (2006). RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol, 13*(12), 1097-1101.

Breast Cancer Linkage Consortium. (1999). Cancer risks in BRCA2 mutation carriers. *J Natl Cancer Inst, 91*(15), 1310-1316.

Brennecke, J., Stark, A., Russell, R. B., & Cohen, S. M. (2005). Principles of microRNA-target recognition. *PLoS Biol, 3*(3), e85.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., & Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science, 282*(5393), 1497-1501.

Cahill, D. P., Lengauer, C., Yu, J., Riggins, G. J., Willson, J. K., Markowitz, S. D., Kinzler, K. W., & Vogelstein, B. (1998). Mutations of mitotic checkpoint genes in human cancers. *Nature, 392*(6673), 300-303.

Callow, M. G., Clairvoyant, F., Zhu, S., Schryver, B., Whyte, D. B., Bischoff, J. R., Jallal, B., & Smeal, T. (2002). Requirement for PAK4 in the

anchorage-independent growth of human cancer cell lines. *J Biol Chem, 277*(1), 550-558.

Callow, M. G., Zozulya, S., Gishizky, M. L., Jallal, B., & Smeal, T. (2005). PAK4 mediates morphological changes through the regulation of GEF-H1. *J Cell Sci, 118*(Pt 9), 1861-1872.

Campbell, A. J., Lyne, L., Brown, P. J., Launchbury, R. J., Bignone, P., Chi, J., Roncador, G., Lawrie, C. H., Gatter, K. C., Kusec, R., & Banham, A. H. (2010). Aberrant expression of the neuronal transcription factor FOXP2 in neoplastic plasma cells. *Br J Haematol, 149*(2), 221-230.

Cancer Genome Atlas Network. (2012). Comprehensive molecular portraits of human breast tumours. *Nature, 490*(7418), 61-70.

Castedo, M., Perfettini, J. L., Roumier, T., Andreau, K., Medema, R., & Kroemer, G. (2004). Cell death by mitotic catastrophe: a molecular definition. *Oncogene, 23*(16), 2825-2837.

Chan, D. W., Hui, W. W., Cai, P. C., Liu, M. X., Yung, M. M., Mak, C. S., Leung, T. H., Chan, K. K., & Ngan, H. Y. (2012). Targeting GRB7/ERK/FOXM1 signaling pathway impairs aggressiveness of ovarian cancer cells. *PLoS One, 7*(12), e52578.

Chan, T. A., Hermeking, H., Lengauer, C., Kinzler, K. W., & Vogelstein, B. (1999). 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature, 401*(6753), 616-620.

Chaudhuri, K., & Chatterjee, R. (2007). MicroRNA detection and target prediction: integration of computational and experimental approaches. *DNA Cell Biol, 26*(5), 321-337.

Che, M., Tornos, C., Deavers, M. T., Malpica, A., Gershenson, D. M., & Silva, E. G. (2001). Ovarian mixed-epithelial carcinomas with a microcystic pattern and signet-ring cells. *Int J Gynecol Pathol, 20*(4), 323-328.

Chen, C. H., Shen, J., Lee, W. J., & Chow, S. N. (2005). Overexpression of cyclin D1 and c-Myc gene products in human primary epithelial ovarian cancer. *Int J Gynecol Cancer, 15*(5), 878-883.

Chen, J., Gomes, A. R., Monteiro, L. J., Wong, S. Y., Wu, L. H., Ng, T. T., Karadedou, C. T., Millour, J., Ip, Y. C., Cheung, Y. N., Sunters, A., Chan, K. Y., Lam, E. W., & Khoo, U. S. (2010). Constitutively nuclear FOXO3a localization predicts poor survival and promotes Akt phosphorylation in breast cancer. *PLoS One, 5*(8), e12293.

Chen, L., Tang, Y., Wang, J., Yan, Z., & Xu, R. (2013). miR-421 induces cell proliferation and apoptosis resistance in human nasopharyngeal carcinoma via downregulation of FOXO4. *Biochem Biophys Res Commun, 435*(4), 745-750.

Cheng, W., Liu, J., Yoshida, H., Rosen, D., & Naora, H. (2005). Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. *Nat Med, 11*(5), 531-537.

Chu, Y., Ouyang, Y., Wang, F., Zheng, A., Bai, L., Han, L., Chen, Y., & Wang, H. (2014). MicroRNA-590 Promotes Cervical Cancer Cell Growth and Invasion by Targeting CHL1. *J Cell Biochem*, 115(5), 847-853.

Colombo, N., Guthrie, D., Chiari, S., Parmar, M., Qian, W., Swart, A. M., Torri, V., Williams, C., Lissoni, A., & Bonazzi, C. (2003). International Collaborative Ovarian Neoplasm trial 1: a randomized trial of adjuvant chemotherapy in women with early-stage ovarian cancer. *J Natl Cancer Inst, 95*(2), 125-132.

Come, C., Arnoux, V., Bibeau, F., & Savagner, P. (2004). Roles of the transcription factors snail and slug during mammary morphogenesis and breast carcinoma progression. *J Mammary Gland Biol Neoplasia, 9*(2), 183-193.

Corson, T. W., Zhu, C. Q., Lau, S. K., Shepherd, F. A., Tsao, M. S., & Gallie, B. L. (2007). KIF14 messenger RNA expression is independently prognostic for outcome in lung cancer. *Clin Cancer Res, 13*(11), 3229-3234.

Cotteret, S., Jaffer, Z. M., Beeser, A., & Chernoff, J. (2003). p21-Activated kinase 5 (Pak5) localizes to mitochondria and inhibits apoptosis by phosphorylating BAD. *Mol Cell Biol, 23*(16), 5526-5539.

Courtney, K. D., Corcoran, R. B., & Engelman, J. A. (2010). The PI3K pathway as drug target in human cancer. *J Clin Oncol, 28*(6), 1075-1083.

Cramer, D. W., Kuper, H., Harlow, B. L., & Titus-Ernstoff, L. (2001). Carotenoids, antioxidants and ovarian cancer risk in pre- and postmenopausal women. *Int J Cancer, 94*(1), 128-134.

Cramer, D. W., & Welch, W. R. (1983). Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. *J Natl Cancer Inst, 71*(4), 717-721.

Dan, C., Kelly, A., Bernard, O., & Minden, A. (2001). Cytoskeletal changes regulated by the PAK4 serine/threonine kinase are mediated by LIM kinase 1 and cofilin. *J Biol Chem, 276*(34), 32115-32121.

Dan, C., Nath, N., Liberto, M., & Minden, A. (2002). PAK5, a new brain-specific kinase, promotes neurite outgrowth in N1E-115 cells. *Mol Cell Biol, 22*(2), 567-577.

Daniilidis, A., & Karagiannis, V. (2007). Epithelial ovarian cancer. Risk factors, screening and the role of prophylactic oophorectomy. *Hippokratia, 11*(2), 63-66.

De, S., Cipriano, R., Jackson, M. W., & Stark, G. R. (2009). Overexpression of kinesins mediates docetaxel resistance in breast cancer cells. *Cancer Res, 69*(20), 8035-8042.

DeGraff, D. J., Clark, P. E., Cates, J. M., Yamashita, H., Robinson, V. L., Yu, X., Smolkin, M. E., Chang, S. S., Cookson, M. S., Herrick, M. K., Shariat, S. F., Steinberg, G. D., Frierson, H. F., Wu, X. R., Theodorescu, D., & Matusik, R. J. (2012). Loss of the urothelial differentiation marker FOXA1 is associated with high grade, late stage bladder cancer and increased tumor proliferation. *PLoS One, 7*(5), e36669.

Di Maso, M., Talamini, R., Bosetti, C., Montella, M., Zucchetto, A., Libra, M., Negri, E., Levi, F., La Vecchia, C., Franceschi, S., Serraino, D., & Polesel, J. (2013). Red meat and cancer risk in a network of case-control studies focusing on cooking practices. *Ann Oncol*, 24(2), 3107-3112.

Ding, S., Xing, N., Lu, J., Zhang, H., Nishizawa, K., Liu, S., Yuan, X., Qin, Y., Liu, Y., Ogawa, O., & Nishiyama, H. (2011). Overexpression of Eg5 predicts

unfavorable prognosis in non-muscle invasive bladder urothelial carcinoma. *Int J Urol, 18*(6), 432-438.

Downing, K. H. (2000). Structural basis for the interaction of tubulin with proteins and drugs that affect microtubule dynamics. *Annu Rev Cell Dev Biol, 16*, 89-111.

Eulalio, A., Mano, M., Dal Ferro, M., Zentilin, L., Sinagra, G., Zacchigna, S., & Giacca, M. (2012). Functional screening identifies miRNAs inducing cardiac regeneration. *Nature, 492*(7429), 376-381.

Farrell, C., Lyman, M., Freitag, K., Fahey, C., Piver, M. S., & Rodabaugh, K. J. (2006). The role of hereditary nonpolyposis colorectal cancer in the management of familial ovarian cancer. *Genet Med, 8*(10), 653-657.

Fathalla, M. F. (1971). Incessant ovulation--a factor in ovarian neoplasia? *Lancet, 2*(7716), 163.

Fendler, A., Jung, M., Stephan, C., Erbersdobler, A., Jung, K., & Yousef, G. M. (2013). The Antiapoptotic Function of miR-96 in Prostate Cancer by Inhibition of FOXO1. *PLoS One, 8*(11), e80807.

Feng, J., Zhang, X., Zhu, H., Wang, X., Ni, S., & Huang, J. (2012). High expression of FoxP1 is associated with improved survival in patients with non-small cell lung cancer. *Am J Clin Pathol, 138*(2), 230-235.

