A MUTATION IN ALK6B CAUSES IMPAIRED GERM CELL DIFFERENTATION

AND TESTICULAR GERM CELL TUMORS IN ZEBRAFISH

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DEDICATION

I would like to dedicate this dissertation to my husband, Roland Esparza and to my daughters, Shelley, Emily, and Liliana for the constant love, encouragement, and support they have given to me, each and every day!

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A MUTATION IN ALK6B CAUSES IMPAIRED GERM CELL DIFFERENTATION AND TESTICULAR GERM CELL TUMORS IN ZEBRAFISH

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A MUTATION IN ALK6B CAUSES IMPAIRED GERM CELL DIFFERENTATION AND TESTICULAR GERM CELL TUMORS IN ZEBRAFISH

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Germ cell tumors (GCTs) affect infants, children and young adults and are increasing in incidence worldwide. GCTs arise from pluripotent germ cells and can exhibit differentiated and undifferentiated histologies, which vary in their malignant potential and response to treatment. The pathways that determine tumor cell differentiation are not known, impeding the development of new therapies. Thus, the treatment of GCTs has remained static since the introduction 30 years ago of cisplatin which, while effective, causes severe side effects including hearing loss, infertility and kidney damage.

We identified a zebrafish mutant line with a high incidence of GCT during a forward genetic screen to identify cancer susceptibility loci. Homozygous adult males develop tumors consisting of undifferentiated spermatogonia by 4 months of age while heterozygous males develop tumors around 7 to 9 months of age. We used interval haplotype analysis and high-resolution recombinational mapping to localize the mutation to a 0.82 cM interval on zebrafish chromosome 10. We identified a premature termination codon in Alk6b (Activin Receptor-like Kinase 6b) in the mutant animals.

Alk6b is a member of the TGF-beta/BMP superfamily of receptors. BMP signaling has diverse roles including regulation of cell proliferation, differentiation, embryonic development, germ cell specification and gonadogenesis. Misregulation of the BMP signaling pathway has been implicated in various human cancers. In agreement with a critical role for Alk6b in controlling germ cell differentiation, we find evidence of impaired BMP signal transduction in the zebrafish GCTs, as well as evidence of alterations in the expression level of BMP target genes.

We have also examined BMP signaling in a series of 40 clinically-annotated human GCTs of diverse histologic subtypes. In agreement with the predictions made from our zebrafish model, we find that undifferentiated GCTs such as dysgerminomas lack BMP signaling activity, whereas signaling is maintained in the differentiated subtype of Yolk Sac Tumors. These results confirm the relevance of the zebrafish model for understanding germ cell tumorigenesis, and will foster the development of improved, targeted therapy of human GCTs.

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PRIOR PUBLICATIONS

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LIST OF ABBREVIATIONS

ActR – Activin Receptor

Alk – Activin-receptor Like Kinase

BMP – Bone Morphogenetic Protein

BMPR – Bone Morphogenetic Protein Receptor

CC – Choriocarcinoma

GCT– Germ Cell Tumor

GDF – Growth/Differentiation Factor

GDNF – Glial Derived Neurotrophic Factor

GSK3 – Glycogen Synthase Kinase 3

ID – Inhibitor of Differentiation/DNA binding

MAPK – Mitogen Activated Protein Kinase

Mdkb – midkine-B

MH1 & MH2 – Mad Homology 1 & 2

NE - Nonseminoma

NLS – Nuclear Localization Signal

PGC – Primordial Germ Cell

RT-PCR – Reverse Transcription Polymerase Chain Reaction

SE– Seminoma

Smad – SMA – *mothers against decapapentaplegic*

SS – Spermatocytic Seminoma

SSLP – Simple Sequence Length Polymorphism

TE – Teratoma

TGCT – Testicular Germ Cell Tumor

TGF-β – Transforming Growth Factor - Beta

YST – Yolk Sac Tumor

CHAPTER ONE
INTRODUCTION – GERMLINE DEVELOPMENT AND GERM CELL TUMORS

Germline stem cells are totipotent cells that contain all the necessary genetic information to give rise to a multicellular organism and ensure the continuation of the species. They are specified early in embryogenesis as primordial germ cells (PGCs) and must migrate from their site of origin to the genital ridges where they are referred to as gonocytes. In the gonads, the gonocytes receive instructive signals and undergo epigenetic reprogramming to differentiate into either spermatogonia or oogonia, depending upon their chromosomal constitution. The spermatogonia/oogonia will undertake gametogenesis, the production of fully mature haploid cells (spermatozoa or ova). This vital process occurs in an orderly series of mitotic and meiotic divisions, resulting in gametes that are capable of giving rise to the next generation upon fertilization. Improper germ cell development, regulation, and gametogenesis are associated with infertility, inherited chromosomal abnormalities, gonadal dysgenesis, and germ cell tumors.

In this chapter, I will review proper germline development including important genes, pathways, and events that are involved in germ cell specification, migration, and gametogenesis with a selective focus on spermatogenesis. I will follow up with a discussion on germ cell tumors which is an example of what can result from improper germline development.

GERMLINE DEVELOPMENT

Primordial germ cells are the founders of the germ cell lineage. They are distinctive for their vital role in the production and development of mature gametes that will give rise to the next generation. Primordial germ cells are also unique in that after specification they must migrate from the border of the embryonic and extraembryonic ectoderm at which they originated to the genital ridge (Kunwar et al 2006; Molyneaux & Wylie 2004; Molyneaux et al 2001; Wylie 2000). Throughout migration and development PGCs are able to maintain their underlying pluripotency program while repressing somatic differentiation (van de Geijn et al 2009; Western 2009). This specialized function enables PGCs to ultimately fulfill their role when, upon fertilization, they reactivate their differentiation program to give rise to the next generation.

Multiple model organisms have been used to study PGC development. However, we have gained the most understanding from research conducted in *Drosophila melanogaster*, zebrafish, and mice (Kunwar et al 2006; Raz 2003; Richardson & Lehmann 2010; Williamson & Lehmann 1996). Each of these animal models have important differences that is vital to their own PGC development but they also share critical functions for proper germ cell development. Here I will review important differences and similarities between zebrafish and mouse PGC specification, migration, and epigenetic reprogramming.

Primordial Germ Cell Specification

Primordial germ cell specification in most organisms requires two conserved molecular mechanisms: repression of the somatic differentiation program and reactivation of pluripotency-associated gene expression. In zebrafish, primordial germ cell specification is predetermined by the inheritance of maternal RNA and proteins located in the germ plasm (Houston & King 2000; Raz 2003; Saffman & Lasko 1999; Williamson & Lehmann 1996). In contrast to other organisms, zebrafish PGCs arise in four distinct random locations with respect to the developmental axis of the embryo (Raz 2003; Weidinger et al 1999; Weidinger et al 2002; Yoon et al 1997). The identification of *vasa* as a germline cell marker in zebrafish was an important discovery that facilitated the study of PGC/germline development (Olsen et al 1997; Yoon et al 1997). In zebrafish, *vasa* expression is first detected in four strips of electron-dense germ plasm along the first two cleavage planes in the embryo. By the 4K cell stage, the *vasa* enriched germ plasm is distributed into the cytoplasm of four closely associated cells that then become PGCs. The four newly specified PGCs undergo multiple rounds of division to generate 25-50 PGCs that migrate to the genital ridges by the end of the first day (Braat et al 1999; Knaut et al 2000; Weidinger et al 1999; Yoon et al 1997).

Mice and other mammals lack germ plasm and require inductive signaling for PGC specification (Lawson et al 1999; Tam & Zhou 1996; Ying et al 2001; Ying & Zhao 2001). At E6.5, bone morphogenetic proteins 4, 8b, and 2 (BMP4/8b/2) and unidentified proteins signal from the extraembryonic ectoderm and visceral endoderm to pluripotent epiblast cells to induce *fragilis/Ifitm3* expression (Saitou et al 2002; Ying et al 2001;

Ying & Zhao 2001; Zhao & Garbers 2002). *fragilis/Ifitm3* expression is required for the proximal epiblast cells to achieve competence to become PGC precursor cells (Lange et al 2003; Saitou et al 2002; Tanaka & Matsui 2002; Tanaka et al 2004; Tanaka et al 2005). BMP4, BMP2, and BMP8b null mice lack or have severely reduced numbers of PGCs due to the failure to generate PGC precursor cells (de Sousa Lopes et al 2004; Itman et al 2006; Lawson et al 1999; Ying et al 2001; Ying & Zhao 2001; Zhao & Garbers 2002 2004). Loss of Smad1 and Smad5, downstream transducers of BMP signaling, also result in complete or significantly reduced numbers of PGCs (Chang & Matzuk 2001; Hayashi et al 2002; Tremblay et al 2001).

An important molecular mechanism for PGC specification that is common to many organisms is the transcriptional silencing of somatic gene expression (Ohinata et al 2005; Saitou et al 2002; Yabuta et al 2006). The *fragilis/Ifitm*-positive proximal epiblast cells also express somatic mesodermal genes including *Hoxb1, Fgf8,* and *Snail* (Ancelin et al 2006; Hayashi et al 2007; Yabuta et al 2006). In these cells, B lymphocyte-induced maturation protein 1 (BLIMP1, also known as PRDM1), a transcriptional repressor, plays significant roles in the somatic gene repression as well as promoting upregulation of PGC-specific genes such as *stella* (Ohinata et al 2005; Saitou et al 2005; Vincent et al 2005). The loss of Blimp1 in mutant mice results in reduced somatic gene silencing, loss of founder PGCs, and lack of PGC migration (Kurimoto et al 2008; Yamaji et al 2008). Another PR-domain containing protein, Prdm14, also plays key roles in PGC specification similar to Blimp1. Mice that lack Prdm14 exhibit a similar PGC phenotype as the Blimp1 mutant mice (Kurimoto et al 2008; Yamaji et al 2008). Blimp1 expression is initially found in only six proximal epiblast cells destined to become PGCs. By E7.25,

there are approximately 40 Blimp1 positive, specified PGCs. (Ohinata et al 2005). These cells are characterized by their transcriptional silencing of somatic genes, the expression of PGC-specific genes, and maintenance or upregulation of pluripotency-associated genes such as Oct4, Sox2, and Nanog (Saitou et al 2002; Scholer et al 1990; Yabuta et al 2006; Yamaguchi et al 2005; Yeom et al 1996).

