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Loss of SKI Expression in Testicular Cancer Leads to an Enhanced Invasive Phenotype Through Both BMP-Dependent and BMP-Independent Pathways

Amy N. Nash

LOSS OF SKI EXPRESSION IN TESTICULAR CANCER LEADS TO AN ENHANCED INVASIVE PHENOTYPE THROUGH BOTH BMP-DEPENDENT AND BMP-INDEPENDENT PATHWAYS

by

Amy N. Nash

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Approved by:

Dr. Laura Richardson, Ph.D., Committee Chairperson
Dr. Beverly Delidow, Ph.D.
Dr. Elaine Hardman, Ph.D.
Dr. Kelley Kiningham, Ph.D.
Dr. Donald Primerano, Ph.D.

Marshall University
Huntington, WV

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ABSTRACT

LOSS OF SKI EXPRESSION IN TESTICULAR CANCER LEADS TO AN ENHANCED INVASIVE PHENOTYPE THROUGH BOTH BMP-DEPENDENT AND BMP-INDEPENDENT PATHWAYS

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The proto-oncogene SKI is a transcription factor and a co-repressor of the TGFβ superfamily, including TGFβ and BMP. However, additional data suggests that SKI may function as a tumor suppressor in some cell types. The TGFβ superfamily has been implicated in cancer progression and germ cell migration. Testicular cancer afflicts men during their peak reproductive years and is the most common cancer among men of this age group. Cisplatin-based chemotherapy is the standard treatment for testicular cancer. This treatment can lead to undesirable side effects, including infertility. We have shown that SKI expression is decreased in testicular germ cell tumors compared to normal testis. The hypothesis of this work is that decreased *SKI* expression promotes testicular cancer progression by allowing unregulated TGFβ superfamily signaling, conferring an increased ability of testicular cancer cells to invade and metastasize. To test the effects of decreased SKI in testicular cancer, we used a human embryonal carcinoma cell line, NCCIT. We found that transient SKI-knockdown confers an increased ability to migrate and invade, but does not affect proliferation, in NCCIT cells. Forced overexpression of SKI in NCCIT cells resulted in decreased migration and invasion compared to control cells. We next investigated whether TGFβ or BMP enhanced the invasive phenotype of stable SKI-knockdown NCCIT cells. NCCIT cells stably transfected with nonspecific shRNA were used as a control. Addition of TGFβ or TGFβ blocking antibody had no effect on the migration of either SKI-knockdown cells or control cells. Addition of BMP4 enhanced migration in SKI-deficient NCCIT cells but had no effect on control cells. Treatment with noggin, a BMP inhibitor, reduced the migration of SKI-deficient NCCIT cells back to the level of control cells. These data imply that in NCCIT cells with decreased *SKI* expression, the BMP pathway, but not the TGFβ pathway, promotes migration. Microarray analysis was performed to identify downstream targets of SKI that may be involved in cellular invasion. *CXCR4* expression was increased in SKI-knockdown cells compared to control cells, but *CXCR4* expression was not affected by treatment with BMP. Signaling partners CXCR4 and SDF1 have been implicated in germ cell migration and cancer metastasis. Addition of SDF1 enhanced the invasive potential of SKI-deficient cells but had no effect on control cells. Addition of AMD3100, a potent and specific inhibitor of CXCR4, blocked the effects seen with SDF1. These data, taken together with the BMP data, imply that decreased *SKI* expression in NCCIT cells leads to enhanced invasion through both BMP-dependent and BMP-independent mechanisms. This research provides insight into the mechanism behind testicular cancer metastasis, and it identifies the BMP signaling pathway and CXCR4/SDF1 as potential targets for the development of new therapies to treat patients with metastatic testicular cancer.

DEDICATION

This work is dedicated to my husband, Jordan. His constant support, love, and encouragement have kept me going during the pursuit of my Ph.D.

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LIST OF SYMBOLS, ABBREVIATIONS

AJCC – American Joint Committee on Cancer
BMP – Bone morphogenetic protein
CCND – cyclin D
ECM – Extracellular matrix
ELR - glutamic acid-leucine-arginine
EMT – epithelial to mesenchymal transition
FBS – fetal bovine serum
GCT – germ cell tumor
GSEA – gene set enrichment analysis
HRP – Horseradish peroxidase
ITGCN – Intratubular germ cell neoplasia
NER – nucleotide excision repair
OMIM – Online Mendelian Inheritance in Man
PBS – phosphate buffered saline
PGC – Primordial germ cell
Rb – retinoblastoma
SCF – Stem cell factor
SDF1 – Stromal derived factor-1
TGFβ – Transforming growth factor β
TGCT – Testicular germ cell tumor

CHAPTER I

INTRODUCTION

Testicular cancer is the most common cancer in American males between the ages of 18 and 35; however, the molecular mechanisms involved in the development and progression of testicular cancer are poorly understood. In recent decades, the incidence of testicular germ cell tumors (TGCTs) has increased annually at a rate of 3 - 6 percent in Caucasian populations (Ulbright, 1993). The precursor lesions to testicular cancer, termed intratubular germ cell neoplasia (ITGCN), are thought to arise from primitive germ cells that have evaded normal differentiation. There are three categories of testicular germ cell tumors; Type II is the most prevalent type and, therefore, the most widely studied and will be the main focus of this thesis.

Cancer is a multi-step disease, typically involving alterations in multiple signaling pathways that eventually lead to cellular transformation. Several genes have been identified that may be involved in the development and progression of testicular cancer. However, very few studies have been performed to identify the mechanism by which these genes promote testicular tumorigenesis.

The proto-oncogene *SKI* is a transcription factor that can function as a co-repressor of the transforming growth factor β (TGFβ) superfamily, including TGFβ and bone morphogenetic protein (BMP), by binding to SMADs, the downstream signaling molecules of TGFβ and BMP. The TGFβ superfamily has

been implicated in cancer growth and metastasis as well as germ cell migration during development, making them of particular interest in testicular cancer research. The role of the TGFβ superfamily in cancer is complicated, with several studies suggesting that TGFβ and BMP may have both tumor suppressor and tumor promoting functions, which may depend on both cell type and the stage of cancer progression. Similarly, the role of SKI in cancer is still incompletely defined and may also be tissue-dependent. Some tumors express high levels of *SKI*, however, loss of SKI function has been linked to tumorigenesis in mice heterozygous for a null mutation in the *SKI* gene, suggesting *SKI* may also function as a tumor suppressor.

Purpose of the Research

The objective of the following body of research is to determine the role that SKI, which we have found to be decreased in testicular tumor samples compared to normal testis, plays in testicular cancer. SKI was originally identified as an oncogene based on its ability to transform avian fibroblasts (Stavnezer 1981, Colmenares 1989); however, other data has provided evidence that SKI may have tumor suppressor activities (Shinagawa 2001). The purpose of the following research is 1) to identify the role of SKI in testicular cancer progression using biological assays; 2) to determine how upstream signals from the TGFβ superfamily contribute to the effects of decreased SKI in testicular cancer; and 3) to identify downstream target genes of SKI that may also contribute to testicular cancer progression.

Significance of the Research

The data presented in this text addresses an area that is lacking in testicular cancer research by studying the mechanism by which SKI contributes to the progression of testicular cancer. Furthermore, this research has been focused on testicular cancer metastasis, an area of testicular cancer that has not been widely researched. The TGFβ superfamily and SKI have previously been reported to play conflicting roles in tumorigenesis, apparently depending on the stage of cancer progression and tissue type; thus, we have sought to define the specific role that SKI and TGFβ superfamily members play in testicular tumorigenesis.

This research is significant, not only because it fills a gap in the research, but also because it will allow a better understanding of testicular cancer progression. This has been accomplished by investigating SKI and its potential role as a tumor suppressor of testicular cancer and also by considering the upstream and downstream signals of SKI that contribute to the overall mechanism of testicular cancer progression. By providing further insight into testicular cancer progression, this research may contribute to the development of more efficient treatments that better preserve male fertility and provide a better quality of life for patients afflicted with the disease.

CHAPTER II

LITERATURE REVIEW

Male Germ Cell Development

Very early in mammalian embryonic development, a distinct cell population arises that has the ability to transmit genetic information from one generation to the next. These pluripotent cells are primordial germ cells (PGCs), the fetal precursors to sperm and oocytes, and they are derived from the proximal epiblasts in response to signals from the extraembryonic ectoderm (Lawson, et al., 1999; Lawson and Hage, 1994). In the mouse, the specification of a population of about 45 cells to the germ cell lineage occurs at approximately embryonic day 7.2; at this time the cell cluster is located in the extraembryonic mesoderm (Lawson and Hage, 1994). Lawson, et. al. (1999) showed that BMP4, secreted first from the extraembryonic ectoderm, and later from the extraembryonic mesoderm, is required for the allocation of the PGC population. BMP8b is also expressed by the extraembryonic ectoderm, and appears to act synergistically with BMP4 in the generation of PGCs (Ying, et al., 2000; Ying, et al., 2001). These cells can be identified by staining for alkaline phosphatase, which continues to be expressed in these cells during their migratory phase (Ginsburg, et al., 1990).

Primordial Germ Cell Migration

During embryonic development, PGCs migrate through the primitive streak, into the allantois, and then enter the embryonic endoderm, which gives rise to the hindgut (Anderson, et al., 2000). In the mouse, PGCs migrate through the hindgut and come to rest in the genital ridge at around embryonic day 11.5 (Anderson, et al., 2000). The migration of the PGCs is dependent upon interaction with the extracellular matrix (ECM) and specific signals secreted from the genital ridge. There are several factors associated with the ECM that influence PCG migration. Fibronectin, an adhesive glycoprotein found in the ECM, stimulates the migration of PGCs from the hindgut (Ffrench-Constant, et al., 1991). In the mouse, PGCs lacking β1-integrin, which is involved in cell-ECM attachment and cell migration, have a reduced ability to colonize the gonad (Anderson, et al., 1999). During migration, PCGs associate with one another via cytoplasmic processes. E-cadherin appears to play a role in this connection as it is upregulated in PGCs when they are leaving the hindgut, and blocking E-cadherin prevented PGC aggregation by preventing their formation of tight clusters in the genital ridges in vitro (Bendel-Stenzel, et al., 2000). The genital ridge also secretes factors that influence the direction of PGC migration in vitro, and TGFβ has been shown to act as a chemoattractant of PGCs in culture, indicating that it may be one of the factors secreted by the genital ridge (Godin and Wylie, 1991). The ligand stem cell factor (SCF) and its receptor, c-kit, are required for PGC survival, migration, and colonization of the hindgut (Besmer, et al., 1993; Buehr and McLaren, 1993). PGCs express c-kit, and cells of the

hindgut express SCF (Besmer, et al., 1993). Signaling partners, stromal derived factor-1 (SDF1) and its receptor, CXCR4, are also required for PGC migration and survival (Molyneaux, et al., 2003; Stebler, et al., 2004). Migrating PGCs express CXCR4, and SDF is expressed in the genital ridges (Molyneaux, et al., 2003). PGCs with mutations in CXCR4 do not colonize the gonad normally (Stebler, et al., 2004). BMP may also be involved in regulating PGC motility. PGCs express BMP receptors, and BMP4 increased PGC numbers, while noggin, a BMP inhibitor, reduced PGC numbers and slowed their migration. Furthermore, BMPs induced, and noggin suppressed, the expression of SCF in the genital ridges (Dudley, et al., 2007).

Overview of Spermatogenesis

Once the PGCs enter the genital ridges, they are no longer migratory, and they are termed gonocytes. Before birth, the gonocytes undergo a few rounds of mitosis before entering a period of mitotic arrest as prospermatogonia (McLaren, 2003). Spermatogenesis begins at puberty with type A spermatogonia, which are capable of self-renewal to maintain the germ cell population throughout life. These cells divide asymmetrically to form type A and type B spermatogonia, the precursors of the spermatocytes. Type B spermatogonia undergo differentiation to form primary spermatocytes. Primary spermatocytes enter meiosis I, followed by spermatocytes undergoing meiosis II to form spermatids (Figure 2.1). Spermatids then undergo a differentiation process to form mature spermatozoa. Spermatogenesis is a complicated process that is dependent on specific signals,

many of which come from Sertoli cells, which provide nutrients and growth factors to the maturing germ cells (Puglisi, et al., 2004).

Intratubular Germ Cell Neoplasia

While there are distinct groups of testicular cancer with histological differences, all are believed to be of germ cell origin (Figure 2.1). TGCTs arise from precursor lesions, known as intratubular germ cell neoplasia (ITGCN) (also referred to as carcinoma in situ), which are thought to arise from primitive germ cells that have evaded normal differentiation (Chaganti and Houldsworth, 2000; Oosterhuis and Looijenga, 2005). ITGCNs, PGCs, and gonocytes have similar morphologies and some similar patterns of gene expression, including positive staining for alkaline phosphatase and c-kit expression (Kristensen, et al., 2008).

Testicular Germ Cell Tumor Classifications

Testicular germ cell tumors (TGCTs) have been divided into three classifications based on epidemiology, their histology, and the age group in which they occur: Type I – teratomas and yolk sac tumors of newborns and infants; Type II - seminomatous and non-seminomatous tumors of adolescents and young adults; and Type III – spermatocytic seminomas of the elderly (Oosterhuis and Looijenga, 2005). Of these three groups, Type II TGCTs are the most common and account for approximately 60% of malignancies diagnosed in men between the ages of 20 – 40 years old (Oosterhuis and Looijenga, 2005; Ulbright, 1993), also making testicular cancer the most prevalent cancer among

men of this age group (Chaganti and Houldsworth, 2000). Type I pediatric GCTs are rarer, constituting about 3% of childhood tumors (Bussey, et al., 1999). In addition to the testis, type I GCTs can be found in sacrocoxygeal region, head and neck, and midline of the brain (Looijenga, et al., 2007). Type III TGCTs are the rarest type, occurring predominantly as benign tumors in males over 50 years of age (Oosterhuis and Looijenga, 2005). Type III tumors are found primarily in the testis and rarely metastasize (Looijenga, et al., 2007).

Type II TGCTs consist of seminomas and embryonal carcinomas (also referred to as nonseminomas) (Figure 2.1). Of these seminomas are the most common type, comprising about 50% of TGCT (Bahrami, et al., 2007). Seminomas are rarely seen in pre-pubertal males; they most typically occur in men between the ages of 34 and 45 (Bahrami, et al., 2007). Seminomas are morphologically similar to spermatogonial germ cells and generally respond very well to radiation and chemotherapy (Chaganti and Houldsworth, 2000). Embryonal carcinomas also encompass tumor types with varying degrees of differentiation, including teratomas, yolk sack tumors, and choriocarcinomas. Embryonal carcinomas occur most frequently in men between the ages of 25 and 35 (Bahrami, et al., 2007). Embryonal carcinomas are comprised of undifferentiated pluripotent stem cells (Looijenga and Oosterhuis, 1999). Yolk sac tumors and choriocarcinomas exhibit extraembryonic differentiation patterns (Chaganti and Houldsworth, 2000). Teratomas display the most complete differentiation and are comprised of a variety of somatic tissues (Chaganti and Houldsworth, 2000).

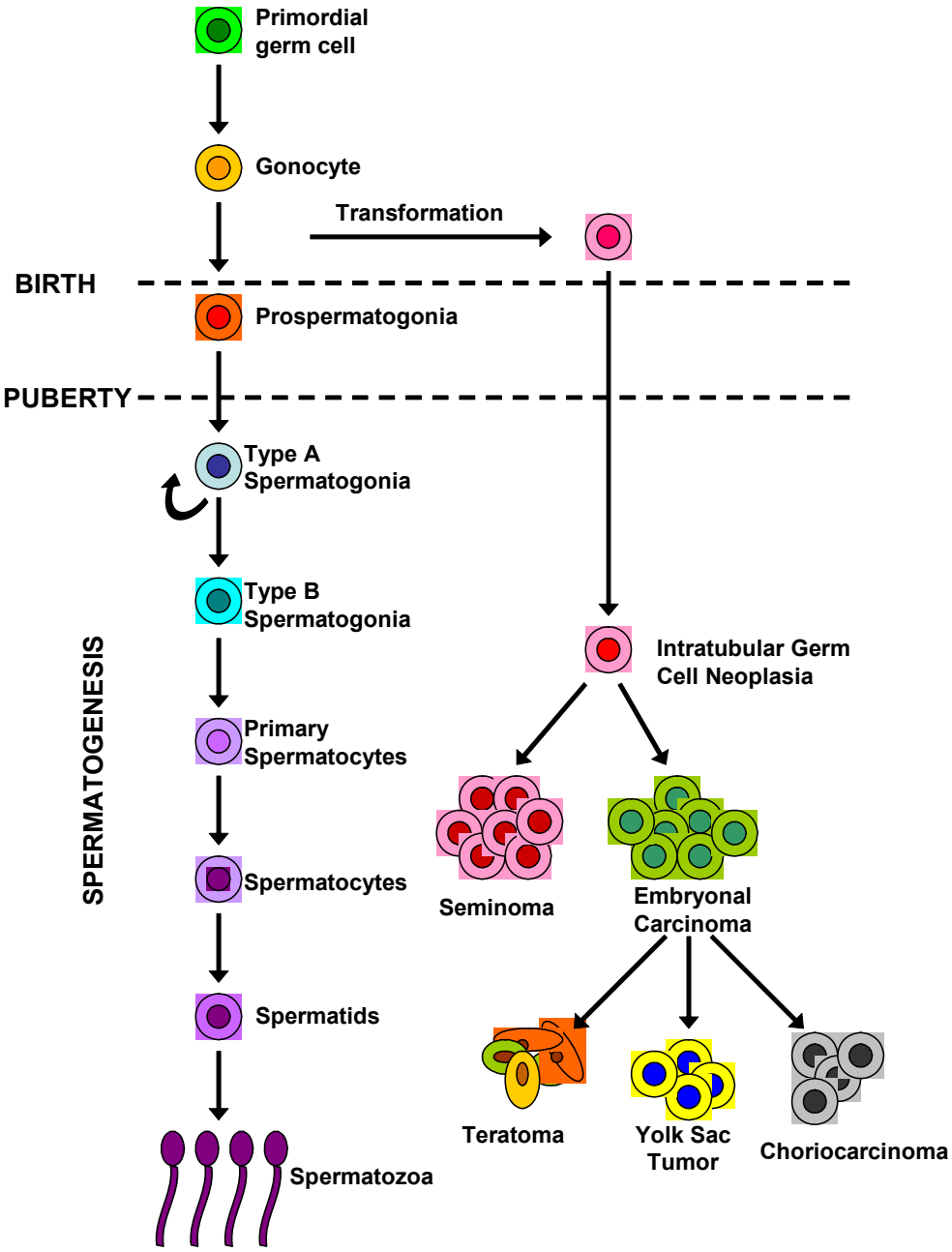

Figure 2.1. Normal germ cell development vs. germ cell tumorigenesis. Normal germ cell development (left) begins during embryogenesis with primordial germ cells, which are allocated in the extraembryonic mesoderm and migrate to the genital ridge, where they are termed gonocytes. Gonocytes undergo a few rounds of mitosis and then enter mitotic arrest as prospermatogonia before birth. These cells divide asymmetrically to form type A and type B spermatogonia. Type B spermatogonia undergo differentiation, forming primary spermatocytes. Primary spermatocytes enter meiosis I, and then spermatocytes undergo meiosis II, forming spermatids. Spermatids undergo a differentiation process to form spermatozoa. Testicular tumorigenesis (right) is thought

to begin during embryonic development with the transformation with a primitive germ cell, possibly the primordial germ cell or the gonocyte. Testicular germ cell tumors arise from intratubular germ cell neoplasia, most commonly during peak reproductive years. Testicular germ cell tumors consist of seminomas and embryonal carcinomas. Embryonal carcinomas also encompass tumors with various levels of differentiation, including teratomas, yolk sac tumors, and choriocarcinomas (adapted from Kristensen, et al., 2008).

Risk Factors Associated with Testicular Cancer

There are several risk factors associated with the development of TGCTs, including both environmental and genetic influences. Testicular cancer generally occurs at an earlier stage of life than many other types of cancer, thus it is thought that tumorigenesis may begin during fetal life. It has been proposed that hormone levels in utero may have some effect on the development of TGCTs. TGCTs are much more common in Caucasian men than black men, and it has been suggested that this may be due to higher levels of testosterone during pregnancy in black women compared to Caucasian women (Looijenga and Oosterhuis, 1999). There appears to be a genetic component to the development of TGCTs. TGCT patients with an affected family member generally develop TGCTs at a younger age and are more likely to have bilateral disease (Looijenga and Oosterhuis, 1999). Skakkebaek et. al. (2001) proposed a theory of testicular dysgenesis syndrome, which includes embryonic exposure to endogenous or environmental factors that affect gonadal development. Risk factors associated with testicular dysgenesis syndrome and the development of testicular cancer include cryptorchidism, poor semen quality, Klinefelter's syndrome, prior history of a GCT, and family history of GCTs (Chaganti and Houldsworth, 2000; Oosterhuis and Looijenga, 2005; Skakkebaek, 2004).

Molecular basis of testicular cancer

Most TGCTs display a high degree of aneuploidy. Although various regions of chromosomal imbalance have been linked to the development of

testicular germ cell tumors (TGCTs) using microarray and FISH analysis (Korkola, et al., 2006; McIntyre, et al., 2004; Summersgill, et al., 2001; Zahn, et al., 2006), little is known about how these chromosomal regions contribute to tumorigenesis in male germ cells. The most common chromosomal aberration associated with TGCTs is the occurrence of isochromosome 12p (Chaganti and Houldsworth, 2000; Summersgill, et al., 2001). However, various other chromosomal alterations are correlated with TGCTs (McIntyre, et al., 2004), including a frequently observed deletion in the short arm of chromosome 1, where the *SKI* gene is located. Chromosome location 1p36 often contains deletions in testicular cancer samples and is a candidate region for tumor suppressor genes of testicular cancer (Zahn, et al., 2006). While several studies have looked at expression profiles in TGCTs in order to identify possible oncogenes and tumor suppressors involved in testicular cancer, the field is lacking in studies of the mechanisms of particular genes to discover the specific role they might play in testicular tumorigenesis. However, the following review of the literature provides a brief discussion of genes that have been more thoroughly investigated in testicular cancer research and have been presented as potential tumor suppressor genes and oncogenes based primarily on their expression pattern and previously known functions.

OCT3/4

OCT3/4 is a transcription factor that functions to maintain pluripotency and promotes survival in embryonic stem cells and primordial germ cells (Gidekel, et

al., 2003; Nichols, 1998; Pesce and Scholer, 2001). OCT3/4 is expressed in PGCs of the human testis, and its expression gradually decreases with differentiation into spermatogonia (Rajpert-De Meyts, et al., 2004). This gene is expressed in germ cell tumors with greater differentiation potential, such as seminomas and embryonal carcinomas, and has been identified through immunohistochemistry and microarray studies as a diagnostic marker for ITGCN (Almstrup, et al., 2007; Cheng, et al., 2007; Oosterhuis and Looijenga, 2005). While OCT3/4 is useful as a marker for both ITGCN and for identifying metastatic tumors of unknown origin, it is unknown whether OCT3/4 plays a causal role in testicular cancer or if it merely reflects the origin and developmental potential of TGCTs.

CCND2

Nearly all invasive type II TGCTs are positive for overexpression of chromosome 12p, most commonly due to the presence of one or more copies of isochromosome 12p (Oosterhuis and Looijenga, 2005). This makes the 12p region popular for studying potential oncogenes of testicular cancer. One such gene is cyclin D2 (CCND2), which maps to 12p13, and has been shown through both immunohistochemistry and RNA studies to be overexpressed in ITGCN and in most TGCTs (Bartkova, et al., 1999; Houldsworth, et al., 1997; Schmidt and Fan, 2001). Houldsworth, et. al. (1997) found that CCND2 mRNA and protein were deregulated in a panel of germ cell tumor (GCT) cell lines and was aberrantly expressed in all ITGCN studied. Furthermore, CCND2 was more

highly expressed in GCTs with a less differentiated phenotype. CCND2 was present in all ITGCNs but was not expressed in normal germ cells. These data suggest that CCND2 overexpression is an early event in male germ cell tumorigenesis. It has been suggested that increased expression of CCND2 may promote cell cycle progression by driving the cell through the G1 checkpoint, which may confer genomic instability (Houldsworth, et al., 1997).

c-KIT/SCF

The aberrant expression of chromosome 12p due to isochromosome 12p in TGCTs can result in an under-representation of chromosome 12q, making chromosome 12q a candidate region for tumor suppressor genes. Murty et. al. (1992) evaluated 45 TGCT DNA samples with allelic loss at 12q and identified a high frequency of loss of heterozygosity at 12q13 and 12q22. This group identified stem cell factor (SCF, also known as MGF), a ligand signaling partner for KIT, to be located within these chromosomal regions. KIT is expressed in the primordial germ cells (PGC) during embryogenesis and in proliferating spermatogonia in the post-natal and adult testis; SCF is secreted from Sertoli cells (Devouassoux-Shisheboran, et al., 2003). KIT and SCF are regulators of spermatogenesis; they are involved in PGC migration, cell adhesion, proliferation, and cell survival (Devouassoux-Shisheboran, et al., 2003). In addition to decreased expression of SCF, KIT has also been shown to be over-expressed in some types of TGCTs, especially seminomas (Looijenga, et al., 2003). McIntyre et. al. (2004) identified an amplification of 4q12, the region in which KIT is

encoded, and other activating mutations of KIT have been identified (Kemmer, et al., 2004; Looijenga, et al., 2003; Sakuma, et al., 2003). These activating mutations have primarily been observed in seminomas as well as ITGCNs and are absent in non-seminomas (Devouassoux-Shisheboran, et al., 2003; Kemmer, et al., 2004). KIT and SCF are known to play important roles in spermatogenesis; however, their function in TGC tumorigenesis has not been fully investigated.

Rb

Another potential tumor suppressor gene of testicular cancer is retinoblastoma (Rb). While the more differentiated cells of teratocarcinomas and mixed tumors retain some expression of Rb, Rb mRNA and protein expression is undetectable in undifferentiated TGCTs, however no mutations in Rb have been observed at the DNA level (Strohmeyer, et al., 1991). Rb is expressed during adult male spermatogenesis, but its expression is absent in PGCs, the precursor cells for TGCTs, and it is also absent in ITGCNs and most seminomas and embryonal carcinomas (Bartkova, et al., 2003). Since no mutations have been detected in the DNA sequence of Rb, Bartkova et. al. (2003) suggested that the lack of expression of Rb in TGCTs may be a reflection of its developmental control and the cell of origin for TGCTs rather than a tumorigenesis promoting event.

GDF3

GDF3 is another candidate oncogene for testicular cancer as it has been shown to be overexpressed specifically in embryonal carcinomas (Caricasole, et al., 1998; Clark, et al., 2004; Korkola, et al., 2006; Skotheim, et al., 2005). GDF3 is a TGFβ superfamily member with stem cell specific expression (Caricasole, et al., 1998). GDF3 expression is low in seminomas but is abundantly expressed in non-seminomas, especially within embryonal carcinomas and yolk sac components of tumors. Interestingly, GDF3 maps to the short arm of human chromosome 12, which, as previously mentioned, is regularly overexpressed in TGCTs (Caricasole, et al., 1998).

While all TGCTs are thought to arise from a common precursor, ITGCN, the different tumor types appear to differ in their gene expression profiles. This would suggest that different types of mutations may predispose the ITGCN toward the development of a seminomatous tumor over an embryonal carcinoma or vice versa. Over-representation of chromosome 12p occurs in most ITGCN and is also common to the various types of TGCTs, further indicating that this may be a very early event in testicular germ cell tumorigenesis. KIT, on the other hand, seems to be overexpressed primarily in seminomas and ITGCNs, while GDF3 is overexpressed in embryonal carcinomas.

Through various other expression based profiles, several other genes have been identified as possible tumor suppressors or oncogenes of testicular cancer (Korkola, et al., 2006; McIntyre, et al., 2004; Oosterhuis and Looijenga, 2005; Rodriguez, et al., 2003; Skotheim, et al., 2005; Sugimura, et al., 2004).

These genes have primarily been identified based on differential expression, and mutations have been identified in some. However, there is a lack of research investigating the mechanism by which possible tumor suppressor and oncogenes may contribute to testicular tumorigenesis.

Treatment for Testicular Cancer

In 1970, metastatic TGCTs claimed the lives of 95 percent of men afflicted, however, cisplatin-based combination chemotherapy is effective in ablating TGCTs in more than 80 percent of men with metastatic germ cell tumors (Masters and Koberle, 2003). The TNM staging system is the most commonly used method for determining the clinical stage of a testicular germ cell tumor (AJCC). This system takes into account the size of the primary tumor (T), involvement of pelvic and abdominal lymph nodes (N), and metastasis (M) of the cancer to distant sites (Table 2.1). Additionally, the patient's serum (S) marker levels are considered. Men with stage I disease have tumors that are confined to the testis with no evidence of metastasis. A diagnosis of stage II involves metastasis to the lymph nodes in the abdomen or pelvis, and stage III refers to cancer that has spread to more distant lymph nodes in the chest, lungs, liver or brain. High blood marker levels may also result in the diagnosis of stage III. The serum markers that are evaluated for TNM staging include lactate dehydrogenase, human chorionic gonadotropin, and alpha-fetoprotein (AJCC). The standard treatment for TGCTs is combination chemotherapy, containing bleomycin, etoposide, and cisplatin, followed by surgical resection of the

remaining tumor (Dearnaley, et al., 2001; Droz and Rivoire, 2001). Stage I TGCTs, those that are confined to the testis at the time of diagnosis, can usually be cured surgically (Masters and Koberle, 2003). However, the more differentiated teratomas are more resistant to chemotherapy (Masters and Koberle, 2003).