Feng, Y., Wang, L., Zeng, J., Shen, L., Liang, X., Yu, H., Liu, S., Liu, Z., Sun, Y., Li, W., Chen, C., & Jia, J. (2013). FoxM1 is overexpressed in Helicobacter pylori-induced gastric carcinogenesis and is negatively regulated by miR-370. *Mol Cancer Res, 11*(8), 834-844.

Francis, R. E., Myatt, S. S., Krol, J., Hartman, J., Peck, B., McGovern, U. B., Wang, J., Guest, S. K., Filipovic, A., Gojis, O., Palmieri, C., Peston, D., Shousha, S., Yu, Q., Sicinski, P., Coombes, R. C., & Lam, E. W. (2009). FoxM1 is a downstream target and marker of HER2 overexpression in breast cancer. *Int J Oncol, 35*(1), 57-68.

Furuyama, T., Kitayama, K., Shimoda, Y., Ogawa, M., Sone, K., Yoshida-Araki, K., Hisatsune, H., Nishikawa, S., Nakayama, K., Ikeda, K., Motoyama, N., & Mori, N. (2004). Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. *J Biol Chem, 279*(33), 34741-34749.

Galanski, M. (2006). Recent developments in the field of anticancer platinum complexes. *Recent Pat Anticancer Drug Discov, 1*(2), 285-295.

Galluzzi, L., Senovilla, L., Vitale, I., Michels, J., Martins, I., Kepp, O., Castedo, M., & Kroemer, G. (2012). Molecular mechanisms of cisplatin resistance. *Oncogene, 31*(15), 1869-1883.

Ganguly, A., Yang, H., & Cabral, F. (2011). Overexpression of mitotic centromere-associated Kinesin stimulates microtubule detachment and confers resistance to paclitaxel. *Mol Cancer Ther, 10*(6), 929-937.

Gao, J., Sai, N., Wang, C., Sheng, X., Shao, Q., Zhou, C., Shi, Y., Sun, S., Qu, X., & Zhu, C. (2011). Overexpression of chromokinesin KIF4 inhibits proliferation of human gastric carcinoma cells both in vitro and in vivo. *Tumour Biol, 32*(1), 53-61.

Gao, N., Zhang, J., Rao, M. A., Case, T. C., Mirosevich, J., Wang, Y., Jin, R., Gupta, A., Rennie, P. S., & Matusik, R. J. (2003). The role of hepatocyte nuclear factor-3 alpha (Forkhead Box A1) and androgen receptor in transcriptional regulation of prostatic genes. *Mol Endocrinol, 17*(8), 1484-1507.

Gao, W., He, H. W., Wang, Z. M., Zhao, H., Lian, X. Q., Wang, Y. S., Zhu, J., Yan, J. J., Zhang, D. G., Yang, Z. J., & Wang, L. S. (2012). Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease. *Lipids Health Dis, 11*, 55.

Gasnereau, I., Boissan, M., Margall-Ducos, G., Couchy, G., Wendum, D., Bourgain-Guglielmetti, F., Desdouets, C., Lacombe, M. L., Zucman-Rossi, J., & Sobczak-Thepot, J. (2012). KIF20A mRNA and its product MKlp2 are increased during hepatocyte proliferation and hepatocarcinogenesis. *Am J Pathol, 180*(1), 131-140.

Gedeon, A. K., Nelson, J., Gecz, J., & Mulley, J. C. (2003). X-linked mild non-syndromic mental retardation with neuropsychiatric problems and the missense mutation A365E in PAK3. *Am J Med Genet A, 120A*(4), 509-517.

Giannakakou, P., Poy, G., Zhan, Z., Knutsen, T., Blagosklonny, M. V., & Fojo, T. (2000). Paclitaxel selects for mutant or pseudo-null p53 in drug resistance associated with tubulin mutations in human cancer. *Oncogene, 19*(27), 3078-3085.

Giatromanolaki, A., Koukourakis, M. I., Sivridis, E., Gatter, K. C., Harris, A. L., & Banham, A. H. (2006). Loss of expression and nuclear/cytoplasmic localization of the FOXP1 forkhead transcription factor are common events in early endometrial cancer: relationship with estrogen receptors and HIF-1alpha expression. *Mod Pathol, 19*(1), 9-16.

Gifford, G., Paul, J., Vasey, P. A., Kaye, S. B., & Brown, R. (2004). The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. *Clin Cancer Res, 10*(13), 4420-4426.

Gnesutta, N., Qu, J., & Minden, A. (2001). The serine/threonine kinase PAK4 prevents caspase activation and protects cells from apoptosis. *J Biol Chem, 276*(17), 14414-14419.

Gong, W., An, Z., Wang, Y., Pan, X., Fang, W., Jiang, B., & Zhang, H. (2009). P21-activated kinase 5 is overexpressed during colorectal cancer progression and regulates colorectal carcinoma cell adhesion and migration. *Int J Cancer, 125*(3), 548-555.

Grooteclaes, M. L., & Frisch, S. M. (2000). Evidence for a function of CtBP in epithelial gene regulation and anoikis. *Oncogene, 19*(33), 3823-3828.

Gu, J., Li, K., Li, M., Wu, X., Zhang, L., Ding, Q., Wu, W., Yang, J., Mu, J., Wen, H., Lu, J., Hao, Y., Chen, L., Zhang, W., Li, S., & Liu, Y. (2013). A role for p21-activated kinase 7 in the development of gastric cancer. *FEBS J, 280*(1), 46-55.

Gwinn, M. L., Lee, N. C., Rhodes, P. H., Layde, P. M., & Rubin, G. L. (1990). Pregnancy, breast feeding, and oral contraceptives and the risk of epithelial

ovarian cancer. *J Clin Epidemiol, 43*(6), 559-568.
Hamroun, D., Kato, S., Ishioka, C., Claustres, M., Beroud, C., & Soussi, T. (2006). The UMD TP53 database and website: update and revisions. *Hum Mutat, 27*(1), 14-20.
Han, Z. X., Wang, X. X., Zhang, S. N., Wu, J. X., Qian, H. Y., Wen, Y. Y., Tian, H., Pei, D. S., & Zheng, J. N. (2013). Downregulation of PAK5 inhibits glioma cell migration and invasion potentially through the PAK5-Egr1-MMP2 signaling pathway. *Brain Tumor Pathol*. Epub ahead of print.
Harrap, K. R. (1985). Preclinical studies identifying carboplatin as a viable cisplatin alternative. *Cancer Treat Rev, 12 Suppl A*, 21-33.
Hart, W. R. (2005). Mucinous tumors of the ovary: a review. *Int J Gynecol Pathol, 24*(1), 4-25.
Hashimoto, S., Tsubouchi, A., Mazaki, Y., & Sabe, H. (2001). Interaction of paxillin with p21-activated Kinase (PAK). Association of paxillin alpha with the kinase-inactive and the Cdc42-activated forms of PAK3. *J Biol Chem, 276*(8), 6037-6045.
Hirokawa, N. (1998). Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science, 279*(5350), 519-526.
Ho, S. M. (2003). Estrogen, progesterone and epithelial ovarian cancer. *Reprod Biol Endocrinol, 1*, 73.
Hollier, B. G., Tinnirello, A. A., Werden, S. J., Evans, K. W., Taube, J. H., Sarkar, T. R., Sphyris, N., Shariati, M., Kumar, S. V., Battula, V. L., Herschkowitz, J. I., Guerra, R., Chang, J. T., Miura, N., Rosen, J. M., & Mani, S. A. (2013). FOXC2 expression links epithelial-mesenchymal transition and stem cell properties in breast cancer. *Cancer Res, 73*(6), 1981-1992.
Holohan, C., Van Schaeybroeck, S., Longley, D. B., & Johnston, P. G. (2013). Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer, 13*(10), 714-726.
Hong Kong Cancer Registry. (2013). Summary of cancer statistics in Hong Kong in 2010 Retrieved 26 November, 2013, from http://www3.ha.org.hk/cancereg/Summary%20of%20CanStat%202010.pdf
Honrado, E., Benitez, J., & Palacios, J. (2005). The molecular pathology of hereditary breast cancer: genetic testing and therapeutic implications. *Mod Pathol, 18*(10), 1305-1320.
Hou, J., Lin, L., Zhou, W., Wang, Z., Ding, G., Dong, Q., Qin, L., Wu, X., Zheng, Y., Yang, Y., Tian, W., Zhang, Q., Wang, C., Zhuang, S. M., Zheng, L., Liang, A., Tao, W., & Cao, X. (2011). Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. *Cancer Cell, 19*(2), 232-243.
Hui, R. C., Gomes, A. R., Constantinidou, D., Costa, J. R., Karadedou, C. T., Fernandez de Mattos, S., Wymann, M. P., Brosens, J. J., Schulze, A., & Lam, E. W. (2008). The forkhead transcription factor FOXO3a increases phosphoinositide-3 kinase/Akt activity in drug-resistant leukemic cells through induction of PIK3CA expression. *Mol Cell Biol, 28*(19), 5886-5898.
Iliopoulos, D., Drosatos, K., Hiyama, Y., Goldberg, I. J., & Zannis, V. I. (2010).

MicroRNA-370 controls the expression of microRNA-122 and Cpt1alpha and affects lipid metabolism. *J Lipid Res, 51*(6), 1513-1523.

Imai, K., Hirata, S., Irie, A., Senju, S., Ikuta, Y., Yokomine, K., Harao, M., Inoue, M., Tomita, Y., Tsunoda, T., Nakagawa, H., Nakamura, Y., Baba, H., & Nishimura, Y. (2011). Identification of HLA-A2-restricted CTL epitopes of a novel tumour-associated antigen, KIF20A, overexpressed in pancreatic cancer. *Br J Cancer, 104*(2), 300-307.