Primordial Germ Cell Migration

In most organisms the PGCs arise in a location distal to the genital ridges where the PGCs will eventually reside. To arrive at the gonads the PGCs must gain motility and migrate through the embryo to their final location. Similar to PGC specification, there are conserved mechanisms for migration amongst different organisms, but there are also important distinctions and modes of migration.

Zebrafish have four clusters of PGCs originating in dispersed locations in the embryo that must migrate to the genital ridges (Weidinger et al 2003; Weidinger et al 1999; Weidinger et al 2002; Yoon et al 1997). The PGCs use a "run" and "tumble" system in which they migrate "run" short distances to intermediate stops where they remain stationary "tumble" for a short period of time to realign themselves to attractant chemokine signals that are guiding them through the embryo (Raz & Reichman-Fried 2006; Reichman-Fried et al 2004).

The initiation of migration requires multiple steps in which the PGCs gain motility. Initially, zebrafish PGCs are morphologically indistinguishable from the surrounding somatic cells, exhibiting a smooth, round morphology. However, in the first

30 minutes after specification the PGCs start to exhibit a ruffled edge appearance with the extension of short cellular protrusions in all directions. At this time, the PGCs have not gained the ability to migrate and these protrusions are eventually lost as the PGCs divide. This is followed by a one hour transitional phase in which the PGCs become polarized and extend out broad pseudopodia for directional migration (Blaser et al 2005). An RNA binding protein, Dead End (Dnd), is critical for PGC polarization and extension of the broad directional protrusions (Weidinger et al 2003). The loss of Dnd in PGCs results in absence of PGC migration due to loss of polarization and protrusion extension (Blaser et al 2005).

After gaining of motility, zebrafish PGCs start to actively migrate towards attractant signals provided by somatic cells. A striking difference in zebrafish PGC migration is that the PGCs migrate as individual cells and do not require cell-to-cell contact with other PGCs (Kunwar et al 2006; Reichman-Fried et al 2004). Stromal-derived factor-1alpha (SDF-1a) has been identified as a critical component guiding the PGCs along the migratory pathway. SDF-1a binds to chemokine receptor, CXCR4b, which is expressed in PGCs. It was demonstrated that PGCs will migrate to ectopic locations in response to aberrant SDF-1a secreting cells (Doitsidou et al 2002; Knaut et al 2003).

Mouse PGCs are initially located in the primitive streak and must follow a migratory path through the posterior embryonic endoderm, extraembryonic endoderm, and finally through the allantois and hindgut to reach the genital ridges (Anderson et al 2000). Several molecules/pathways have been identified as important mediators of proper PGC migration. Similar to zebrafish PGCs, guidance is provided by the SDF1

chemokine and CXCR4 interaction (Ara et al 2003; Molyneaux et al 2003). The c-kit receptor tyrosine kinase and its ligand *steel* were discovered to facilitate migration by regulating PGC motility (Gu et al 2009). In addition, E-cadherin and β-1 integrin are required for proper exiting from the hindgut and migration into the gonads (Anderson et al 1999a; Anderson et al 1999b; Bendel-Stenzel et al 2000).

Epigenetic Reprogramming of Primordial Germ Cells

Until the time of PGC migration, all cells in a developing embryo have a bi-parental pattern of genomic imprinting. Genomic imprinting is an epigenetic phenomenon in which DNA methylation controls expression of a limited number of genes that are dependent on parental origin (McLaren 2003; Surani 2001). Approximately 100-200 genes in the human genome are imprinted such that only one allele, either maternal or paternal, is expressed (Lucifero et al 2004; Paoloni-Giacobino & Chaillet 2004). In PGCs, the bi-parental pattern of genomic imprinting must be erased, followed by the establishment of a uni-parental pattern in order to ensure that proper sex-specific imprinting is passed on to the next generation. This process, called genome-wide epigenetic reprogramming, occurs in migratory and post-migratory PGCs.

In mice, several key events occur in migratory and gonadal PGCs that are indicative of epigenetic reprogramming. At E8.0, X-chromosome reactivation is initiated in female PGCs, concomitant with the gradual repression of Xist (inactive X-specific transcript), a key regulator of X-inactivation (Sugimoto & Abe 2007). Changes in histone methylation also occur as H3K9me2 is reduced and H3K27me3 is increased (Erhardt et

al 2003; O'Carroll et al 2001; Silva et al 2003). Soon after E11.5, PGCs begin to undergo dramatic changes to erase the bi-parental pattern of genomic imprinting. Along with a widespread reduction in DNA methylation, a lack of DNA methyltransferases, DNMT3a and 3b occurs within the nucleus and X-chromosome reactivation is completed (Seki et al 2007; Sugimoto & Abe 2007; Yabuta et al 2006). Once erasure is complete, establishment of uni-parental, sex-specific imprinting occurs through DNMT3L and DNMT3a activity (Bourc'his et al 2001; Hata et al 2002; Sakai et al 2004).

SPERMATOGENESIS

Once the PGCs (now referred to as gonocytes) arrive at the gonads, they begin differentiating into either pro-spermatogonia or oogonia based on their chromosomal constitution (XY or XX) and the signals they receive from the gonadal microenvironment and surrounding somatic cells. Then at the appropriate time, depending on the sex of the organism, the process of gametogenesis (spermatogenesis if male and oogenesis if female) is initiated to generate fully mature gametes that are capable of producing the next generation upon fertilization. In this next section, I will review the process of spermatogenesis.

Spermatogenesis

Spermatogenesis is a dynamic, intricate process occurring in a series of highly organized steps that culminates in the production of fully mature spermatozoa.

Spermatogenesis is initiated during puberty and is maintained continuously throughout the life of most organisms.

In most vertebrates, spermatogenesis occurs within seminiferous tubules that are composed of a basement membrane, basal compartment, and adluminal compartment. Blood vessels, Leydig cells, lymphatic epithelium, and macrophages surround each tubule in the interstitial space (as reviewed inYoshida 2008a). On the inside of the basement membrane reside the Sertoli cells. Representing the only somatic cell within the tubules, Sertoli cells support the germ cells by providing nourishment, growth factors, and function to organize the spermatogenic process (Griswold 1998). One distinguishing characteristic of spermatogenesis in lower vertebrates (also known as anamniotes) including fish is that in the seminiferous tubules, spermatogenesis occurs within cysts that are formed by Sertoli cell cytoplasmic extensions (Schulz et al 2010). In contrast to higher vertebrates (retiles, birds, and mammals) where one Sertoli cell is associated with several spermatogonial germ cells at different developmental stages, cystic spermatogenesis is represented by having one Sertoli cell associated with a single spermatogonial germ cell (Matta et al 2002; Schulz et al 2005).

The process of spermatogenesis is much conserved amongst different species including flies, fish, and mammals with relatively few minor distinctions. In the mouse, Type A undifferentiated spermatogonia (A_{single}) are believed to be the stem cells of the testis and are closely associated with the basement membrane in the basal compartment (Chiarini-Garcia et al 2001; Chiarini-Garcia & Russell 2001; Hess et al 2006; Oatley & Brinster 2006; Ogawa et al 2005; Ryu et al). The A_{single} spermatogonia are thought to undergo self-renewal as well as to give rise to specialized daughter cells that will divide

and differentiate to ultimately become spermatozoa (Oatley & Brinster 2006). The delicate balance between self-renewal and committed differentiation to become spermatozoa must be tightly maintained in order to ensure proper spermatogenesis and fertility.

A_{single} spermatogonia give rise to the undifferentiated Type A spermatogonia (A_{undiff}), A_{paired} and $A_{aligned}$. These undifferentiated cells, including the A_{single} cells are tightly clustered together and only account for 1% of testicular cells. The A_{undiff} cells will give rise to the next class of spermatogonia, the differentiated Type A_1 spermatogonia. The differentiated Type A_1 spermatogonia will undergo successive rounds of mitosis. The number of rounds is dependent on the organism. In rodents there are typically six rounds of mitotic division which results in Type A_{2-4} differentiated spermatogonia, intermediate spermatogonia, Type B spermatogonia, and finally the preleptotene primary spermatocytes (de Rooij & Russell 2000). Zebrafish has a total of nine rounds of mitosis that is initiated by type A undifferentiated primary spermatogonia and results in three type A differentiated spermatogonia generations, five type B spermatogonia generations, and one generation of primary preleptotene spermatocytes (Leal et al 2009).

Still closely associated with the basement membrane, the preleptotene spermatocytes initiate meiosis. During a long prophase, the leptotene and zygotene spermatocytes will move away from the basement membrane and will enter the adluminal compartment through the tight junctions that are formed by the Sertoli cells. The exchange of genetic information occurs during the recombination event in pachytene spermatocytes, after which the completion of meiosis I results in 2N secondary

spermatocytes. These will then undergo meiosis II to produce four haploid (1N) round spermatids (Hess & Renato de Franca 2008; Schulz et al 2010; Yoshida 2008b).