TGCTs are more sensitive to chemotherapy than other types of cancer. This sensitivity to cisplatin-based chemotherapy may be due to defective DNA repair mechanisms. TGCTs have been shown to be deficient in nucleotide excision repair (NER). Specifically they have decreased levels of some NER proteins, including XPA and ERCC1-XPF proteins, and adding these proteins to testis tumor extracts restored NER activity to normal levels (Koberle, et al., 1999). However, forcing the expression of XPA in human testis tumors cells did not confer an increased resistance to cisplatin (Koberle, et al., 2008), indicating that other genes, and possibly other DNA repair pathways, are likely involved in cisplatin sensitivity seen in TGCTs.

While cisplatin-based chemotherapy has proven to be exceedingly successful in the treatment of patients with testicular germ cell tumors, patients who relapse after treatment with cisplatin-based chemotherapy have a poor prognosis. Patients with metastases, especially those with late relapse disease, defined as tumor recurrence more than two years after successful treatment without the presence of a contralateral tumor, have a worse prognosis (Sharp, et al., 2008). The tumors in patients with late relapse are more resistant to chemotherapy (Sharp, et al., 2008). About ten percent of testicular cancer patients are either

unresponsive to chemotherapy or relapse after treatment (Mayer, et al., 2003). These patients are typically treated with intensive chemotherapy, surgical resection, and, possibly, radiotherapy (Dearnaley*, et al.*, 2001). This more extensive treatment is effective in about 30% of patients (Dearnaley*, et al.*, 2001).

Though the treatments for testicular cancer described above are highly effective, they are not without side-effects. The more common side effects of chemotherapy, also seen in treatment of testicular cancer, include nausea, vomiting, alopecia, fatigue, neutropenia, and sepsis. Lung toxicity is also of concern because of the use of bleomycin (Dearnaley*, et al.*, 2001). Testicular cancer most commonly afflicts men during their peak reproductive years. Therefore, infertility is an obvious area of concern in the treatment of TGCTs because chemotherapy frequently results in azoospermia. This is reversible in 70-80% of patients, and patients are usually counseled about the option of storing sperm (Dearnaley*, et al.*, 2001). However, the potential of treatment for testicular cancer resulting in infertility is still a major issue when considering quality of life for patients after treatment.

Table 2.1. TNM Staging System for Testicular Germ Cell Tumors

Primary Tumor (T) Categories:	
T0	No evidence of cancer
Tis	Intratubular germ cell neoplasia (Carcinoma in situ); not invasive
T1	Tumor spread to the membrane surrounding the testis
T2	Tumor limited to testis with vascular/lymphatic invasion
T3	Invasion of the spermatic cord with/without vascular/lymphatic invasion
T4	Invasion of the scrotum with/without vascular/lymphatic invasion
Regional Lymph Nodes (N) Categories:	
N0	No cancer cells present in lymph nodes
N1	Metastases with lymph node mass(es) $\leq$ 2cm
N2	Metastases with lymph node mass(es) > 2cm, but < 5cm
N3	Metastases with lymph node mass(es) > 5cm
Metastasis (M) Categories:	
M0	No distant metastasis
M1	Distant metastasis present
M1a	Metastasis to lungs or lymph nodes beyond the abdomen and pelvis
M1b	Metastasis to more distant organs, such as the lung and brain
Serum (S) Marker Levels Categories:	
S0	Serum marker levels are normal
S1	Serum markers are slightly elevated
S2	Moderate increase in serum markers
S3	Marker levels are very high

Testicular cancer metastasis

Approximately 70 percent of testicular germ cell tumors metastasize, with the most frequent site of metastasis being the retroperitoneal lymph nodes (Jones, et al., 2006). In non-seminomatous tumors, metastases are more likely to occur in the lungs, liver, and brain (Dearnaley, et al., 2001). TGCTs are also less commonly found in the thymus and the pineal gland; it has been questioned whether TGCTs arising in these areas are due to metastases or if they are derived from PGCs that failed to complete their migration during embryonic development (Chaganti and Houldsworth, 2000). Another hypothesis suggested for the presence of TGCTs located in the mediastinal region is the reverse migration of early stage TGCTs to these locations; this hypothesis is supported by data showing that many of the chromosomal aberrations seen in TGCTs of the gonad, including overrepresentation of chromosome 12p, were also present in TGCTs located in the mediastinal region (Chaganti, et al., 1994).

Very few studies have been published on genes involved in TGCT metastasis. SDF1 (also known as CXCL12) and CXCR4 signaling partners appear to play a role in the pathology of TGCT metastasis (Gilbert, et al., 2009). CXCR4 and SDF1 belong to the CXC subgroup of a family of chemokines and their receptors (Vandercappellen, et al., 2008); this group is further divided based on the presence of an ELR (glutamic acid-leucine-arginine) motif; SDF1 lacks the ELR motif and binds to receptors, CXCR4 and CXCR7 (Vandercappellen, et al., 2008). Chemokines and their receptors function in regulating the migration of cells to a particular destination (Zlotnik, 2008). SDF1 is angiogenic and has been

shown to be involved in cancer metastasis (Vandercappellen, et al., 2008; Zlotnik, 2008).

SDF1 and CXCR4 have been shown to be involved in PGC migration in the mouse and zebrafish (Doitsidou, et al., 2002; Molyneaux, et al., 2003). PGCs express CXCR4, the receptor for the SDF1 ligand, and migrate towards regions where SDF1 is expressed, including the genital ridge, the final destination of the PGCs (Doitsidou, et al., 2002; Molyneaux, et al., 2003). Gilbert, et. al. (2009) have presented data showing that SDF1 is expressed in adult human Sertoli cells, and CXCR4 is expressed in germ cells, with the exception of mature sperm. Furthermore, *in vitro,* SDF1 stimulated invasion and migration in a seminomatous TGCT cell line (Gilbert, et al., 2009). These data provide evidence that CXCR4/SDF1 signaling may promote TGCT metastasis.

TGFβ Superfamily

The transforming growth factor-β (TGFβ) superfamily is a cytokine signaling family made up of more than 40 members in the human genome. The most prominent members of this family include TGFβ, bone morphogenetic protein (BMPs), nodal, and activin (Massague and Gomis, 2006). The superfamily members are found in a wide variety of cell types, and are involved in diverse biological processes, including, but not limited to, differentiation, migration, cellular division, and adhesion. TGFβ and BMP have been implicated in cancer growth and metastasis (Massague and Gomis, 2006) as well as germ cell allocation and migration during development (Dudley, et al., 2007; Godin and

Wylie, 1991), making these two members of the superfamily of interest in testicular cancer research. Furthermore, these two members of the TGFβ superfamily are negatively regulated by SKI (Akiyoshi, et al., 1999; Luo, et al., 1999).

SMADs

TGFβ and BMP signal through cell surface receptors and downstream SMAD molecules (Figure 2.2). TGFβ and BMP bind to transmembrane serine/threonine receptors, causing them to form a heteromeric complex composed of type I and type II receptors. When these receptors are activated by BMP or TGFβ, they become phosphorylated, and in turn, they phosphorylate the downstream signaling molecules, the R-SMADs. SMAD1, SMAD5, and SMAD8 are phosphorylated by activated BMP receptors (Yamashita, et al., 1996), while SMAD2 and SMAD3 transmit TGFβ signals. Upon activation, the R-SMAD forms a complex with the Co-SMAD, SMAD4; this complex translocates to the nucleus and activates transcription of BMP or TGFβ target genes (Luo, 2003).

PGCs express high quantities of SMAD4, and SMAD1 is found in both PGCs and gonadal somatic cells (Pesce, et al., 2002). SMAD1 may play a role in PGC specification. SMAD1 knockout embryos die by 10.5 dpc, but, interestingly, these mice produce very few, if any, PGCs (Tremblay, et al., 2001). SMAD5 knockout is also embryonic lethal, and, these mice also display a great reduction in PGC number or none at all (Chang and Matzuk, 2001). Overexpression of SMAD4 in male transgenic mice causes a primary testicular

defect which results in Leydig cell hyperplasia and germ cell ablation, leading to infertility (Narula, et al., 2002). In light of the fact that the occurrence of testicular germ cell tumors (TGCTs) is frequently associated with abnormalities of testicular development, including cryptorchidism, testicular atrophy, and infertility (Kanto, et al., 2004), this observation further implies that the BMP/SMAD signaling pathway is important in the development of germ cells and possibly testicular cancer.

Transforming Growth Factor β

As mentioned, TGFβ has been implicated in germ cell development and migration. TGFβ inhibits murine PGC proliferation *in vitro* (Godin and Wylie, 1991; Richards, et al., 1999) and also acts as a chemo-attractant for murine PGCs *in vitro* (Godin and Wylie, 1991). TGFβ1 and 2 are produced by the Sertoli cells of the fetal and newborn rodent testis, while TGFβ2 and 3 are produced in Leydig cells, and TGFβ3 has been seen in gonocytes and spermatogonia (Loveland, et al., 2007). TGFβ3 mRNA is the most abundantly expressed TGFβ isoform in the pre-pubertal and adult rat testis, and increased expression of TGFβ3 correlates with the onset of puberty and the induction of spermatogenesis (Mullaney and Skinner, 1993).

For a cell to become malignant, it must acquire several characteristics that allow it to evade normal regulation. The hallmark of cancer is uncontrolled proliferation, which involves autocrine growth stimulation, loss of contact inhibition, and protection from antiproliferative signaling. In addition to this, the cell must acquire a mechanism for evading programmed cell death, or apoptosis.

Cancer cells also must become immortalized, meaning they are no longer limited to a certain number of cell divisions; they do not enter senescence. In later stages of tumorigenesis, cancer cells form new vasculature to support the growth of the tumor, and, finally, the cancerous cells can gain the ability to invade the surrounding tissue and metastasize to distant sites.

TGFβ plays diverging roles in the acquisition of these properties. In the early stages of cancer, TGFβ may act as a tumor suppressor by inhibiting proliferation, while TGFβ signaling has been shown to promote invasion and metastasis in cancer progression, indicating that TGFβ signaling may also promote cancer progression (Leivonen and Kahari, 2007; Zhu, et al., 2007). TGFβ is known to inhibit growth in many epithelial cell types, including keratinocytes, breast cancer cells, and colon cells (Jakowlew, 2006). However in some cell types, high concentrations of TGFβ induce growth arrest, while low concentrations actually stimulate proliferation (Jakowlew, 2006). Nevertheless, loss of response to TGFβ-induced growth inhibition is a mechanism by which many cancerous cells achieve increased proliferation. Another tumor suppressing activity in which TGFβ is involved is apoptosis. This action of TGFβ has not been as widely studied as the growth inhibitory actions, but TGFβ has been shown to induce apoptosis in various epithelial cells and has been implicated in controlling liver size through apoptosis (Jakowlew, 2006).

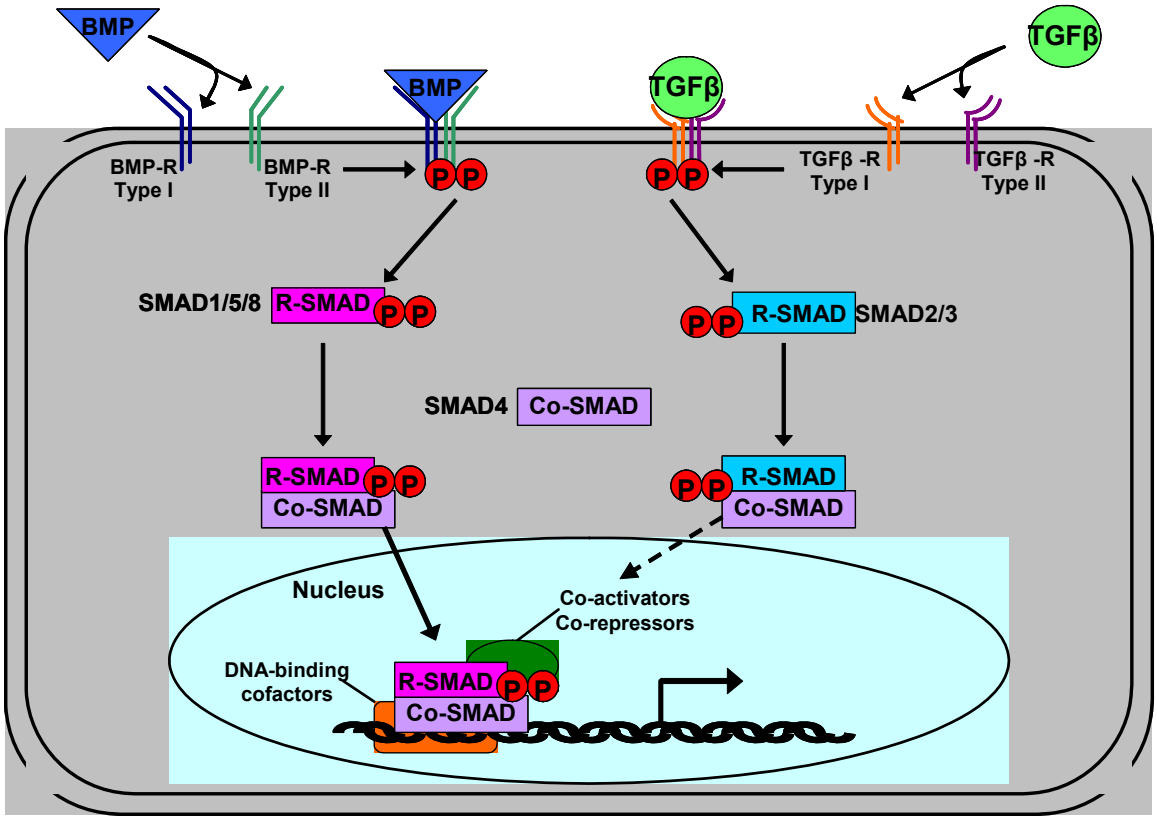

Figure 2.2. TGFβ superfamily signaling cascade. TGFβ and BMP bind to cell surface transmembrane serine/threonine receptors. This binding induces the formation of heterodimers, consisting of type-I and type-II receptors. The activated receptors induce phosphorylation of the R-SMADs, which, in turn, form a complex with the co-SMAD, SMAD4. This complex then translocates to the nucleus where it activates transcription of TGFβ or BMP-specific target genes. This transcriptional activation is mediated by co-activators and co-repressors.

In contrast to the tumor suppressing activities that TGFβ displays in the early stages of tumorigenesis, TGFβ appears to promote tumor progression by stimulating invasion and metastasis. Specifically, TGFβ is involved in epithelial to mesenchymal transition (EMT), which is a process by which cells become more migratory by losing some characteristics of epithelial cells and gaining some of the characteristics of mesenchymal cells. Increasing TGFβ levels in cell lines causes increased tumor growth and metastasis in animal models (Jakowlew, 2006). In addition, TGFβ is more highly expressed in breast and colon metastases compared to the primary tumors. This observation is also true for several other types of epithelial cancers (Jakowlew, 2006). Tang et. al. (2003) reported evidence that TGFβ switches from a tumor suppressor to a prometastatic factor during breast cancer progression. In their experiments they used four breast epithelial lines, all of which were derivatives of the "normal" immortalized MCF10A cell line, that spanned the progression from "normal" to highly malignant metastatic cancer cells. Their data supports the hypothesis that TGFβ switches from acting as a tumor suppressor in the early stages of breast cancer when it is anti-proliferative, to promoting metastasis in later stages of breast cancer. Their data suggest that the switch occurs at the transition from histologically low-grade to high-grade breast cancer (Tang, et al., 2003).

Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs) are members of the TGFβ superfamily of growth factor signaling molecules. At least 20 members of the

BMP subfamily have been identified, and they are expressed in a wide variety of tissues (Gazzerro and Canalis, 2006). The BMP subfamily was originally named based on their role in bone formation; these proteins induced ectopic bone formation when implanted under the skin of rodents (Ducy and Karsenty, 2000). However, BMPs are now known to play various roles in embryonic development, organogenesis, differentiation, and cancer. Several of the BMPs are expressed very early in embryogenesis, around the time of gastrulation, and are involved in organogenesis of various tissue types (Ducy and Karsenty, 2000). While the expression pattern of BMPs is broad, they are often localized at areas of epithelial-mesenchymal interaction (Ducy and Karsenty, 2000).

BMPs are also very important in germ cell development and spermatogenesis. Mouse studies have shown that BMP4 and BMP8b are necessary for the allocation of PGCs during embryonic development (Lawson, et al., 1999; Ying, et al., 2000; Ying, et al., 2001). Additional evidence has revealed that BMP signaling contributes to the maintenance of germ stem cells in the *Drosophila* testis (Kawase, et al., 2004). BMP4 is also expressed in the early postnatal mouse testis but is downregulated prior to puberty (Pellegrini, et al., 2003). BMP signaling promotes proliferation of germ cells during spermatogenesis (Puglisi, et al., 2004).

BMPs also appear to play conflicting roles in cancer progression. BMPs have been shown to play a role in regulating stem cell differentiation (Varga and Wrana, 2005). BMPs influence the differentiation of mesenchymal stem cells down the chondrogenic and osteogenic lineages, but BMPs have also been

shown to block neural differentiation (Ying, et al., 2003). BMP2 and BMP4 promote tumor growth through a variety of mechanisms, including angiogenesis, invasion, cell growth, and cell survival (Langenfeld, et al., 2005; Langenfeld, et al., 2006; Montesano, 2007; Raida, et al., 2005; Rothhammer, et al., 2005). About 98% of lung carcinomas display increased BMP2 expression (Langenfeld, et al., 2005). Lung cancer cells with forced expression of BMP2 had increased expression of ID1 and displayed enhanced tumor growth in the lungs following intravenous injection into athymic nude mice (Langenfeld, et al., 2006). This BMP-induced stimulation of tumor growth is associated with activation of SMAD1/5 (Langenfeld, et al., 2006). BMP6 has been implicated in prostate cancer development and metastasis to bone (Darby, et al., 2008). In PC3M prostate cancer cells, treatment with BMP6 did not affect proliferation; however, it did result in increased expression of matrix metalloproteinases, MMP-1 and MMP-9, and also caused increased migration and invasion (Darby, et al., 2008). However, BMP9 and BMP10 expression were found to be low or absent in prostate tumors, and forced expression of BMP9 or -10 in PC3 prostate cancer cells resulted in a decrease in growth, cell matrix adhesion, invasion, and migration (Ye, et al., 2009). BMP9 and BMP10 also induced apoptosis in these cells via a SMAD-independent pathway (Ye, et al., 2008; Ye, et al., 2009). This suggests that BMP6 has tumor promoting activities, while BMPs 9 and 10 may act as tumor suppressors in prostate cancer. BMP2 promotes motility and invasion in MDA-231-D breast cancer cells and appears to be involved in metastasis to bone, but also did not affect their proliferation (Katsuno, et al.,

2008). BMP4 and BMP7 are increased in melanoma cells compared to normal melanocytes, and they appear to upregulate the expression of MMP-1, -2, -3, and -9, suggesting that BMPs may also promote metastasis in melanoma (Rothhammer, et al., 2008; Rothhammer, et al., 2005). In colorectal cancer, expression of BMP7 is correlated with liver metastasis and poor prognosis (Motoyama, et al., 2008). On the other hand, BMP3 expression was found to be down-regulated in 50 out of 56 primary colorectal tumors examined. Bisulfite sequencing revealed hypermethylation of the BMP3 promoter in both human colorectal tumors and HT29 colorectal carcinoma cells. Restoration of BMP3 expression in colorectal cancer cell lines resulted in growth suppression (Loh, et al., 2008). Upon review of this collection of data, it is evident that BMPs play diverse roles in cancer progression. Again, the role of BMP in tumorigenesis may be dependent on the cell type and stage of cancer progression.

Inhibitors of TGFβ and BMP Signaling

The activity of TGFβ and BMP is regulated at multiple levels to maintain coordinated cellular functions, including regulation of ligand activity, extracellular antagonists, dominant negative pseudo-receptors, and blocking of downstream signaling by SMADs (Gazzerro and Canalis, 2006; Massague and Chen, 2000). TGFβ is secreted in a latent form and must be cleaved to be activated and recognized by cell surface receptors (Massague and Chen, 2000). Several different molecules are involved in the activation of latent TGFβ. This is a

complicated process that is not completely understood, but thrombospondin 1 appears to be an important participator (Massague and Chen, 2000).

Extracellular antagonists prevent signaling by binding to TGFβ or BMP and interfering with their interaction with cell surface receptors. There are four groups of extracellular BMP antagonists: noggin, the chordin family, twisted gastrulation, and the Dan family (Gazzerro and Canalis, 2006). Noggin is secreted as a glycosylated protein and binds to BMP2, 4, 5, 6, and 7 with various degrees of affinity and prevents interaction with the BMP receptors. Noggin does not interact with TGFβ (Gazzerro and Canalis, 2006). Chordin is also a secreted glycoprotein and interacts specifically with BMP2, 4, and 7 (Gazzerro and Canalis, 2006). Twisted gastrulation is another secreted glycoprotein that interacts with BMP2 and 4; it can act as both a BMP antagonist and a BMP agonist (Gazzerro and Canalis, 2006). The Dan family is a family of glycoprotiens, consisting of at least nine members, that is capable of binding BMPs. Most members of the Dan family are expressed during embryogenesis, but two members, gremlin and sclerostin, mediate BMP activity in the adult skeleton (Gazzerro and Canalis, 2006).

BAMBI (BMP and activin membrane-bound inhibitor) is a pseudo-receptor with extracellular domains similar to TGFβ Type I receptors but lacks the intracellular kinase domain (Onichtchouk, et al., 1999). BAMBI can inhibit signaling by forming heterodimers with TGFβ Type I receptors and inhibiting their activation (Massague and Chen, 2000). BAMBI is capable of inhibiting both

TGFβ and BMP signals. Transcription of BAMBI is upregulated by BMP, TGFβ, and Wnt (Gazzerro and Canalis, 2006).

An additional way to regulate TGFβ superfamily signaling is by controlling the activity of the downstream signaling molecules, the SMADs. There are several mechanisms that regulate SMAD signaling. SMURF1 (SMAD ubiquitination regulator factor 1) is an E3 ubiquitin ligase that interacts with SMAD1 and 5 but not SMAD2 or 3 (Massague and Chen, 2000; Zhu, et al., 1999). Interaction with SMURF1 targets SMAD1 and 5 for ubiquitin-mediated degradation, inhibiting BMP signaling (Massague and Chen 2000). BMP and TGFβ signaling is also mediated by a group of inhibitory SMADS (I-SMADs), SMAD6 and 7. The I-SMADs bind to Type I receptors and inhibit the phosphorylation of R-SMADs. SMAD6 more specifically inhibits BMP signaling, while SMAD7 inhibits both BMP and TGFβ signaling (Gazzerro and Canalis, 2006). Finally, co-repressors function to inhibit TGFβ superfamily signaling by binding to SMADs and preventing the transcription of target genes; these include TGIF, p300/CBP, and the SKI family (Gazzerro and Canalis, 2006; Massague and Wotton, 2000). TGIF (transforming growth factor interacting factor) interacts with SMAD2 and 3, and prevents TGFβ induced gene transcription by recruiting histone deacetylases to SMAD-responsive DNA elements (Massague and Wotton, 2000). In addition, LEFTY1 represses TGFβ and BMP signaling by inhibiting the receptor-induced phosphorylation of the R-SMADs (Ulloa and Tabibzadeh, 2001). The SKI family, including SKI and SNO, are also potent

inhibitors of TGFβ and BMP signaling that function as transcriptional co-repressors of SMAD proteins.

SKI

SKI was originally identified as a viral oncogene, *v-ski,* which is a 49 kDa polypeptide capable of inducing transformation in avian fibroblasts (Colmenares and Stavnezer, 1989; Stavnezer, et al., 1981). Interestingly, *v-ski* is also capable of inducing myogenic differentiation in quail embryonic fibroblasts (Colmenares and Stavnezer, 1989). *c-Ski,* the cellular homologue of *v-ski,* consists of eight coding exons, five of which are seen in *v-ski* (Stavnezer, et al., 1981). SKI functions as a transcriptional regulator; the first exon of *SKI* contains motifs that are common to transcription factors, including a proline-rich region, a nuclear localization sequence, cysteine-histidine-rich metal-binding motifs, and paired amphipathic alpha-helices (Colmenares and Stavnezer, 1990). This region of SKI is highly conserved in SNO (SKI-related novel), another member of the SKI family (Nomura, et al., 1989).

SKI and SNO can function as negative regulators of the TGFβ superfamily, through interaction with SMADs, the downstream signaling molecules of the TGFβ superfamily (Akiyoshi, et al., 1999; Luo, et al., 1999). There are several mechanisms by which the SKI family functions to repress TGFβ superfamily signaling. SKI and SNO can repress TGFβ superfamily signaling by binding to SMAD complexes and recruiting other repressors, including the NCoR/Sin3/HDAC complex (Luo, et al., 1999; Massague and Chen, 2000).

Additionally, SKI and SNO can block the binding of the co-activator p300/CBP to the TGFβ-mediated R-SMADs (Akiyoshi, et al., 1999; Luo, et al., 1999). SKI can also bind to MeCP2, a methyl-CpG-binding protein that functions in transcriptional repression (Kokura, et al., 2001). Furthermore, interaction between SKI or SNO and SMAD4 can prevent the formation of functional complexes between SMAD4 and the R-SMADs (Luo, 2003). SKI, but not SNO, has been shown to interact directly with the SMAD1/5-SMAD4 complexes to repress transcriptional activation of BMP target genes (Takeda, et al., 2004; Wang, et al., 2000).

The role of SKI in cancer is complex and appears to be tissue and context-specific. As previously mentioned, SKI was classified as a proto-oncogene based on its ability to induce transformation in avian fibroblasts; yet SKI can also simultaneously induce muscle differentiation in quail embryo fibroblasts (Colmenares and Stavnezer, 1989; Stavnezer, et al., 1981). In accordance with the transforming capability of *SKI*, some tumors, including melanoma, esophageal cancer, colon cancer, and pancreatic cancer, express high levels of *SKI* (Buess, et al., 2004; Fukuchi, et al., 2004; Heider, et al., 2007; Reed, et al., 2005). SKI has also been considered pro-oncogenic because its upregulation in some cancers, such as melanoma and prostate, results in a loss of response to TGFβ-induced growth inhibition (Medrano, 2003; Xu, et al., 2000). SKI has been shown to be upregulated in pancreatic cancer, and siRNA-knockdown of *SKI* in a PANC-1 pancreatic cancer cells restored TGFβ-induced growth inhibition (Heider, et al., 2007).

In contrast, loss of *SKI* function has been linked to tumorigenesis in mice heterozygous for a null mutation in the *SKI* gene (Shinagawa, et al., 2001), suggesting *SKI* may also act as a tumor suppressor. Mice lacking *SKI* display defects in neural and muscle development (Berk, et al., 1997; Colmenares, et al., 2002), further indicating the importance of SKI in differentiation. Recently, it has been reported that loss of *SKI* function promotes cancer progression by allowing TGFβ to promote metastasis in lung and breast cancer cells (Le Scolan, et al., 2008). In these studies, decreased SKI expression did not affect the proliferation of the lung and breast cancer cells (Le Scolan et al., 2008; Zhu et al., 2008). Thus, the role of SKI in cancer is not yet fully defined and appears to be dependent upon the cell type and possibly the stage of cancer.

Hypothesis

The rationale for the research presented in this text is that determining the role of SKI in testicular cancer will provide a better understanding of testicular cancer progression. With a better understanding of the mechanisms behind testicular cancer progression, scientists will be able to design treatments that are aimed at the specific causes of testicular cancer, and these treatments may allow patients a better quality of life by potentially causing less severe side effects, such as preventing loss of fertility. It is the hypothesis of this research that decreased *SKI* expression promotes testicular cancer progression by allowing unregulated TGFβ superfamily signaling, conferring an increased ability of testicular cancer cells to invade and metastasize.

CHAPTER III

RESULTS

SKI expression is decreased in TGCTs compared to normal testis

Although *SKI* has been reported to have oncogenic activity in various cancers (Buess, et al., 2004; Reed, et al., 2005), *SKI*-deficient heterozygous mice have an increased susceptibility to tumorigenesis (Shinagawa, et al., 2001), indicating that SKI may have tumor suppressor activity. RNA isolated from both normal human testis tissue and human tumor samples, including seminoma, yolk sac, and mixed germ cell tumors was analyzed for *SKI, SNO,* and *CCND2* mRNA expression using quantitative real time PCR. *SKI* mRNA expression was decreased in all types of testicular tumors compared to normal testis samples (Figure 3.1 A). The expression of *SNO* (Figure 3.1 B), another member of the *SKI* family, was not significantly different in tumor and normal testis samples. *CCND2* was used as a positive control, as its expression is known to increase in several types of cancer, and has specifically been shown to be increased in testicular cancer (Bartkova, et al., 1999). As expected, *CCND2* expression was elevated in testicular tumors compared to normal tissue (Figure 3.1 C).