Inui, M., Martello, G., & Piccolo, S. (2010). MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol, 11*(4), 252-263.

Iorio, M. V., Visone, R., Di Leva, G., Donati, V., Petrocca, F., Casalini, P., Taccioli, C., Volinia, S., Liu, C. G., Alder, H., Calin, G. A., Menard, S., & Croce, C. M. (2007). MicroRNA signatures in human ovarian cancer. *Cancer Res, 67*(18), 8699-8707.

Ishida, S., Lee, J., Thiele, D. J., & Herskowitz, I. (2002). Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proc Natl Acad Sci U S A, 99*(22), 14298-14302.

Ishikawa, K., Kamohara, Y., Tanaka, F., Haraguchi, N., Mimori, K., Inoue, H., & Mori, M. (2008). Mitotic centromere-associated kinesin is a novel marker for prognosis and lymph node metastasis in colorectal cancer. *Br J Cancer, 98*(11), 1824-1829.

Jakobi, R., Moertl, E., & Koeppel, M. A. (2001). p21-activated protein kinase gamma-PAK suppresses programmed cell death of BALB3T3 fibroblasts. *J Biol Chem, 276*(20), 16624-16634.

Jasurda, J. S., Jung, D. O., Froeter, E. D., Schwartz, D. B., Hopkins, T. D., Farris, C. L., McGee, S., Narayan, P., & Ellsworth, B. S. (2013). The Forkhead Transcription Factor, FOXP3: A Critical Role in Male Fertility. *Biol Reprod*.

Jazaeri, A. A., Yee, C. J., Sotiriou, C., Brantley, K. R., Boyd, J., & Liu, E. T. (2002). Gene expression profiles of BRCA1-linked, BRCA2-linked, and sporadic ovarian cancers. *J Natl Cancer Inst, 94*(13), 990-1000.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., & Thun, M. J. (2009). Cancer statistics, 2009. *CA Cancer J Clin, 59*(4), 225-249.

Jiang, L., Siu, M. K., Wong, O. G., Tam, K. F., Lu, X., Lam, E. W., Ngan, H. Y., Le, X. F., Wong, E. S., Monteiro, L. J., Chan, H. Y., & Cheung, A. N. (2011). iASPP and chemoresistance in ovarian cancers: effects on paclitaxel-mediated mitotic catastrophe. *Clin Cancer Res, 17*(21), 6924-6933.

John, E. M., Whittemore, A. S., Harris, R., & Itnyre, J. (1993). Characteristics relating to ovarian cancer risk: collaborative analysis of seven U.S. case-control studies. Epithelial ovarian cancer in black women. Collaborative Ovarian Cancer Group. *J Natl Cancer Inst, 85*(2), 142-147.

Jordan, S. J., Green, A. C., Whiteman, D. C., & Webb, P. M. (2007). Risk factors for benign, borderline and invasive mucinous ovarian tumors: epidemiological evidence of a neoplastic continuum? *Gynecol Oncol, 107*(2), 223-230.

Kaestner, K. H., Knochel, W., & Martinez, D. E. (2000). Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev, 14*(2), 142-146.

Kanehira, M., Katagiri, T., Shimo, A., Takata, R., Shuin, T., Miki, T., Fujioka, T., & Nakamura, Y. (2007). Oncogenic role of MPHOSPH1, a cancer-testis antigen specific to human bladder cancer. *Cancer Res, 67*(7), 3276-3285.

Kaur, R., Yuan, X., Lu, M. L., & Balk, S. P. (2008). Increased PAK6 expression in prostate cancer and identification of PAK6 associated proteins. *Prostate, 68*(14), 1510-1516.

Khoo, U. S., Ozcelik, H., Cheung, A. N., Chow, L. W., Ngan, H. Y., Done, S. J., Liang, A. C., Chan, V. W., Au, G. K., Ng, W. F., Poon, C. S., Leung, Y. F., Loong, F., Ip, P., Chan, G. S., Andrulis, I. L., Lu, J., & Ho, F. C. (1999). Somatic mutations in the BRCA1 gene in Chinese sporadic breast and ovarian cancer. *Oncogene, 18*(32), 4643-4646.

Kim, D. H., Zhang, T., Lee, S., & Dong, H. H. (2013). FoxO6 in glucose metabolism (FoxO6). *J Diabetes, 5*(3), 233-240.

King, A. J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S., & Marshall, M. S. (1998). The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. *Nature, 396*(6707), 180-183.

King, M. G., Olson, S. H., Paddock, L., Chandran, U., Demissie, K., Lu, S. E., Parekh, N., Rodriguez-Rodriguez, L., & Bandera, E. V. (2013). Sugary food and beverage consumption and epithelial ovarian cancer risk: a population-based case-control study. *BMC Cancer, 13*, 94.

Koberle, B., Tomicic, M. T., Usanova, S., & Kaina, B. (2010). Cisplatin resistance: preclinical findings and clinical implications. *Biochim Biophys Acta, 1806*(2), 172-182.

Koo, C. Y., Muir, K. W., & Lam, E. W. (2012). FOXM1: From cancer initiation to progression and treatment. *Biochim Biophys Acta, 1819*(1), 28-37.

Kovalchuk, O., Filkowski, J., Meservy, J., Ilnytskyy, Y., Tryndyak, V. P., Chekhun, V. F., & Pogribny, I. P. (2008). Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. *Mol Cancer Ther, 7*(7), 2152-2159.

Kumar, R., Gururaj, A. E., & Barnes, C. J. (2006). p21-activated kinases in cancer. *Nat Rev Cancer, 6*(6), 459-471.

Kumar, S., Mahdi, H., Bryant, C., Shah, J. P., Garg, G., & Munkarah, A. (2010). Clinical trials and progress with paclitaxel in ovarian cancer. *Int J Womens Health, 2*, 411-427.

Kuper, H., Titus-Ernstoff, L., Harlow, B. L., & Cramer, D. W. (2000). Population based study of coffee, alcohol and tobacco use and risk of ovarian cancer. *Int J Cancer, 88*(2), 313-318.

Kupryjanczyk, J., Thor, A. D., Beauchamp, R., Merritt, V., Edgerton, S. M., Bell, D. A., & Yandell, D. W. (1993). p53 gene mutations and protein accumulation in human ovarian cancer. *Proc Natl Acad Sci U S A, 90*(11), 4961-4965.

Kushi, L. H., Mink, P. J., Folsom, A. R., Anderson, K. E., Zheng, W., Lazovich, D., & Sellers, T. A. (1999). Prospective study of diet and ovarian cancer. *Am J Epidemiol, 149*(1), 21-31.

Kwok, J. M., Myatt, S. S., Marson, C. M., Coombes, R. C., Constantinidou, D., &

Lam, E. W. (2008). Thiostrepton selectively targets breast cancer cells through inhibition of forkhead box M1 expression. *Mol Cancer Ther, 7*(7), 2022-2032.

Kwok, J. M., Peck, B., Monteiro, L. J., Schwenen, H. D., Millour, J., Coombes, R. C., Myatt, S. S., & Lam, E. W. (2010). FOXM1 confers acquired cisplatin resistance in breast cancer cells. *Mol Cancer Res, 8*(1), 24-34.

La Vecchia, C., Decarli, A., Negri, E., Parazzini, F., Gentile, A., Cecchetti, G., Fasoli, M., & Franceschi, S. (1987). Dietary factors and the risk of epithelial ovarian cancer. *J Natl Cancer Inst, 79*(4), 663-669.

Lam, E. W., Brosens, J. J., Gomes, A. R., & Koo, C. Y. (2013). Forkhead box proteins: tuning forks for transcriptional harmony. *Nat Rev Cancer, 13*(7), 482-495.

Lam, E. W., Shah, K., & Brosens, J. J. (2012). The diversity of sex steroid action: the role of micro-RNAs and FOXO transcription factors in cycling endometrium and cancer. *J Endocrinol, 212*(1), 13-25.

Laoukili, J., Kooistra, M. R., Bras, A., Kauw, J., Kerkhoven, R. M., Morrison, A., Clevers, H., & Medema, R. H. (2005). FoxM1 is required for execution of the mitotic programme and chromosome stability. *Nat Cell Biol, 7*(2), 126-136.

Laoukili, J., Stahl, M., & Medema, R. H. (2007). FoxM1: at the crossroads of ageing and cancer. *Biochim Biophys Acta, 1775*(1), 92-102.

Lee, A. H., Su, D., Pasalich, M., & Binns, C. W. (2013a). Preserved foods associated with increased risk of ovarian cancer. *Gynecol Oncol, 129*(3), 570-573.

Lee, A. H., Su, D., Pasalich, M., & Binns, C. W. (2013b). Tea consumption reduces ovarian cancer risk. *Cancer Epidemiol, 37*(1), 54-59.

Lee, C. S., Friedman, J. R., Fulmer, J. T., & Kaestner, K. H. (2005). The initiation of liver development is dependent on Foxa transcription factors. *Nature, 435*(7044), 944-947.

Leitzmann, M. F., Koebnick, C., Danforth, K. N., Brinton, L. A., Moore, S. C., Hollenbeck, A. R., Schatzkin, A., & Lacey, J. V., Jr. (2009). Body mass index and risk of ovarian cancer. *Cancer, 115*(4), 812-822.

Levine, A. J., Momand, J., & Finlay, C. A. (1991). The p53 tumour suppressor gene. *Nature, 351*(6326), 453-456.

Levine, D. A., Bogomolniy, F., Yee, C. J., Lash, A., Barakat, R. R., Borgen, P. I., & Boyd, J. (2005). Frequent mutation of the PIK3CA gene in ovarian and breast cancers. *Clin Cancer Res, 11*(8), 2875-2878.