The round spermatids are transformed into elongated, highly condensed, mature spermatozoa through a process called spermiogenesis that includes four different stages; golgi, capping, acrosomal, and maturation (Hess & Renato de Franca 2008). The mature spermatozoa are released into the lumen and upon ejaculation exit the body through the rete testes, epididymis, and vas deferens. In zebrafish, mature spermatozoa are present in the lumen and are released through the efferent duct (Leal et al 2009).

Germ Cell Tumors (GCTs)

Germline development is an intricate process that requires precise regulation to control germ cell proliferation, differentiation, and survival. Disruption of this regulation can lead to disorders such as infertility, chromosomal abnormalities, and germ cell tumors (GCTs). GCTs are a group of heterogeneous neoplasms that arise primarily in the testes and ovaries, though they also occur in extragonadal sites along the midline of the body and the brain. GCTs differ in clinical and histological presentation and are classified into five distinct groups based on differences in age of onset, histology, cell of origin, chromosomal constitution, and pattern of genomic imprinting (Table 1) (Oosterhuis & Looijenga 2005). Type I GCTs consist of the teratomas and yolk sac tumors of neonates and young children. Type II GCTs encompass the seminomas and non-seminomas of adolescents and adults. Spermatocytic seminomas that affect men > 50 years old make up the Type III GCT. Finally, the ovarian dermoid cyst and uterine

hydatidiform mole are classified as the type IV and type V GCTs respectively. Here I will review types I, II, and III GCTs with a primary focus on the type II testicular GCTs (TGCTs).

Type	Anatomical site	Phenotype	Age	Originating cell	Genomic imprinting	Genotype	Animal model
I	Testis/ovary/ sacral region/ retroperitoneum/ mediastinum/ neck/midline brain/other rare sites	(Immature) teratoma/ yolk-sac tumour	Neonates and children	Early PGC/ gonocyte	Biparental, partially erased	Diploid (teratoma). Aneuploid (yolk-sac tumour): gain of 1q, 12(p13) and 20q, and loss of 1p,4 and 6q	Mouse teratoma
II	Testis	Seminoma/ non-seminoma	>15 years (median age 35 and 25 years)	PGC/gonocyte	Erased	Aneuploid (+/– triploid): gain of X, 7, 8, 12p and 21; and loss of Y, 1p, 11, 13 and 18	Not available
	Ovary	Dysgerminoma/ non-seminoma	>4 years	PGC/gonocyte	Erased	Aneuploid	Not available
	Dysgenetic gonad	Dysgerminoma/ non-seminoma	Congenital	PGC/gonocyte	Erased	Diploid/tetraploid	Not available
	Anterior mediastinum (thymus)	Seminoma/ non-seminoma	Adolescents	PGC/gonocyte	Erased	Diploid/tri-tetraploid	Not available
	Midline brain (pineal gland/ hypothalamus)	Germinoma/ non-seminoma	Children (median age 13 years)	PGC/gonocyte	Erased	Diploid/tri-tetraploid	Not available
III	Testis	Spermatocytic seminoma	>50 years	Spermatogonium/ spermatocyte	Partially complete paternal	Aneuploid: gain of 9	Canine seminoma
IV	Ovary	Dermoid cyst	Children/adults	Oogonia/oocyte	Partially complete maternal	(Near) diploid, diploid/tetraploid, peritriploid (gain of X, 7, 12 and 15)	Mouse gynogenote
V	Placenta/uterus	Hydatidiform mole	Fertile period	Empty ovum/ spermatozoa	Completely paternal	Diploid (XX and XY)	Mouse androgenote

PGC, primordial germ cell.

Table 1: Germ cell tumor classification
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Type I Germ Cell Tumors

Type I germ cell tumors include teratomas and yolk sac tumors that affect neonates and children < 5 years of age (Oosterhuis & Looijenga 2005). They most

commonly occur in the testes, ovaries, sacrococcygeal area, retroperitoneum, head and neck, and in the hypophyseal region of the brain. The type I GCTs are relatively rare with an incidence of 0.12/100,000; sacral teratomas are the most commonly diagnosed type I GCT (Rescorla 1999). Based on a partially erased biparental pattern of genomic imprinting, an early primordial germ cell is thought to be the cell of origin in type I germ cell tumors (Bussey et al 2001; Schneider et al 2001). Typically, the type I teratomas are benign; however, ovarian teratomas can be malignant and teratomas that are incompletely surgically removed can progress to yolk sac tumors, which have the potential to be metastatic (Gobel et al 2000). Type I teratomas primarily have a normal chromosomal constitution while type I yolk sac tumors are characteristically aneuploid. Recurrent chromosomal abnormalities in yolk sac tumors include loss of 1p, 4, and 6q and gain of 1q, 12(p13), 20q, and 22 (Mostert et al 2000; Perlman et al 2000; Schneider et al 2001)

Type I animal models include various mouse models of teratomas. Spontaneous testicular teratomas arise in the inbred 129-strain of mice that closely resemble human type I teratomas (Noguchi & Noguchi 1985; Stevens 1973). Mutations in *Dead-end/Ter* in the 129-strain mice cause significant PGC loss and increased type I testicular germ cell tumor susceptibility (Youngren et al 2005). Targeted deletion of *Pten* in mouse PGCs leads to greater risk for testicular teratomas, increased germ cell proliferation, and greater capacity to generate embryonic germ cells in culture; thus indicating an important role for *Pten* in regulating germ cell proliferation and differentiation (Kimura et al 2003).

Type II Germ Cell Tumors

Epidemiology

Type II GCTs occur in adolescents and adults and testicular GCTs represent the most common malignancy found in men 20-40 years of age (McIntyre et al 2008). Type II GCTs largely occur in the testes and ovaries but also occur in extragonadal sites such as the mediastinum and in the brain (Oosterhuis & Looijenga 2005). Although type II GCTs are often diagnosed in females, they predominantly affect males and are referred to as testicular germ cell tumors (TGCTs).

The incidence for TGCTs is 6-11/100,000 with an annual increase in incidence of 3-6%; primarily in Caucasian males of European descent. Men of Asian and African descent have a lower rate of incidence (Giwercman et al 1993; Oosterhuis & Looijenga 2005; Rapley 2007). TGCTs have an unusually high relative risk of 8-10 fold for brothers of an affected patient and 4-6 fold for father/son, thus suggesting a strong genetic component associated with risk (Forman et al 1992; Heimdal et al 1996a; b; Rapley 2007). TGCTs are often diagnosed in patients with infertility, cryptorchidism, and testicular atrophy (Brown et al 1987; Petersen et al 1998; Verp & Simpson 1987). There is also evidence that indicates that endogenous and environmental factors affect the risk and incidence for TGCTs. In Sweden, immigrants from Finland, who classically exhibit a low incidence and risk for TGCT, show increased risk in the second generation (Hemminki & Li 2002; Hemminki et al 2002).

Type II GCTs are further divided into two subgroups based on histological and clinical variations; seminomas and nonseminomas (Figure 1). Seminomas (SE) are composed of primitive, undifferentiated germ cells that resemble PGCs/gonocytes and occur in the testes. They are also called dysgerminomas when present in the ovaries and germinomas when found extragonadally in the brain. Nonseminomas (NS) include GCTs that are further along the differentiation program than SE such as embryonal carcinomas (EC), choriocarcinomas (CC), yolk sac tumors (YST), and mature teratomas (TE) (Oosterhuis & Looijenga 2005; van de Geijn et al 2009).

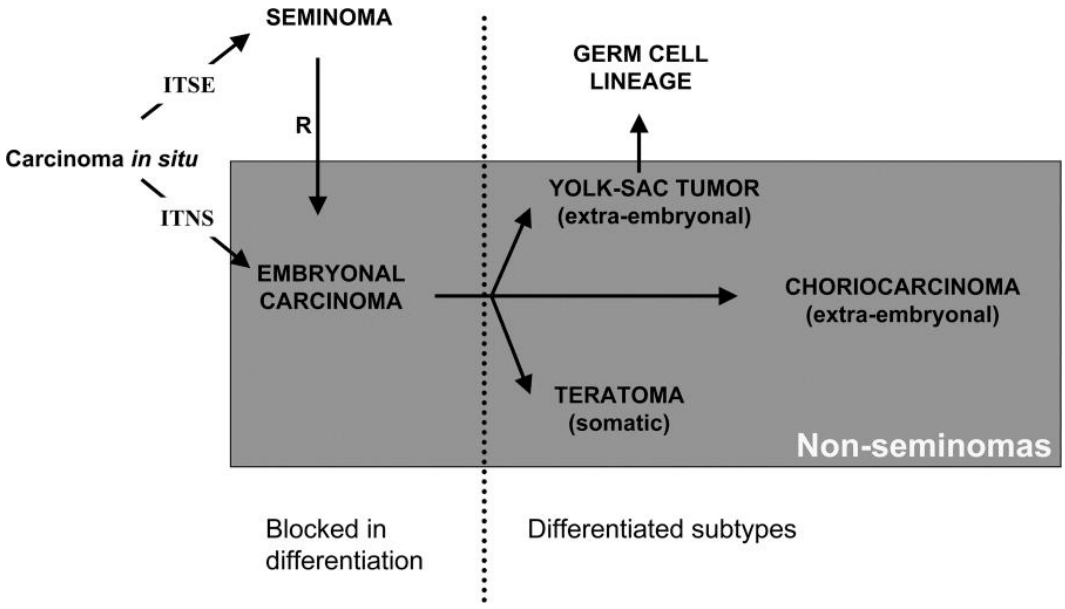


Figure 1: Type II germ cell tumor histologies. Seminomas and nonseminomas both develop from the preinvasive lesion, carcinoma *in situ*, through intermediate stages, intratubular seminoma (ITSE) and intratubular nonseminoma (ITNS) respectively. Nonseminomas (in shaded box) including yolk sac tumors, choriocarcinomas, and teratomas arise from embryonal carcinomas which develop from ITNS or may develop from a seminoma that has undergone "reprogramming" (R). (Reprinted by permission from John Wiley & Sons: Birth Defects Research, van de Geijn et al, copyright 2009)