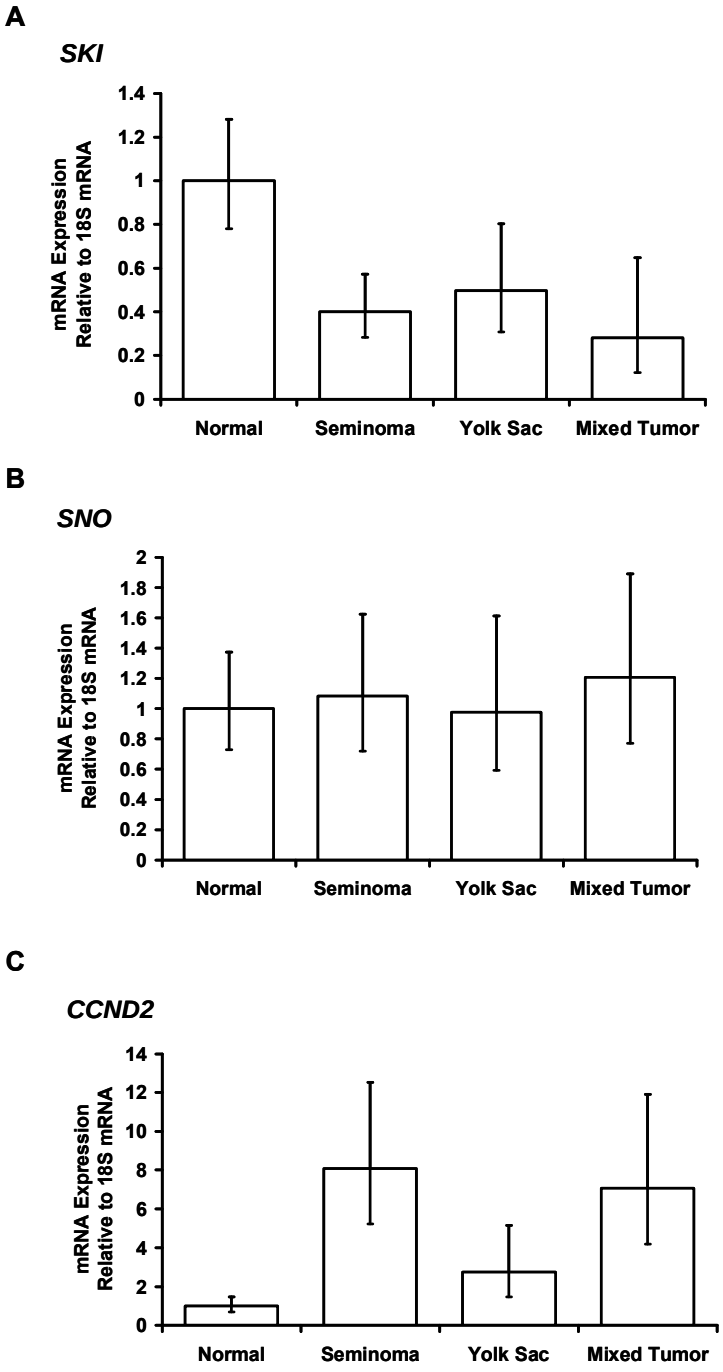

Figure 3.1. Decreased *SKI* expression in human testicular germ cell tumors. Real time PCR analysis comparing mRNA levels in human testicular germ cell tumors, including seminoma (n = 16), yolk sac tumors (n = 11), and mixed tumors (n = 7), to normal testicular tissue samples (n = 20). (A) *SKI* mRNA expression in germ cell tumors compared to normal testis. (B) *SNO,* a member of the SKI family, was also measured. (C) *CCND2* is used as a control. mRNA levels were normalized to 18S RNA. Data were analyzed using $2^{-\Delta\Delta Ct}$ method, and error bars indicate the 95% confidence interval.

SKI expression varies among different human cell types

SKI expression has previously been shown to vary among different cancerous cell types (Buess, et al., 2004; Fukuchi, et al., 2004; Heider, et al., 2007; Reed, et al., 2005; Zhu, et al., 2007), suggesting that SKI may play diverging roles in the development and progression of cancer dependent on cell type and environment. In order to determine the role that SKI plays in testicular cancer, a human embryonal carcinoma cell line, NCCIT, was used. To determine the relative level of SKI protein expression in NCCIT testicular cancer cells, we compared SKI expression in NCCIT cells to various other human cancerous and normal cells (Figure 3.2). These cell lines included human hepatocelluar carcinoma (HEPG2), human normal mammary cells (HMEC), human breast cancer cells (MCF7 and T47D), human lung adenocarcinoma (A549), human melanoma cells (SbCl2, WM1366, and WM9), human colorectal carcinoma (HCT116), human neuroblastoma (SK-N-AS and SK-N-SH), and human embryonal carcinoma (NT2/D1 and NCCIT).

In accordance with previously published data, we found that SKI is expressed at varying levels in different cancerous and normal human cell types. It has previously been reported that SKI expression increases with cancer progression in human melanoma (Reed, et al., 2001). In support of this, we found that SKI expression is higher in WM1366 human melanoma cells, which are categorized in the vertical growth phase, compared to expression in SbCl2 human melanoma cells, which are primary melanoma cells in the radial growth phase. SKI expression is higher still in WM9 human melanoma cells, which are

categorized as metastatic melanoma (Figure 3.2). SKI expression was relatively low in A549 human lung carcinoma cells (Figure 3.2), which was also previously observed (Zhu, et al., 2007). Increased expression of SKI has previously been reported in human colorectal carcinoma (Buess, et al., 2004), and, in accordance with this, SKI expression was found to be high in HCT116 human colorectal carcinoma cells (Figure 3.2). SKI was expressed at similar levels in HMEC human normal mammary cells and MCF7 human mammary adenocarcinoma, derived from a pleural effusion, but SKI expression was lower in T47D human ductal carcinoma, also derived from a pleural effusion. In comparison to these cell lines, SKI was expressed at an intermediate level in NCCIT and NT2/D1 human embryonal carcinoma cells (Figure 3.2), both of which were derived from mediastinal metastases.

Manipulation of *SKI* expression in NCCIT cells

Our data shows that *SKI* expression is reduced in human testicular germ cell tumors, therefore, we wanted to compare the effects of decreased *SKI* and forced *SKI* expression using an *in vitro* model of testicular cancer. For these experiments we used the human testicular cancer cell line, NCCIT, which is classified as embryonal carcinoma (or non-seminoma). Since some *SKI* expression was detected in NCCIT cells via western blotting, siRNA was used to knockdown the levels of *SKI* to determine the effects of decreased *SKI*. Three siRNAs against different regions of the *SKI* gene (designated siSKI-1, siSKI-2, and siSKI-3) were used individually to knockdown *SKI* levels. A non-specific

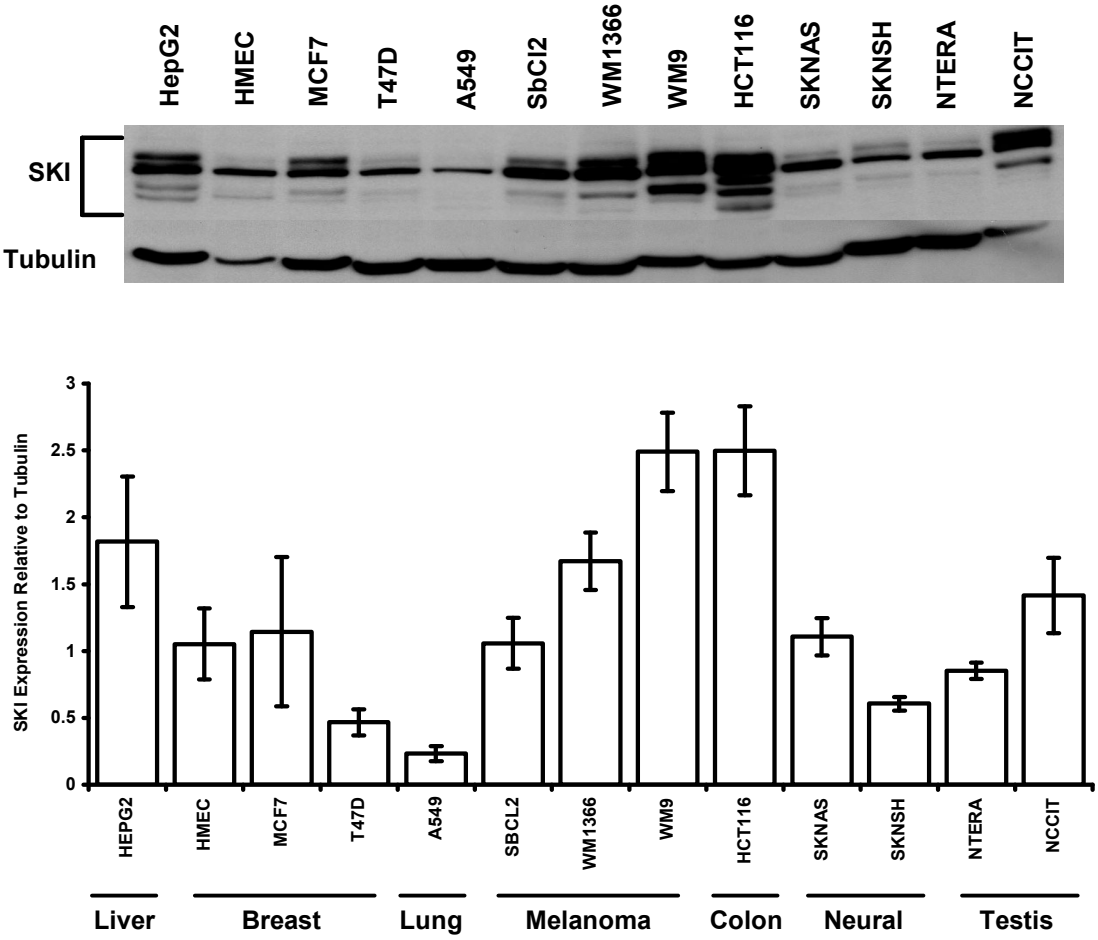

Figure 3.2. SKI Expression in Human Cell Lines. *SKI* protein levels were analyzed by western blotting (upper panel) in various human normal and cancerous cell lines. α-tubulin was used as a loading control. Protein levels were quantified by densitometry (lower panel) and expressed relative to α-tubulin. Data represents three combined trials; error bars indicate SEM.

siRNA was used as a control, and cells treated with the transfection reagent alone (mock transfection) were used to ensure that there were no adverse consequences from the transfection procedure. Cells were treated with siRNA over a 72 hr time period. To assess SKI knockdown, SKI mRNA and protein levels were measured using real-time PCR and western blotting at 24, 48, and 72 hrs. In NCCIT cells, siRNAs siSKI-2 and siSKI-3 were able to knockdown *SKI* mRNA and protein levels by 45-60% 48 h after transfection, while siSKI-1 siRNA was less effective in knocking down *SKI* expression in NCCIT cells (Figure 3.3 A and B).

To assess the effects of forced *SKI* expression on NCCIT cells, the cells were transfected with a *SKI* expression vector, and the empty vector was used as a control. SKI protein levels were assessed at 24, 48, and 72 hrs. In NCCIT cells transfected with the SKI expression vector, SKI protein levels were increased by 43% at 48 hrs (Figure 3.3 C).

Effects of SKI on migration in NCCIT cells

NCCIT cells were plated on Matrigel-coated plates and were treated with one of the three *SKI* siRNAs or the non-specific control siRNA. A scrape was made in the cell monolayer 48 hrs after siRNA transfection, and the cells were analyzed for percent closure of the scrape after a 20 hr incubation period. NCCIT cells treated with siSKI-2 and siSKI-3 siRNA accomplished a 39.1% ($p < 0.05$) and 40.6% ($p < 0.05$) increase, respectively, in percent closure of the scraped area compared to NCCIT cells treated with the non-specific control

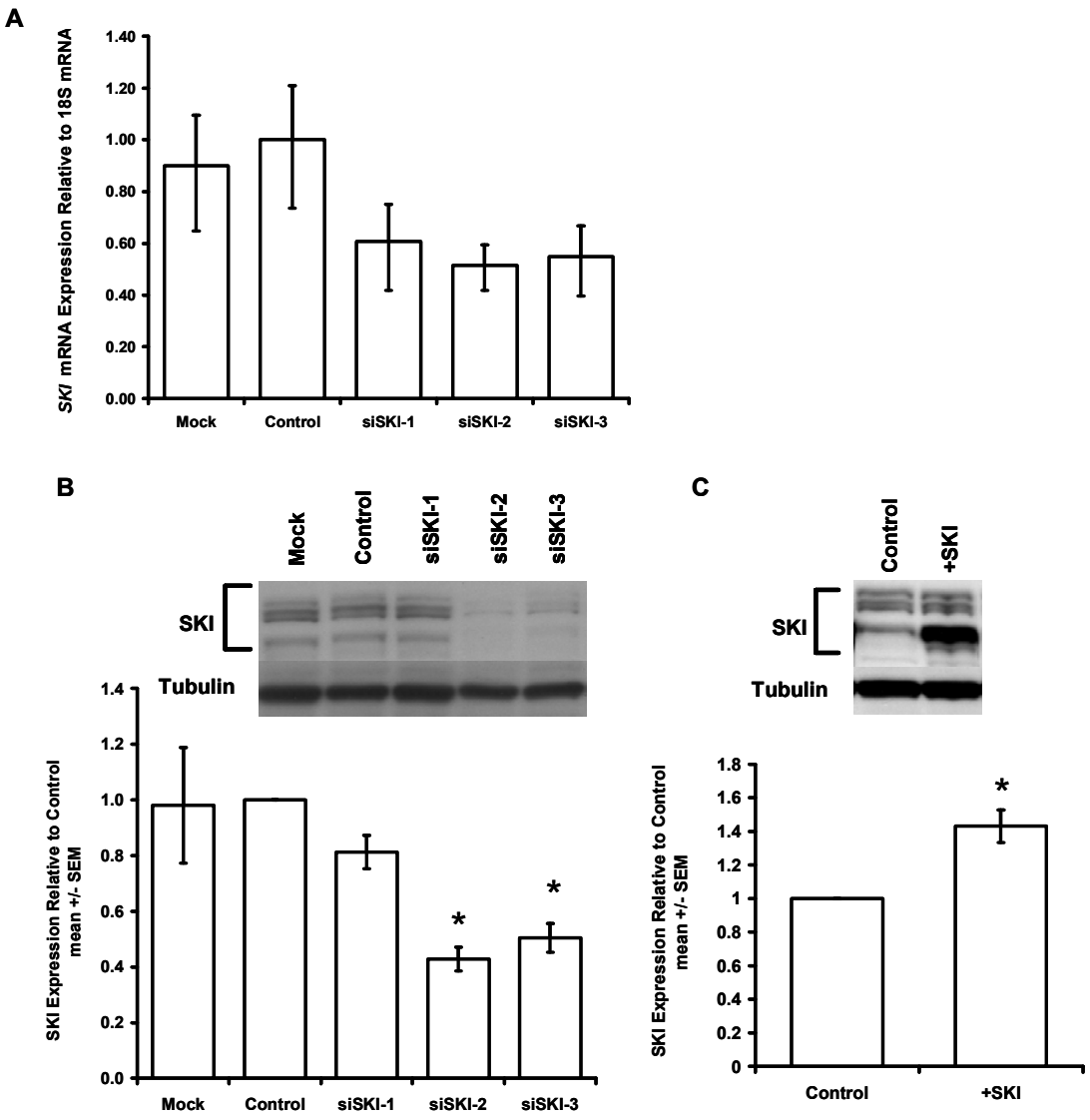

Figure 3.3. siRNA Knockdown and Forced Expression of *SKI* in NCCIT Cells. *SKI* mRNA and protein levels were measured at 48 hrs after transfection with one of three *SKI* siRNAs, a control siRNA, a *SKI* expression vector, or the control vector. (A) siRNA knockdown of *SKI* confirmed by real time PCR. Data were analyzed using $2^{-\Delta\Delta Ct}$ method; data represents three individual trials; error bars indicate the 95% confidence intervals. (B) siRNA knockdown of SKI in NCCIT cells confirmed by western blotting; α-tubulin was used as a loading control. Protein levels were quantified by densitometry and are expressed relative to α-tubulin. Data represents three combined trials; error bars indicate SEM; * indicates a significant difference with $p < 0.05$ as assessed by One-way ANOVA with Bonferroni adjustment. Mock – mock transfection; Control – non-specific siRNA; siSKI-1, siSKI-2, and siSKI-3 represent individual siRNAs. (C) Forced SKI expression in NCCIT cells confirmed by western blotting; α-tubulin was used as a loading control. Protein levels were quantified by densitometry and are expressed relative to α-tubulin. Data represents three combined trials; error bars indicate SEM; * indicates a significant difference with $p < 0.05$, as assessed by T-test.

siRNA (Figure 3.4, panel A), while NCCIT cells treated with siSKI-1 siRNA achieved a 19.7% increase ($p < 0.05$) in percent closure of the scraped area compared to the control siRNA. The greater increase in migration seen in cells treated with siSKI-2 and siSKI-3 compared to that seen with siSKI-1 correlates with the data showing that siSKI-2 and siSKI-3 were more efficient than siSKI-1 in knocking down the level of *SKI* mRNA and protein.

If decreased *SKI* levels play an important role in promoting the metastasis of testicular cancer cells, one would expect that forcing the expression of *SKI* in these cells would have the opposite effect, inhibiting the migration of these cells. To test this hypothesis, we forced expression of *SKI* in NCCIT cells by transfecting them with a vector containing the *SKI* gene and performed cell scrape assays. Cells transfected with the empty vector were used as a control. We found that forcing expression of *SKI* in NCCIT cells does, indeed, significantly decrease the percent closure of the scraped area by approximately 20% relative to the control-transfected cells (Figure 3.4, panel B). Therefore, the level of *SKI* expression does appear to directly affect the ability of NCCIT cells to migrate.

Effects of SKI on invasion in NCCIT cells

To confirm the observations seen in the cell scrape assays, Matrigel invasion chambers were used to further assess the invasive potential of NCCIT cells in response to the level of *SKI* expression. NCCIT cells treated with siSKI-2 and siSKI-3 siRNA displayed a 75% and 71% increase ($p < 0.05$), respectively, in

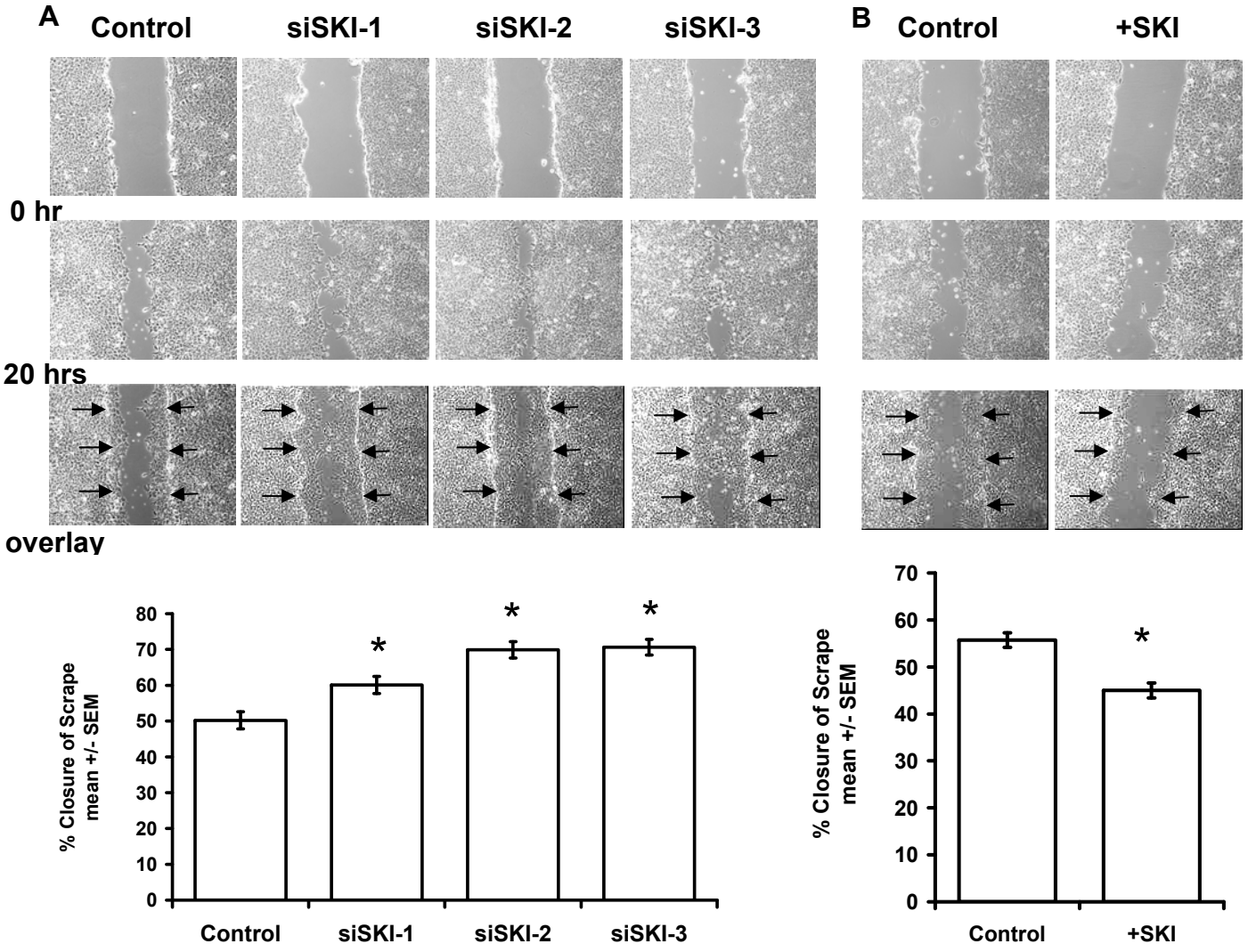
A
Control
siSKI-1
siSKI-2
siSKI-3
B
Control
+SKI
0 hr
20 hrs
overlay
% Closure of Scrape
mean +/- SEM
80
70
60
50
40
30
20
10
0
*
*
*
Control
siSKI-1
siSKI-2
siSKI-3
% Closure of Scrape
mean +/- SEM
70
60
50
40
30
20
10
0
*
Control
+SKI

Figure 3.4. Cell Scrape Migration Assay on NCCIT Cells. (A) Phase contrast images of siRNA-treated NCCIT cells at 0 hr (first row) and 20 hrs (second row) after scratch and an overlay of the 0 and 20 hr images (third row). The arrows in the overlay figures point out the edge of the original scrape at the 0 hr time point. Bar graph represents % closure of scratch. Data represents three trials; error bars indicate SEM; *indicates a significant difference compared to Control siRNA with $p < 0.05$, as assed by One-way ANOVA with Bonferroni adjustment. (B) Phase contrast images of NCCIT cells treated with SKI expression vector or control vector at 0 hr (first row) and 20 hrs (second row) after scratch and an overlay of the 0 and 20 hr images (third row). Bar graph represents % closure of scratch; data representative of three trials; error bars indicate SEM; *indicates a significant difference compared to Control vector with $p < 0.05$, as assessed by T-test.

the number of invading cells compared to NCCIT cells treated with the control siRNA (Figure 3.5A). NCCIT cells treated with the siSKI-1 siRNA did not display a significant change in the number of invading cells, which, again, correlates with the level of knockdown seen in the cells treated with siSKI-1 siRNA. Similar to data collected in the cell scrape assays, forcing the expression of *SKI* in NCCIT cells resulted in a 44% decrease in the number of invading cells compared to the cells treated with the control vector (Figure 3.5B).

SKI expression does not affect the growth rate of NCCIT cells

SKI expression has previously been reported to affect growth in some cancers, including pancreatic cancer (Heider, et al., 2007). In order to determine if SKI expression affects cellular proliferation in NCCIT cells, the growth rate of NCCIT cells treated with *SKI* siRNA was determined via trypan blue dye exclusion cell counts 24, 48, and 72 hours after transfection with siRNA, the *SKI* expression vector, or the control vector (Figure 3.6A, B). There was no significant change in the rate of growth in either the NCCIT cells treated with *SKI* siRNA compared to cells treated with the control siRNA or NCCIT cells treated with the *SKI* expression vector compared to cells treated with the control vector (Figure 3.6A, B). In addition, cell counts were performed after each cell scrape assay (data not shown), and these cell counts also revealed no difference in growth among cells with different levels of SKI expression. These data indicate that SKI expression does not affect proliferation in NCCIT cells.

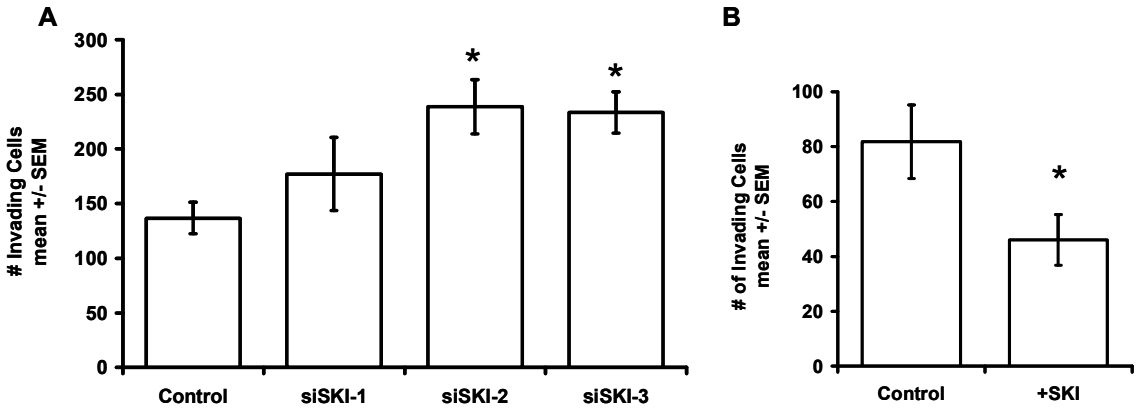

Figure 3.5. Matrigel Invasion Assay on NCCIT cells. (A) Number of siRNA-treated NCCIT cells migrating through the Matrigel invasion chamber, error bars represent SEM, *indicates a significant difference compared to Control siRNA with $p < 0.05$. (B) Number of NCCIT cells treated with either the SKI expression vector or the control vector migrating through the Matrigel invasion chamber, error bars represent SEM, *indicates a significant difference compared to Control vector with $p < 0.05$ as assessed by One-way ANOVA with Bonferroni adjustment.

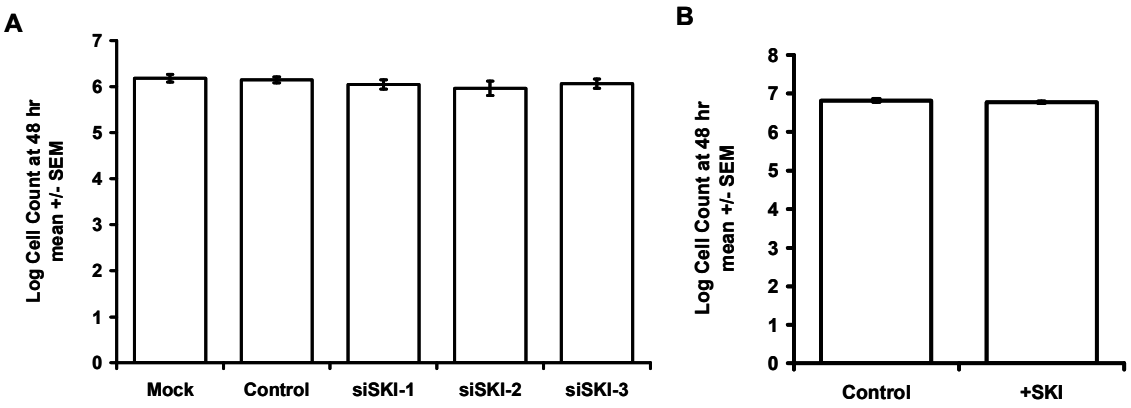

Figure 3.6. Cell Growth Measured by Trypan Blue Cell Counts. Cells were collected at 48hrs after transfection and were counted using trypan blue dye exclusion. Data are reported as the log of the total cell number; error bars represent SEM. (A) NCCIT cells receiving mock transfection (Mock), treatment with the non-specific control siRNA (Control), or treatment with one of the three SKI siRNAs (siSKI-1, siSKI-2, or siSKI-3). (B) NCCIT cells transfected with the SKI expression vector (+SKI) or the control vector (Control). There were no significant differences among the treatment groups as assessed by One-way ANOVA with Bonferroni adjustment.