Lewin, G. R., & Barde, Y. A. (1996). Physiology of the neurotrophins. *Annu Rev Neurosci, 19*, 289-317.

Li, D., Yao, X., & Zhang, P. (2013). The overexpression of P21-activated kinase 5 (PAK5) promotes paclitaxel-chemoresistance of epithelial ovarian cancer. *Mol Cell Biochem, 383*(1-2), 191-199.

Li, F., Adam, L., Vadlamudi, R. K., Zhou, H., Sen, S., Chernoff, J., Mandal, M., & Kumar, R. (2002). p21-activated kinase 1 interacts with and phosphorylates histone H3 in breast cancer cells. *EMBO Rep, 3*(8), 767-773.

Li, G., Luna, C., Qiu, J., Epstein, D. L., & Gonzalez, P. (2010). Targeting of integrin beta1 and kinesin 2alpha by microRNA 183. *J Biol Chem, 285*(8), 5461-5471.

Li, Y., Shao, Y., Tong, Y., Shen, T., Zhang, J., Gu, H., & Li, F. (2012).

Nucleo-cytoplasmic shuttling of PAK4 modulates beta-catenin intracellular translocation and signaling. *Biochim Biophys Acta, 1823*(2), 465-475.

Li, Z., Tuteja, G., Schug, J., & Kaestner, K. H. (2012). Foxa1 and Foxa2 are essential for sexual dimorphism in liver cancer. *Cell, 148*(1-2), 72-83.

Liao, X., Siu, M. K., Au, C. W., Wong, E. S., Chan, H. Y., Ip, P. P., Ngan, H. Y., & Cheung, A. N. (2009). Aberrant activation of hedgehog signaling pathway in ovarian cancers: effect on prognosis, cell invasion and differentiation. *Carcinogenesis, 30*(1), 131-140.

Liedert, B., Materna, V., Schadendorf, D., Thomale, J., & Lage, H. (2003). Overexpression of cMOAT (MRP2/ABCC2) is associated with decreased formation of platinum-DNA adducts and decreased G2-arrest in melanoma cells resistant to cisplatin. *J Invest Dermatol, 121*(1), 172-176.

Lin, L., Miller, C. T., Contreras, J. I., Prescott, M. S., Dagenais, S. L., Wu, R., Yee, J., Orringer, M. B., Misek, D. E., Hanash, S. M., Glover, T. W., & Beer, D. G. (2002). The hepatocyte nuclear factor 3 alpha gene, HNF3alpha (FOXA1), on chromosome band 14q13 is amplified and overexpressed in esophageal and lung adenocarcinomas. *Cancer Res, 62*(18), 5273-5279.

Liu, R. X., Wang, W. Q., Ye, L., Bi, Y. F., Fang, H., Cui, B., Zhou, W. W., Dai, M., Zhang, J., Li, X. Y., & Ning, G. (2010). p21-activated kinase 3 is overexpressed in thymic neuroendocrine tumors (carcinoids) with ectopic ACTH syndrome and participates in cell migration. *Endocrine, 38*(1), 38-47.

Liu, X., Gong, H., & Huang, K. (2013). Oncogenic role of kinesin proteins and targeting kinesin therapy. *Cancer Sci, 104*(6), 651-656.

Liu, Y., Xiao, H., Tian, Y., Nekrasova, T., Hao, X., Lee, H. J., Suh, N., Yang, C. S., & Minden, A. (2008). The pak4 protein kinase plays a key role in cell survival and tumorigenesis in athymic mice. *Mol Cancer Res, 6*(7), 1215-1224.

Lo, S. S., Hung, P. S., Chen, J. H., Tu, H. F., Fang, W. L., Chen, C. Y., Chen, W. T., Gong, N. R., & Wu, C. W. (2012). Overexpression of miR-370 and downregulation of its novel target TGFbeta-RII contribute to the progression of gastric carcinoma. *Oncogene, 31*(2), 226-237.

Lu, W., Xia, Y. H., Qu, J. J., He, Y. Y., Li, B. L., Lu, C., Luo, X., & Wan, X. P. (2013). p21-activated kinase 4 regulation of endometrial cancer cell migration and invasion involves the ERK1/2 pathway mediated MMP-2 secretion. *Neoplasma, 60*(5), 493-503.

Luan, N. N., Wu, Q. J., Gong, T. T., Vogtmann, E., Wang, Y. L., & Lin, B. (2013). Breastfeeding and ovarian cancer risk: a meta-analysis of epidemiologic studies. *Am J Clin Nutr, 98*(4), 1020-1031.

Lupien, M., Eeckhoute, J., Meyer, C. A., Wang, Q., Zhang, Y., Li, W., Carroll, J. S., Liu, X. S., & Brown, M. (2008). FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell, 132*(6), 958-970.

Madureira, P. A., Varshochi, R., Constantinidou, D., Francis, R. E., Coombes, R. C., Yao, K. M., & Lam, E. W. (2006). The Forkhead box M1 protein regulates the transcription of the estrogen receptor alpha in breast cancer cells. *J Biol Chem, 281*(35), 25167-25176.

Maehama, T., Taylor, G. S., & Dixon, J. E. (2001). PTEN and myotubularin: novel phosphoinositide phosphatases. *Annu Rev Biochem, 70*, 247-279.

Mak, G. W., Chan, M. M., Leong, V. Y., Lee, J. M., Yau, T. O., Ng, I. O., & Ching, Y. P. (2011). Overexpression of a novel activator of PAK4, the CDK5 kinase-associated protein CDK5RAP3, promotes hepatocellular carcinoma metastasis. *Cancer Res, 71*(8), 2949-2958.

Maney, T., Hunter, A. W., Wagenbach, M., & Wordeman, L. (1998). Mitotic centromere-associated kinesin is important for anaphase chromosome segregation. *J Cell Biol, 142*(3), 787-801.

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M., Campbell, L. L., Polyak, K., Brisken, C., Yang, J., & Weinberg, R. A. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell, 133*(4), 704-715.

Manning, B. D., & Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. *Cell, 129*(7), 1261-1274.

Marchbanks, P. A., Wilson, H., Bastos, E., Cramer, D. W., Schildkraut, J. M., & Peterson, H. B. (2000). Cigarette smoking and epithelial ovarian cancer by histologic type. *Obstet Gynecol, 95*(2), 255-260.

Marlow, L. A., von Roemeling, C. A., Cooper, S. J., Zhang, Y., Rohl, S. D., Arora, S., Gonzales, I. M., Azorsa, D. O., Reddi, H. V., Tun, H. W., Doppler, H. R., Storz, P., Smallridge, R. C., & Copland, J. A. (2012). Foxo3a drives proliferation in anaplastic thyroid carcinoma through transcriptional regulation of cyclin A1: a paradigm shift that impacts current therapeutic strategies. *J Cell Sci, 125*(Pt 18), 4253-4263.

Maroto, B., Ye, M. B., von Lohneysen, K., Schnelzer, A., & Knaus, U. G. (2008). P21-activated kinase is required for mitotic progression and regulates Plk1. *Oncogene, 27*(36), 4900-4908.

Marugame, T., & Hirabayashi, Y. (2007). Comparison of time trends in ovary cancer incidence (1973-1997) in East Asia, Europe, and the USA, from Cancer Incidence in Five Continents Vols IV VIII. *Jpn J Clin Oncol, 37*(10), 802-803.

Mazumdar, A., & Kumar, R. (2003). Estrogen regulation of Pak1 and FKHR pathways in breast cancer cells. *FEBS Lett, 535*(1-3), 6-10.

McGovern, U. B., Francis, R. E., Peck, B., Guest, S. K., Wang, J., Myatt, S. S., Krol, J., Kwok, J. M., Polychronis, A., Coombes, R. C., & Lam, E. W. (2009). Gefitinib (Iressa) represses FOXM1 expression via FOXO3a in breast cancer. *Mol Cancer Ther, 8*(3), 582-591.

Miki, H., Okada, Y., & Hirokawa, N. (2005). Analysis of the kinesin superfamily: insights into structure and function. *Trends Cell Biol, 15*(9), 467-476.

Millour, J., Constantinidou, D., Stavropoulou, A. V., Wilson, M. S., Myatt, S. S., Kwok, J. M., Sivanandan, K., Coombes, R. C., Medema, R. H., Hartman, J., Lykkesfeldt, A. E., & Lam, E. W. (2010). FOXM1 is a transcriptional target of ERalpha and has a critical role in breast cancer endocrine sensitivity and resistance. *Oncogene, 29*(20), 2983-2995.

Millour, J., de Olano, N., Horimoto, Y., Monteiro, L. J., Langer, J. K., Aligue, R.,

Hajji, N., & Lam, E. W. (2011). ATM and p53 regulate FOXM1 expression via E2F in breast cancer epirubicin treatment and resistance. *Mol Cancer Ther, 10*(6), 1046-1058.

Minden, A. (2012). PAK4-6 in cancer and neuronal development. *Cell Logist, 2*(2), 95-104.

Mo, M., Peng, F., Wang, L., Peng, L., Lan, G., & Yu, S. (2013). Roles of mitochondrial transcription factor A and microRNA-590-3p in the development of bladder cancer. *Oncol Lett, 6*(2), 617-623.

Modugno, F., Ness, R. B., & Cottreau, C. M. (2002). Cigarette smoking and the risk of mucinous and nonmucinous epithelial ovarian cancer. *Epidemiology, 13*(4), 467-471.

Molli, P. R., Li, D. Q., Murray, B. W., Rayala, S. K., & Kumar, R. (2009). PAK signaling in oncogenesis. *Oncogene, 28*(28), 2545-2555.