Cell of Origin & TGCT Precursor Lesion

A fully erased pattern of biparental genomic imprinting suggests that type II GCTs arise from slightly later PGCs/gonocytes than the type I GCTs (Bussey et al 2001; Oosterhuis & Looijenga 2005; Schneider et al 2001). Carcinoma *in situ* (CIS) is a noninvasive precursor lesion that gives rise to all type II TGCTs (Hoei-Hansen et al 2005; Rajpert-De Meyts et al 2003a). CIS has an incidence rate similar to the type II GCTs and indicates that all CIS lesions will eventually progress to invasive TGCTs (Oosterhuis & Looijenga 2005; van de Geijn et al 2009). CIS cells show phenotypic characteristics similar to PGCs such as morphology, pluripotent gene expression, and genomic imprinting. A confirmed marker for CIS is Oct3/4, a gene that is known for a role in maintaining pluripotency. Oct3/4 is expressed in all CIS, seminomas, and the embryonal carcinoma component of nonseminomas which suggests that all SE and NS share similar pathogenesis pathways (Hoei-Hansen et al 2005; Rajpert-De Meyts et al 2003a). Oct3/4 is expressed in normal PGCs during development but then decreases in the germ cells post-natally (Honecker et al 2004; Rajpert-De Meyts et al 2004; Stoop et al 2005). The decrease in Oct3/4 can be delayed in disorders such as cryptorchidism and dysgenesis syndromes and the delayed expression of OCT3/4 often leads to misdiagnosis of CIS. CIS also exhibits expression of pluripotency markers PLAP, Sox17, and c-Kit (Honecker et al 2004; Rajpert-De Meyts et al 2004; Stoop et al 2005).

Histology/Phenotypic Characteristics

Seminomas exhibit an accumulation of undifferentiated PGC/gonocyte-like germ cells that share a similar morphology to CIS cells. Nonseminomas can be composed of different histological components that represent the differentiation of a truly totipotent cell. Embryonal carcinomas (EC) consist of undifferentiated stem cells and it is thought that ECs may arise from seminomas that have undergone reprogramming to activate underlying pluripotency to become ECs (Looijenga et al 1999; Oosterhuis et al 2003). ECs undergo differentiation to give rise to other nonseminoma components including choriocarcinomas and yolk sac tumors that are differentiated into extraembryonic components (trophoblast and yolk sac, respectively) and mature teratomas that have undergone somatic differentiation. Thus the EC represents the neoplastic counterpart to embryonic germ cells and the true cancer stem cell.

Chromosomal Constitution and Mutations

Type II TGCTs are typically aneuploid and show a consistent pattern of recurrent chromosomal abnormalities including the loss of chromosomes 4, 5, 11, 13, 18, and Y, and gain of chromosomes 7, 8, 12p, and X (Castedo et al 1989; Looijenga et al 2000; Oosterhuis & Looijenga 2005; Ottesen et al 1997; Summersgill et al 1998). The gain of 12p is characteristic of all invasive type II TGCTs and interestingly is not consistently present in the preinvasive lesion, CIS (Looijenga et al 2007a; Looijenga et al 2000;

Oosterhuis et al 1997; Summersgill et al 2001; van Echten et al 1995). This indicates that gain of 12p plays a significant role in the transformation of CIS into invasive TGCTs.

Very few mutations associated with TGCT development have been identified, primarily due to lack of large pedigrees for analysis and the lack of suitable animal models for type II TGCTs (Oosterhuis & Looijenga 2005). However, activating mutations in c-Kit exon17, in particular at codon 816, are associated predominantly with bilateral TGCTs, which only account for up to 5% of TGCTs (Dieckmann et al 2007a; Dieckmann et al 2007b; Kemmer et al 2004; Looijenga et al 2003; Nakai et al 2005; Oosterhuis & Looijenga 2005; Sakuma et al 2003; Tian et al 1999).

Type III Germ Cell Tumors

Spermatocytic seminomas, a testicular GCT that affects men typically > 50 years of age, represents the type III GCTS (Oosterhuis & Looijenga 2005). Once thought to be a variant of seminomas, spermatocytic seminomas are now recognized as a morphologically and pathogenetically distinct entity. Spermatocytic seminomas have a relatively low and steady incidence of 0.20 per 100,000 (Chung et al 2004; Cummings et al 1994; Eble 1994) and are typically benign (Floyd et al 1988; Matoska et al 1988; True et al 1988). Morphologically, spermatocytic seminomas are quite distinct from type II TGCTs in that they exhibit the presence of small, intermediate, and large tumor cells that are similar in appearance to spermatogonia and spermatocytes (Looijenga et al 2007b; Oosterhuis & Looijenga 2005). Spermatocytic seminomas are also distinguished from seminomas by positive staining for markers XPA, SCP3, CHK2, and SSX, and negative

staining for c-Kit, Oct3/4, and PLAP (Dekker et al 1992; Rajpert-De Meyts et al 2003b; Stoop et al 2001).

A paternal pattern of genomic imprinting and morphology indicates that spermatocytic seminomas arise from a more mature germ cell (Oosterhuis & Looijenga 2005; Verkerk et al 1997). Also, a precursor lesion called intratubular spermatocytic seminomas, indicates that the cell of origin is located on the luminal side of the Sertoli cell tight junctions and thus representing a more mature germ cell (Muller et al 1987).

In striking contrast to type II TGCTs, chromosomal abnormalities are rare for spermatocytic seminomas with the exception of supernumerical copies of chromosome 9 (Maiolino et al 2004; Rosenberg et al 1998). Recently the gene *DMRT1*, a transcription factor, was identified as a candidate gene on chromosome 9 for the development of spermatocytic seminomas. *DMRT1* was found to be overexpressed in spermatocytic seminomas relative to seminomas and positive-staining for *DMRT1* was found in spermatogonia and spermatocytes (Looijenga et al 2007b).

Interestingly, a canine animal model of spermatocytic seminoma, also exhibited positive staining for *DMRT1* (Looijenga et al 2007b). Other animal models for spermatocytic seminomas include a Puf-8 knockout mutant in *Caenorhabditis elegans* and a rhinoceros model (Portas et al 2005; Subramaniam & Seydoux 2003).

CHAPTER TWO

Bone Morphogenetic Protein (BMP) Signaling

Bone morphogenetic proteins (BMPs) are extracellular signaling molecules that belong to the transforming growth factor β (TGF-β) superfamily. BMPs have functionally diverse roles in cellular proliferation, differentiation, and apoptosis. They were initially identified for their role in inducing bone formation but they have subsequently been recognized for their significant involvement in development.

BMP Pathway Components

There are over twenty identified BMP growth factor ligands that are classified into subgroups based upon their amino acid sequence similarity (Figure 2) (Derynck & Zhang 2003; Miyazono et al 2010; Schmierer & Hill 2007). BMPs are initially expressed as large precursor monomers containing an N-terminal signal peptide domain, a pro-domain, and a mature C-terminal growth factor domain (Constam & Robertson 1999; Kingsley 1994; Sebald et al 2004; Walsh et al 2010). The monomer contains a region of seven highly conserved cysteines that facilitates the formation of disulfide bonds and dimerization between two BMP molecules (Kawabata et al 1998). The mature BMP dimer is secreted upon proteolytic cleavage of the pro-domain at a conserved RXXR consensus site (Ducy & Karsenty 2000). Although BMP homodimers are the most common dimers found, heterodimers are also formed and can have more potent signaling capabilities (Aono et al 1995; Israel et al 1996; Little & Mullins 2009; Zhu et al 2006).

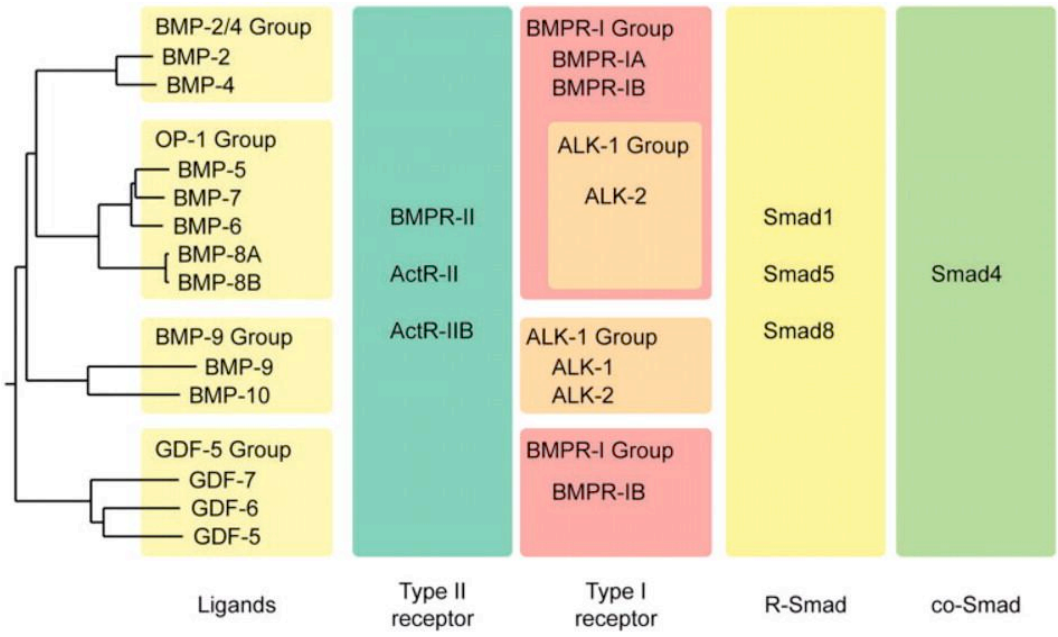


Figure 2: Members of the BMP signaling family. Based on amino acid homology, BMPs are classified into subgroups and interact with specific BMP receptors and Smads (Miyazono et al 2010. Bone morphogenetic protein receptors and signal transduction. J Biochem 147:35-51. By permission of Oxford University Press).