Effect of TGFβ on cell migration in SKI-knockdown cells

SKI is a negative regulator of the TGFβ superfamily signaling molecules, including TGFβ and BMP. We have shown that SKI expression is decreased in human testicular cancer samples compared to normal human testis samples. We have also shown that transiently reducing the levels of SKI in NCCIT human testicular cancer cells enhances their ability to migrate and invade in vitro. Therefore, we sought to determine if decreased SKI expression in NCCIT testicular cancer cells affects migration by allowing increased signaling from TGFβ superfamily signaling molecules.

To determine this, we performed stable transfections with a SKI shRNA vector to generate stable SKI-knockdown NCCIT cells (Figure 3.7). These cells were exposed to TGFβ and/or TGFβ blocking antibody, and their ability to migrate was assessed via cell scrape assays. As we previously observed with transient SKI knockdown, cells with decreased SKI expression had significantly increased migration compared to cells treated with the control shRNA vector (Figure 3.8). If reduced SKI expression enhanced migration by allowing increased TGFβ signaling, one would expect that adding TGFβ would further enhance the migration of shSKI NCCIT cells, but adding TGFβ to shNC NCCIT cells would not necessarily have an effect. However, treating either shSKI or shNC NCCIT cells with TGFβ does not appear to affect their migration, nor does treating shSKI or shNC NCCIT cells with TGFβ blocking antibody have an effect on their migration (Figure 3.8).

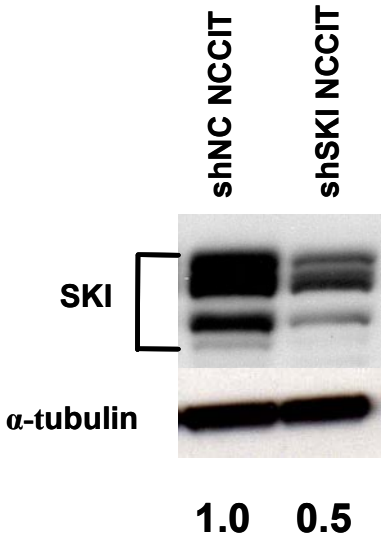

Figure 3.7. SKI knockdown in shSKI NCCIT cells. Representative western blot showing the expression of SKI in shNC NCCIT cells and shSKI NCCIT cells. α-tubulin was used as a loading control. Numbers indicate the level of SKI expression relative to the level in shNC NCCIT cells.

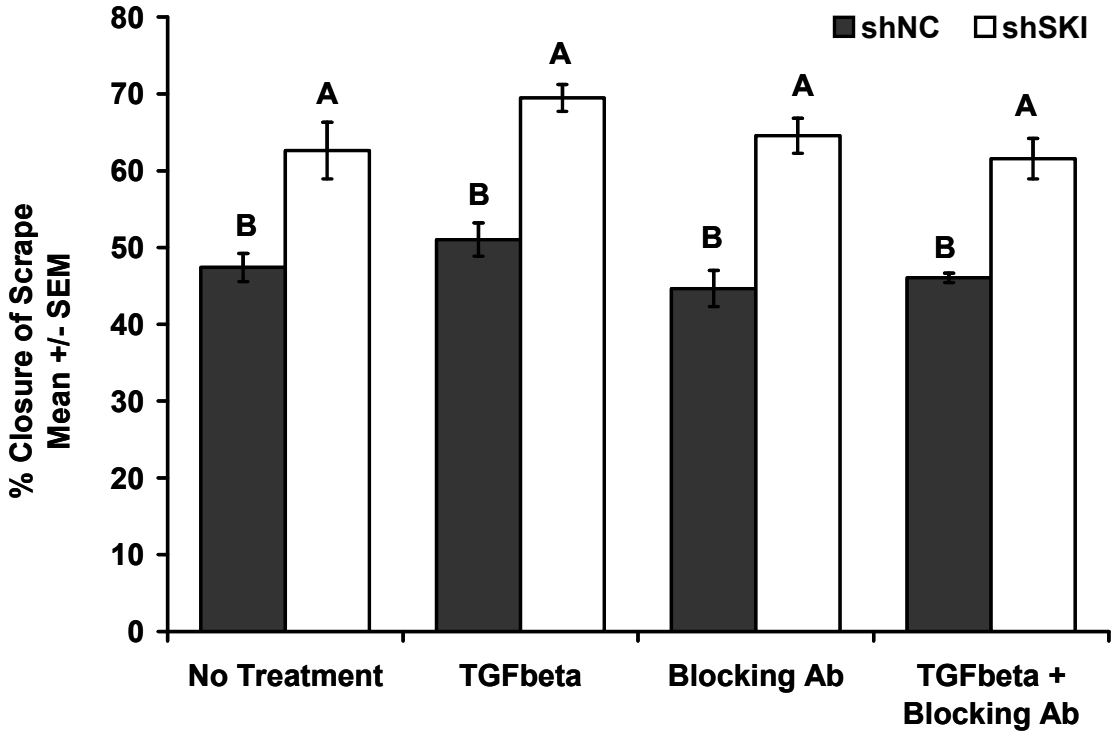

Figure 3.8. Effect of TGFβ on the migration of shNC and shSKI NCCIT cells. Bar graph representing the % closure in cell scrape migration assays using shNC NCCIT cells (black bars) and shSKI NCCIT cells (white bars) ± 0.1nM TGFβ ± 200nM TGFβ blocking antibody. Data represents three trials; error bars indicate SEM; Groups labeled **A** and **B** are significantly different from each other with $p < 0.05$ as assessed by One-way ANOVA with Bonferroni adjustment.

Since addition of TGFβ to SKI-knockdown NCCITs does not appear to affect the migration of these cells, one would question whether the TGFβ pathway is active in these cells. To explore this question, we tested TGFβ-induced phosphorylation of SMAD2 in shNC and shSKI NCCIT cells. SMAD2 was readily detected by western blotting and was expressed at a constant level regardless of the addition of TGFβ. However, p-SMAD2 was only faintly detected by western blotting, and addition of 0.1nM TGFβ for 2 hours to either shNC or shSKI NCCIT cells resulted in a slight induction of SMAD2 phosphorylation (1.6 fold in shNC NCCIT cells and 1.8 fold in shSKI NCCIT cells) (Figure 3.9).

Effect of BMP on cell migration in SKI-knockdown cells

SKI is known to repress both TGFβ and BMP signaling by interacting with SMAD proteins (Akiyoshi, et al., 1999; Luo, et al., 1999). SKI can interact with both TGFβ-specific SMADs, SMADs 2 and 3, and BMP-specific SMADs, SMADs 1, 5, and 8, as well as the co-SMAD, SMAD4. Hence, we treated shSKI and shNC NCCIT cells with BMP and/or noggin, a BMP inhibitor, to determine if increased BMP signaling affects migration in SKI-deficient testicular cancer cells. This was also assessed via cell scrape migration assays. ShSKI NCCIT cells treated with BMP had a significant increase in percent closure of the cell scrape compared to both untreated shSKI cells and shNC cells, untreated or treated with BMP (Figure 3.10). Treating shNC NCCIT cells with BMP did not affect their migration (Figure 3.10). In addition, treating shSKI cells with noggin, a BMP

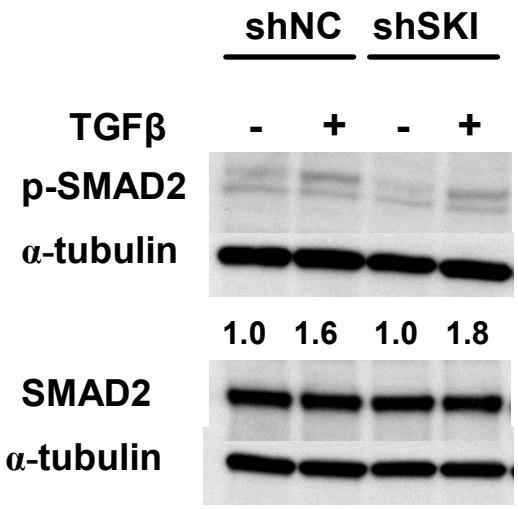

Figure 3.9. Induction of SMAD2 phosphorylation by TGFβ in shNCCIT cells. Upper panel is a representative western blot showing the expression of phospho-SMAD2 in shNCCIT cells ± TGFβ treatment (0.1nM) for 2 hrs. α-tubulin was used as a loading control. Numbers indicate the level of p-SMAD2 relative to the level in shNC NCCIT cells – TGFβ. Lower panel is a representative western blot showing the expression of SMAD2 in shNCCCIT cells ± TGFβ treatment. α-tubulin was used as a loading control.

inhibitor, reduced their level of migration back to the level of untreated shNC NCCIT cells (Figure 3.10). Treating shNC NCCIT cells with noggin also slightly reduced their migration. This indicates that in NCCIT cells, reduced SKI expression enhances BMP signaling, which promotes migration.

To demonstrate the activity of the BMP pathway in NCCIT cells, shNC or shSKI NCCIT cells were treated with BMP, noggin, or both and assessed for phosphorylation of SMAD1/5/8. Treating shNC or shSKI NCCIT cells with BMP induced the phosphorylation of SMAD1/5/8 (Figure 3.11). This effect was inhibited in the presence of noggin. As expected, SMAD1/5/8 phosphorylation was induced by BMP regardless of SKI expression. P-SMAD1/5/8 expression was approximately 2.3 and 2.5 fold higher in shNC NCCIT and shSKI NCCIT cells, respectively, treated with BMP compared to untreated cells. Treatment with BMP, noggin, or both did not affect the level of SMAD5 expression (Figure 3.11 lower panel). As expected, the presence of SKI did not affect SMAD phosphorylation because SKI does not act at the level of inhibiting SMAD phosphorylation, but, rather, prevents SMAD-induced gene transcription.

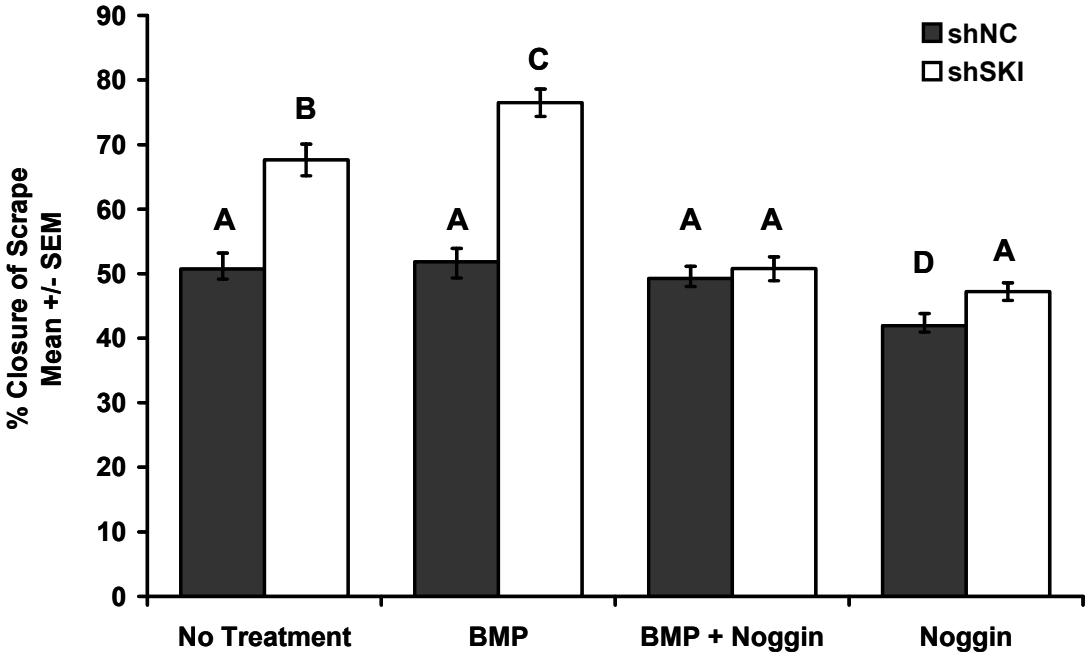

Figure 3.10. Effect of BMP on migration of shNC and shSKI NCCIT cells. Bar graph representing the percent closure in cell scrape migration assays using shNC NCCIT cells (black bars) and shSKI NCCIT cells (white bars) ± 10ng/mL BMP, ± 0.1µg/mL noggin. Data represents the mean of three trials; error bars indicate SEM; Groups labeled **A, B, C** and **D** are each significantly different from each other with $p < 0.05$ as assessed by One-way ANOVA with Bonferroni adjustment.

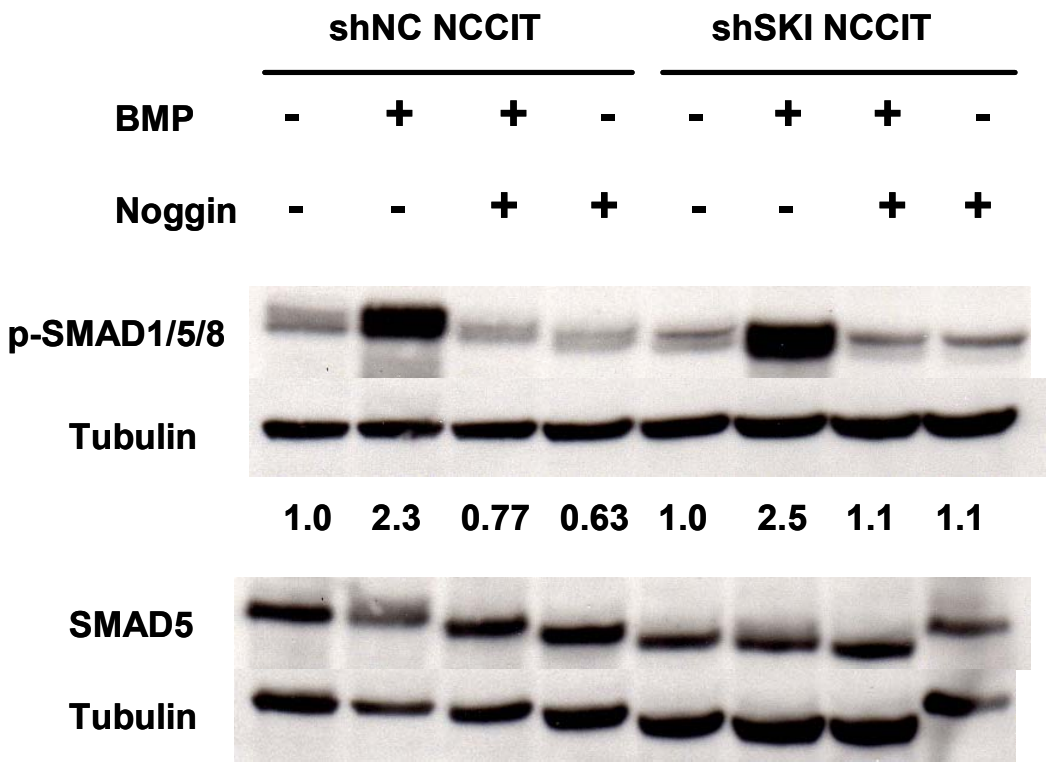

Figure 3.11. Phosphorylation of SMAD1/5/8 by BMP4. The upper panel is a representative western blot showing the expression of phospho-SMAD1/5/8 in shNCCIT cells ± 10ng/mL BMP, ± 0.1µg/mL noggin treatment for 2 hrs. Numbers indicate the level of p-SMAD1/5/8 relative to p-SMAD1/5/8 levels in shNC NCCIT cells + BMP. The lower panel shows SMAD5 expression in shNCCIT cells ± 10ng/mL BMP, ± 0.1µg/mL noggin treatment for 2 hrs. α-tubulin was used as a loading control for both western blots..

SKI Expression in shNC and shSKI NCCIT cells treated with BMP and/or Noggin

Real time PCR analysis was used to test the level of *SKI* knockdown in shSKI NCCIT cells compared to shNC NCCIT cells and to determine if addition of BMP or noggin affects the level of *SKI*. *SKI* mRNA expression was significantly reduced by about 80% in shSKI NCCIT cells compared to shNC NCCIT cells (Figure 3.12). Addition of BMP and/or noggin did not significantly alter the level of SKI mRNA in these cells.

Microarray Analysis

We found that experimentally reducing SKI expression in NCCIT cells resulted in an enhanced invasive phenotype. Therefore we next sought to determine if reducing SKI expression in these NCCIT cells allowed an upregulation of genes involved in promoting metastasis and downregulation of genes that function to inhibit cellular invasion. Furthermore, since SKI is a transcription factor and a co-repressor of the TGFβ superfamily, we were interested in identifying changes in the expression patterns of genes involved in TGFβ superfamily signaling, especially genes involved in BMP signaling because we have shown that BMP enhances the invasive phenotype seen in shSKI NCCIT cells. In order to identify potential downstream targets of SKI that may play a role in metastasis, microarray analysis was performed to compare the gene expression profile of shSKI NCCIT cells to that of the shNC NCCIT cells. Statistical analysis of microarray (SAM) and gene set enrichment analysis (GSEA)

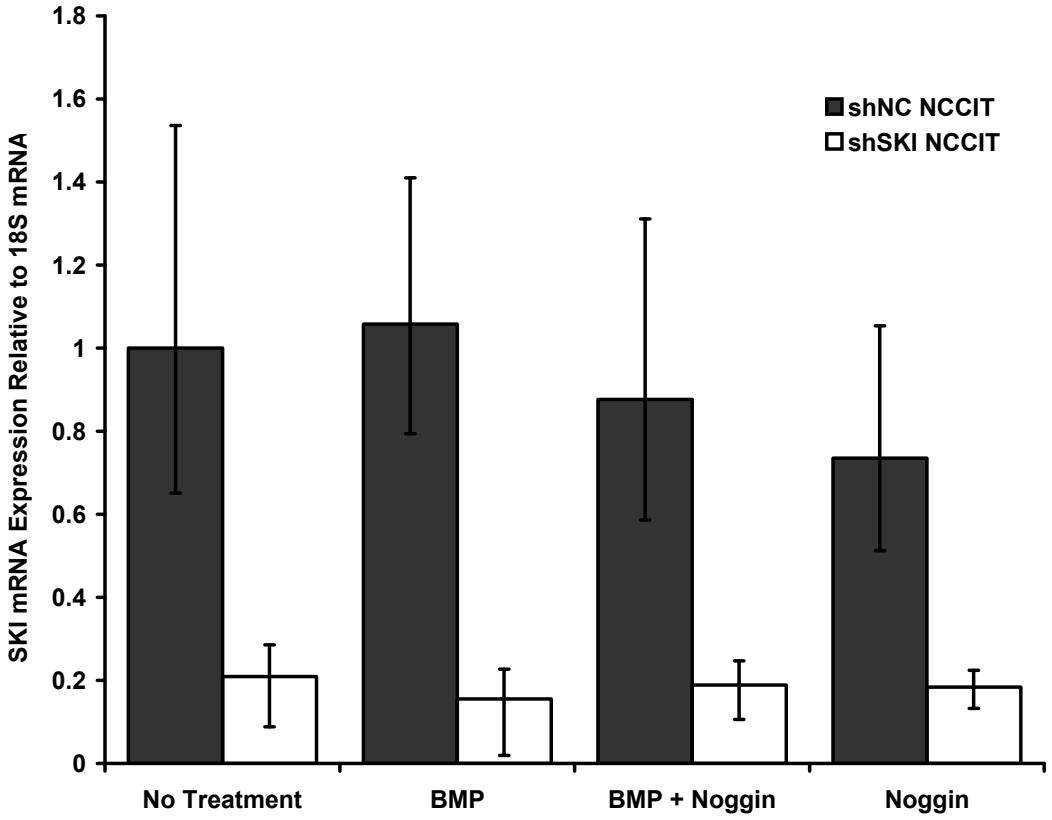

Figure 3.12. *SKI* mRNA expression in shNCCIT cells treated with BMP and/or noggin. Real time PCR analysis comparing *SKI* mRNA expression in shNC NCCIT cells ± 10ng/mL BMP, ± 0.1µg/mL noggin compared to shSKI NCCIT cells ± 10ng/mL BMP, ± 0.1µg/mL noggin. RNA levels were normalized to 18S RNA. Data represents three individual trials and was analyzed using $2^{-\Delta\Delta Ct}$ method; error bars indicate the 95% confidence interval.

(http://www.broadinstitute.org/gsea) were used to identify significant genes and gene families that were differentially expressed in shSKI NCCIT cells compared to shNC NCCIT cells.

Statistical analysis of microarrays (SAM) revealed 963 human gene probes were differentially expressed in shSKI and shNC NCCIT cells. Of these differentially expressed genes, 593 probes were upregulated by at least 1.5 fold in shSKI NCCIT cells, and 370 probes were downregulated by at least 1.5 fold in shSKI NCCIT cells compared to shNC NCCIT cells. The differentially expressed genes belong to diverse signaling pathways and are involved in a variety of cellular processes.

Gene Set Enrichment Analysis (GSEA) was used to identify gene sets involved in specific biological processes that were significantly upregulated in shSKI NCCIT cells compared to shNC NCCIT cells. GSEA uses four key statistics for determining gene set enrichment. These include the enrichment score (ES), the normalized enrichment score (NES), the false discovery rate (FDR), and the nominal p-value. The ES value ranks the gene sets according to the degree to which they are over-represented at the top or bottom of a ranked list of genes. A positive ES indicates a correlation with the shSKI phenotype, while a negative ES indicates a correlation with the control phenotype. If several genes from a gene set are clustered at the top of the ranked list, the ES value would be higher, and that gene set would be more strongly correlated with the shSKI phenotype. The NES can be used to compare analysis results across gene sets because it accounts for differences in gene set size and in correlations

between gene sets and the expression dataset. The FDR is the estimated probability that a gene set with a given NES represents a false positive finding. The nominal p value provides an evaluation of the statistical significance of the enrichment score for a single gene set (http://www.broadinstitute.org/gsea). The FDR is adjusted for gene set size and multiple hypothesis testing, but the nominal p value is not. The family-wise error rate (FWER) is a more stringent measure of significance that corrects for multiple hypothesis testing. This more conservative cutoff resulted in only a few statistically significant results with FWER less than 0.05. In our analysis we were primarily interested in identifying potential targets of SKI that may play a role in metastasis, therefore we considered gene sets with both an FDR less than 0.25 and a nominal p value less than 0.05.

Within the gene sets, GSEA provides the rank in gene list, the rank metric score, and the running ES. GSEA assigns each gene a rank metric score, which is computed using a “Signal2Noise” measurement that uses the difference of means scaled by the standard deviation. This provides a measurement of the gene’s correlation with the phenotype being studied and is used to position the gene in the ranked list. The rank values go from positive to negative as you move down the list, with a higher positive value indicating a stronger correlation with the shSKI phenotype and a negative value indicating a correlation with the control phenotype. The rank in gene list indicates the position of the gene in a ranked list of genes. Thus a lower value indicates a higher position on the ranked list and a stronger correlation with the shSKI phenotype. The running ES

is the enrichment score at this point in the ranked list of genes. During the analysis, GSEA creates a running sum statistic by walking down the ranked list of genes, and this value is increased when the gene belongs to the gene set being tested. The magnitude of the ES from zero is indicative of the correlation of that gene with the phenotype, with an ES closer to one being a strong correlation.

GSEA identified 101 gene sets that were enriched in shSKI NCCIT cells compared to shNC NCCIT cells with a nominal p-value (p-nominal) less than 0.05 and a false discovery rate (FDR) less than 0.25 (Table 3.1). Of these 101 gene sets, the gene set "Regulation of MAP Kinase Activity" contained 20 genes that were enriched in shSKI NCCIT cells compared to shNC NCCIT cells with a family-wise error rate p-value (FWER) of less than 0.05, indicating a higher level of significance (Table 3.2). Some interaction between the MAPK and BMP pathways has previously been shown to be involved in cell fate determination. FGF8 and IGF2, both of which activate MAPK signaling, have been shown to inhibit nuclear localization of SMAD1 (Massague, et al., 2005). However, these specific genes were not identified through GSEA as being enriched in the SKI-knockdown cells. It is possible that upregulation of MAPK signaling may be a compensation mechanism to the increased activity of the BMP pathway in SKI-knockdown cells. Activation of the Ras/MAPK pathway has also been shown to inhibit SMAD2 nuclear localization, preventing TGFβ signaling (Massague, 2003). It is possible that alterations in MAPK signaling may contribute to the lack of response seen with the addition of TGFβ to the shSKI NCCIT cells. A specific link between SKI and the MAPK pathway has not been shown, thus our data

suggest that there may be further interaction between MAPK signaling and TGFβ superfamily signaling.

Also of interest to this research is the gene set “Transforming Growth Factor Beta Receptor Signaling Pathway,” which met the dual criteria of nominal p-value less than 0.05 and FDR less than 0.25 (Table 3.3). This signaling pathway is of interest because SKI is known to be a co-repressor of the TGFβ superfamily. Both SMAD7 and LEFTY1, which are known to inhibit TGFβ signaling, were enriched in SKI knockdown cells. This was also seen in the SAM analysis (Table 3.6). Upregulation of these genes may contribute to the lack of response seen with the addition of TGFβ to shSKI NCCIT cells.

In review of the SAM data set, other signaling families of interest were identified, including the WNT signaling family, genes belonging to the family of chemokines and their receptors, and additional TGFβ superfamily signaling genes. Several genes from the WNT signaling family (Table 3.4) were differentially expressed in shSKI and shNC NCCIT cells. The importance of the WNT signaling pathway in colorectal cancer has been widely reported, and the WNT pathway has also been implicated in epithelial to mesenchymal transition (reviewed in (Huang and Du, 2008), which suggests that this signaling family may play a role in the enhanced invasive phenotype seen in the shSKI NCCIT cells.

Additionally, several chemokines and chemokine receptors were differentially expressed in shSKI and shNC NCCIT cells (Table 3.5). Chemokines and their receptors are known to play an important role in

tumorigenesis, especially in tissue directed metastasis (Vandercappellen, et al., 2008). Based on our observed phenotype in SKI-knockdown cells, CXCR4 is of particular interest in this group of chemokines because CXCR4 has previously been implicated in testicular cancer metastasis. CXCR4 was upregulated in shSKI NCCIT cells compared to shNC NCCIT cells. This finding supports our hypothesis that decreased SKI expression promotes metastasis in testicular cancer, and it also suggests that SKI may regulate the expression of CXCR4 in testicular cancer cells.

Table 3.1. Gene Sets Enriched in shSKI NCCIT Cells Identified by GSEA.