Monsalve, M., & Olmos, Y. (2011). The complex biology of FOXO. *Curr Drug Targets, 12*(9), 1322-1350.

Monteiro, L. J., Khongkow, P., Kongsema, M., Morris, J. R., Man, C., Weekes, D., Koo, C. Y., Gomes, A. R., Pinto, P. H., Varghese, V., Kenny, L. M., Charles Coombes, R., Freire, R., Medema, R. H., & Lam, E. W. (2013). The Forkhead Box M1 protein regulates BRIP1 expression and DNA damage repair in epirubicin treatment. *Oncogene, 32*(39), 4634-4645.

Mraz, M., & Pospisilova, S. (2012). MicroRNAs in chronic lymphocytic leukemia: from causality to associations and back. *Expert Rev Hematol, 5*(6), 579-581.

Murdoch, W. J., Townsend, R. S., & McDonnel, A. C. (2001). Ovulation-induced DNA damage in ovarian surface epithelial cells of ewes: prospective regulatory mechanisms of repair/survival and apoptosis. *Biol Reprod, 65*(5), 1417-1424.

Myatt, S. S., & Lam, E. W. (2007). The emerging roles of forkhead box (Fox) proteins in cancer. *Nat Rev Cancer, 7*(11), 847-859.

Nakamura, Y., Tanaka, F., Haraguchi, N., Mimori, K., Matsumoto, T., Inoue, H., Yanaga, K., & Mori, M. (2007). Clinicopathological and biological significance of mitotic centromere-associated kinesin overexpression in human gastric cancer. *Br J Cancer, 97*(4), 543-549.

Narod, S. A., Risch, H., Moslehi, R., Dorum, A., Neuhausen, S., Olsson, H., Provencher, D., Radice, P., Evans, G., Bishop, S., Brunet, J. S., & Ponder, B. A. (1998). Oral contraceptives and the risk of hereditary ovarian cancer. Hereditary Ovarian Cancer Clinical Study Group. *N Engl J Med, 339*(7), 424-428.

Nekrasova, T., & Minden, A. (2012). Role for p21-activated kinase PAK4 in development of the mammalian heart. *Transgenic Res, 21*(4), 797-811.

Ness, R. B., Cramer, D. W., Goodman, M. T., Kjaer, S. K., Mallin, K., Mosgaard, B. J., Purdie, D. M., Risch, H. A., Vergona, R., & Wu, A. H. (2002). Infertility, fertility drugs, and ovarian cancer: a pooled analysis of case-control studies. *Am J Epidemiol, 155*(3), 217-224.

Nishidate, T., Katagiri, T., Lin, M. L., Mano, Y., Miki, Y., Kasumi, F., Yoshimoto, M.,

Tsunoda, T., Hirata, K., & Nakamura, Y. (2004). Genome-wide gene-expression profiles of breast-cancer cells purified with laser microbeam microdissection: identification of genes associated with progression and metastasis. *Int J Oncol, 25*(4), 797-819.

Noda, Y., Okada, Y., Saito, N., Setou, M., Xu, Y., Zhang, Z., & Hirokawa, N. (2001). KIFC3, a microtubule minus end-directed motor for the apical transport of annexin XIIIb-associated Triton-insoluble membranes. *J Cell Biol, 155*(1), 77-88.

Obata, K., Morland, S. J., Watson, R. H., Hitchcock, A., Chenevix-Trench, G., Thomas, E. J., & Campbell, I. G. (1998). Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. *Cancer Res, 58*(10), 2095-2097.

Obaya, A. J., Mateyak, M. K., & Sedivy, J. M. (1999). Mysterious liaisons: the relationship between c-Myc and the cell cycle. *Oncogene, 18*(19), 2934-2941.

Oberlies, N. H., & Kroll, D. J. (2004). Camptothecin and taxol: historic achievements in natural products research. *J Nat Prod, 67*(2), 129-135.

Ofir, R., Seidman, R., Rabinski, T., Krup, M., Yavelsky, V., Weinstein, Y., & Wolfson, M. (2002). Taxol-induced apoptosis in human SKOV3 ovarian and MCF7 breast carcinoma cells is caspase-3 and caspase-9 independent. *Cell Death Differ, 9*(6), 636-642.

Oh, K. J., Han, H. S., Kim, M. J., & Koo, S. H. (2013). CREB and FoxO1: two transcription factors for the regulation of hepatic gluconeogenesis. *BMB Rep*.

Okabe, H., Satoh, S., Kato, T., Kitahara, O., Yanagawa, R., Yamaoka, Y., Tsunoda, T., Furukawa, Y., & Nakamura, Y. (2001). Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. *Cancer Res, 61*(5), 2129-2137.

Oki, E., Hisamatsu, Y., Ando, K., Saeki, H., Kakeji, Y., & Maehara, Y. (2012). Clinical aspect and molecular mechanism of DNA aneuploidy in gastric cancers. *J Gastroenterol, 47*(4), 351-358.

Olsen, C. M., Nagle, C. M., Whiteman, D. C., Ness, R., Pearce, C. L., Pike, M. C., Rossing, M. A., Terry, K. L., Wu, A. H., Risch, H. A., Yu, H., Doherty, J. A., Chang-Claude, J., Hein, R., Nickels, S., Wang-Gohrke, S., Goodman, M. T., Carney, M. E., Matsuno, R. K., Lurie, G., Moysich, K., Kjaer, S. K., Jensen, A., Hogdall, E., Goode, E. L., Fridley, B. L., Vierkant, R. A., Larson, M. C., Schildkraut, J., Hoyo, C., Moorman, P., Weber, R. P., Cramer, D. W., Vitonis, A. F., Bandera, E. V., Olson, S. H., Rodriguez-Rodriguez, L., King, M., Brinton, L. A., Yang, H., Garcia-Closas, M., Lissowska, J., Anton-Culver, H., Ziogas, A., Gayther, S. A., Ramus, S. J., Menon, U., Gentry-Maharaj, A., & Webb, P. M. (2013). Obesity and risk of ovarian cancer subtypes: evidence from the Ovarian Cancer Association Consortium. *Endocr Relat Cancer, 20*(2), 251-262.

Ong, C. C., Jubb, A. M., Haverty, P. M., Zhou, W., Tran, V., Truong, T., Turley, H., O'Brien, T., Vucic, D., Harris, A. L., Belvin, M., Friedman, L. S., Blackwood,

E. M., Koeppen, H., & Hoeflich, K. P. (2011). Targeting p21-activated kinase 1 (PAK1) to induce apoptosis of tumor cells. *Proc Natl Acad Sci U S A, 108*(17), 7177-7182.

Ouyang, W., Beckett, O., Ma, Q., Paik, J. H., DePinho, R. A., & Li, M. O. (2010). Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. *Nat Immunol, 11*(7), 618-627.

Pan, S. Y., Ugnat, A. M., Mao, Y., Wen, S. W., & Johnson, K. C. (2004). Association of cigarette smoking with the risk of ovarian cancer. *Int J Cancer, 111*(1), 124-130.

Park, J., Ko, Y. S., Yoon, J., Kim, M. A., Park, J. W., Kim, W. H., Choi, Y., Kim, J. H., Cheon, Y., & Lee, B. L. (2013). The forkhead transcription factor FOXO1 mediates cisplatin resistance in gastric cancer cells by activating phosphoinositide 3-kinase/Akt pathway. *Gastric Cancer*.

Park, Y. Y., Jung, S. Y., Jennings, N. B., Rodriguez-Aguayo, C., Peng, G., Lee, S. R., Kim, S. B., Kim, K., Leem, S. H., Lin, S. Y., Lopez-Berestein, G., Sood, A. K., & Lee, J. S. (2012). FOXM1 mediates Dox resistance in breast cancer by enhancing DNA repair. *Carcinogenesis, 33*(10), 1843-1853.

Parkin, D. M., Pisani, P., & Ferlay, J. (1999). Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer, 80*(6), 827-841.

Piek, J. M., Kenemans, P., Zweemer, R. P., van Diest, P. J., & Verheijen, R. H. (2007). Ovarian carcinogenesis, an alternative theory. *Gynecol Oncol, 107*(2), 355.

Pilarsky, C., Wenzig, M., Specht, T., Saeger, H. D., & Grutzmann, R. (2004). Identification and validation of commonly overexpressed genes in solid tumors by comparison of microarray data. *Neoplasia, 6*(6), 744-750.

Pirruccello, M., Sondermann, H., Pelton, J. G., Pellicena, P., Hoelz, A., Chernoff, J., Wemmer, D. E., & Kuriyan, J. (2006). A dimeric kinase assembly underlying autophosphorylation in the p21 activated kinases. *J Mol Biol, 361*(2), 312-326.

Pisani, P., Bray, F., & Parkin, D. M. (2002). Estimates of the world-wide prevalence of cancer for 25 sites in the adult population. *Int J Cancer, 97*(1), 72-81.

Qinyu, L., Long, C., Zhen-dong, D., Min-min, S., Wei-ze, W., Wei-ping, Y., & Cheng-hong, P. (2013). FOXO6 promotes gastric cancer cell tumorigenicity via upregulation of C-myc. *FEBS Lett, 587*(14), 2105-2111.

Qu, J., Cammarano, M. S., Shi, Q., Ha, K. C., de Lanerolle, P., & Minden, A. (2001). Activated PAK4 regulates cell adhesion and anchorage-independent growth. *Mol Cell Biol, 21*(10), 3523-3533.

Qu, J., Li, X., Novitch, B. G., Zheng, Y., Kohn, M., Xie, J. M., Kozinn, S., Bronson, R., Beg, A. A., & Minden, A. (2003). PAK4 kinase is essential for embryonic viability and for proper neuronal development. *Mol Cell Biol, 23*(20), 7122-7133.