BMPs signal by forming a heterotetrameric signaling complex with type I and type II BMP receptors (Figure 3) (Derynck & Zhang 2003; Heldin et al 1997). There are three type II receptors, Bmp receptor type II (Bmpr2), Activin receptor type IIa (ActRIIa), and Activin receptor type IIb (ActRIIb) (Miyazono et al 2005). Type II receptors are responsible for transphosphorylating and activating the type I receptors upon complex formation. Type II receptors are constitutively active though they require interaction with the type I receptors to stably bind BMPs. The type II receptors are composed of an extracellular ligand binding domain, a single transmembrane domain, and an intracellular serine/threonine kinase domain (Derynck & Feng 1997; Derynck &

Zhang 2003; Massague 2000). There are two known splice variants of type II receptors; the long form which has an extra long C-terminal tail rich in serine/threonine residues and the short form which lacks this tail (Liu et al 1995; Nohno et al 1995; Rosenzweig et al 1995). The long form is the most commonly expressed splice variant.

There are also three type I BMP receptors; Bmp receptor type1a (Bmpr1a or Alk3), Bmp receptor type 1b (Bmpr1b or Alk6), and Activin receptor type 1a (ActR1a or Alk2). The type I receptors are key components of the BMP signaling pathway in that they transduce the BMP signal. They are also important for BMP signal specificity. BMPs are functionally diverse and achieve this diversity by preferentially binding to type I receptors (Derynck & Feng 1997; Derynck & Zhang 2003; Massague 2000). For example, BMP2 and BMP4 bind to either Bmpr1a or Bmpr1b whereas BMP7 preferentially binds to ActR1a (Kawabata et al 1998; ten Dijke et al 2003). GDF5 only binds strongly to Bmpr1b (Nishitoh et al 1996). Similar to the type II receptors, the type I receptors consist of an extracellular ligand binding domain, a single transmembrane domain, and an intracellular serine/threonine kinase domain. In addition, the type I receptors also have a region N-terminal of the kinase domain that is rich in glycines and serines called the GS domain. Type I receptors are activated upon transphosphorylation of the GS domain by the type II receptors. The GS domain in conjunction with the kinase domain is also vital to the recruitment and activation of receptor-activated Smads1/5/8 as well as other downstream mediators (Derynck & Feng 1997; Derynck & Zhang 2003; Massague 2000).

BMPs are known to primarily activate the canonical Smad pathway but they also activate non-Smad pathways such as the p38/MAPK pathway (Derynck & Zhang 2003;

Hassel et al 2003; Kimura et al 2000; Nohe et al 2002; Nohe et al 2003). The method of the heterotetrameric complex formation plays a role in determining which pathway is activated. The signal is transduced through a non-Smad pathway when the BMP dimer first binds to the type I receptor dimer which then recruits and forms the complex with the type II receptor dimer (Derynck & Zhang 2003; Hassel et al 2003). The Smad pathway is activated when the BMP dimer binds with a preformed type I and II receptor complex (Gilboa et al 2000; Nohe et al 2004; Sieber et al 2009).

There are three subgroups of Smads in BMP signaling, receptor-activated Smads1/5/8, common mediator Smad4 (co-Smad4), and inhibitory Smads6/7 (Derynck & Zhang 2003; Massague 1998; Nishimura et al 2003). With the exception of Smads6/7, all other Smads contain two conserved domains, the N-terminal MH1 domain and the C-terminal MH2 domain linked together by a proline rich linker region. The inhibitory Smads lack the MH1 domain but retain the MH2 domain. The MH1 domain is important for DNA binding and interaction with other DNA-binding proteins. The MH2 domain contains the nuclear transport signal and the conserved SXSS motif that is required for phosphorylation and activation by the type I receptor. The domain is also important for oligomerization, binding to co-Smad4, and interaction with other transcription factors in the nucleus. In the Smad inactive state, the two domains are bound together, preventing interaction with other Smads and transport into the nucleus. Upon type I receptor activation, Smads are recruited to the receptor where a Smad L3 loop within the MH2 domain interacts with the type I receptor L45 loop located in the kinase domain. This initiates a conformational change in the Smad and the two domains are separated to expose the NLS signal, the binding site for oligomerization and complex formation with

co-Smad4, and allow Smad activation. In the nucleus, Smads will associate with other transcription factors to regulate gene transcription (Derynck & Zhang 2003; Massague 1998; Nishimura et al 2003).

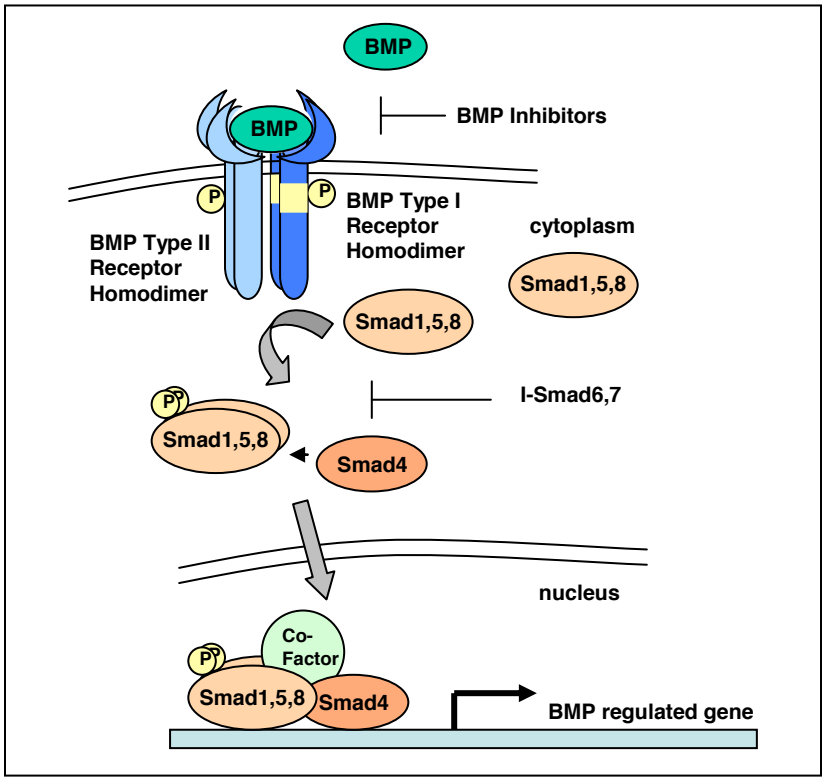


Figure 3: The BMP pathway. BMP signaling is initiated when BMP ligand dimers bind to a BMP receptor homodimer. This allows the type II receptor to activate the type I receptor. The type I and II receptor homodimers form a stable, active heterotetrameric complex with the BMP ligands. The type I receptor kinase domain phosphorylates and activates an rSmad which then disassociates from the receptor to form a complex with other rSMADs and co-Smad4 The complex translocates into the nucleus to act as a transcription factor to regulate gene expression

BMP Signaling Regulation

Pathways that are heavily involved in cell proliferation, differentiation, and apoptosis must be tightly regulated to prevent developmental disorders and disease. With BMPs playing vital roles in cell regulation during development and in adult processes, the pathway is carefully monitored and controlled on multiple levels. Regulation of BMP signaling occurs extracellularly, from membrane-bound proteins, as well as in the intracellular compartment (Massague 2000; Sieber et al 2009; Umulis et al 2009; Walsh et al 2010). Other pathways and molecules such as Wnt, MapK, and ubiquitin ligases are often involved in BMP regulation (Fuentealba et al 2007; Sapkota et al 2007; Sapkota et al 2006). The BMP pathway is also involved in self-regulation by positive and negative-feedback loops (Miyazono 2000).

Extracellularly, the BMP ligands are limited by BMP antagonists such as Chordin, Noggin, Follistatin, and members of the Cerberus and Dan family (Walsh et al 2010) (Miyazono 2000). The antagonists negatively regulate BMP signaling by either binding to and sequestering the BMPs or by competitive binding to the BMP receptors. Bambi, a membrane bound BMP pseudoreceptor, is able to form the heterotetrameric complex with BMPs and BMP receptors (Miyazono 2000; Onichtchouk et al 1999). However, Bambi lacks the serine/threonine kinase domain and acts as a dominant-negative receptor to abrogate BMP signaling. There are also membrane bound receptors (sometimes referred to as BMP/TGF-β type III receptors) such as Endoglin and Betaglycan that are known to enhance BMP signaling by promoting ligand:receptor binding (Barbara et al 1999; Halbrooks et al 2007; Kirkbride et al 2008)

There are multiple intracellular mechanisms of BMP regulation. Inhibitory Smads6/7 (I-Smads) play key roles to reduce BMP signal transduction. Both of the I-Smads are able to bind type I BMP receptors to prevent R-Smad binding and activation. Smad6 is additionally able to compete for binding with co-Smad4 and therefore reducing the number of functional trimeric Smad complexes. Smad7 aids in recruiting ubiquitin ligases, Smurf1 and Smurf2, to target R-Smads and BMP receptors for degradation (Hayashi et al 1997; Imamura et al 1997; Miyazono 2000; Nakao et al 1997. The MAPK and Wnt pathways also play a role in R-Smad regulation. The R-Smad linker region contains conserved sites for sequential phosphorylation by MAP kinases and GSK3, leading to R-Smad degradation (Fuentealba et al 2007; Sapkota et al 2007). Finally, within the nucleus, BMP components are regulated by epigenetic modifications, including demethylation and hypermethylation of BMP pathway component promoters. Aberrant epigenetic regulation of BMP pathway components has been reported in cancers (Kimura et al 2008; Lee et al 2008; Wen et al 2006; Zhang et al 2007).