NAME OF GENE SET	# of GENES	ES	NES	p-nominal	FDR	FWER	RANK AT MAX
Transcription Initiation	35	0.5738	2.1093	0.0000	0.0138	0.0110	1717
Protein DNA Complex Assembly	48	0.5146	1.9813	0.0000	0.0308	0.0530	833
Positive Regulation of MAP Kinase Activity	46	0.5157	1.9244	0.0000	0.0367	0.0990	4160
Transcription Initiation from RNA Polymerase II Promoter	29	0.5998	1.9896	0.0000	0.0376	0.0440	1717
Activation of MAPK Activity	40	0.5216	1.9299	0.0000	0.0396	0.0890	2645
Regulation of Mitotic Cell Cycle	22	0.5415	1.8599	0.0000	0.0415	0.1650	4833
Cell Structure Disassembly during Apoptosis	18	0.5250	1.9436	0.0000	0.0421	0.0810	1920
Apoptotic Nuclear Changes	19	0.4939	1.8827	0.0000	0.0427	0.1440	1920
Amino Acid Transport	26	0.5672	1.8621	0.0000	0.0450	0.1610	971
Negative Regulation of MAP Kinase Activity	17	0.6590	1.8910	0.0000	0.0452	0.1380	3196
Amine Transport	38	0.5268	1.8231	0.0000	0.0464	0.2480	971
Transforming Growth Factor Beta Receptor Signaling Pathway	36	0.5141	1.8143	0.0000	0.0477	0.2640	2714
Glycoprotein Metabolic Process	90	0.4294	1.8248	0.0000	0.0487	0.2430	1069
Regulation of MAP kinase Activity	66	0.5468	2.0014	0.0000	0.0500	0.0370	3718
Negative Regulation of Transferase Activity	35	0.5344	1.8266	0.0000	0.0510	0.2330	3581
Glycoprotein Biosynthetic Process	74	0.4304	1.8295	0.0000	0.0521	0.2260	1069
Regulation of Organelle Organization and Biogenesis	40	0.4514	1.7190	0.0000	0.0696	0.4680	3877
Central Nervous System Development	121	0.3945	1.7582	0.0000	0.0696	0.3680	4987
DNA Catabolic Process	23	0.4721	1.7627	0.0000	0.0702	0.3610	1802
Transmembrane Receptor Protein Serine Threonine Kinase Signaling Pathway	47	0.4056	1.7196	0.0000	0.0718	0.4670	2714
Response to Oxidative Stress	45	0.4269	1.7649	0.0000	0.0718	0.3500	1842
Regulation of Cytoskeleton Organization and Biogenesis	30	0.4925	1.7260	0.0000	0.0758	0.4500	3877
Regulation of Hydrolase Activity	75	0.4409	1.7318	0.0000	0.0774	0.4360	4997

NAME OF GENE SET	# of GENES	ES	NES	p-nominal	FDR	FWER	RANK AT MAX
Positive Regulation of Catalytic Activity	158	0.4067	1.7270	0.0000	0.0783	0.4480	4433
Negative Regulation of Catalytic Activity	68	0.3798	1.6866	0.0000	0.0815	0.5600	3581
Positive Regulation of Transferase Activity	85	0.4080	1.6769	0.0000	0.0821	0.5790	2645
Protein Kinase Cascade	286	0.3542	1.6784	0.0000	0.0829	0.5780	3866
Activation of Immune Response	16	0.6843	1.6813	0.0000	0.0832	0.5700	3006
Apoptotic Program	59	0.4217	1.6514	0.0000	0.0866	0.6340	4433
Regulation of Catalytic Activity	268	0.3336	1.6316	0.0000	0.0909	0.6780	4917
Organic Acid Transport	42	0.4124	1.6202	0.0000	0.0926	0.7050	971
Regulation of Transferase Activity	159	0.3424	1.6027	0.0000	0.1006	0.7430	3718
Carboxylic Acid Transport	41	0.4180	1.5956	0.0000	0.1050	0.7580	971
Enzyme Linked Receptor Protein Signaling Pathway	139	0.3190	1.5842	0.0000	0.1131	0.7750	4401
Protein Secretion	31	0.3958	1.5519	0.0000	0.1283	0.8370	3322
Homeostasis of Number of Cells	20	0.5226	1.7366	0.0020	0.0778	0.4240	3790
Cell Substrate Adhesion	37	0.4951	1.6765	0.0040	0.0780	0.5790	3216
Cell Matrix Adhesion	36	0.4787	1.6333	0.0040	0.0911	0.6730	3216
Response to Abiotic Stimulus	87	0.4134	1.6947	0.0040	0.0776	0.5220	5363
Regulation of Signal Transduction	219	0.3099	1.4788	0.0041	0.1600	0.9360	3826
Regulation of Kinase Activity	155	0.3331	1.5612	0.0041	0.1250	0.8200	3718
MAPKKK Cascade GO 0000165	103	0.4030	1.6765	0.0041	0.0801	0.5790	2645
Mesoderm Development	21	0.5195	1.5343	0.0042	0.1349	0.8740	6231
Chromosome Segregation	32	0.5086	1.7238	0.0059	0.0739	0.4550	472
Glycolipid Metabolic Process	16	0.5776	1.8312	0.0059	0.0551	0.2230	601
Organelle Organization and Biogenesis	469	0.2734	1.5045	0.0061	0.1484	0.9070	2644
Secretion	174	0.3212	1.4673	0.0062	0.1583	0.9450	3334
Regulation of Protein Kinase Activity	153	0.3286	1.5380	0.0062	0.1335	0.8650	3718
Oxygen and Reactive Oxygen Species Metabolic Process	20	0.6436	1.7050	0.0063	0.0760	0.5030	4224
Positive Regulation of Transcription from RNA Polymerase II Promoter	65	0.3795	1.5523	0.0078	0.1299	0.8370	3508

NAME OF GENE SET	# of GENES	ES	NES	p-nominal	FDR	FWER	RANK AT MAX
Positive Regulation of Immune Response	26	0.6119	1.6596	0.0080	0.0884	0.6060	3191
Regulation of Molecular Function	316	0.3036	1.5693	0.0082	0.1256	0.8020	2645
Positive Regulation of RNA Metabolic Process	118	0.3576	1.5650	0.0099	0.1255	0.8150	5734
Mitochondrial Transport	20	0.4701	1.6558	0.0101	0.0871	0.6190	588
Actin Filament Organization	24	0.5038	1.6566	0.0120	0.0886	0.6150	3877
Positive Regulation of Hydrolase Activity	51	0.4937	1.6456	0.0121	0.0883	0.6470	4997
Brain Development	51	0.4229	1.6102	0.0138	0.0978	0.7220	2507
Positive Regulation of Signal Transduction	122	0.3390	1.4179	0.0145	0.1946	0.9730	4260
Cellular Cation Homeostasis	96	0.4445	1.6229	0.0150	0.0925	0.7000	4105
Mitotic Sister Chromatid Segregation	16	0.5633	1.7426	0.0154	0.0763	0.4110	2181
Sulfur Metabolic Process	35	0.4868	1.7202	0.0160	0.0741	0.4670	6219
Response to UV	25	0.4798	1.6534	0.0162	0.0868	0.6300	3222
Axon Guidance	22	0.4497	1.5003	0.0162	0.1472	0.9140	1582
Mitochondrial Organization and Biogenesis	47	0.4152	1.6108	0.0180	0.0992	0.7210	3644
ER to Golgi Vesicle Mediated Transport	18	0.6646	1.7031	0.0210	0.0747	0.5040	2953
Cation Homeostasis	99	0.4364	1.6043	0.0213	0.1007	0.7390	4105
Meiotic Cell Cycle	33	0.3765	1.5473	0.0216	0.1279	0.8490	3063
Nucleotide Excision Repair	20	0.5083	1.6299	0.0220	0.0902	0.6840	1481
Secretion by Cell	112	0.3361	1.4592	0.0220	0.1668	0.9630	3334
IkappaB Kinase NFkappaB Cascade	109	0.3689	1.4697	0.0222	0.1572	0.9410	3826
Sister Chromatid Segregation	17	0.5310	1.6345	0.0230	0.0915	0.6720	2181
Positive Regulation of Nucleobase Nucleoside Nucleotide and Nucleic Acid Metabolic Process	150	0.3355	1.4911	0.0240	0.1497	0.9200	5078
Lipid Biosynthetic Process	96	0.3520	1.4942	0.0266	0.1496	0.9180	4453
Transmembrane Receptor Protein Tyrosine Kinase Signaling Pathway	82	0.3016	1.3972	0.0276	0.2054	0.9840	3697
Positive Regulation of Transcription DNA Dependent	116	0.3411	1.4917	0.0279	0.1506	0.9200	5734
Activation of NFkappaB Transcription Factor	18	0.4725	1.5628	0.0293	0.1254	0.8190	2277
Membrane Lipid Biosynthetic Process	48	0.4475	1.5510	0.0295	0.1275	0.8410	4213

NAME OF GENE SET	# of GENES	ES	NES	p-nominal	FDR	FWER	RANK AT MAX
Regulation of GTPase Activity	16	0.6576	1.6401	0.0296	0.0910	0.6660	2501
Regulation of IkappaB Kinase NFkappaB Cascade	89	0.3705	1.4337	0.0297	0.1832	0.9680	3826
Regulation of Cytokine Production	25	0.4534	1.4718	0.0306	0.1613	0.9400	4084
Maintenance of Localization	22	0.4646	1.5074	0.0308	0.1486	0.9050	1919
Positive Regulation of Caspase Activity	30	0.4728	1.5094	0.0319	0.1480	0.9040	4433
Cellular Component Disassembly	33	0.3383	1.4397	0.0332	0.1803	0.9680	3644
Positive Regulation of Cytokine Production	15	0.5410	1.5394	0.0333	0.1338	0.8630	4084
Ion Homeostasis	119	0.4165	1.5551	0.0356	0.1289	0.8310	4105
Negative Regulation of Cellular Component Organization and Biogenesis	28	0.4108	1.4703	0.0364	0.1597	0.9410	3877
Regulation of Intracellular Transport	25	0.5016	1.5508	0.0365	0.1260	0.8450	7129
Centrosome Organization and Biogenesis	15	0.5518	1.5332	0.0367	0.1343	0.8750	3099
Cellular Homeostasis	137	0.3971	1.5662	0.0392	0.1268	0.8100	4105
Response to Organic Substance	29	0.3699	1.4530	0.0394	0.1686	0.9640	4987
Response to Radiation	58	0.4089	1.5287	0.0395	0.1371	0.8800	3445
Leukocyte Migration	16	0.5836	1.5133	0.0397	0.1453	0.8990	1968
Negative Regulation of Cell Cycle	79	0.3370	1.4102	0.0413	0.1977	0.9800	6373
Regulation of Protein Import into Nucleus	16	0.5630	1.6280	0.0425	0.0900	0.6870	5632
Protein Amino Acid N-Linked Glycosylation	30	0.4339	1.4089	0.0426	0.1977	0.9810	704
Regulation of Nucleocytoplasmic Transport	22	0.5176	1.6371	0.0431	0.0918	0.6710	5970
Microtubule Organizing Center Organization and Biogenesis	16	0.5404	1.5061	0.0441	0.1481	0.9050	3099
Positive Regulation of IkappaB Kinase NFkappaB Cascade	83	0.3872	1.4363	0.0457	0.1828	0.9680	3826
Positive Regulation of Response to Stimulus	38	0.4759	1.4736	0.0482	0.1624	0.9390	3191
Response to Chemical Stimulus	305	0.3074	1.3973	0.0487	0.2067	0.9840	5257
Cellular Protein Complex Assembly	33	0.4256	1.4955	0.0494	0.1503	0.9180	3355

ES = enrichment score; NES = normalized enrichment score; p-nominal = nominal p-value; FDR = false discovery rate; FWER = family wise error rate. Gene sets highlighted in red are further discussed.

Table 3.2. Regulation of MAP Kinase Activity Genes Upregulated in shSKI NCCIT Cells.

REGULATION OF MAP KINASE ACTIVITY					
GENE NAME	**GENE TITLE**	**RANK IN GENE LIST**	**RANK METRIC SCORE**	**RUNNING ES**	**CORE ENRICHMENT**
ADRA2C	adrenergic, alpha-2C-, receptor	28	3.569720984	0.099149056	Yes
DUSP6	dual specificity phosphatase 6	118	2.6670959	0.17067271	Yes
CXCR4	chemokine (C-X-C motif) receptor 4	175	2.362784386	0.23489298	Yes
SOD1	superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	383	1.804534674	0.27777663	Yes
ZAK	-	716	1.414972186	0.30503437	Yes
MDFI	MyoD family inhibitor	846	1.300608873	0.3367004	Yes
DUSP22	dual specificity phosphatase 22	876	1.286507726	0.37172356	Yes
TPD52L1	tumor protein D52-like 1	1252	1.064876556	0.38754058	Yes
DUSP16	dual specificity phosphatase 16	1346	1.026440144	0.4128619	Yes
GPS2	G protein pathway suppressor 2	1498	0.969061494	0.434396	Yes
MADD	MAP-kinase activating death domain	1564	0.940572977	0.4583579	Yes
RGS4	regulator of G-protein signaling 4	1806	0.858003378	0.47339702	Yes
CD24	CD24 molecule	1851	0.845130682	0.49546805	Yes
MAP3K5	Mitogen-activated protein kinase kinase kinase 5	2135	0.774060726	0.5065747	Yes
MAP4K5	mitogen-activated protein kinase kinase kinase kinase 5	2584	0.679615498	0.50883806	Yes
FGF2	fibroblast growth factor 2 (basic)	2645	0.669743836	0.5253856	Yes
DUSP9	dual specificity phosphatase 9	3196	0.583537579	0.5211242	Yes
ADRB2	adrenergic, beta-2-, receptor, surface	3355	0.560504258	0.5309276	Yes
TRIB3	tribbles homolog 3 (Drosophila)	3559	0.534068823	0.53830016	Yes
PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	3718	0.514676154	0.54681724	Yes

“Rank in gene list” indicates the position of the gene in a ranked list of genes. “Rank metric score” is a score used to position the gene in the ranked list and is computed using a “Signal2Noise” measurement that uses the difference of means scaled by the standard deviation. The “Running ES” is the enrichment score at this point in the ranked list of genes. Genes with a Yes value in the “Core enrichment” column contribute to the leading-edge subset within the gene set. This is the subset of genes that contributes most to the enrichment result.

Table 3.3. Transforming Growth Factor Beta Receptor Signaling Pathway Genes Upregulated in shSKI NCCIT Cells.

TRANSFORMING GROWTH FACTOR BETA RECEPTOR SIGNALING PATHWAY					
GENE NAME	**GENE TITLE**	**RANK IN GENE LIST**	**RANK METRIC SCORE**	**RUNNING ES**	**CORE ENRICHMENT**
SMAD7	SMAD, mothers against DPP homolog 7 (Drosophila)	32	3.454453945	0.14178942	Yes
MAP3K7	mitogen-activated protein kinase kinase kinase 7	245	2.102769136	0.22088137	Yes
EID2	EP300 interacting inhibitor of differentiation 2	284	2.000285625	0.302254	Yes
LEFTY1	left-right determination factor 1	494	1.66031909	0.36314425	Yes
FMOD	Fibromodulin	613	1.515063643	0.4214333	Yes
TGFBRAP1	transforming growth factor, beta receptor associated protein 1	1009	1.202226281	0.4563893	Yes
SMAD3	SMAD, mothers against DPP homolog 3 (Drosophila)	1927	0.828692615	0.4563156	Yes
TGFBR1	transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kDa)	2340	0.730790496	0.47112036	Yes
ACVR1	activin A receptor, type I	2391	0.717254817	0.4989351	Yes
SNX6	sorting nexin 6	2714	0.65763092	0.5140854	Yes

“Rank in gene list” indicates the position of the gene in a ranked list of genes. “Rank metric score” is a score used to position the gene in the ranked list and is computed using a “Signal2Noise” measurement that uses the difference of means scaled by the standard deviation. The “Running ES” is the enrichment score at this point in the ranked list of genes. Genes with a Yes value in the “Core enrichment” column contribute to the leading-edge subset within the gene set. This is the subset of genes that contributes most to the enrichment result.

As expected based on SKI's known function as a co-repressor of the TGFβ superfamily, genes belonging to this signaling pathway were differentially expressed in shSKI NCCIT cells compared to shNC NCCIT cells as well (Table 3.3). The expression pattern of the genes in the TGFβ superfamily may contribute to our finding that addition of BMP into our cell scrape assays resulted in an enhanced invasive phenotype of the shSKI NCCIT cells, while addition of TGFβ had no effect. For example, the microarray data shows that LEFTY1 and SMAD7, both of which can function as inhibitors of TGFβ signaling, were upregulated in shSKI NCCIT cells compared to shNC NCCIT cells. Furthermore, FST (follistatin), which specifically represses BMP signaling, was downregulated in shSKI NCCIT cells. These data suggest that SKI may regulate the expression of these negative regulators of the TGFβ superfamily signaling molecules.

The data indicate that decreased SKI may promote metastasis in testicular cancer. Therefore, we were interested in differentially expressed genes that may be involved in cellular migration and invasion in addition to genes that may be involved in TGFβ superfamily signaling. We selected certain genes from the list of differentially expressed genes, based on a review of the literature, which we believed to be potentially involved in the mechanism behind which decreased SKI expression promotes invasion of testicular cancer cells. Real time PCR analysis was used to confirm the changes in gene expression of LEFTY1 (Figure 3.13A), ADAMTS1 (Figure 3.13B), and CXCR4 (Figure 3.14). Real time PCR analysis showed that these genes were, indeed, upregulated in shSKI NCCIT cells compared to shNC NCCIT cells by 1.7, 2.6, and 5.5 fold, respectively. This

Table 3.4. WNT signaling family genes identified by microarray analysis.

Gene	Gene Description	Gene Function	Fold change
HHIP	Hedgehog interacting protein	Regulator of vertebrate hedgehog signaling pathway	2.6
WNT3	WNT family member 3	Involved in primary axis formation in the mouse	2.4
PPP3CC	Protein phosphatase 3 (formerly B2) gamma isoform	Ca(2+) dependent modifier of phosphorylation status	2.2
FZD2	Frizzled homolog 2	G-protein coupled receptor; binds WNT protein and induces intracellular calcium release	2.1
MAP3K7	Mitogen activated protein kinase kinase kinase 7 (TGFβ activated kinase 1)	Stimulated in response to TGFβ and BMP; mediator of TGFβ superfamily transcription	1.7
PPP2R1B	Protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform	Downregulates the MAPK cascade	1.7
TCF7	Transcription factor 7	May act as a feedback repressor of β-catenin target genes	0.6
AXIN2	Axis inhibitor 2 (conductin)	role in β-catenin stability	0.5
PLCB4	Phospholipase C beta 4	Plays a role in the light response in retinas	0.5
PTCH1	Patched 1 transmembrane protein	Represses transcription in certain cells of genes from the TGFβ and WNT families	0.4
FZD8	Frizzled homolog 8	Receptor for secreted WNT glycoproteins	0.3

Red numbers indicate increased expression and blue numbers indicate decreased expression in shSKI NCCIT cells compared to shNC NCCIT cells. Gene functions descriptions are from Online Mendelian Inheritance in Man (OMIM) and Gene Entrez.

Table 3.5. Chemokine genes identified by microarray analysis.

Gene	Gene Description	Gene Function	Fold change
CXCR4	Chemokine (C-X-C motif) receptor 4	Receptor for SDF1; invovled in HIV infection; implicated in cancer metastasis	**6.5**
CCL26	Chemokine (C-C motif) ligand 26	Member of chemokine family; plays a role in cell trafficking and immune system function	**2.3**
CCL2	Chemokine (C-C motif) ligand 2	Recruitment of monocytes to sites of injury and infection	**2.1**
CXCR7	Chemokine (C-X-C motif) receptor 7	Alternate receptor for SDF1; involved in cell growth, survival, and adhesion	**1.9**
CX3CL1	Chemokine (C-X3-C motif) ligand 1	Mediator of cellular migration; attracts natural killer cells to sites of infection	**0.5**

Red numbers indicate increased expression and blue numbers indicate decreased expression in shSKI NCCIT cells compared to shNC NCCIT cells. Gene functions descriptions are from Online Mendelian Inheritance in Man (OMIM) and Gene Entrez.

Table 3.6. TGFβ superfamily signaling genes identified by microarray analysis.

Gene	Gene Description	Gene Function	Fold Change
GDF3	Growth differentiation factor 3	BMP family member; expression decreases with differentiation of cultured human embryonic stem cells	**2.0**
LEFTY1	L-R determination factor 1	TGFβ superfamily inhibitor; also involved in left-right polarity in early development	**1.7**
MAP3K7	Mitogen activated protein kinase kinase kinase 7 (TGFβ activated kinase 1)	Stimulated in response to TGFβ and BMP; mediator of TGFβ superfamily transcription	**1.7**
SMAD7	Inhibitory SMAD 7	TGFβ superfamily inhibitor by inhibition of R-SMAD phosphorylation	**1.5**
SPP1	Secreted phosphoprotein 1 (osteopontin)	Cytokine involved in bone formation and immune response	**0.5**
FST	Follistatin	Negative regulator of the TGFβ superfamily	**0.5**
SKI	SKI protooncogene	Negative regulator of the TGFβ superfamily	**0.4**
ACVR1C	Activin A receptor, type 1C	Receptor for the TGFβ superfamily signaling molecules	**0.3**

Red numbers indicate increased expression and blue numbers indicate decreased expression in shSKI NCCIT cells compared to shNC NCCIT cells. Gene functions descriptions are from Online Mendelian Inheritance in Man (OMIM) and Gene Entrez.

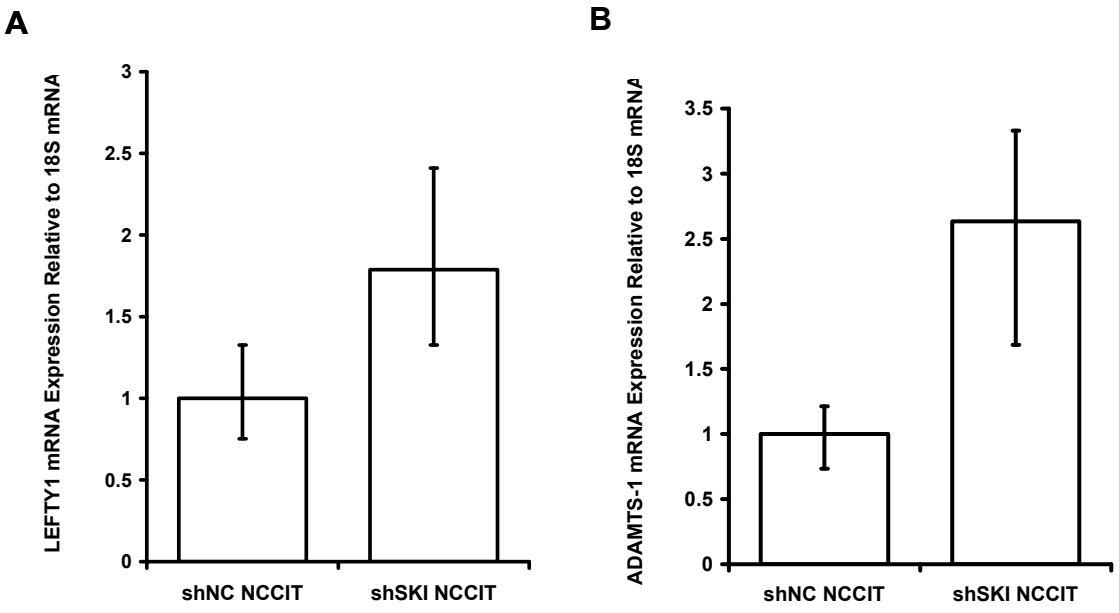

Figure 3.13. *LEFTY1* and *ADAMTS-1* expression in shSKI NCCIT cells compared to shNC NCCIT cells. Real time PCR analysis comparing *LEFTY1* (A) and *ADAMTS1 (*B) expression in shSKI NCCIT cells and shNC NCCIT cells. RNA levels were normalized to 18S RNA. Data represents six individual trials and was analyzed using $2^{-\Delta\Delta Ct}$ method; error bars indicate the 95% confidence interval.

confirmed the microarray SAM results showing that LEFTY1, ADAMTS1, and CXCR4 were upregulted in shSKI NCCIT cells by 1.7, 2.1, and 6.5 fold, respectively.

SMAD Binding Elements in Promoters of Target Genes

In order to determine if potential target genes of SKI that were identified by microarray analysis were transcriptionally regulated by SMADs, promoter analysis of specific genes was performed to identify the presence of BMP and/or TGFβ regulatory elements. BMP and TGFβ signaling is transmitted inside the cell by SMADs. R-SMADs, SMAD 1, 5, and 8, are phosphorylated in response to BMP signals, while R-SMADs, SMADs 2 and 3, are phosphorylated in response to TGFβ signals. These phosphorylated R-SMADs form a complex with the co-SMAD, and these complexes are able to initiate target gene transcription by binding to the promoter region with additional co-factors (Massague and Wotton, 2000). SKI is known to repress both BMP and TGFβ signaling by interfering with SMAD-mediated gene transcription (Akiyoshi, et al., 1999; Luo, et al., 1999; Stroschein, et al., 1999). Cell scrape migration experiments revealed that addition of BMP to SKI-knockdown NCCIT cells resulted in an enhanced invasive phenotype, while addition of TGFβ had no effect. Therefore, we were interested in identifying SKI-responsive genes that are regulated by BMP. The presence of SMAD 1/5/8 binding elements in the promoter region of a gene would be evidence of regulation by BMP. TGFβ-regulated SMAD binding elements were also considered to determine the specificity of gene regulation.

A literature search revealed several potential SMAD regulatory elements for both BMP and TGFβ responsive genes. GCCGnCGC has been reported as a SMAD1 binding motif (Benchabane and Wrana, 2003; Ishida, et al., 2000; Kim, et al., 1997; Kusanagi, et al., 2000). Kusanagi, et. al. (2000) provided evidence that a reporter gene construct containing multiple copies of this motif was stimulated in response to BMP, but not in response to TGFβ or activin. It has also previously been reported that SMAD1 binds to this sequence in the SMAD7 promoter (Benchabane et al., 2003).

GTCTAGAC has been shown to be a SMAD5 binding sequence (Li, et al., 2001; Zawel, et al., 1998) and has also been reported to be TGFβ-responsive (Stopa, et al., 2000). The CAGACA motif is also not specific to BMP-activated SMADs. It has been reported to be BMP-responsive in the ID1 promoter, and both SMAD4 and SMAD1 can bind this sequence (Katagiri, et al., 2002). This element was also identified in the promoter of JunB, and expression of JunB was induced by TGFβ, activin, and BMP (Jonk, et al., 1998). However, Dennler et al. (1998) reported that this sequence was specific to TGFβ and activin responses.

Several other TGFβ response sequences have been reported. Song et al. (1998) reported that both SMAD3 and SMAD4 bind to the motif AGACAAGGTTGT in the promoter of human type 1 plasminogen activator inhibitor (PAI1), and activate transcription in response to TGFβ. ACAGACAGACAC has been identified as a binding site for the SMAD3-SMAD4 complex in the promoter of JunB and is TGFβ responsive (Wong 1999). Additionally TGFβ has been shown to enhance the expression of type VII

collagen (COL7A1) by inducing the binding of the SMAD3-SMAD4 complex to the sequence, GCCCACCAGACAGATGGCTGAATCACAGGAGTGGCCGGCG GGACCCATGGCCT within the promoter of COL7A1 (Kon et al., 1999, Vindevoghel et al., 1998). Hua et al. (1998, 1999) identified two sequences in the PAI1 promoter that contained multiple AGAC SMAD-binding element repeats, AGACAGACAAAACCTAGACAATCACGTGGCTGGCTGCATGCCCTGTGGCTG TTGGGC and CCTAGACAGACAAAACCTAGACAATCACGTGGCTGG; these sequences also contain a binding site for transcription factor μE3 (TFE3), which was determined to be an essential binding partner in the SMAD transcriptional complex for TGFβ-induced transcription of PAI1 (Hua et al., 1999). Lindemann et al. (2001) described a TGFβ-responsive SMAD3-SMAD4 binding element in the promoter of parathyroid hormone-related protein (PTHrP), GAGGAGGTAGA CAGACAGCTATGT, which aso contained multiple AGAC SMAD-binding element repeats, as well as an Ets transcription factor binding domain. Finally, Shi et al. (1998) performed cocrystallization studies and electrophoretic mobility shift assays to identify a the SMAD3 DNA binding element, AGTATGTCTAGAC TGA.

Using the Motif Locator tool on the MOtif Discovery (MoD) tools website (http://159.149.109.9/modtools/; Pavesi 2006), the promoter sequences of specific genes, chosen based on our microarray analysis and previous reports associating these genes with metastasis, migration, or TGFβ superfamily signaling, were searched for the presence of each of the SMAD binding elements (Table 3.7). ADAMTS1, CXCR4, LEFTY1, SMAD7, MAP3K7, and WNT3 were

all upregulated in SKI knockdown cells according to our microarray data. CXCR4, SMAD7, and WNT3 promoters contained all three of the BMP response elements. CXCR4 and SMAD7 both contained multiple copies of each of the three BMP response elements, and it has been reported that multiple copies of the consensus sequence promotes SMAD binding (Ishida, et al., 2000; Kusanagi, et al., 2000). The WNT3 promoter had multiple copies of the two motifs that are not BMP-specific, and only one copy of the GCCGnCGC motif. The MAP3K7 promoter contained multiple copies of the BMP-specific motif and the non-specific motif, CAGACA. The ADAMTS1 promoter contained multiple copies both the GTCTAGAC and the CAGACA motif; these two motifs are responsive to both TGFβ and BMP. The LEFTY1 promoter contained multiple copies of the TGFβ/BMP-response element CAGACA motif as well as multiple copies of the BMP-specific GCCGnCGC motif. The ADAMTS1 promoter was the only promoter that contained TGFβ-specific response element, the ACAGACAGACAC motif.

FST (follistatin) and SPARC (secreted protein acidic and rich in cysteine) were both downregulated in SKI knockdown cells compared to control cells. The FST promoter contained multiple copies of both of the TGFβ/BMP response motifs as well as the BMP-specific motif. The SPARC promoter also contained multiple copies of both of the TGFβ/BMP response motifs, but the BMP-specific motif was not present.

SMAD6 was not differentially expressed in shSKI compared to shNC NCCIT cells according to our microarray data; however its expression has

Table 3.7. SMAD Promoter Elements in Potential Target Genes.

		ADAMTS1	CXCR4	FST	LEFTY1	MAP3K7	MMP9	SMAD6	SMAD7	SPARC	WNT3
BMP Elements	GTCTAGAC*	+ (2)	+ (4)	+ (6)	-	-	+ (6)	+ (2)	+ (4)	+ (6)	+ (2)
	CAGACA**	+ (69)	+ (84)	+ (66)	+ (78)	+ (30)	+ (66)	+ (79)	+ (72)	+ (113)	+ (92)
	GCCGnCGC	-	+ (13)	+ (63)	+ (29)	+ (2)	-	+ (71)	+ (2)	-	+ (1)
TGFβ Elements	GTCTAGAC*	+ (2)	+ (4)	+ (6)	-	-	+ (6)	+ (2)	+ (4)	+ (6)	+ (2)
	AG(C/A)CAGACA**	+ (69)	+ (84)	+ (66)	+ (78)	+ (30)	+ (66)	+ (79)	+ (72)	+ (113)	+ (92)
	AGACAAGGTTGT	-	-	-	-	-	-	-	-	-	-
	ACAGACAGACAC	+ (1)	-	-	-	-	-	-	-	-	-
	GCCCACCAGACA GATGGCTGAATCA CAGGAGTGGCCG GCGGGACCCATG GCCT	-	-	-	-	-	-	-	-	-	-
	AGACAGACAAAAC CTAGACAATCACG TGGCTGGCTGCAT GCCCTGTGGCTG TTGGGC	-	-	-	-	-	-	-	-	-	-
	CCTAGACAGACAA AACCTAGACAATC ACGTGGCTGG	-	-	-	-	-	-	-	-	-	-
	GAGGAGGTAGAC AGACAGCTATGT	-	-	-	-	-	-	-	-	-	-
	AGTATGTCTAGAC TGA	-	-	-	-	-	-	-	-	-	-

* and ** sequences have been described to be both BMP and TGFβ responsive. + indicates the response element was present in the promoter sequence; - indicates the response element was not present. Numbers in ( ) indicate the number of times the response element was detected within the promoter sequence.

previously been described to be regulated by BMP (Ishida, et al., 2000), and thus served as a positive control. The SMAD6 promoter contained multiple copies of both of the TGFβ/BMP response motifs and the BMP-specific motif. MMP9 is a matrix remodeling protein, and, thus, thought to be involved in metastasis. MMP9 also was not differentially expressed in shSKI compared to shNC NCCIT cells according to our microarray data, and, therefore, appears to be SKI non-responsive. However, it also contained multiple copies of the BMP-specific motif as well as multiple copies of the TGFβ/BMP response motif, CAGACA.