Ray, P. S., Wang, J., Qu, Y., Sim, M. S., Shamonki, J., Bagaria, S. P., Ye, X., Liu, B., Elashoff, D., Hoon, D. S., Walter, M. A., Martens, J. W., Richardson, A. L., Giuliano, A. E., & Cui, X. (2010). FOXC1 is a potential prognostic biomarker with functional significance in basal-like breast cancer. *Cancer Res, 70*(10), 3870-3876.

Rescigno, P., Cerillo, I., Ruocco, R., Condello, C., De Placido, S., & Pensabene, M. (2013). New hypothesis on pathogenesis of ovarian cancer lead to future tailored approaches. *Biomed Res Int, 2013*, 852839.

Reynolds, E. A., & Moller, K. A. (2006). A review and an update on the screening of epithelial ovarian cancer. *Curr Probl Cancer, 30*(5), 203-232.

Riman, T., Dickman, P. W., Nilsson, S., Correia, N., Nordlinder, H., Magnusson, C. M., Weiderpass, E., & Persson, I. R. (2002). Hormone replacement therapy and the risk of invasive epithelial ovarian cancer in Swedish women. *J Natl Cancer Inst, 94*(7), 497-504.

Risch, H. A. (1998). Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. *J Natl Cancer Inst, 90*(23), 1774-1786.

Robbins, C. M., Tembe, W. A., Baker, A., Sinari, S., Moses, T. Y., Beckstrom-Sternberg, S., Beckstrom-Sternberg, J., Barrett, M., Long, J., Chinnaiyan, A., Lowey, J., Suh, E., Pearson, J. V., Craig, D. W., Agus, D. B., Pienta, K. J., & Carpten, J. D. (2011). Copy number and targeted mutational analysis reveals novel somatic events in metastatic prostate tumors. *Genome Res, 21*(1), 47-55.

Rodriguez-Burford, C., Barnes, M. N., Oelschlager, D. K., Myers, R. B., Talley, L. I., Partridge, E. E., & Grizzle, W. E. (2002). Effects of nonsteroidal anti-inflammatory agents (NSAIDs) on ovarian carcinoma cell lines: preclinical evaluation of NSAIDs as chemopreventive agents. *Clin Cancer Res, 8*(1), 202-209.

Rodriguez, C., Calle, E. E., Fakhrabadi-Shokoohi, D., Jacobs, E. J., & Thun, M. J. (2002). Body mass index, height, and the risk of ovarian cancer mortality in a prospective cohort of postmenopausal women. *Cancer Epidemiol Biomarkers Prev, 11*(9), 822-828.

Rodriguez, C., Patel, A. V., Calle, E. E., Jacob, E. J., & Thun, M. J. (2001). Estrogen replacement therapy and ovarian cancer mortality in a large prospective study of US women. *JAMA, 285*(11), 1460-1465.

Roig, J., & Traugh, J. A. (1999). p21-activated protein kinase gamma-PAK is activated by ionizing radiation and other DNA-damaging agents. Similarities and differences to alpha-PAK. *J Biol Chem, 274*(44), 31119-31122.

Rosen, D. G., Yang, G., Liu, G., Mercado-Uribe, I., Chang, B., Xiao, X. S., Zheng, J., Xue, F. X., & Liu, J. (2009). Ovarian cancer: pathology, biology, and disease models. *Front Biosci (Landmark Ed), 14*, 2089-2102.

Roth, L. M., Emerson, R. E., & Ulbright, T. M. (2003). Ovarian endometrioid tumors of low malignant potential: a clinicopathologic study of 30 cases with comparison to well-differentiated endometrioid adenocarcinoma. *Am J Surg Pathol, 27*(9), 1253-1259.

Rousso, D. L., Pearson, C. A., Gaber, Z. B., Miquelajauregui, A., Li, S., Portera-Cailliau, C., Morrisey, E. E., & Novitch, B. G. (2012). Foxp-mediated suppression of N-cadherin regulates neuroepithelial character and progenitor maintenance in the CNS. *Neuron, 74*(2), 314-330.

Salih, D. A., Rashid, A. J., Colas, D., de la Torre-Ubieta, L., Zhu, R. P., Morgan, A. A., Santo, E. E., Ucar, D., Devarajan, K., Cole, C. J., Madison, D. V., Shamloo, M., Butte, A. J., Bonni, A., Josselyn, S. A., & Brunet, A. (2012). FoxO6 regulates memory consolidation and synaptic function. *Genes Dev, 26*(24), 2780-2801.

Sanhaji, M., Friel, C. T., Wordeman, L., Louwen, F., & Yuan, J. (2011). Mitotic centromere-associated kinesin (MCAK): a potential cancer drug target. *Oncotarget, 2*(12), 935-947.

Sato, M., Matsuda, Y., Wakai, T., Kubota, M., Osawa, M., Fujimaki, S., Sanpei, A., Takamura, M., Yamagiwa, S., & Aoyagi, Y. (2013). P21-activated kinase-2 is a critical mediator of transforming growth factor-beta-induced hepatoma cell migration. *J Gastroenterol Hepatol, 28*(6), 1047-1055.

Schmidt, M., Lu, Y., Liu, B., Fang, M., Mendelsohn, J., & Fan, Z. (2000). Differential modulation of paclitaxel-mediated apoptosis by p21Waf1 and p27Kip1. *Oncogene, 19*(20), 2423-2429.

Schouten, L. J., Zeegers, M. P., Goldbohm, R. A., & van den Brandt, P. A. (2004). Alcohol and ovarian cancer risk: results from the Netherlands Cohort Study. *Cancer Causes Control, 15*(2), 201-209.

Schubbert, S., Shannon, K., & Bollag, G. (2007). Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer, 7*(4), 295-308.

Schurmann, A., Mooney, A. F., Sanders, L. C., Sells, M. A., Wang, H. G., Reed, J. C., & Bokoch, G. M. (2000). p21-activated kinase 1 phosphorylates the death agonist bad and protects cells from apoptosis. *Mol Cell Biol, 20*(2), 453-461.

Schwanhausser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., & Selbach, M. (2011). Global quantification of mammalian gene expression control. *Nature, 473*(7347), 337-342.

SEER Cancer Statistics Factsheets: Ovary Cancer. National Cancer Institute. Bethesda, MD, Retrieved 26 November 2013 from http://seer.cancer.gov/statfacts.html/ovary.html.

Seidman, J. D., Horkayne-Szakaly, I., Haiba, M., Boice, C. R., Kurman, R. J., & Ronnett, B. M. (2004). The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin. *Int J Gynecol Pathol, 23*(1), 41-44.

Seo, S., & Kume, T. (2006). Forkhead transcription factors, Foxc1 and Foxc2, are required for the morphogenesis of the cardiac outflow tract. *Dev Biol, 296*(2), 421-436.

Shan, X., Miao, Y., Fan, R., Qian, H., Chen, P., Liu, H., Yan, X., Li, J., & Zhou, F. (2013). MiR-590-5P Inhibits Growth of HepG2 Cells via Decrease of S100A10 Expression and Inhibition of the Wnt Pathway. *Int J Mol Sci, 14*(4), 8556-8569.

Sharp, D. J., Rogers, G. C., & Scholey, J. M. (2000). Microtubule motors in mitosis. *Nature, 407*(6800), 41-47.

Shih Ie, M., & Kurman, R. J. (2004). Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. *Am J Pathol, 164*(5), 1511-1518.

Shimo, A., Tanikawa, C., Nishidate, T., Lin, M. L., Matsuda, K., Park, J. H., Ueki, T., Ohta, T., Hirata, K., Fukuda, M., Nakamura, Y., & Katagiri, T. (2008). Involvement of kinesin family member 2C/mitotic centromere-associated kinesin overexpression in mammary carcinogenesis. *Cancer Sci, 99*(1), 62-70.

Shu, W., Lu, M. M., Zhang, Y., Tucker, P. W., Zhou, D., & Morrisey, E. E. (2007). Foxp2 and Foxp1 cooperatively regulate lung and esophagus development. *Development, 134*(10), 1991-2000.

Siegenthaler, J. A., Choe, Y., Patterson, K. P., Hsieh, I., Li, D., Jaminet, S. C., Daneman, R., Kume, T., Huang, E. J., & Pleasure, S. J. (2013). Foxc1 is required by pericytes during fetal brain angiogenesis. *Biol Open, 2*(7), 647-659.

Singer, G., Stohr, R., Cope, L., Dehari, R., Hartmann, A., Cao, D. F., Wang, T. L., Kurman, R. J., & Shih Ie, M. (2005). Patterns of p53 mutations separate ovarian serous borderline tumors and low- and high-grade carcinomas and provide support for a new model of ovarian carcinogenesis: a mutational analysis with immunohistochemical correlation. *Am J Surg Pathol, 29*(2), 218-224.

Siu, M. K., Chan, H. Y., Kong, D. S., Wong, E. S., Wong, O. G., Ngan, H. Y., Tam, K. F., Zhang, H., Li, Z., Chan, Q. K., Tsao, S. W., Stromblad, S., & Cheung, A. N. (2010b). p21-activated kinase 4 regulates ovarian cancer cell proliferation, migration, and invasion and contributes to poor prognosis in patients. *Proc Natl Acad Sci U S A, 107*(43), 18622-18627.

Siu, M. K., Wong, E. S., Chan, H. Y., Kong, D. S., Woo, N. W., Tam, K. F., Ngan, H. Y., Chan, Q. K., Chan, D. C., Chan, K. Y., & Cheung, A. N. (2010a). Differential expression and phosphorylation of Pak1 and Pak2 in ovarian cancer: effects on prognosis and cell invasion. *Int J Cancer, 127*(1), 21-31.