BMP Functions

BMP Signaling During Development

During early embryogenesis, BMPs are required for PGC specification (see Chapter 1) and for patterning the embryo. Studies in *Xenopus, Drosophila*, and zebrafish have established a critical role for BMPs in dorsoventral patterning and left-right

asymmetry (Hogan 1996a; b; Kishimoto et al 1997; Little & Mullins 2009; Monteiro et al 2008; Schier & Talbot 2005; Suzuki et al 1994). In most organisms, BMPs have ventralizing activity and BMP antagonists secreted from the Spemann organizer and presumptive dorsal regions are required to set up a BMP signaling gradient for proper dorsoventral patterning (Dale et al 1992; Graff 1997; Hogan 1996a; Piccolo et al 1996; Sasai et al 1996; Zimmerman et al 1996). Mutations in several BMP pathway components were identified in zebrafish mutant lines that exhibited moderate to severe dorsoventral patterning defects (Kondo 2007; Little & Mullins 2006; Schier & Talbot 2005). *swirl* (BMP2b), *snailhouse* (BMP7), and *somitabun* (Smad5) mutants are severely dorsalized with paraxial mesoderm and neuroectoderm expansion, accompanied by lack of ventral structures such as epidermis, neural crest, blood, heart, and tail (Dick et al 2000; Dick et al 1999; Hild et al 1999; Kishimoto et al 1997; Mullins et al 1996; Schmid et al 2000). Recently in zebrafish, redundant roles in early dorsoventral patterning were identified for BMP type I receptors (Alk3a/b and Alk6a/b) through BMP2 and BMP7 ligands (Little & Mullins 2009). In contrast, loss of BMP antagonist, Chordin, in zebrafish leads to a ventralized phenotype with enlarged ventral and posterior structures and severely reduced or absence of dorsal structures (Hammerschmidt et al 1996; Schulte-Merker et al 1997).

Along with their essential roles in early embryogenesis, BMPs also are crucial to other developmental processes. BMPs were first identified for their vital and unique role in cartilage and bone formation (Urist 1965; Wozney 1998; Wozney & Rosen 1998) but also have crucial roles in organogenesis (Hogan 1996a). Targeted inactivation of BMP components in mice has provided a greater understanding of BMP developmental

roles (Zhao 2003). BMP4 and BMP2 inactivation in embryonic stem cells results in early embryonic lethality (starting at the time of gastrulation and up to E10), thus indicating critical roles during early embryogenesis (Winnier et al 1995; Ying et al 2001; Ying & Zhao 2001; Zhang & Bradley 1996). Bmpr1a, the suggested downstream receptor for BMP2/4 is also embryonic lethal starting at the time of gastrulation (Mishina et al 1995). BMP4, which is key during gastrulation and for primordial germ cell specification is also involved in hematopoetic cell formation and mesoderm differentiation into teeth, limb, and bone structures (Dunn et al 1997; Lawson et al 1999; Winnier et al 1995). BMP2 is indicated for vital roles in dorsoventral patterning, osteoblast differentiation, and cardiac development (Ying et al 2001; Ying & Zhao 2001; Zhang & Bradley 1996). BMP7 shares a redundant role with BMP2 in osteoblast differentiation but also has distinct roles for cell proliferation, survival, and/or differentiation during lens placode and kidney development (Dudley et al 1995; Luo et al 1995; Wawersik et al 1999). Homozygous BMP7 mutant mice die postnatally due to renal failure. BMP7 mutants also exhibit defects in pre-axial polydactyly (Dudley et al 1995; Luo et al 1995). Mice with targeted inactivation of Bmpr1b exhibit appendicular skeletal defects due to reduced prechondrogenic and chondrocyte differentiation in the phalangeal region. *Gdf5* single and *Bmpr1b; Gdf5* double mutant mice share a similar phenotype as *Bmpr1b* single mutants indicating Gdf5 as a potential ligand for Bmpr1b (Yi et al 2000).

The BMP pathway is crucial to the developing embryo and is also critical for initiation and maintenance of adult functions such as gametogenesis.

BMP Signaling During Spermatogenesis

BMP signaling is required for proper germline development and regulation of proliferation, differentiation, and survival of germ cells during spermatogenesis. *In vitro* analysis indicates that exogenous BMP4 appears to drive the differentiation of spermatogonial stem cells in enriched germ cell cultures (Pellegrini et al 2003). Also, during early postnatal testis development, BMP2 and BMP7 are important for spermatogonia and Sertoli cell proliferation respectively (Puglisi et al 2004). *In vivo* expression analysis and targeted inactivation of BMP ligands in mice have been instrumental for demonstrating roles of BMP signaling in spermatogenesis initiation and maintenance. BMP8b inactivation leads to reduced or delayed germ cell proliferation and differentiation during the first initial wave of spermatogenesis during puberty and increased apoptosis of pachytene spermatocytes leading to reduced fertility in the adult (Zhao et al 1996). Targeted inactivation of BMP8a in mice likewise results in increased meiotic germ cell apoptosis and consequently causes germ cell degeneration while inactivation of BMP7 in the BMP8a deficient background exacerbates the phenotype, suggesting that both BMP7 and BMP8a play a role in maintenance of spermatogenesis (Zhao et al 1998). BMP8a, BMP7, and BMP4 are also involved in maintaining epididymal integrity. The loss of BMP8a and BMP7 leads to granuloma formation through the breakdown and collapse of the distal caput and cauda region of the epididymis (Zhao et al 2001; Zhao et al 1998). BMP4 loss causes degeneration of the corpus area of the epididymal epithelium and although this does not lead to granulomas, it does affect the fertility of the mutant mice (Hu et al 2004). Glial-derived neurotrophic

factor (GDNF), a distant relative of the TGF-β pathway, has been implicated in the regulation of spermatogonial stem cell self-renewal (Meng et al 2000; Puglisi et al 2004). Taken together, these studies have established the BMP signaling pathway as an important component in the initiation and maintenance of spermatogenesis.

BMP Signaling in Cancer

Many pathways that have significant roles in development and cell regulation are often disrupted in cancers and thus, it is not surprising to find that recent evidence suggests that the BMP pathway can be an important factor in tumorigenesis. In the last decade, increased focus on BMP signaling in cancer has identified potential roles for disrupted BMP signaling in several cancers including colorectal, prostate, and breast cancer (Alarmo & Kallioniemi 2010; Hardwick et al 2008; Kim et al 2000; Kim et al 2004).

The BMP pathway was implicated in early stage colorectal cancer with the discovery of germline mutations in BMP receptor 1a (*BMPR1A*) and common mediator *SMAD4* in juvenile polyposis syndromes (Howe et al 2001; Howe et al 1998a; Howe et al 1998b; Zhou et al 2001). Together, *BMPR1A* and *SMAD4* mutations account for nearly half of all juvenile polyposis cases (Howe et al 2004). Deregulation of BMP ligands has also been detected in colorectal cancers. Increased expression of BMP4 and BMP7 is associated with adenoma-carcinoma colorectal cancer progression (Deng et al 2007; Motoyama et al 2008; Nosho et al 2005). In contrast, BMP2, BMP3, and BMP5 have

been found to be downregulated in colorectal cancers indicating a potential tumor suppressor role for certain BMP components in colorectal cancer (Beck et al 2006; Hardwick et al 2004; Koehler et al 2004; Koinuma et al 2005; Loh et al 2008).

Variable expression of BMP receptors type II, IA, and IB was identified in progressive tumor grades of prostate cancer (Kim et al 2000). Positive expression of all three receptors was found in well differentiated, benign prostate tumor samples whereas, loss of expression of one or more of the BMP receptors was seen in progressively worse tumor samples. In addition, only 1 of 10 high-grade, poorly differentiated tumor samples exhibited expression of only one BMP receptor (Kim et al 2000). It is suggested that BMP signaling acts as a growth-inhibitory factor in prostate cells and that loss of active BMP signaling may lead to cancer progression. Similar to colorectal cancers, variable deregulation of BMP ligands has also been found in prostate cancers. Loss of BMP2 expression correlated to prostate cancer progression (Horvath et al 2004) and in contrast, higher expression of BMP6 and BMP7 in prostate cancer samples is associated with bone metastasis (Autzen et al 1998; Hamdy et al 1997; Masuda et al 2003).