SDF/CXCR4: Role in invasion of SKI-knockdown cells

According to the microarray data, *CXCR4* mRNA expression was upregulated in shSKI NCCIT cells compared to shNC NCCIT cells. CXCR4 and its signaling ligand, SDF1, have previously been implicated in metastasis, and are also important regulators of germ cell migration. Therefore, we sought to determine if this upregulation of CXCR4 contributes to the increased invasive potential seen in SKI-knockdown NCCIT cells.

The microarray results that *CXCR4* mRNA is upregulated in shSKI NCCIT cells compared to shNC NCCIT cells were first confirmed using real time PCR analysis (Figure 3.14). *CXCR4* is, indeed, upregulated 5.5 fold in shSKI NCCIT cells compared to shNC cells, however, addition of BMP or its inhibitor, noggin, did not significantly alter the levels of *CXCR4* mRNA in either shSKI or shNC NCCIT cells. SDF1, the signaling ligand partner of CXCR4, has previously been shown to be upregulated by BMP (Yang, et al., 2008). However, at the

concentration used, real time PCR showed no change in *SDF1* mRNA expression in response to treatment of either shNC or shSKI NCCIT cells with BMP4 (Figure 3.15). This indicates that, at the concentrations used, BMP is not able to induce transcription of *CXCR4* or *SDF1* in either shNC or shSKI NCCIT cells.

Since *CXCR4* is increased in shSKI NCCIT cells compared to shNC NCCIT cells, Matrigel invasion assays were used to assess whether this upregulation of *CXCR4* enhanced the invasive phenotype of the shSKI NCCIT cells. SDF1, the signaling ligand for CXCR4, and/or AMD3100, a potent and specific inhibitor of CXCR4 (Broxmeyer, et al., 2005; Hatse, et al., 2002), were added to the companion wells of the Matrigel invasion chambers to determine their effects on the invading cells. Addition of 25 ng/mL SDF1 to the companion wells significantly increased the invasion of shSKI NCCIT cells, but had no effect on the invasion of shNC NCCIT cells (Figure 3.16). When 50 ng/mL AMD3100 was added in combination with SDF1, it was able to significantly reduce the effects of SDF1, almost to the level of invasion seen in shNC NCCIT cells (Figure 3.16). Addition of 50 ng/mL AMD3100 alone significantly reduced the level of invasion in shSKI NCCIT cells to the level of invasion seen in shNC NCCIT cells. However, addition of AMD3100 to shNC NCCIT cells did not affect invasion of these cells, indicating that AMD3100, alone, does not affect invasion. These data suggest that the CXCR4/SDF1 signaling partners enhance the invasive phenotype of NCCIT cells with reduced levels of SKI.

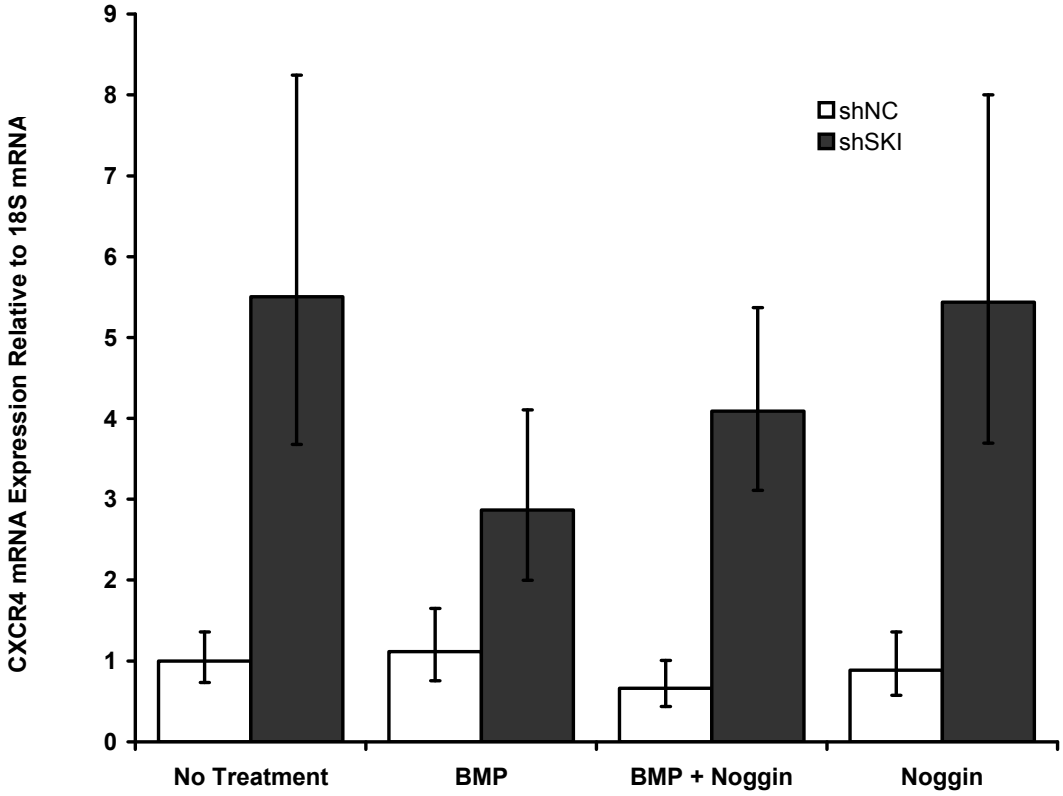

Figure 3.14. *CXCR4* mRNA expression in shNCCIT cells treated with BMP and/or noggin. Real time PCR analysis comparing *CXCR4* mRNA expression in shNC NCCIT cells ± 10ng/mL BMP, ± 0.1µg/mL noggin compared to shSKI NCCIT cells ± 10ng/mL BMP, ± 0.1µg/mL noggin. RNA levels were normalized to 18S RNA. Data represents three individual trials and was analyzed using $2^{-\Delta\Delta Ct}$ method; error bars indicate the 95% confidence interval.

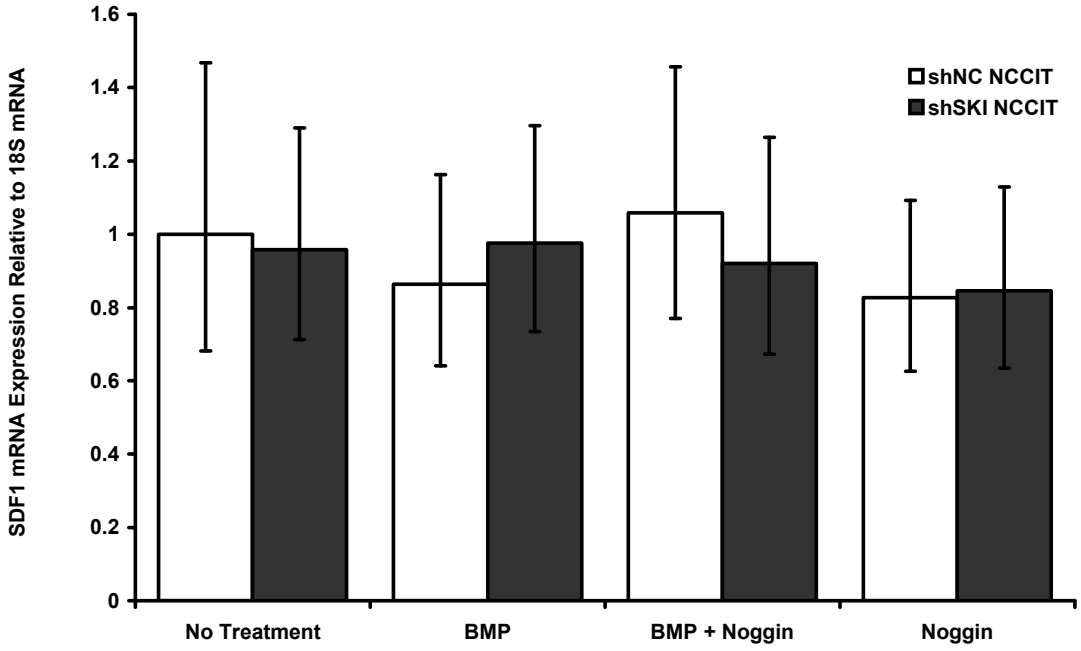

Figure 3.15. *SDF1* mRNA expression in shNCCIT cells treated with BMP and/or noggin. Real time PCR analysis for *SDF1* mRNA in shNC NCCIT cells ± 10ng/mL BMP, ± 0.1µg/mL noggin compared to shSKI NCCIT cells ± 10ng/mL BMP, ± 0.1µg/mL noggin. RNA levels were normalized to 18S RNA. Data represents three individual trials and was analyzed using $2^{-\Delta\Delta Ct}$ method; error bars indicate the 95% confidence interval.

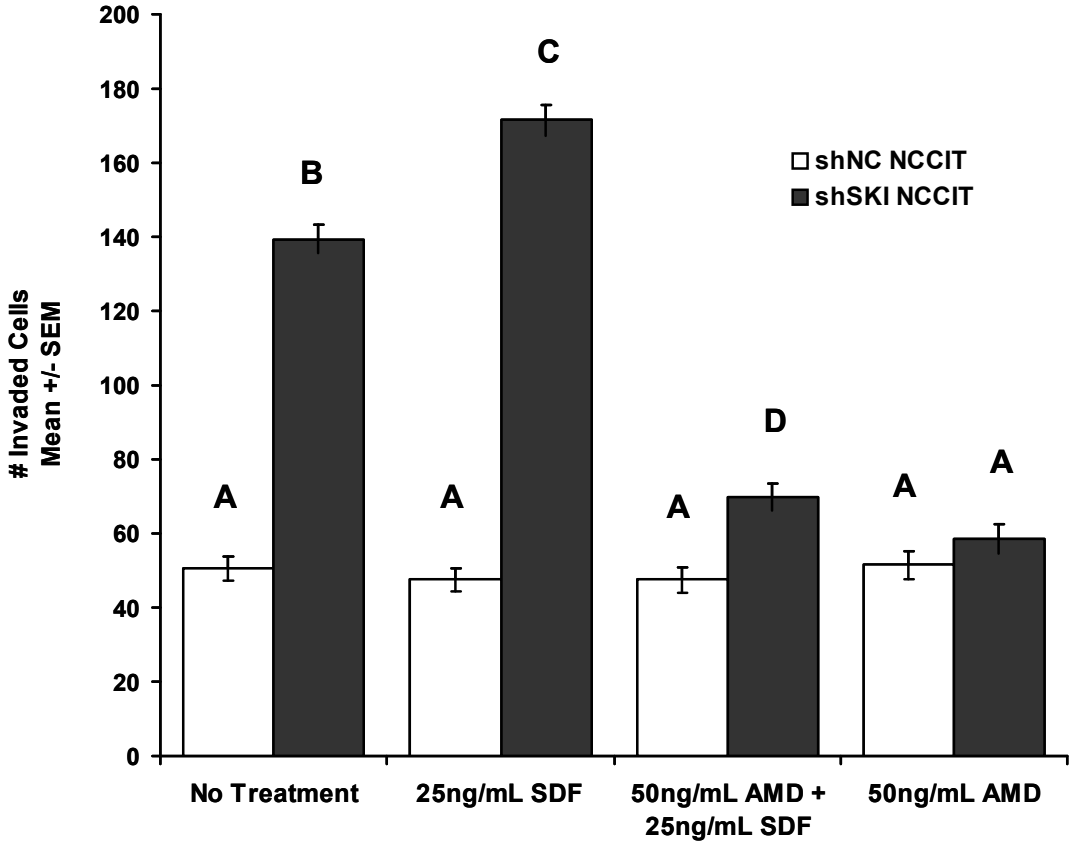

Figure 3.16. Role of CXCR4/SDF1 in the invasive potential of shNCCIT cells. Bar graph representing the number of migrating cells in Matrigel invasion assays using shNC NCCIT cells and shSKI NCCIT cells ± 25ng/mL SDF, ± 50ng/mL AMD 3100 to the medium in the companion wells. Data represents five trials; error bars indicate SEM; Groups labeled **A, B,** and **C,** and **D** are each significantly different from each other with $p < 0.05$.

CHAPTER IV

DISCUSSION

SKI has previously been described as an oncogene based on its ability to transform avian fibroblasts (Colmenares and Stavnezer, 1989). However, the role of *SKI* in mammalian tumorigenesis is not yet fully characterized. The available literature on *SKI* suggests that *SKI* plays dual roles in tumorigenesis and that *SKI's* involvement in cancer progression may be cell type and context dependent. For example, *SKI* has been shown to promote proliferation in pancreatic cancer cells (Heider, et al., 2007), yet other studies have shown that *SKI* does not affect the rate of proliferation in lung and breast cancer cell lines (Le Scolan, et al., 2008; Zhu, et al., 2007). In addition, various studies have shown that *SKI* is over-expressed in some tumor types including melanoma, esophageal, colon, and pancreatic cancer (Buess, et al., 2004; Fukuchi, et al., 2004; Heider, et al., 2007; Reed, et al., 2005). In contrast, data presented by Shinagawa et. al.(2001), showed that mice heterozygous for a null mutation in the *SKI* gene were more susceptible to tumorigenesis, which suggests that *SKI* may function as a tumor suppressor.

The data presented within this text supports the idea that SKI may play different roles in tumorigenesis in different cell types. Analysis of a panel of human cell lines for SKI expression showed that SKI expression varies widely in

different types of cancer. In support of previous data (Reed, et al., 2005), SKI expression increases in progressing stages of melanoma, with the highest expression seen in WM9 metastatic melanoma cells. On the other hand, SKI expression appears to be lower in T47D ductal carcinoma cells compared to HMEC normal breast cells; while the level of SKI expression in MCF7 mammary adenocarcinoma cells was very similar to that of HMEC cells. Both of these cell lines were derived from metastatic pleural effusions, and are described as weakly metastatic *in vitro* (Lacroix and Leclercq, 2004). HCT116 cells, derived from a primary colorectal carcinoma (Brattain, et al., 1981), expressed high levels of SKI, while A549 primary lung carcinoma cells have very low SKI expression. The level of SKI expression in NCCIT embryonal carcinoma cells, derived from a mediastinal metastasis, was intermediate compared to other cell lines studied. Taken together, these data indicate that SKI may, indeed, act as an oncogene in some tissues, but may play an anti-tumorigenic role in others.

The data presented here indicate that *SKI* expression is decreased in human testicular germ cell tumors compared to normal testis samples. Previous studies have shown that CCND2 mRNA and protein expression are increased in TGCT samples compared to normal testis (Bartkova, et al., 1999; Houldsworth, et al., 1997; Schmidt, et al., 2001). Our real time PCR data confirms this observation, and *CCND2* served as a positive control in these experiments. *SKI* mRNA expression decreased in the same tumor samples, while *SNO* was not differentially expressed. Furthermore, our data also show that proliferation is not affected by *SKI* expression in NCCIT testicular cancer cells. However, the level

of *SKI* expression in NCCIT cells does influence the ability of these cells to migrate and invade since experimentally reducing SKI expression in NCCIT cells resulted in increased invasive potential. These data suggest that in testicular germ cell tumors SKI does not act as an oncogene, but rather promotes tumor progression to a metastatic state.

SKI has been identified as a negative regulator of the TGFβ superfamily. Previous data indicate that TGFβ has diverging roles in tumorigenesis. While TGFβ may act as a tumor suppressor in the early stages of tumorigenesis by inhibiting cellular proliferation, evidence suggests that TGFβ superfamily signaling promotes cancer invasion and metastasis, permitting cancer progression (Derynck, et al., 2001; Le Scolan, et al., 2008; Leivonen and Kahari, 2007; Rothhammer, et al., 2005; Tang, et al., 2003; Wakefield and Roberts, 2002; Zhu, et al., 2007). A recent study reported that forced over-expression of *SKI* in JygMC(A) mouse mammary carcinoma cells decreased the metastatic potential of these cells *in vivo* (Azuma, et al., 2005). In accordance with this finding, Le Scolan et al. (2008) recently reported that experimentally reducing *SKI* expression in breast and lung cancer cell lines promoted TGFβ-mediated cancer metastasis. Similarly, we found that reducing SKI expression in NCCIT testicular cancer cells resulted in a more invasive phenotype. In the same study by Le Scolan et. al. (2008), they found that *SKI* was abundantly expressed in a panel of metastatic melanoma cells and breast cancer cell lines. We also found that SKI expression is high in metastatic melanoma and variable in other cell lines derived from metastatic and primary tumors. These data suggest that the role of *SKI* in

tumorigenesis is complex and may be dependent on the tissue type and other factors present within the cell and microenvironment as well as the levels of SKI.

The data presented in this body of work support the role of *SKI* as an inhibitor of metastasis in TGCTs. In human embryonal carcinoma NCCIT cells, decreasing the expression of *SKI* resulted in enhanced migration and invasion, while forcing the expression of *SKI* significantly reduced the invasive potential of these cells, as assessed by both cell scrape assays and Matrigel invasion assays. Interestingly, it has also been reported that experimentally reducing *SNO,* another member of the *SKI* gene family, in breast and lung cancer cell lines resulted in increased metastatic potential in these cells in response to TGFβ (Zhu, et al., 2007). In contrast to reduced *SKI* levels in TGCTs, our data show that *SNO* expression was not significantly different in TGCTs and normal testis samples, indicating that *SKI*, but not *SNO,* may be involved in testicular cancer progression and metastasis. These data, taken together with the tumor data showing decreased *SKI* expression in human TGCTs, suggest that *SKI* may function to prevent testicular cancer metastasis by repressing signals from the TGFβ superfamily.

In the current experiments, decreased *SKI* expression was sufficient to promote invasion in the testicular cancer cell lines without the addition of growth factors, suggesting that the cells may be responding to an autocrine loop of TGFβ superfamily signaling. Although the *SKI*-knockdown cells were cultured in low-serum medium, it is also possible that factors in the serum may contribute to the increased metastasis observed in these studies. In addition to being a co-

repressor of the TGFβ superfamily, SKI is able to interact with transcription factors other than SMADs, including MyoD (Engert, et al., 1995) and nuclear factor I (NF1) (Tarapore, et al., 1997), and therefore, the increase in invasive potential seen in SKI-deficient cells could also partially be due to changes in gene expression that are independent of TGFβ superfamily signaling.

It is possible that the level of closure observed in the cell scrape assays could be due in part to proliferation of the cells at the border of the scrape; however, it is unlikely that the closure could be due to proliferation alone. The rate of proliferation in the *SKI*-knockdown cells was not significantly different from that of the control treated cells. The NCCIT cells have a population doubling time of approximately 18 hours (data not shown). Migration was observed over a 20 hour time period, thus the NCCIT cells barely had time to complete one round of division. In the cell scrape assays, the distance that cells have migrated into the scrape area is greater than one cell diameter in thickness at 20 hours compared to the scrape at 0 hours. Furthermore, the data from the cell scrape assays are complemented by the Matrigel invasion assays, which require cells to migrate through a membrane coated with Matrigel. Therefore, cellular proliferation cannot fully account for the percentage closure observed.

There is further evidence to suggest that TGFβ superfamily signaling molecules may be involved in metastasis of testicular cancer. The TGFβ superfamily has been implicated in the allocation and migration of primordial germ cells (PGCs), the precursors to adult germ cells, during development. BMP4 and BMP8b, members of the TGFβ superfamily, have been shown to be

required for the allocation of PGCs in the mouse and also appear to be involved in PGC migration (Dudley, et al., 2007; Lawson, et al., 1999; Pesce, et al., 2002; Ying, et al., 2000; Ying, et al., 2001). Additionally, TGFβ1 has a chemotropic affect on PGCs, and, thus, may be involved in germ cell migration (Godin and Wylie, 1991). This suggests that loss of function of SKI may contribute to the metastasis of testicular cancer by allowing uncontrolled signaling from the TGFβ superfamily, which may function to increase the migration of TGCTs through a chemotropic effect, promoting the dissemination of tumor cells. It has been proposed that testicular cancer metastasis is related to the migration process of PGCs, partially based on common sites of metastasis being related to the path of migrating PGCs (Chaganti, et al., 1994). Thus genes involved in PGC migration are of interest as potential target genes promoting testicular cancer metastasis.

The data presented within text show that decreased SKI expression appears to promote a more invasive phenotype in NCCIT cells. Since SKI is known to be a co-repressor of the TGFβ superfamily, the next goal of this research was to determine if loss of function of SKI contributes to testicular cancer metastasis by allowing uncontrolled signaling from the TGFβ superfamily. To address this question, we studied the response of SKI- knockdown NCCIT cells to both TGFβ and BMP to determine if these members of the TGFβ superfamily further enhanced the increased invasive potential seen in NCCIT cells with reduced SKI expression.

TGFβ has been shown to promote metastasis in the later stages of some cancers (Jakowlew, 2006; Tang, et al., 2003). Le Scolan et. al. (2008) reported

that SKI plays an antitumorigenic role in MDA-MB-231 breast cancer cells and A549 lung cancer cells. Expression of SKI was high in MDA-MB-231 breast cancer cells and low in A549 lung cancer cells. Experimentally reducing levels of SKI in these cells resulted in an enhanced ability of these cells to invade. Furthermore, they found that treating these cells with TGFβ reduced SKI expression through E3 ubiquitin ligase-mediated degradation, and they suggest that this pathway plays an important role in the ability of SKI to repress metastasis. Taken together, these data suggest that reduced levels of SKI in cancer cells may promote metastasis by allowing unregulated signaling of TGFβ. However, our data shows that addition of TGFβ to shSKI NCCIT cells did not affect the migration of these cells *in vitro*. Furthermore, the addition of TGFβ blocking antibody also did not affect the migration of shSKI NCCIT cells. This indicates that in NCCIT cells with reduced SKI expression, TGFβ does not affect migration.

There are several possible reasons to explain why TGFβ may not have an effect on the migration of SKI-deficient NCCIT cells. One possible explanation is that the components of the TGFβ signaling pathway are not present or are not active in NCCIT cells. This prompted us to look at the activity of the TGFβ pathway in these cells by investigating SMAD phosphorylation. TGFβ signals through intracellular transcription factors, SMAD2 and SMAD3. When TGFβ activates the cell surface receptors, these receptors induce the phosphorylation of SMAD2 and SMAD3, which then form a complex with SMAD4. This complex is able to translocate to the nucleus and activate transcription of TGFβ target

genes (Luo, 2003). Western blots showed that SMAD2 is expressed in shNCCIT cells. However, even after a two hour incubation with TGFβ, phosphorylated SMAD2 (p-SMAD2) was only detected at very low levels. Based on microarray data, ELISA, and Superarray PCR data (data not shown) other components of the TGFβ signaling pathway, including TGFβ receptors and TGFβ, are expressed in NCCIT cells. Treatment with TGFβ did result in a slight induction of p-SMAD2. However, because this induction was only slight, it is possible that an inhibitor of TGFβ may be preventing strong signaling from this pathway, even in the absence of SKI.

LEFTY1 is an inhibitor of the TGFβ superfamily which functions by preventing SMAD phosphorylation (Ulloa, et al., 2001). LEFTY1 was shown via the microarray analysis, and confirmed by real time PCR analysis, to be upregulated in shSKI NCCIT cells compared to shNC NCCIT cells. Increased LEFTY1 expression may contribute to the lack of response seen in shSKI NCCIT cells treated with TGFβ. TGIF, which interacts with SMAD2 and SMAD3 to prevent TGFβ signaling, was also shown to be expressed in shSKI NCCIT cells by Superarray PCR analysis (data not shown). In addition, the inhibitory SMAD, SMAD7, was also increased in shSKI NCCIT cells compared to shNC NCCIT cells according to microarray analysis. Although SMAD7 has been shown to interfere with both TGFβ and BMP signaling, there may be multiple inhibitors acting against TGFβ signaling that weaken that branch of the TGFβ superfamily while allowing BMP signaling. Finally, our data showed that *SKI* mRNA expression is decreased in TGCTs compared to normal testis samples, but *SNO*

mRNA levels were not altered. Both SKI and SNO have been shown to repress TGFβ signaling (Akiyoshi, et al., 1999; Luo, et al., 1999), but only SKI has been shown to interfere with BMP signal transduction (Takeda, *et al.*, 2004; Wang, *et al.*, 2000). This suggests that SNO may also function to inhibit TGFβ signaling, even in the absence of SKI, but BMP signaling could still be active.

In addition to inhibiting TGFβ signaling, SKI also functions as a co-repressor of BMP, another member of the TGFβ superfamily, through its interaction with SMAD1/5/8 – SMAD4 complex (Takeda, et al., 2004; Wang, et al., 2000). Therefore, we next tested the effect of adding BMP to our cell scrape assays to determine if this enhanced migration in SKI knockdown NCCIT cells. When exogenous BMP4 was added to cell scrape assays, migration was significantly increased in shSKI NCCIT cells, but not in shNC NCCIT cells. Furthermore, addition of noggin, an extracellular BMP inhibitor, was able to reverse the effects of BMP seen in shSKI cells, and addition of noggin alone reduced migration in shSKI NCCIT cells back to the level of migration seen in untreated shNC NCCIT cells. Noggin treatment, alone, also slightly reduced migration in shNC NCCIT cells. This suggests that treating both shSKI and shNC NCCIT cells with noggin may inhibit the effects of any endogenous BMP expressed by NCCIT cells. This data indicates that reduced levels of SKI in NCCIT cells enhance the migration of these cells by allowing increased BMP signaling.

The activity of the BMP pathway was also considered by testing the cells for the induction of phosphorylated R-SMADs. BMP signals are transmitted

intracellularly by SMADs 1, 5, and 8 (Yamashita, et al., 1996). Activation of cell surface receptors by BMP results in phosphorylation of SMAD1/5/8, which then forms a complex with SMAD4, and this complex activates the transcription of BMP target genes. ShSKI and shNC NCCIT cells incubated with BMP4 for two hours showed strong expression of phospho-SMAD1/5/8 (p-SMAD1/5/8). Phosphorylation of SMAD1/5/8 was inhibited in the presence of noggin. This indicates that the BMP pathway is active in shNCCIT cells. As expected, phosphorylation of SMAD1/5/8 was not affected by SKI expression because SKI's inhibition of BMP signaling acts downstream of SMAD phosphorylation. The data collected on the effects of BMP in SKI-deficient NCCIT cells suggest that the BMP family may be a potential target to consider in the development of new therapies to treat patients with metastatic testicular cancer. However, further research is necessary to determine the efficacy of using BMP inhibitors to prevent testicular cancer metastasis *in vivo.*

Because SKI functions primarily as a transcription factor and co-repressor, microarray analysis was performed to identify potential target genes with altered expression in SKI-deficient cells that may contribute to an invasive phenotype and play a role in testicular cancer progression. As expected, one of the gene sets identified through microarray analysis by GSEA as being upregulated in SKI-knockdown cells compared to control cells was the "Transforming Growth Factor Beta Receptor Signaling Pathway" (Table 3.3). Several genes involved in TGFβ superfamily signaling were also identified by SAM analysis as being differentially expressed in shSKI NCCIT cells compared to shNC NCCIT cells (Table 3.6).

SKI is a co-repressor of the TGFβ superfamily, therefore, it would be expected that some members of this signaling pathway would be affected by depleting SKI. In studying the results from the GSEA and SAM analyses, some common genes were identified as being upregulated in SKI-knockdown NCCIT cells, including SMAD7, LEFTY1, and MAP3K7. As previously discussed, SMAD7 and LEFTY1 are both inhibitors of TGFβ signaling, thus increased expression of these genes may contribute to the lack of response seen with the addition of TGFβ to shSKI NCCIT cells in the cell scrape migration assays.