Sorrentino, A., Liu, C. G., Addario, A., Peschle, C., Scambia, G., & Ferlini, C. (2008). Role of microRNAs in drug-resistant ovarian cancer cells. *Gynecol Oncol, 111*(3), 478-486.

Soslow, R. A. (2008). Histologic subtypes of ovarian carcinoma: an overview. *Int J Gynecol Pathol, 27*(2), 161-174.

Sotiropoulou, G., Pampalakis, G., Lianidou, E., & Mourelatos, Z. (2009). Emerging roles of microRNAs as molecular switches in the integrated circuit of the cancer cell. *RNA, 15*(8), 1443-1461.

Spiteri, E., Konopka, G., Coppola, G., Bomar, J., Oldham, M., Ou, J., Vernes, S. C., Fisher, S. E., Ren, B., & Geschwind, D. H. (2007). Identification of the transcriptional targets of FOXP2, a gene linked to speech and language, in developing human brain. *Am J Hum Genet, 81*(6), 1144-1157.

Stratton, J. F., Pharoah, P., Smith, S. K., Easton, D., & Ponder, B. A. (1998). A systematic review and meta-analysis of family history and risk of ovarian cancer. *Br J Obstet Gynaecol, 105*(5), 493-499.

Stumm, L., Burkhardt, L., Steurer, S., Simon, R., Adam, M., Becker, A., Sauter, G., Minner, S., Schlomm, T., Sirma, H., & Michl, U. (2013). Strong expression of the neuronal transcription factor FOXP2 is linked to an increased risk of early

PSA recurrence in ERG fusion-negative cancers. *J Clin Pathol, 66*(7), 563-568.

Sturgill, E. G., & Ohi, R. (2013). Kinesin-12 differentially affects spindle assembly depending on its microtubule substrate. *Curr Biol, 23*(14), 1280-1290.

Sun, D., Lu, J., Ding, K., Bi, D., Niu, Z., Cao, Q., Zhang, J., & Ding, S. (2013). The expression of Eg5 predicts a poor outcome for patients with renal cell carcinoma. *Med Oncol, 30*(1), 476.

Sun, X. D., Shi, X. J., Sun, X. O., Luo, Y. G., Wu, X. J., Yao, C. F., Yu, H. Y., Li, D. W., Liu, M., & Zhou, J. (2011). Dimethylenastron suppresses human pancreatic cancer cell migration and invasion in vitro via allosteric inhibition of mitotic kinesin Eg5. *Acta Pharmacol Sin, 32*(12), 1543-1548.

Takata, R., Akamatsu, S., Kubo, M., Takahashi, A., Hosono, N., Kawaguchi, T., Tsunoda, T., Inazawa, J., Kamatani, N., Ogawa, O., Fujioka, T., Nakamura, Y., & Nakagawa, H. (2010). Genome-wide association study identifies five new susceptibility loci for prostate cancer in the Japanese population. *Nat Genet, 42*(9), 751-754.

Takayama, K., Horie-Inoue, K., Ikeda, K., Urano, T., Murakami, K., Hayashizaki, Y., Ouchi, Y., & Inoue, S. (2008). FOXP1 is an androgen-responsive transcription factor that negatively regulates androgen receptor signaling in prostate cancer cells. *Biochem Biophys Res Commun, 374*(2), 388-393.

Tan, Y., Raychaudhuri, P., & Costa, R. H. (2007). Chk2 mediates stabilization of the FoxM1 transcription factor to stimulate expression of DNA repair genes. *Mol Cell Biol, 27*(3), 1007-1016.

Taniuchi, K., Nakagawa, H., Nakamura, T., Eguchi, H., Ohigashi, H., Ishikawa, O., Katagiri, T., & Nakamura, Y. (2005). Down-regulation of RAB6KIFL/KIF20A, a kinesin involved with membrane trafficking of discs large homologue 5, can attenuate growth of pancreatic cancer cell. *Cancer Res, 65*(1), 105-112.

Taniwaki, M., Takano, A., Ishikawa, N., Yasui, W., Inai, K., Nishimura, H., Tsuchiya, E., Kohno, N., Nakamura, Y., & Daigo, Y. (2007). Activation of KIF4A as a prognostic biomarker and therapeutic target for lung cancer. *Clin Cancer Res, 13*(22 Pt 1), 6624-6631.

Terry, P. D., Miller, A. B., Jones, J. G., & Rohan, T. E. (2003). Cigarette smoking and the risk of invasive epithelial ovarian cancer in a prospective cohort study. *Eur J Cancer, 39*(8), 1157-1164.

Theriault, B. L., Pajovic, S., Bernardini, M. Q., Shaw, P. A., & Gallie, B. L. (2012). Kinesin family member 14: an independent prognostic marker and potential therapeutic target for ovarian cancer. *Int J Cancer, 130*(8), 1844-1854.

Thiel, D. A., Reeder, M. K., Pfaff, A., Coleman, T. R., Sells, M. A., & Chernoff, J. (2002). Cell cycle-regulated phosphorylation of p21-activated kinase 1. *Curr Biol, 12*(14), 1227-1232.

Thiery, J. P. (2003). Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol, 15*(6), 740-746.

Thompson, D., & Easton, D. F. (2002). Cancer Incidence in BRCA1 mutation carriers. *J Natl Cancer Inst, 94*(18), 1358-1365.

Timm, T., Matenia, D., Li, X. Y., Griesshaber, B., & Mandelkow, E. M. (2006). Signaling from MARK to tau: regulation, cytoskeletal crosstalk, and pathological phosphorylation. *Neurodegener Dis, 3*(4-5), 207-217.

Turner, N., Tutt, A., & Ashworth, A. (2005). Targeting the DNA repair defect of BRCA tumours. *Curr Opin Pharmacol, 5*(4), 388-393.

Uddin, S., Ahmed, M., Hussain, A., Abubaker, J., Al-Sanea, N., AbdulJabbar, A., Ashari, L. H., Alhomoud, S., Al-Dayel, F., Jehan, Z., Bavi, P., Siraj, A. K., & Al-Kuraya, K. S. (2011). Genome-wide expression analysis of Middle Eastern colorectal cancer reveals FOXM1 as a novel target for cancer therapy. *Am J Pathol, 178*(2), 537-547.

Uehara, R., Tsukada, Y., Kamasaki, T., Poser, I., Yoda, K., Gerlich, D. W., & Goshima, G. (2013). Aurora B and Kif2A control microtubule length for assembly of a functional central spindle during anaphase. *J Cell Biol, 202*(4), 623-636.

UK Cancer Registry (2012). Stages of ovarian cancer Retrieved 27 November, 2013, from http://www.cancerresearchuk.org/cancer-help/type/ovarian-cancer/treatment/stages-of-ovarian-cancer

UK Cancer Registry (2013). Ovarian cancer incidence statistics Retrieved 26 November, 2013, from http://www.cancerresearchuk.org/cancer-info/cancerstats/types/ovary/incidence/#trends

Vadlamudi, R. K., Bagheri-Yarmand, R., Yang, Z., Balasenthil, S., Nguyen, D., Sahin, A. A., den Hollander, P., & Kumar, R. (2004). Dynein light chain 1, a p21-activated kinase 1-interacting substrate, promotes cancerous phenotypes. *Cancer Cell, 5*(6), 575-585.

Vale, R. D. (2003). The molecular motor toolbox for intracellular transport. *Cell, 112*(4), 467-480.

Vale, R. D., Reese, T. S., & Sheetz, M. P. (1985). Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell, 42*(1), 39-50.

Vilchez, D., Boyer, L., Lutz, M., Merkwirth, C., Morantte, I., Tse, C., Spencer, B., Page, L., Masliah, E., Berggren, W. T., Gage, F. H., & Dillin, A. (2013). FOXO4 is necessary for neural differentiation of human embryonic stem cells. *Aging Cell, 12*(3), 518-522.

Walker, G. R., Schlesselman, J. J., & Ness, R. B. (2002). Family history of cancer, oral contraceptive use, and ovarian cancer risk. *Am J Obstet Gynecol, 186*(1), 8-14.

Walle, T., Walle, U. K., Kumar, G. N., & Bhalla, K. N. (1995). Taxol metabolism and disposition in cancer patients. *Drug Metab Dispos, 23*(4), 506-512.

Walter, B. A., Valera, V. A., Pinto, P. A., & Merino, M. J. (2013). Comprehensive microRNA Profiling of Prostate Cancer. *J Cancer, 4*(5), 350-357.

Wang, C. Q., Qu, X., Zhang, X. Y., Zhou, C. J., Liu, G. X., Dong, Z. Q., Wei, F. C., & Sun, S. Z. (2010). Overexpression of Kif2a promotes the progression and metastasis of squamous cell carcinoma of the oral tongue. *Oral Oncol, 46*(1), 65-69.

Wang, F., Zheng, Z., Guo, J., & Ding, X. (2010). Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor. *Gynecol Oncol, 119*(3), 586-593.

Wang, L., Gu, F., Liu, C. Y., Wang, R. J., Li, J., & Xu, J. Y. (2013). High level of FOXC1 expression is associated with poor prognosis in pancreatic ductal adenocarcinoma. *Tumour Biol, 34*(2), 853-858.

Wang, R. A., Zhang, H., Balasenthil, S., Medina, D., & Kumar, R. (2006). PAK1 hyperactivation is sufficient for mammary gland tumor formation. *Oncogene, 25*(20), 2931-2936.

Wang, X., Gong, W., Qing, H., Geng, Y., Zhang, Y., Peng, L., Zhang, H., & Jiang, B. (2010). p21-activated kinase 5 inhibits camptothecin-induced apoptosis in colorectal carcinoma cells. *Tumour Biol, 31*(6), 575-582.

Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P., & McPhail, A. T. (1971). Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *J Am Chem Soc, 93*(9), 2325-2327.

Wei, L. X., Zhou, R. S., Xu, H. F., Wang, J. Y., & Yuan, M. H. (2013). High expression of FOXC1 is associated with poor clinical outcome in non-small cell lung cancer patients. *Tumour Biol, 34*(2), 941-946.

Wen, X., Li, X., Liao, B., Liu, Y., Wu, J., Yuan, X., Ouyang, B., Sun, Q., & Gao, X. (2009). Knockdown of p21-activated kinase 6 inhibits prostate cancer growth and enhances chemosensitivity to docetaxel. *Urology, 73*(6), 1407-1411.

Whittemore, A. S., Harris, R., & Itnyre, J. (1992). Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. II. Invasive epithelial ovarian cancers in white women. Collaborative Ovarian Cancer Group. *Am J Epidemiol, 136*(10), 1184-1203.

Wiehagen, K. R., Corbo-Rodgers, E., Li, S., Staub, E. S., Hunter, C. A., Morrisey, E. E., & Maltzman, J. S. (2012). Foxp4 is dispensable for T cell development, but required for robust recall responses. *PLoS One, 7*(8), e42273.

Williams, C. J. (2001). Tamoxifen for relapse of ovarian cancer. *Cochrane Database Syst Rev*(1), CD001034.

Wilson, M. S., Brosens, J. J., Schwenen, H. D., & Lam, E. W. (2011). FOXO and FOXM1 in cancer: the FOXO-FOXM1 axis shapes the outcome of cancer chemotherapy. *Curr Drug Targets, 12*(9), 1256-1266.

Wolf, J. K., Mills, G. B., Bazzet, L., Bast, R. C., Jr., Roth, J. A., & Gershenson, D. M. (1999). Adenovirus-mediated p53 growth inhibition of ovarian cancer cells is independent of endogenous p53 status. *Gynecol Oncol, 75*(2), 261-266.

Wong, K. K., Tsang, Y. T., Deavers, M. T., Mok, S. C., Zu, Z., Sun, C., Malpica, A., Wolf, J. K., Lu, K. H., & Gershenson, D. M. (2010). BRAF mutation is rare in advanced-stage low-grade ovarian serous carcinomas. *Am J Pathol, 177*(4), 1611-1617.

Wong, L. E., Chen, N., Karantza, V., & Minden, A. (2013). The Pak4 protein kinase is required for oncogenic transformation of MDA-MB-231 breast cancer cells. *Oncogenesis, 2*, e50.

Wonsey, D. R., & Follettie, M. T. (2005). Loss of the forkhead transcription factor

FoxM1 causes centrosome amplification and mitotic catastrophe. *Cancer Res, 65*(12), 5181-5189.

World Health Organization (2013). Incidence, mortality and survival databases Retrieved 27 November, 2013, from http://www.who.int/cancer/resources/incidences/en/

Xiao, X., Tang, C., Xiao, S., Fu, C., & Yu, P. (2013). Enhancement of proliferation and invasion by MicroRNA-590-5p via targeting PBRM1 in clear cell renal carcinoma cells. *Oncol Res, 20*(11), 537-544.

Xu, L., Panel, V., Ma, X., Du, C., Hugendubler, L., Gavrilova, O., Liu, A., McLaughlin, T., Kaestner, K. H., & Mueller, E. (2013). The winged helix transcription factor Foxa3 regulates adipocyte differentiation and depot-selective fat tissue expansion. *Mol Cell Biol, 33*(17), 3392-3399.

Xu, Y., Takeda, S., Nakata, T., Noda, Y., Tanaka, Y., & Hirokawa, N. (2002). Role of KIFC3 motor protein in Golgi positioning and integration. *J Cell Biol, 158*(2), 293-303.

Yamakawa, N., Takahashi, A., Mori, E., Imai, Y., Furusawa, Y., Ohnishi, K., Kirita, T., & Ohnishi, T. (2008). High LET radiation enhances apoptosis in mutated p53 cancer cells through Caspase-9 activation. *Cancer Sci, 99*(7), 1455-1460.

Yan, G. R., Zou, F. Y., Dang, B. L., Zhang, Y., Yu, G., Liu, X., & He, Q. Y. (2012). Genistein-induced mitotic arrest of gastric cancer cells by downregulating KIF20A, a proteomics study. *Proteomics,* 12(14), 2391-2399.

Yang, F., Li, X., Sharma, M., Zarnegar, M., Lim, B., & Sun, Z. (2001). Androgen receptor specifically interacts with a novel p21-activated kinase, PAK6. *J Biol Chem,* 276(18), 15345-15353.

Yang, L., Belaguli, N., & Berger, D. H. (2009). MicroRNA and colorectal cancer. *World J Surg, 33*(4), 638-646.

Ye, D. Z., & Field, J. (2012). PAK signaling in cancer. *Cell Logist, 2*(2), 105-116.

You, W., Fan, L., Duan, D., Tian, L., Dang, X., Wang, C., & Wang, K. (2014). Foxc2 over-expression in bone marrow mesenchymal stem cells stimulates osteogenic differentiation and inhibits adipogenic differentiation. *Mol Cell Biochem*, 386(1-2), 125-134.

Yu, Y. H., Chen, H. A., Chen, P. S., Cheng, Y. J., Hsu, W. H., Chang, Y. W., Chen, Y. H., Jan, Y., Hsiao, M., Chang, T. Y., Liu, Y. H., Jeng, Y. M., Wu, C. H., Huang, M. T., Su, Y. H., Hung, M. C., Chien, M. H., Chen, C. Y., Kuo, M. L., & Su, J. L. (2013). MiR-520h-mediated FOXC2 regulation is critical for inhibition of lung cancer progression by resveratrol. *Oncogene,* 32(4), 431-443.

Yungang, W., Xiaoyu, L., Pang, T., Wenming, L., & Pan, X. (2014). miR-370 targeted FoxM1 functions as a tumor suppressor in laryngeal squamous cell carcinoma (LSCC). *Biomed Pharmacother*, 68(2), 149-154.

Zaino, R. J., Brady, M. F., Lele, S. M., Michael, H., Greer, B., & Bookman, M. A. (2011). Advanced stage mucinous adenocarcinoma of the ovary is both rare and highly lethal: a Gynecologic Oncology Group study. *Cancer, 117*(3), 554-562.

Zhang, H. Y., & Sun, H. (2010). Up-regulation of Foxp3 inhibits cell proliferation,

migration and invasion in epithelial ovarian cancer. *Cancer Lett, 287*(1), 91-97.

Zhang, N., Wu, X., Yang, L., Xiao, F., Zhang, H., Zhou, A., Huang, Z., & Huang, S. (2012). FoxM1 inhibition sensitizes resistant glioblastoma cells to temozolomide by downregulating the expression of DNA-repair gene Rad51. *Clin Cancer Res, 18*(21), 5961-5971.

Zhang, X., Zeng, J., Zhou, M., Li, B., Zhang, Y., Huang, T., Wang, L., Jia, J., & Chen, C. (2012). The tumor suppressive role of miRNA-370 by targeting FoxM1 in acute myeloid leukemia. *Mol Cancer, 11*, 56.

Zhao, F., & Lam, E. W. (2012). Role of the forkhead transcription factor FOXO-FOXM1 axis in cancer and drug resistance. *Front Med, 6*(4), 376-380.

Zhao, F., Siu, M. K., Jiang, L., Tam, K. F., Ngan, H. Y., Le, X. F., Wong, O. G., Wong, E. S., Chan, H. Y., & Cheung, A. N. (2011). Overexpression of dedicator of cytokinesis I (Dock180) in ovarian cancer correlated with aggressive phenotype and poor patient survival. *Histopathology, 59*(6), 1163-1172.

Zhao, W., Yang, J., Shi, W., Wu, X., Shao, B., Wu, Q., Chen, J., & Ni, L. (2011). Upregulation of p21-activated Kinase 6 in rat brain cortex after traumatic brain injury. *J Mol Histol, 42*(3), 195-203.

Zhao, Z. S., Lim, J. P., Ng, Y. W., Lim, L., & Manser, E. (2005). The GIT-associated kinase PAK targets to the centrosome and regulates Aurora-A. *Mol Cell, 20*(2), 237-249.

Zhu, G., Wang, Y., Huang, B., Liang, J., Ding, Y., Xu, A., & Wu, W. (2012). A Rac1/PAK1 cascade controls beta-catenin activation in colon cancer cells. *Oncogene, 31*(8), 1001-1012.

Zografos, G. C., Panou, M., & Panou, N. (2004). Common risk factors of breast and ovarian cancer: recent view. *Int J Gynecol Cancer, 14*(5), 721-740.

Zou, Y., Tsai, W. B., Cheng, C. J., Hsu, C., Chung, Y. M., Li, P. C., Lin, S. H., & Hu, M. C. (2008). Forkhead box transcription factor FOXO3a suppresses estrogen-dependent breast cancer cell proliferation and tumorigenesis. *Breast Cancer Res, 10*(1), R21.

Zuo, T., Liu, R., Zhang, H., Chang, X., Liu, Y., Wang, L., & Zheng, P. (2007b). FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2. *J Clin Invest, 117*(12), 3765-3773.

Zuo, T., Wang, L., Morrison, C., Chang, X., Zhang, H., Li, W., Liu, Y., Wang, Y., Liu, X., Chan, M. W., Liu, J. Q., Love, R., Liu, C. G., Godfrey, V., Shen, R., Huang, T. H., Yang, T., Park, B. K., Wang, C. Y., & Zheng, P. (2007a). FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. *Cell, 129*(7), 1275-1286.