The study of BMP signaling in breast cancer has increased significantly in recent years leading to the discovery of potential roles for BMP signaling in both the promotion and inhibition of breast cancer progression (Alarmo & Kallioniemi 2010). The same BMP pathway components have, in fact, been shown to either contribute to or suppress breast cancer progression depending on the cell type and context. For example, BMP2 has the ability to suppress breast cancer cell proliferation (Ghosh-Choudhury et al 2000a; Ghosh-Choudhury et al 2000b; Pouliot & Labrie 2002) but also confers resistance to apoptosis (Raida et al 2005a; Raida et al 2005b; Steinert et al 2008) and promotes

migration and invasion of breast cancer cells (Clement et al 2005). In addition, variable expression of *BMPR1B* has been reported in high-grade, poor prognosis breast cancer patients. *BMPR1B* overexpression is associated with high-tumor grade and high proliferation index in estrogen receptor-positive breast cancer samples (Helms et al 2005) while, in contrast, loss of *BMPR1B* was found in the high grade, poor prognosis estrogen receptor-negative samples (Bokobza et al 2009). However, downstream negative-regulation of receptor activated *SMADs* by estrogen in the estrogen receptor-positive samples may confer similar BMP signaling activity as in estrogen receptor-negative samples (Bokobza et al 2009; Kusumegi et al 2004; Takahashi et al 2008).

Disruption and variable expression of the BMP signaling has been seen in several other cancers including lung, glioblastomas, and germ cell tumors (Bouras et al 2000; Chen et al 2010; Lee et al 2008). Certainly, a greater understanding of the involvement of the BMP pathway in tumorigenesis is needed.

CHAPTER THREE

A MUTATION IN *ALK6B* CAUSES IMPAIRED GERM CELL DIFFERENTIATION AND TESTICULAR GERM CELL TUMORS IN ZEBRAFISH

Abstract

The development and differentiation of germ cells must be carefully controlled to ensure fertility and continuation of the species. Maturation and differentiation of the germ cells in the developing gonad results from reciprocal signaling interactions between germ cells and surrounding somatic cells. The TGF-β/BMP pathway has been implicated in germ cell differentiation through studies in flies, mice and humans. However, the precise nature of how these pathways contribute to differentiation in vertebrate gonads has remained unclear, in part because of a lack of available genetic mutants. We previously identified a zebrafish mutant line, tgct, which exhibits impaired germ cell differentiation that leads to the development of germ cell tumors. We now report that an inactivating mutation in alk6b, a Type IB BMP Receptor, is responsible for the tgct phenotype. Alk6b is expressed in spermatogonia and early oocytes, and alk6b mutant gonads display impaired BMP signal transduction, altered expression of BMP target genes and abnormal germ cell differentiation. These results indicate a germ cell-autonomous role for BMP signal transduction in germ cell differentiation.

Introduction

Germline stem cells are totipotent cells that contain all the necessary genetic information to give rise to a multicellular organism. In most metazoans, the germline is specified early in embryogenesis as primordial germ cells (PGCs), which must migrate from their site of origin to the developing gonad. In the gonads, the gonocytes receive signals that instruct them to differentiate into either spermatogonia or oogonia. Improper germ cell development, regulation, and gametogenesis are associated with infertility, inherited chromosomal abnormalities, and germ cell tumors.

Several embryonic signaling pathways have been implicated in the development of germ cells, somatic cells or both, including wnt, Hedgehog, Notch, and FGF (Cook & Capel 2010). An especially prominent role in gonadogenesis has emerged for the Bone Morphogenetic Protein (BMP) signaling pathway, a member of the TGF-β superfamily of growth factors and receptors. Over 20 components of this pathway have been identified including bone morphogenetic protein (BMP) growth factors, bone morphogenetic receptors type I and type II, and receptor-activated Smad 1/5/8 transcription factors as well as various BMP pathway antagonists.

A mutant zebrafish line that develops highly penetrant, dominantly inherited testicular germ cell tumors (TGCTs) was identified through an ethylnitrosourea (ENU) based forward genetic screen (Neumann et al 2009). The TGCTs in the affected male fish display disrupted testicular architecture, accumulation of spermatogonial-like cells, and impaired germ cell differentiation. Here we report the identification of the mutation responsible for TGCT phenotype in activin receptor-like kinase 6b (*alk6b*), the zebrafish

homolog to human Bmpr1b. A premature termination codon that truncates the kinase domain results in the abrogation of active BMP signaling in the testis and leads to impaired differentiation of spermatogonial stem cells. Further understanding of the role of the BMP/TGF-beta signaling pathway in germ cell differentiation may provide insight to the development of human GCTs.

Results:

Adult zebrafish males carrying the tgct mutation develop spontaneous testicular tumors consisting of undifferentiated, primitive germ cells.

Adult homozygous and heterozygous males from the testicular germ cell tumor (*tgct*) mutant line develop visible abdominal tumors that are remarkable for the presence of large cells with enlarged nuclei, open chromatin and pale cytoplasm that resemble

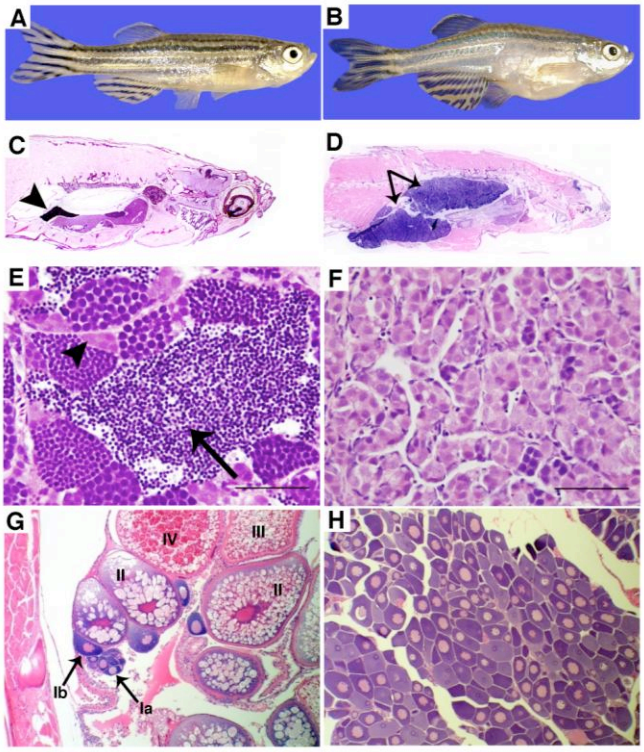


Figure 4: *tgct* mutants develop germ cell tumors due to impaired spermatocytic differentiation. Compared to adult wildtype males (A), adult *tgct* males (B) display marked abdominal distension. Low-power images of sagittal sections (C,D) shows normal testicular morphology in wildtypes (C; arrowhead) but marked testicular enlargement in *tgct* mutant males (D; arrow). E, F: cross-sectional anatomy of normal and mutant testis. In wildtype (E) spermatogenesis proceeds through ordered stages of differentiation from spermatogonia (arrowhead) to fully differentiated spermatozoa (arrow). In *tgct* mutant testis (F), differentiation is impaired and testicular architecture is disrupted by the accumulation of spermatogonial-like cells. (G) cross-section of normal ovary showing various stages of oocyte maturation. (H) ovary from a *tgct* homozygous female showing an oocyte maturation defect where further maturation of stage 1 oogonia is halted.

undifferentiated spermatogonia. *tgct* homozygous males develop tumors as early as 3 months of age with 100% penetrance by 5 months of age (figure 4). In contrast, heterozygous male carriers are initially fertile and there is usually a small amount of

residual spermatogenesis associated with the tumors; however by approximately 8 months of age the tumor cells disrupt normal testis tissue architecture, and spermatogenesis is lost. Homozygous *tgct* females exhibit a profound oocyte maturation defect and are infertile due to the arrest of differentiation in stage 1 of oogenesis (figure 4D). Heterozygous *tgct* females are phenotypically normal but are carriers of the *tgct* mutation (Neumann, 2009).

Based on the resemblance of the tumor cells to spermatogonia, we suspected that the cells were early, pre-meiotic germ cells. To test this idea we stained sections of normal and tumor testis with an antibody to the phosphorylated form of zebrafish histone H2AX (pH2AX) (figure 5). Histone H2AX is a variant histone that is reversibly phosphorylated at sites of DNA double-strand breaks resulting from DNA damage or meiotic recombination. In the testis, H2AX phosphorylation strongly marks primary pachytene stage spermatocytes that are undergoing meiosis (Cabrero et al 2007; Mahadevaiah et al 2001; Viera et al 2004). In the wildtype zebrafish testis, synchronously developing groups of primary and secondary spermatocytes exhibit strong pH2AX immunoreactivity (figure 5A). In contrast, the mutant testis exhibits only scattered groups of pH2AX-positive cells. The large tumor cells that make up the majority of the tumor testis do not stain (figure 5B), suggesting that the tumor cells are pre-meiotic germ cells.