"Regulation of MAP kinase activity" was one of only three gene sets identified by GSEA that met not only the dual criteria of FDR less than 0.25 and nominal p-value less than 0.05, but also the more stringent test of FWER less than 0.05. Interaction between the MAPK and BMP pathways has previously been shown to be involved in cell fate determination. FGF8 and IGF2, both of which activate MAPK signaling, have been shown to inhibit nuclear localization of the BMP-responsive SMAD1; this interaction can function to inhibit BMP signaling, preventing differentiation down the mesoderm lineage and favoring neural differentiation (Massague, 2003). However, these specific genes were not identified through GSEA as being enriched in the SKI-knockdown cells. Since MAPK signaling can function to inhibit SMAD1-mediated transcription by blocking nuclear localization of SMAD1, it is possible that upregulation of MAPK signaling may be a compensation mechanism for the increased activity of the BMP pathway in SKI-knockdown cells. Activation of the Ras/MAPK pathway has also been shown to inhibit SMAD2 nuclear localization, preventing TGFβ signaling

(Massague, 2003). This suggests that upregulation of MAPK signaling in SKI-knockdown cells may contribute to the lack of response seen with the addition of TGFβ to the shSKI NCCIT cells. A specific link between SKI and the MAPK pathway has not been shown. Thus our data suggest that there may be further interaction between MAPK signaling and TGFβ superfamily signaling.

One of the genes identified by GSEA as belonging to the gene group "Regulation of MAP kinase activity" was CXCR4. CXCR4 was also shown to be upregulated by SAM analysis in shSKI NCCIT cells compared to shNC NCCIT cells, and this increased expression was confirmed via real time PCR. CXCR4/SDF1 signaling partners have previously been shown to be involved in both PGC migration (Doitsidou, et al., 2002; Molyneaux, et al., 2003) and testicular cancer metastasis (Gilbert, et al., 2009); SDF1 acts as a chemoattractant for the germ cells and cancer cells, which express CXCR4. SDF1 is known to be expressed in sites of the body to which testicular cancer frequently metastasizes, including lymph nodes, lung, liver, and brain (Gilbert, et al., 2009; Muller, et al., 2001), further implicating these signaling partners as potential mediators of testicular cancer metastasis.

We were interested to know if *CXCR4* is a possible target of BMP signaling. Real time PCR analysis showed that, at the concentration used, *CXCR4* expression was not significantly changed when shNC or shSKI NCCIT cells were incubated with either BMP or noggin. These data suggest that the increase in *CXCR4* expression in shSKI NCCIT cells compared to shNC NCCIT cells may be independent of BMP signaling. SDF1, the signaling ligand partner

of CXCR4, has previously been shown to be upregulated by BMP (Yang, et al., 2008). However, at the concentration used, real time PCR showed no change in *SDF1* mRNA expression in response to treatment of either shNC or shSKI NCCIT cells with BMP4.

Although *CXCR4* and *SDF1* expression were not affected by BMP4 treatment of shNCCIT cells, *CXCR4* expression was upregulated in SKI-knockdown NCCIT cells compared to NCCIT cells stably transfected with the negative control shRNA vector. Thus, the effect of SDF1 on the ability of these cells to invade *in vitro* was tested using a Matrigel invasion assay. Addition of SDF1 to the companion wells of the Matrigel invasion chambers significantly enhanced the invasion of shSKI NCCIT cells but had no effect on shNC NCCIT cells. Furthermore, AMD3100, a potent and specific inhibitor of CXCR4 (Broxmeyer, et al., 2005; Hatse, et al., 2002), was able to reverse the effects of SDF1 in shSKI NCCIT cells, but, again, no effect was seen in shNC NCCIT cells. These data provides good evidence that increased expression of CXCR4 in SKI-knockdown NCCIT cells contributes to the enhanced invasive phenotype seen in these cells.

Previous data have implicated the CXCR4/SDF1 signaling partners in testicular cancer metastasis (Gilbert, et al., 2009). Our data support the role of CXCR4/SDF1 signaling in testicular cancer metastasis, and our data also adds additional information on the upstream regulation of CXCR4 expression, which gives a better overall picture of the mechanism by which testicular cancer cells may acquire a more invasive phenotype. These data, taken together with

previous data showing that BMP also enhances migration in shSKI NCCIT cells, suggests that decreased SKI expression in NCCIT testicular cancer cells promotes migration and invasion through both BMP-dependent and BMP-independent pathways. This collection of data also implicates CXCR4 and SDF1 as potential targets for testicular cancer therapies. Again, further research is needed to determine the effects of using inhibitors CXCR4 and SDF1 signaling *in vivo*.

Several genes belonging to the WNT signaling family were identified by SAM analysis as being differentially expressed in shSKI compared to shNC NCCIT cells. Interaction between SKI and the WNT family has previously been shown (Chen, et al., 2003). In melanoma cells, forcing the expression of SKI stimulated cellular proliferation by activating the WNT pathway. Chen et. al. (2003) found that SKI binds to FHL2 (four-and-a-half lim domains 2), which binds β-catenin and can either repress or activate β-catenin, depending upon the cell type (Martin, et al., 2002). Forced SKI expression resulted in the activation of FHL2 and β-catenin-responsive gene promoters. These data suggest that, in addition to mediating TGFβ superfamily target gene transcription, SKI can act as a transcriptional regulator of the WNT signaling family by acting as a binding partner for FHL2. This could be a BMP-independent mechanism for gene regulation in our system which merits further exploration.

While several individual genes and gene families of interest were identified as potential participants in testicular cancer metastasis by microarray analysis, there are some notable absences in groups of genes that one might expect to be

involved in the observed invasive phenotype. For example, GSEA did not reveal significant differences in gene sets such as “Cell migration” and “Cell-cell adhesion”; nor were there any significant changes in matrix metalloproteinases (MMPs). Genes in these groups would likely be involved in invasion and metastasis, so one might expect these sets to be significantly altered in the shSKI NCCIT cells, which had enhanced invasive potential. There are several possible explanations for the absence of a significant difference in these gene sets. (1) There are limitations in the GSEA software in that it is not yet capable of identifying significant changes in gene sets that have both up- and down-regulated genes. The software currently only recognizes significantly up-regulated genes. It is quite possible that certain genes involved in the prevention of cellular migration, for example, may be down-regulated in SKI-knockdown cells, contributing to the enhanced invasive phenotype. However, these changes, though potentially important to the observed phenotype, would not be recognized by GSEA. (2) The gene sets used in this software are constructed based on the available literature. They are not all-inclusive, and it is possible that there are additional genes involved in cellular migration or cell-cell adhesion that are not represented in the GSEA gene sets, but that may be important in promoting invasion in NCCIT cells. (3) GSEA considers the gene set as a whole entity. While several members of the gene set may not be significantly enhanced, there still may be a few members of that gene set that are significantly altered in the shSKI NCCIT cells. (4) The absence of significant changes in groups of genes such as MMPs may be explained by the fact that these genes may not be

transcriptionally regulated by SKI, but could possibly be altered at the level of protein activity. MMPs are made as propeptides and require cleavage for activation (Fanjul-Fernandez, et al., 2009). It is possible that decreased SKI expression my lead to the transcription of certain genes that promote the eventual cleavage and activation of MMPs. Further studies could be performed to determine if the activity of certain MMPs is increased in SKI-knockdown NCCIT cells to assess whether the activation of MMPs plays a role in the enhanced invasive phenotype observed in SKI-deficient NCCIT cells. These studies involve exposing the cells to gels containing the specific substrate for a particular MMP, and measuring the breakdown of that substrate.

Although MMP expression was not significantly changed in shSKI compared to shNC NCCIT cells, the microarray data did show an increase in ADAMTS1, a metalloproteinase from another gene family, in shSKI NCCIT cells compared to shNC NCCIT cells, which was confirmed by real time PCR. ADAMTS1 is a member of the ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin repeats) family. This gene family is involved in proteolytic processing of transmembrane proteins, cell adhesion, and cell signaling (Mochizuki and Okada, 2007). ADAMTS1 has been shown to be upregulated in cancers with advanced metastatic potential, including breast cancer (Kang, et al., 2003) and pancreatic cancer (Masui, et al., 2001). It has also been reported that ADAMTS1 forced overexpression in human lung carcinoma cells promotes tumor development and enhances tumor cell proliferation after injection in into SCID mice (Rocks, et al., 2008). These data imply that increased ADAMTS1

expression may promote cancer progression. However, more research is necessary to determine if ADAMTS1 expression is regulated by SKI and to delineate the role of ADAMTS1 in testicular cancer.

The microarray data revealed several genes and gene families that are potential targets of SKI and/or may be involved in an enhanced metastatic phenotype in testicular cancer. From the list of genes generated by the microarray analysis, a subset of genes associated with invasion, migration, or TGFβ superfamily signaling was chosen to assess whether their promoters contained SMAD binding elements. These genes include ADAMTS1, CXCR4, FST, LEFTY1, MAP3K7, SMAD7, SPARC, and WNT3. In addition, SMAD6 and MMP9 were used as controls. The purpose of this assessment was to determine if these genes might be downstream signaling targets of either BMP or TGFβ. The presence of a SMAD binding element within the promoter region of these genes would indicate that the gene may be transcriptionally activated by BMP or TGFβ. Although no true SMAD consensus sequence has yet emerged, a literature search revealed several potential SMAD 2/3 and SMAD1/5/8 binding sequences. Genes containing binding sites for SMAD2/3 within their promoter region would potentially be activated in response to TGFβ signaling, while genes containing SMAD1/5/8 binding sites would potentially be activated by BMP.

Since BMP treatment of SKI-knockdown cells resulted in an enhanced invasive phenotype, but TGFβ treatment did not elicit a response, we were interested in comparing the presence of BMP and TGFβ response elements in potential target genes identified through the microarray analysis. The literature

search revealed that there is some overlap in the sequences that have been designated as BMP and TGFβ responsive. In this analysis, three BMP-response elements and eight TGFβ-response elements were used. However, 2 of the sequences, GTCTAGAC (Li, et al., 2001; Stopa, et al., 2000; Zawel, et al., 1998) and CAGACA (Dennler, et al., 1998; Jonk, et al., 1998; Katagiri, et al., 2002), have been described in the literature as being both BMP and TGFβ responsive. It was also reported that SMAD4 is able to bind to the sequence GTCTAGAC in the promoter of ID1 (Katagiri, et al., 2002), and this provides an explanation for the lack of specificity in regards to BMP and TGFβ responsiveness. SMAD4 acts as the co-SMAD; it is able to form a complex with either BMP-induced SMAD1/5/8 or TGFβ-induced SMAD2/3 (Luo, 2003). Therefore if the sequence GTCTAGAC is recognized by SMAD4, expression of genes with this element in their promoter could be activated by either BMP or TGFβ or both. The third sequence, GCCGnCGC, has thus far been described to be specific to the BMP-responsive SMADS (Benchabane and Wrana, 2003; Ishida, et al., 2000; Kim, et al., 1997; Kusanagi, et al., 2000).

For the promoter analysis, SMAD6 was used as a positive control. SMAD6 was not differentially expressed in shSKI and shNC NCCIT cells, however, it has been reported that SMAD6 is BMP-responsive and contains the SMAD1 binding motif, GCCGnCGC (Ishida, et al., 2000). The current analysis found the presence of multiple copies of the GCCGnCGC binding motif, as well as multiple copies of the two other BMP/TGFβ response elements, CAGACA and

GTCTAGAC. This shows that the Motif locator tool was capable of recognizing the sequences.

ADAMTS1, CXCR4, LEFTY1, SMAD7, MAP3K7, and WNT3 were all upregulated in SKI knockdown cells according to our microarray data. CXCR4, SMAD7, and WNT3 promoters each contained all three of the BMP response elements, including the BMP-specific response element. This suggests that their up-regulation in shSKI NCCIT cells may be due to increased BMP signaling. In agreement with these data, SMAD7 has previously been shown to be responsive to BMP (Benchabane and Wrana, 2003). Although CXCR4 does appear to contain these BMP response elements, the real time PCR data presented in this text (Figure 3.14) did not show up-regulation of CXCR4 in response to treatment of shSKI NCCIT cells with BMP4. It is possible that the concentration of BMP4 used in these experiments was not sufficient to induce transcription of CXCR4, and further experiments are needed to determine if different concentrations of BMP might elicit an increase in CXCR4 mRNA expression. It is also possible that the induction caused by SKI-knockdown is already maximal. To test this, cell lines with varying levels of SKI expression could be treated with BMP to determine if cells with intermediate SKI knockdown showed a response. Real time PCR could also be done for SMAD7 and WNT3 to determine if their expression is increased in shSKI NCCIT cells upon treatment with BMP.

LEFTY1 and MAP3K7 both contained multiple copies of the BMP-specific response element, GCCGnCGC, and the BMP/TGFβ response element, CAGACA, suggesting they also may be up-regulated by BMP and/ or TGFβ.

ADAMTS1 contained multiple copies of both of the BMP/TGFβ response elements, GTCTAGAC and CAGACA. Additionally, ADAMTS1 was the only gene queried that contained one of the TGFβ-specific response elements, ACAGACAGACAC, further suggesting possible regulation by TGFβ.

FST (follistatin) and SPARC (secreted protein acidic and rich in cysteine) were both down-regulated in shSKI compared to shNC NCCIT cells. Yet both of these genes contained multiple copies of both BMP/TGFβ response elements, and FST also contained multiple copies of the BMP-specific response element, suggesting they also may be regulated by BMP. The presence of BMP response elements within the promoters of these two down-regulated genes would not support the hypothesis that decreasing SKI expression in NCCIT cells confers increased BMP signaling and allows increased expression of BMP target genes. FST is a negative regulator of the TGFβ superfamily, primarily activin (Ogino, et al., 2008). It has previously been shown to be regulated by SMAD3 through a SMAD binding element (Blount, et al., 2009), therefore, it may primarily be regulated by activin and may not be BMP responsive. SPARC is involved in matrix remodeling and has been implicated in cancer progression and metastasis; however, SPARC's role in tumorigenesis appears to be context and cell-type dependent as it appears to act as a tumor suppressor in some tissues (Arnold and Brekken, 2009). SPARC's role in testicular cancer has not been studied to date. Since SPARC did not contain the BMP-specific response element, it may be regulated by TGFβ and not by BMP. It is also possible that there are inhibitors

of FST and SPARC transcription present in shSKI NCCIT cells that prevent their transcription despite the presence of SMAD binding elements within the promoter.

Finally, MMP9 was also used for the promoter analysis. According to the microarray data, MMP9 was not differentially expressed in shSKI and shNC NCCIT cells, and, therefore, does not appear to be responsive to SKI expression. However, the promoter analysis shows that the MMP9 promoter does contain both of the BMP/TGFβ response elements. It does not contain the BMP-specific response element. Again, it is possible that this gene may be regulated by TGFβ, which did not appear to have an effect in our system, and not by BMP. Because two of the sequences used for this analysis have been described to be responsive to both BMP and TGFβ induced SMADs, it is somewhat difficult to draw a conclusion about the genes that have these two motifs in their promoters. They could potentially be activated by TGFβ or BMP or both. The SMAD transcription factor complex requires several binding partners to activate transcription of target genes, and these binding partners account for some of the specificity between TGFβ and BMP activated transcription (Massague and Wotton, 2000). Certain DNA binding cofactors, such as FAST (forkhead activin signal transducer), are specific to SMAD2/3-SMAD4 complexes, while others, such as OAZ (OLF1/EBF-associated zinc finger protein), bind to the BMP responsive SMAD complex, SMAD1/5/8-SMAD4 (Massague and Wotton, 2000). Therefore, future studies should focus on the identification of these binding factors to determine which are present in NCCIT cells and if potential target genes also contain motifs for these cofactors.

From this collection of data, a model for the acquisition of a more invasive phenotype in testicular cancer cells is proposed that involves reduced levels of SKI, which results in aberrant expression of both BMP-dependent and BMP-independent downstream target genes (Figure 4.1). In this model, a primitive germ cell acquires a mutation, such as protection from antiproliferative signaling, forming a precancerous cell. This precancerous cell then undergoes additional transformation to gain the properties of a cancerous cell, including uncontrolled proliferation, evasion from apoptosis, and immortalization. The acquisition of these properties results in the formation of an intratubular germ cell neoplasia, however further mutations are required for the ITGCN to invade the surrounding tissue. The data presented within this text indicate that loss of function of SKI may promote this transition to a more invasive phenotype. As shown in the model, loss of SKI expression results in uncontrolled BMP signaling as well as upregulation of other BMP-independent genes. Uncontrolled BMP signaling allows increased expression of BMP target genes, some of which promote testicular cancer metastasis. Additionally, loss of SKI expression promotes the expression of other genes, such as CXCR4, that promote testicular cancer metastasis. According to this model, the increased expression of CXCR4 promotes metastasis to distant sites where SDF1, the ligand for CXCR4, is expressed; these include the lymph nodes, lung, liver, and brain. The changes in gene expression brought on by loss of SKI result in invasion of the surrounding lymph nodes by the cancerous cells and dissemination to distant organs.

The microarray data presented here indicates that loss of SKI expression enhances the expression of genes in other signaling families, such as the WNT and MAP kinase families, thus these signaling families may also be involved in promoting an invasive phenotype in testicular cancer cells. For example, SKI has been shown to be a binding partner of FHL2, which can activate or repress β-catenin, depending on cell type (Chen, et al., 2003). Therefore, decreased SKI expression could potentially result in lack of interaction with FHL2 and lack of repression of β-catenin in testicular cancer cells. β-catenin has previously been correlated with an invasive phenotype in breast cancer (Niu, et al., 2009) and colon cancer (Suzuki, et al., 2008). Further research on the interactions of these pathways in testicular cancer cells is necessary to determine their importance in testicular cancer metastasis.

The data presented here provide further insight into testicular cancer by identifying SKI as a potential regulator of male germ cell tumor metastasis. These data show that testicular cancer cells with decreased SKI expression have a greater ability to invade and metastasize. Furthermore, the data provide evidence that the BMP pathway plays a role in this process by further increasing the metastatic potential of SKI-deficient testicular cancer cells. Noggin, a BMP inhibitor, was able to reverse the effects of BMP in SKI-knockdown NCCIT cells. This suggests that the BMP pathway may be a potential target for the development of new therapies to treat metastatic testicular cancer. The use of noggin, or other small molecule inhibitors of BMP, may be useful in preventing progression of testicular cancer and warrants further research.

In accordance with previous findings (Gilbert, et al., 2009), these data confirm the importance of CXCR4 and SDF1 signaling partners in testicular cancer metastasis and also, for the first time, indicate that SKI may play a role regulating the expression of CXCR4. This implicates CXCR4 and SDF1 as potential targets for therapies to treat testicular cancer metastasis as well. In the current studies, AMD3100 was effective at blocking the effects of CXCR4/SDF1 signaling partners, revealing AMD3100 as a candidate for the treatment of metastatic testicular germ cell tumors. AMD3100 is a small molecule inhibitor that is highly specific for CXCR4 (Broxmeyer, et al., 2005; Hatse, et al., 2002) and has been used in clinical trials as an anti-HIV drug because CXCR4 is a co-receptor used by HIV to enter T-lymphotropic cells (De Clercq, 2005; De Clercq, 2009; Hendrix, et al., 2000). In these trials, AMD3100 (Plerixafor) was very effective in inhibiting HIV-1 and HIV-2 viral replication. While no toxicity was observed, the main side effect in patients treated with AMD3100 was leukocytosis, or increased white blood cells (De Clercq, 2005). The discovery of this side effect led to clinical trials using AMD3100 as a hematopoetic stem cell mobilizer to treat patients with non-Hodgkins lymphoma and multiple myeloma (Stiff, et al., 2009). These clinical trials have shown that AMD3100 is safe for use in humans, but further studies are necessary to determine the efficacy of this drug in the treatment of testicular cancer metastasis.

Additionally, several potential targets of testicular cancer metastasis were revealed by microarray analysis. These genes were up- or down-regulated in SKI-deficient NCCIT cells and further studies to elucidate the interaction between

these individual genes or signaling families and SKI will likely provide additional targets for the development of gene therapies.

Previous studies have yielded conflicting roles for SKI in the metastatic process (Reed et al., 2001; Le Scolan et al., 2008; Zhu et al., 2008). The studies presented within this text are the first to implicate SKI as regulator of metastasis in testicular cancer; this is in accordance with other studies showing that loss of SKI function promotes invasion in some types of cancer, including lung and breast (Le Scolan, et al., 2008; Zhu, et al., 2007). However, other studies have shown that increased SKI expression correlates with cancer progression in melanoma cells (Reed*, et al.*, 2001). Taken together, these findings reveal that metastasis is a very complex process that depends upon the various signals present within the microenvironment of specific tissues that guide a cancerous cell to invade the basement membrane and metastasize to distant tissues. When compared with previous data, it appears that SKI may function to promote tumor progression in some cell types, such as melanoma cells, but inhibit cancer metastasis in other cell types, such as testicular cancer cells. Furthermore, the findings on CXCR4/SDF1, taken together with previous data (Gilbert*, et al.*, 2009), show that not only are the factors present within the microenvironment of the primary tumor important, but factors, such as SDF1, found in the sites of metastasis must also be considered when attempting to develop therapies for the treatment of malignant disease.

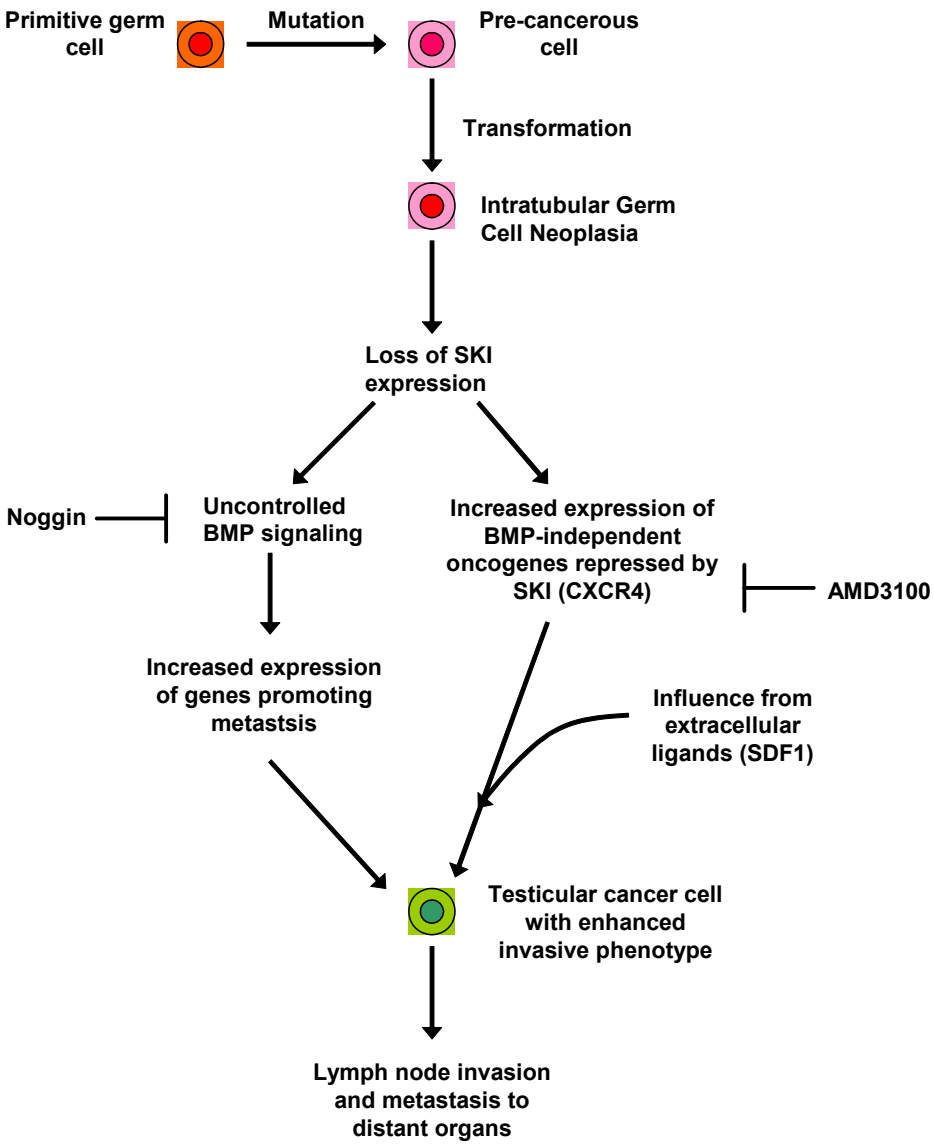

Figure 4.1. Proposed mechanism for SKI's role in testicular tumorigenesis. A primitive male germ cell acquires a mutation, forming a precancerous cell. This precancerous cell then undergoes transformation, gaining additional properties of a cancerous cell, to form an ITGCN. Loss of SKI expression in ITGCN results in changes in gene expression, both BMP-dependent and BMP-independent, that contribute to an invasive phenotype, allowing the cancerous cells to invade the adjacent lymph nodes and disseminate. Noggin is able to abrogate the effects of BMP in this model. BMP-independent changes in gene expression, including increased expression of CXCR4, further contributes to the metastatic potential. CXCR4 interacts with its signaling partner SDF1, to promote metastasis to tissues expressing SDF1. This interaction is inhibited by AMD3100.

SUMMARY

We have shown that *SKI* expression is reduced in human testicular germ cell tumors compared to normal human testis. To our knowledge, this is the first study showing a decrease in *SKI* expression in testicular cancer. Our studies have also shown that experimentally reducing SKI in a testicular cancer cell line, NCCIT, results in an increased ability of these cells to migrate and invade. In parallel with this finding, forcing the overexpression of SKI in NCCIT cells resulted in a less invasive phenotype. This data suggests that SKI may function as an inhibitor of metastasis in testicular cancer.

Through a microarray study, we found that a large number of genes are differentially expressed in NCCIT cells stably transfected with a shRNA vector for SKI knockdown compared to NCCIT cells stably transfected with a negative control shRNA vector. These differentially expressed genes are potential target genes of SKI that may be involved in the increased invasive phenotype of SKI-deficient NCCIT cells. Furthermore, we were able to identify the presence of BMP response elements within the promoter regions of certain genes, including ADAMTS1, CXCR4, LEFTY1, SMAD7, and WNT3, from the microarray, indicating that these genes are potentially upregulated by BMP signaling in SKI-deficient NCCIT cells.

We also found that TGFβ does not enhance migration in SKI-knockdown NCCIT cells. However, BMP does enhance the migration of SKI-knockdown

NCCIT cells, and noggin, a BMP inhibitor, blocks the effect of BMP on SKI-knockdown cells. This indicates that decreased SKI expression in testicular cancer may confer increased sensitivity to BMP-receptor driven signaling, thereby enhancing the metastatic phenotype of these cells. Furthermore, we found that *CXCR4* expression is upregulated in SKI-knockdown cells. CXCR4, and its signaling partner, SDF1, have previously been implicated in testicular cancer metastasis. We found that the CXCR4/SDF1 signaling partners promoted invasion in SKI-deficient cells but not in cells that retained a higher level of SKI expression. This indicates that the absence of SKI is an important factor in testicular cancer metastasis. Furthermore, our data suggest that BMP and CXCR4/SDF1 signaling partners may be potential targets for therapies to treat patients with metastatic testicular cancer. This is an area of research that warrants further investigation.

The microarray data presented in this text also indicate that genes belonging to that the MAP kinase and WNT signaling pathways are up-regulated in SKI-deficient cells. This suggests that these pathways may be regulated at some level by SKI expression. Certain genes in these pathways may also be involved in promoting testicular cancer metastasis. Studying the interaction between these pathways and the BMP pathway, and how SKI regulates their target gene expression will likely further shed light on the mechanisms behind male germ cell tumor metastasis and provide additional targets for testicular cancer therapies.

The data presented in this text identify SKI as a potential tumor suppressor of testicular cancer and provide a mechanism by which the increased invasive phenotype is acquired. This allows a better understanding of the disease process involved in testicular tumorigenesis which may help with the development of treatments for patients with disseminated disease who have a worse prognosis. As scientific advances are made that allow the development of therapies targeted more directly at the testicular cancer cells rather than targeting all rapidly dividing cells, as with chemotherapy and radiation, the patients' quality of life will be enhanced through decreased side effects of cancer treatment.

FUTURE DIRECTIONS

The research presented in this text provides novel information regarding a possible mechanism by which testicular cancer cells acquire an increased ability to invade and metastasize. However, more research is necessary to fully understand the role that SKI plays in testicular cancer. Our data implicates SKI as a tumor suppressor of testicular cancer. There are several mechanisms by which tumor suppressor activity is lost in cancer cells, such as gene silencing modifications to the promoter region, allelic loss due to chromosome instability, and DNA sequence mutation that leads to decreased gene expression or decreased protein activity. Additional studies could be performed to determine the mechanism by which SKI expression is decreased in testicular germ cell tumors compared to normal testis. This information is important because it would further support the role of SKI as a tumor suppressor, and it would allow a better understanding of testicular cancer progression.

Our data suggest that the BMP pathway is involved in the increased invasive phenotype seen in SKI knockdown NCCIT cells. Further investigation into potential BMP target genes would help in understanding exactly how this pathway contributes to invasion in testicular cancer. The microarray analysis provides a good starting point for looking into potential BMP target genes that may promote metastasis of testicular cancer. Identification of these genes may

present new targets for testicular cancer therapies. Determining the importance of SMAD signaling in the increased invasive phenotype observed in SKI-deficient testicular cancer cells would also provide an increased understanding of the importance of the BMP signaling pathway. This could potentially be accomplished using siRNA against SMADs 1, 5, and 8 or dominant negative proteins to determine if SMAD-mediated transduction of BMP signaling is required for the increased ability of SKI-deficient cells to migrate.

In our studies, TGFβ did not affect the invasive potential of SKI-deficient cells. In order to better understand why TGFβ does not function to enhance migration in SKI deficient cells, experiments could be done to identify if this lack of response is due to inhibitors of TGFβ blocking the signal or if some component of the TGFβ signaling pathway is not functioning properly.

Furthermore, several genes were shown to be differentially expressed by microarray analysis in shSKI NCCIT cells compared to shNC NCCIT cells. This microarray analysis was performed on cells that were stably transfected for SKI knockdown or with a negative control vector; thus they do not confirm that loss of SKI directly controls the transcription of these genes. Assays, such as lacz transcription assays, to determine the direct interaction of SKI with the potential target genes would provide a more detailed look at the exact pathways involved in testicular cancer progression. Additionally, the importance of the upregulated target genes identified by microarray analysis in testicular cancer metastasis could be tested by using siRNA or dominant negative proteins to determine if they are required for increased invasive potential in SKI-knockdown NCCIT cells.

Our experiments were performed using NCCIT cells, which are classified as embryonal carcinomas. In reviewing the literature, it appeared that some gene expression profiles were specific to either embryonal carcinomas or seminomas. Our data showed that SKI was decreased in various types of testicular germ cell tumors. Thus, it would be interesting to know if decreased SKI expression had similar effects on seminoma cells as we saw in embryonal carcinoma cells.

Additional experiments studying the effects of the pathways outlined in this text in an *in vivo* model would be very beneficial in the clinical translation of this research to the development of gene-targeted testicular cancer therapies. As mentioned, the data presented here suggest that BMP and the signaling partners, CXCR4/SDF1, promote invasion in SKI-deficient testicular cancer cells, potentially making these molecules attractive targets for the development of new treatments against metastatic testicular cancer. First, the efficacy and safety of inhibitors of these pathways would need to be assessed using an *in vivo* model.

CHAPTER V

METHODS

Tumors

Testicular tumors and normal human testis tissues were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. Other investigators may have received samples from these same tissues. Tissues were frozen in liquid nitrogen and stored at -80°C until analysis. Tumor and normal samples were analyzed for relative gene expression after laser capture microdissection as described below.

Laser Capture Microdissection

Normal and tumor tissue specimens were embedded in Tissue-Tek OCT (VWR, Batavia, IL) medium for frozen sectioning. Five-micrometer sections were processed with the Arcturus HistoGene kit (Molecular Devices, Sunnyvale, CA). Sections were briefly fixed and dehydrated in ethanol, stained with hematoxylin and dried in xylene prior to sample capture using the Arcturus Pixcel IIe (Molecular Devices, Sunnyvale, CA). Tissue captured from normal testes included seminiferous tubules, but not interstitial cells. Tumor cells were isolated avoiding stromal elements. After capture the samples were extracted for RNA using the RNeasy Micro Kit (Qiagen, Valencia, CA). RNA quantity and integrity

were assessed using the 2100 Bioanalyzer and the RNA Pico Kit (Agilent Technologies, Santa Clara, CA).

Cell Culture

NCCIT cells were purchased from American Type Culture Collection (Manassas, VA). NCCIT cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

Materials

TGFβ, TGFβ blocking antibody, BMP4, noggin, and SDF1 were purchased from R & D Systems (Minneapolis, MN). AMD3100 was purchased from SigmaAldrich (St. Louis, MO).

Transient siRNA Transfections

For transfection protocols, NCCIT cells were plated in antibiotic-free RPMI medium, and, on the day of transfection, the medium was changed to Opti-MEM Reduced Serum Medium (Invitrogen, Carlsbad, CA) supplemented with 2% FBS. For siRNA knockdown of *SKI*, three individual siRNAs specific to *SKI* and a non-specific control siRNA were purchased from Invitrogen. Cells were plated in 6 well plates at $5x10^5$ cells/well. Twenty-four hours after plating, when the cells were approximately 60-70% confluent, the cells were transfected with one of the

three *SKI* siRNAs or the non-specific control siRNA at a final concentration of 20 μM siRNA. Mock transfections, using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), were performed to ensure that there were no adverse consequences from the transfection procedure. Transfection efficiency was assessed both visually and through the use of flow cytometry on cells transfected with Block-It (Figure 5.1), a fluorescently tagged oligonucleotide, as described by the manufacturer (Invitrogen, Carlsbad, CA). Transfection efficiencies were routinely greater than 80%. Knockdown of *SKI* mRNA and protein levels were assessed at 24, 48, and 72 hrs, using real time PCR and western blotting, respectively.

Flow Cytometry

Cell culture medium was aspirated from cells, and the cells were washed with PBS prior to collection via trypsinization. Cells were spun down, washed with PBS, and resuspended in 1% paraformaldehyde/PBS solution. Ten thousand events were analyzed using the BD FACSaria with the FITC-A laser. To determine the transfection efficiency, the percentage of cells positive for FITC-A was determined using BD FACSDiva software.

SKI Expression Vector Transient Transfection of NCCIT Cells

To force the expression of SKI in NCCIT cells, a SKI expression vector, generously donated by Dr. Estella Medrano, Baylor College of Medicine, was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to

the manufacturer's protocol. Briefly, NCCIT cells were plated in 6 well plates at $5x10^5$ cells/well in medium without antibiotics. Twenty four hours after plating, the medium was replaced with OptiMEM supplemented with 2% FBS, and the cells were transfected with 5μg of either the SKI expression vector or the empty vector (Invitrogen). *SKI* mRNA and protein levels were assessed at 24, 48, and 72 hours by Real Time PCR and western blotting, respectively.

Generation of Stably Transfected SKI-knockdown NCCIT cells

NCCIT cells were plated at $3.5X10^5$ in antibiotic-free medium, and, on the day of transfection, the medium was changed to Opti-MEM Reduced Serum Medium (Invitrogen, Carlsbad, CA) supplemented with 2% FBS. Transfections were performed on cells that had been passaged less than 5 times. Pre-designed SureSilencing™ shRNA vectors specific to SKI (shSKI) and an shRNA negative control vector (shNC) were purchased from SuperArray Bioscience Corporation (Frederick, MD). The cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), according to manufacturer's suggestions. Transfected cells were selected using 600 μg/mL Geneticin because the shRNA vectors contained a neomycin-resistance gene. Clones were established from single cells and were analyzed for SKI expression via Real Time PCR and western blotting. Stably transfected NCCIT cells were cultured in RPMI 1640, supplemented with 10% FBS and 300 μg/mL Geneticin (Invitrogen, Carlsbad, CA). Stably transfected NCCIT cells are denoted shNCCIT as a group. NCCIT cells stably transfected with the negative control

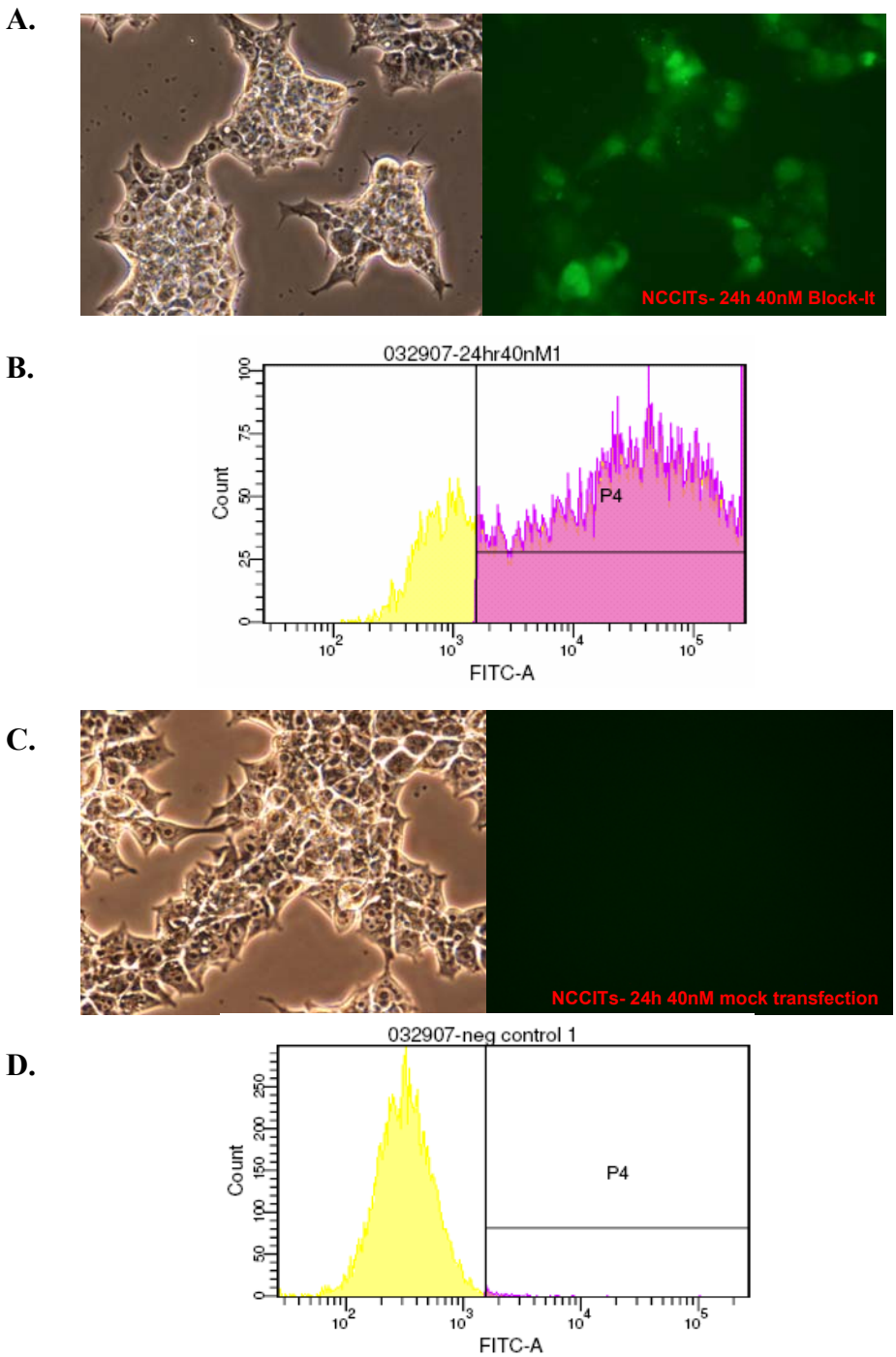

Figure 5.1. Transfection efficiency assessed by Block-It transfection. NCCIT cells transfected with Block-It were assessed both visually and by flow cytometry for transfection efficiency. (A) Microscopic assessment of NCCIT cells transfected with Block-It. (B) Flow cytometry analysis of NCCIT cells transfected with Block-It. (C) Microscopic assessment of NCCIT cells mock-transfected with lipofectamine 2000. (D) Flow cytometry analysis of NCCIT cells mock-transfected with lipofectamine 2000.

shRNA vector are denoted shNC NCCIT, and NCCIT cells stably transfected with the SKI shRNA vector are denoted shSKI NCCIT. SKI knockdown was routinely assessed via real time PCR and western blotting.

Real Time PCR

Total RNA was extracted from NCCIT cells using TRIzol (Invitrogen, Carlsbad, CA). Relative levels of gene expression were measured by real-time polymerase chain reaction (PCR) using Taqman® probes and the 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). First strand cDNA was synthesized from 2 ng total RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Taqman® probes for *SKI, SNO, CCND2 LFT1, ADAMTS1,* and *CXCR4, SDF1,* and *18S* ribosomal RNA were purchased from Applied Biosystems (Foster City, CA). Probes for 18S ribosomal RNA were used as the endogenous control. Relative expression levels for *SKI, SNO, CCND2 LFT1, ADAMTS1,* and *CXCR4, SDF1* were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data for the tumor samples were normalized to the endogenous control and to the normal tissues. Data for siRNA treated cell lines were normalized to the endogenous control and to cells transfected with the non-specific control siRNA.

Western Blotting

Protein was extracted from NCCIT, N-TERA2/D1 (NT2/D1), HepG2, HMEC, MCF7, T47D, A549, SbCl2, WM1366, WM9, HCT116, SK-N-AS, and SK-

N-SH cells with lysis buffer containing 50mM Tris, 150mM NaCl, and 0.1% IGEPAL (Cobb, et al., 1997). Total protein concentration was determined by Bradford assay. Twenty micrograms of total protein lysate were run on a 10% SDS-PAGE gel (BioRad, Hercules, CA) and transferred to a nitrocellulose membrane. Successful transfer of the protein was assessed by staining the blots with Ponceau S (Sigma Aldrich, Saint Louis, MO), and then the stain was washed off with distilled water. Membranes were blocked in a 5% powdered milk/ PBS-Tween (0.5% Tween) (PBST) solution at room temperature for 1hr. SKI and α-tubulin antibodies were purchased from Millipore/Upstate (Charlottesville, VA). SMAD2, p-SMAD2, SMAD 1/5, and p-SMAD 1/5/8 antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Blots were incubated in a primary antibody (Millipore/Upstate, Charlottesville, VA) overnight at 4°C at the following dilutions: SKI 1:2000; SMAD2 1:1000, p-SMAD2 1:1000, SMAD1/5 1:1000, and p-SMAD1/5/8 1:10,000. SKI and α-tubulin blots were incubated in a 1:20,000 dilution of the appropriate HRP-conjugated secondary antibody (Pierce, Rockford, IL) at room temperature for one hour. SMAD and p-SMAD blots were incubated in a 1:2000 dilution of anti-rabbit IgG secondary antibody (Cell Signaling Technology, Danvers, MA) at room temperature for one hour. α-tubulin was used as a loading control for densitometry; blots were reprobed with α-tubulin diluted at 1:10,000 at room temperature for 10 minutes, and then secondary antibody (rabbit-anti-mouse) at 1:40,000 at room temperature for 10 minutes (personal communication with Dr. Beverly Delidow, Marshall University). After primary and secondary antibody

incubations, the blots were washed 4 X 5 minutes. in PBST. ECL Plus chemiluminescent reagent (GE Healthcare) was used for detection of the antibody as described by the manufacturer.

Cell Scrape Migration Assays

NCCIT cells were plated on Matrigel-coated cell culture plates and treated with either siRNA or the expression vector as described. Forty-eight hours after siRNA transfection, when the cells were ≥ 90% confluent, a 20 µL pipette tip was used to create a scratch across the cell monolayer. shNCCIT cells were plated and scraped in the same manner. shNCCIT cells were treated with ± TGFβ (0.1 nM), ±TGFβ blocking antibody (200 nM), or ± BMP4 (10 ng/mL), ± noggin (0.1 µg/mL). The cells were observed over a 20 hour time period, and images were taken of three fields of view per well at 0 hour and 20 hour using a Nikon inverted microscope (model Eclipse TS100-F) with camera attachment (Nikon Digital Sight DS-5M). To ensure that the same fields were analyzed at 0 and 20 hours, identifying marks were made on the bottom surface of the well. The percent closure of the scrape was determined by measuring the mean distance between the edges of the scrape (33 measurements per image; 99 measurements per well) at 0 and 20 hours using Image J 1.37v Analysis Software (Wayne Rasband, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). The percent closure of the scrape was estimated by comparing the measurements at 20 hours to the measurements at 0 hours. Treatments were performed in triplicate, and experiments were repeated at least three times (n=9).

Matrigel Invasion Assays

Forty eight hours after treatment with either siRNA or the expression vector, NCCIT cells were removed from the 6 well plates with trypsin, washed, and resuspended in 1 mL of serum-free Opti-MEM, and plated at $5x10^5$ cells per Matrigel-coated invasion chamber inserts (Falcon cell culture inserts with an 8 micron pore size PET membrane; BD Biosciences, Bedford, MA), containing a total volume of 2 mL serum-free Opti-MEM. The inserts were held in 6-well companion plates, which contained 2.5 mL Opti-MEM with 2% serum (BD Biosciences, Bedford, MA). According to the manufacturer's protocol, after a 22 hour incubation in the Matrigel invasion chambers, the cells were fixed to the membranes and stained using the Diff-Quik Stain Set (Dade Behring, Newark, DE), and the number of migrated cells was determined and compared to the non-specific control siRNA-treated cells. For each well, the number of invading cells was counted using Image J Analysis Software 1.37v in six random fields in each of three wells per treatment, and these numbers were added to obtain the total number of invaded cells for each sample. Each treatment was performed in triplicate, and each experiment was performed three times.

Stably transfected shNCCIT cells were plated at $5x10^5$ in Matrigel-coated invasion chamber inserts in the 6-well companion plates, which contained OptiMEM supplemented with 2% FBS ± SDF1 (25 ng/mL), ± AMD3100 (50 ng/mL).

Microarray Gene Analysis

Stably transfected shSKI and shNC NCCIT cells were plated on Matrigel-coated 6-well plates and scraped as described for Matrigel cell scrape assays. Seventy two hours after plating, the cells were washed with PBS and collected using TRIzol (Invitrogen, Carlsbad, CA). Six experiments were performed with three replicates in each experiment. The three replicates from each experiment were pooled for the microarray analysis for a total of six shNC and six shSKI samples. The samples were given to the Microarray Core Facility at Marshall University, Joan C. Edwards School of Medicine in TRIzol solution. RNA extraction, microarray analysis, and statistical analysis on the microarray data were performed by the Microarray Core Facility at Marshall University, Joan C. Edwards School of Medicine. Total RNA quality was determined using a Model 2100 bioanalyzer (Agilent, Santa Clara, CA). All samples had an RNA integrity number (RIN) greater than 8. Six biological replicates were used in reference microarray design; sample cDNA is hybridized against cDNA derived from universal reference RNA (Stratagene/Agilent, Cedar Creek, Texas). 250 ng of total RNA was used for labeling with the QuickAmp labeling kit (Agilent) and Cy3-CTP and Cy5-CTP from Perkin Elmer as previously described (Syed and Threadgill, 2006). ShNC or shSKI samples were labeled with Cy3-CTP, and universal human reference RNA (Agilent, Santa Clara, CA) was labeled with Cy5-CTP. Following purification of labeled cRNA, 825 ng of Cy3 and Cy5-labeled sample were combined and hybridized on a microarray chip containing 45,016 features that represent 33,000 known and novel human genomes for 17

hours at 65°C in an Agilent hybridization oven. Slides containing arrays were then washed and scanned using the Agilent DNA microarray scanner. Intensity of microarray features was measured using Agilent Feature Extraction software (version 9.5). Extracted data was lowess normalized.

Statistical analysis of microarray (SAM) and gene set enrichment analysis (GSEA) (http://www.broadinstitute.org/gsea) were used to identify significant genes and gene families that were differentially expressed in shSKI NCCIT cells compared to shNC NCCIT cells. SAM was used to determine significant changes in the expression of individual genes in shSKI cells compared to shNC cells. The cutoff for differential expression was a 1.5 fold change.

The GSEA software version 2.0.4 (Subramanian, et al., 2005) from the BROAD Institute (http://www.broadinstitute.org/gsea) was used to perform gene set enrichment analysis. This analysis was used to compare SKI-knockdown, shSKI NCCIT cells, to control cells, shNC NCCIT cells, to identify specific gene sets enriched in the shSKI phenotype. The dataset queried consisted of 26,712 genes. The GSEA software is designed to filter out gene sets containing less than 15 or more than 500 genes, which resulted in 586 gene sets to be used in the analysis. 380 out of the 586 gene sets were enriched in the shSKI NCCIT cells. Of these 380, 147 gene sets met the criteria of a false discovery rate (FDR) less than 0.25, and 101 of these gene sets were significantly enriched at a nominal p-value less than 0.05. The familywise-error rate (FWER) is a more conservative correction that ensures that the results reported do no contain a single false-positive gene set (Subramanian, et al., 2005); using the more

stringent cutoff of FWER less than 0.05 resulted in only a few enriched gene families in shSKI NCCIT cells.

Promoter Analysis

The Motif Locator tool on the MOtif Discovery (MoD) tools website (http://159.149.109.9/modtools/) (Pavesi, et al., 2006) was used to determine if potential target genes of SKI that were identified by microarray analysis contained SMAD binding elements. BMP and TGFβ SMAD binding elements were chosen via a literature search. The following SMAD binding sequences were used: BMP elements: GTCTAGAC, (Li*, et al.*, 2001; Zawel*, et al.*, 1998); CAGACA (Dennler*, et al.*, 1998; Jonk*, et al.*, 1998); GCCGnCGC (Kusanagi 2000, Ishida 2000, Kim 1997, Benchabane 2003); TGFβ elements: GTCTAGAC (Stopa 2000); AG(C/A)CAGACA (Dennler*, et al.*, 1998; Jonk*, et al.*, 1998); AGACAAGGTTGT (Song, et al., 1998); ACAGACAGACAC (Wong, et al., 1999); GCCCACCAGACAGATGGCTGAATCACAGGAGTGGCCGGCGGGACCCATGG CCT (Kon, et al., 1999; Vindevoghel, et al., 1998); AGACAGACAAAACCTAGACAATCACGTG GCTGGCTGCATGCCCTGTGGCTGTTGGGC (Hua, et al., 1998); CCTAGACAGACAA AACCTAGACAATCACGTGGCTGG (Hua*, et al.*, 1998; Hua*, et al.*, 1999); GAGGAGGTAGAC AGACAGCTATGT (Lindemann, et al., 2001); AGTATGTCTAGACTGA (Shi, et al., 1998). Two of the sequences used have been described to be both BMP and TGFβ response sequences. In this analysis, one substitution in the binding element sequence was allowed. The default

minimum match percent of 85% was chosen to report motif matches, and both of the complementary strands of the input promoter sequences were searched.

Promoter sequences were obtained from the Transcriptional Regulatory Element Database (TRED; http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=searchPromForm) developed by Michael Zhang's laboratory at Cold Spring Harbor. The promoter sequences used spanned from -3000 to +300, and the following genes were searched for SMAD binding elements: ADAMTS1, CXCR4, LEFTY1, SMAD7, SMAD6, WNT3, FST, SPARC, and MMP9.

Statistical Analysis

Data are reported as the mean of at least three individual experiments, and error bars represent standard error of the mean, except for the real-time PCR analysis for which error bars represent the 95% confidence intervals. Data were analyzed for significance with SigmaStat using a One Way Analysis of Variance (ANOVA) with Bonferroni adjustment, T-test, or Two-Way ANOVA. For the Two-Way ANOVA tests, all pairwise multiple comparison procedures were assessed for significance with a Tukey test. Significance was assessed at the $p < 0.05$ level.

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CURRICULUM VITAE

Amy N. Nash

wolfe48@marshall.edu
Phone (304)633-0016

2222 Lammermoor Lane
Indianapolis, IN 46214

EDUCATION

Graduate Education:
Joan C. Edwards School of Medicine at Marshall University, Huntington, WV
Major: Biomedical Sciences (Ph.D.), Cancer Biology GPA: 4.0

Undergraduate Education:
Marshall University, Huntington, WV May 2005
Major: Integrated Science and Technology, Biotechnology
Minor: Chemistry GPA: 3.87

AWARDS AND HONORS

Graduate

- 2008 Larry Ewing Memorial Trainee Travel Fund (41st Annual Society for the Study of Reproduction Meeting, May 2008)
- NASA West Virginia Space Grant Graduate Research Fellowship 2008-2009 ($43,320 including cost share)
- Anagene B. Heiner Memorial Basic Science Oral Presentation Winner (21st Annual Marshall University School of Medicine Research Day, March 2008)
- Student Travel Award (16th Annual Short Course on Experimental Models of Human Cancer, August 2007)
- Best Academic Performance of 2005-2006 (Marshall University Biomedical Sciences Program)
- Ph.D. Research Assistantship Stipend (Marshall University Biomedical Sciences Program 2005-Present)

Undergraduate

- Graduated Magna Cum Laude
- American Heart Association Summer Fellowship (Summer 2005)
- NASA Space Grant Undergraduate Scholarship (Spring 2005)
- Summer Undergraduate Research Fellowship Award (Summer 2004)
- West Virginia Engineering, Science, and Technology Scholarship (2003-2005)
- Marshall University Presidential Scholarship (2001-2005)
- Dean's List (2001-2005)

PUBLICATIONS/ ACCEPTED ABSTRACTS

- Invasive Potential of Testicular Cancer Cells: Effects of Bone Morphogenetic Protein (BMP) and Decreased *SKI* Expression, North American Testis Workshop, Philadelphia, PA, April 2009

- Effects of Decreased *SKI* on Invasive Properties of Testicular Cancer Cells, 41st Annual Society for the Study of Reproduction Meeting, Kailua-Kona, HI, May 2008
- The Role of *SKI* in Testicular Germ Cell Tumor Invasion and Metastasis, 21st Annual Marshall University School of Medicine Research Day, Huntington, WV, March 2008
- *SKI* Target Genes Revealed by siRNA Knockdown in NCCIT cells, COBRE/INBRE Annual Meeting, Charleston, WV, November 2007
- Expression of Ski and Sno Oncogenes in Testicular Tumors, 47th Annual Short Course on Medical and Experimental Mammalian Genetics, Bar Harbor, ME, July 2006
- Expression of Neurexins in Cultured Vascular Smooth Muscle Cells, Sigma Xi Research Day, Huntington, WV, April 2005
- Expression of Neurexins in A7R5 Smooth Muscle Cells, Summer Undergraduate Research Fellowship Day, Huntington, WV, August 2004
- Randomly Amplified Polymorphic DNA Profile-Based Measures of Genetic Diversity in Crayfish Correlated with Environmental Impacts, Sigma Xi Research Day, Huntington, WV, April 2004

CONFERENCES/ WORKSHOPS ATTENDED

- North American Testis Workshop, Philadelphia, PA, April 2009
- 41st Annual Society for the Study of Reproduction Meeting, Kailua-Kona, HI, May 2008
- 21st Annual Marshall University School of Medicine Research Day, Huntington, WV, March 2008
- 3rd Annual West Virginia COBRE/INBRE Conference, November 2007
- 16th Annual Short Course on Experimental Models of Human Cancer, Jackson Laboratory, August 2007
- 47th Annual Short Course on Medical and Experimental Mammalian Genetics, Jackson Laboratory, July 2006
- National Institutes of Health Grant Writers' Seminars and Workshops, LLC, 2005
- Accelrys GCG with SeqWeb Wisconsin Package Bioinformatics Training Workshop, 2005

RELEVANT GRADUATE LEVEL COURSES

- Human Genetics
- Principles of Immunology
- Cancer Biology

MEMBERSHIPS

- American Society of Human Genetics
- American Academy for the Advancement of Science
- Society for the Study of Reproduction

SERVICE

- Active member of the Marshall University Biomedical Sciences Graduate Student Organization
- Recruiting trip to the University of Louisville, Louisville, KY (November 2007)
- Recruiting seminar at Bethany College, Wheeling, WV (March 2007)
- Habitat for Humanity Volunteer

RESEARCH EXPERIENCE

Current Research: Graduate Student: Graduate Research Assistant for Dr. Laura Richardson at Joan C. Edwards School of Medicine at Marshall University. Our lab focuses on transcription factors, specifically *SKI* and *SNO*, in both germ cell development and testicular cancer. As part of my dissertation research, I am utilizing siRNA and shRNA to knockdown the expression of *SKI* in testicular cancer cell lines and assessing the invasive potential of these *SKI*-deficient testicular cancer cells. I presented a poster on this research at the 47th Annual Short Course on Medical and Experimental Mammalian Genetics (Jackson Laboratory, 2006). I presented a scientific talk at the 21st Annual MUSOM Research Day in March of 2008 and gave a poster presentations at the 41st Annual SSR Meeting in May, 2008 and at the North American Testis Workshop in April, 2009. I have also obtained funding for the continuation of this project through the NASA West Virginia Space Grant Consortium.

May 2004 – July 2005: Research Assistant: Undergraduate Research Assistant for Dr. Todd Green at the Joan C. Edwards School of Medicine. This project studied obesity-associated hypertension using obese Zucker rats as an animal model. I focused on the expression of several proteins that were up-regulated in obese hypertensive rats, including neurexins and 12-LO. I gave poster presentations on this work at both the Summer Undergraduate Research Fellowship Day in August 2004 and at Sigma Xi Research Day in April 2005. This research was funded by the American Heart Association, SURF, and NASA.

Aug. 2003-May 2004: Research Assistant with Vandalia Project at Marshall University: The focus of the project was developing cost-effective high volume DNA amplification technology. I worked as the lead molecular biology student on the project. I was responsible for ordering and inventorying supplies, planning and carrying out DNA amplification experiments, troubleshooting and maintaining a research notebook. This research project was partially funded by National Collegiate Inventors and Innovators Alliance and National Science Foundation.

Oct. 2003 – May 2004: Research Assistant: Unpaid position, conducting research as part of a team on the impact of environmental stressors on *Orconectes rusticus* using RAPD-PCR analysis. I presented an oral presentation on this research at Sigma Xi Research Day in April 2004.