To further delineate the loss of differentiation of the cells, we removed the testes from wildtype and heterozygous mutant males and cultured the germ cells. Unlike mammalian testis, the zebrafish testis is capable of undergoing transmeiotic differentiation to mature, functional sperm in vitro (Sakai 2002; 2006). 5 to 7 days after

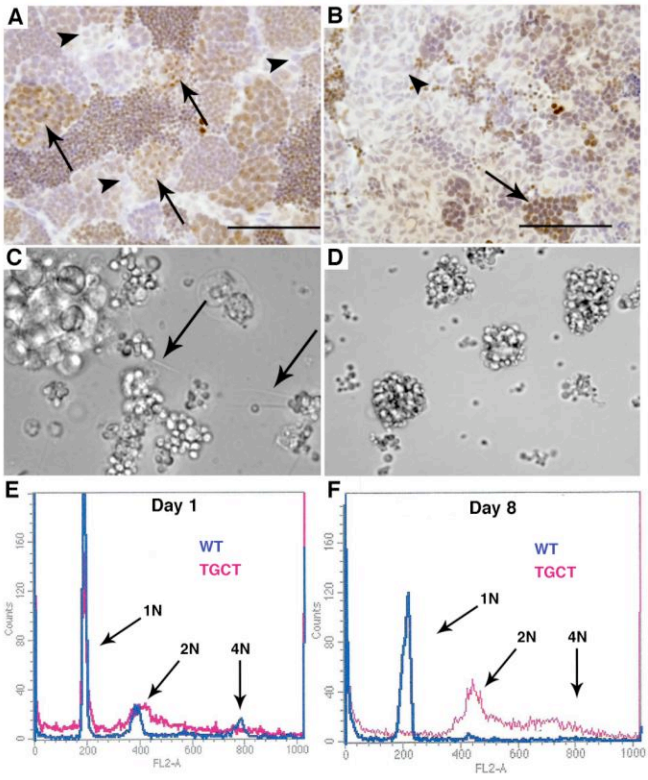


Figure 5: *tgct* mutants exhibit impaired differentiation and reduced meiosis *in vitro* and *in vivo*. A. In normal testis, Phosphohistone H2Ax marks clusters of primary spermatocytes undergoing meiosis (arrows). Spermatogonia (arrowheads) are phosphohistone H2Ax negative. B. *tgct* mutant testis exhibits severely reduced clusters of meiotic spermatocytes. The spermatogonial-like tumor cells are phosphohistone H2AX negative. C. *In vitro* culture of wildtype spermatogonial stem cells undergoing differentiation and meiosis to fully functional spermatozoa (arrows). D. culture of *tgct* mutant testis showing absence of spermatozoa due to the inability of *tgct* spermatogonial-like cells to differentiate and complete meiosis. E. FACS analysis for DNA content of cultured wildtype and *tgct* testis on day 1. F. FACS analysis on day 8 after culturing. Wildtype spermatogonia have completed meiosis to become haploid spermatozoa (1N) while *tgct* spermatogonia remain mostly diploid (2N) and are unable to complete meiosis.

placing explants of wildtype testis in culture, motile sperm can be detected. The mutant testis, however, failed to produce motile sperm. We used flow cytometric DNA content profiling of the testicular explants to assess the ability of the cells to differentiate to haploid spermatids and spermatozoa (figure 5). On initial culturing, wildtype testes exhibit haploid cells (spermatids and spermatozoa) and diploid, mitotic and meiotic cells with 2n and 4n DNA content. Tumor testes contain a small percentage of haploid cells on day 1 of culture, consisting of residual spermatids and spermatozoa; the majority of cells from the tumor testis have 2n DNA content. After 8 days in culture, the wild-type spermatogonia differentiated to haploid cells while the *tgct* cells remained predominantly

diploid and did not complete meiosis. Taken together these results indicate that the testicular tumor cells in the tgct mutant are early, pre-meiotic germ cells that are defective in spermatocytic differentiation. We obtained similar results when we cultured the cells on a supporting layer of somatic cells obtained from a wildtype male gonad (not shown), suggesting that the differentiation defect in the *tgct* mutant testes is cell-autonomous to the germ cells.

Identification of the tgct mutation

To identify the mutation responsible for the germ cell tumor susceptibility in the *tgct* strain, we initiated a positional cloning project. Pedigree analysis indicated that the germ cell tumor susceptibility trait is dominantly inherited (Neumann 2009). Owing to the relatively late onset of the testicular tumor trait, only a small number of affected males were initially available. We crossed a *wik* strain *tgct* founder to the polymorphic AB strain, and used interval haplotype analysis, a highly efficient method for rapid assignment of novel mutations to a defined chromosomal interval (Beier & Herron 2004; Herron et al 2002; Neuhaus & Beier 1998), to position the *tgct* mutation on a chromosome. We identified polymorphic Simple Sequence Length Polymorphism (SSLP) markers for the centromere and telomere of each chromosome based on the zebrafish MGH genetic map (Knapik et al 1998; Shimoda et al 1999) and profiled the markers on genomic DNA from 15 affected males derived from a hybrid backcross of a tgct carrier female. A haplotype on zebrafish chromosome 10 was identified as the most likely location of the mutation based on the low number of nonrecombinant haplotypes

from the non-affected parent. Further linkage tests with Chromosome 10 microsatellite markers on a panel of 88 affected males placed the mutation in the 12 cM interval between markers Z6427 and Z355.

Male heterozygous carriers are fertile prior to developing testicular tumors; therefore using the linked microsatellite markers to identify carriers we could carry out *tgct* heterozygote incrosses. Genotyping of adult progeny from incrosses yielded an average of 23.2% homozygotes, indicating that the *tgct* mutation does not impair viability. To further refine the critical interval containing the *tgct* gene, we used fifteen known and novel polymorphic SSLP markers. We generated the novel markers by identifying Simple Sequence Repeats in the zebrafish genome (Sanger Center assembly Zv6; www.sanger.ac.uk/drerio). In order to confirm the location of the markers, we generated a high resolution meiotic map of the interval between Z9208 and Z7316 by analyzing marker recombination segregation in a panel of 493 F1 progeny of hybrid wik/AB outcrosses (data not shown). Using the markers on a panel of 16 *tgct* recombinants between Z9208 and Z355, we localized the *tgct* mutation to an interval containing three genes: the netrin receptor *unc5c*, the lim/homeobox gene *pdlim5* and *alk6b*, the zebrafish ortholog of human Bone Morphogenetic Receptor Type 1B (*BMPR1B*). Gaps were present in the zebrafish genome assembly in this region; however a similar syntenic arrangement of these three genes is also found in human and mouse and in other fish species such as medaka, making it likely that the assembly is correct. Intragenic recombinants were present in both *unc5c* and *pdlim5*, limiting the critical interval to 0.82 cM.

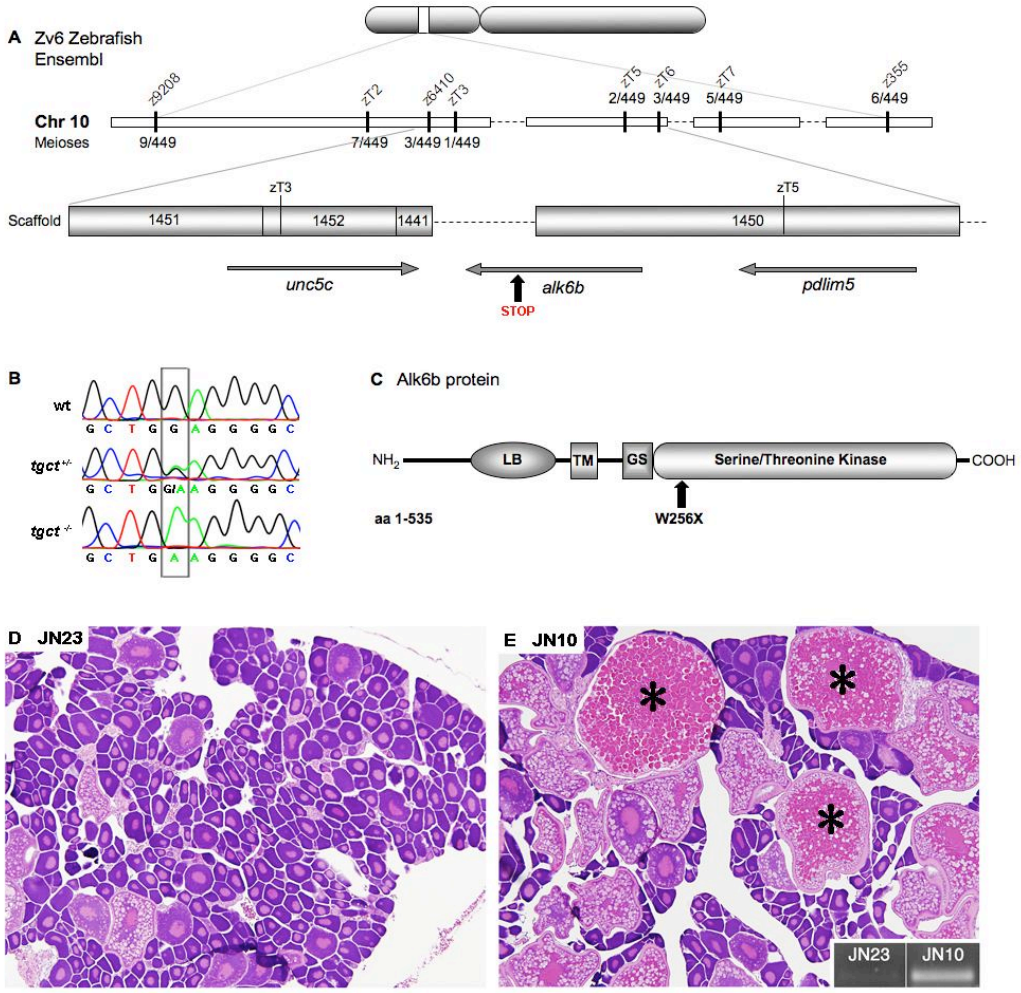


Figure 6: Positional cloning of the *tgct* mutant locus. A. Position of the *tgct* mutation on chromosome 10 between novel SSLP markers, zT3 and zT5 located on Zv6 ensembl scaffolds 1452 and 1450 respectively. B. Chromatogram showing the G to A transition C. Schematic of Alk6b protein illustrating the Activin-like receptor (AcvR) ligand binding (LB), transmembrane (TM), GS box (GS), and serine/threonine kinase domains. The *tgct* mutation is located in the kinase domain at amino acid 256. D. *β-actin-alk6b*^{wt} transgene injected *alk6b*$^{tgct-/-}$ female showing no rescue of the oocyte maturation and very little transgene incorporation (inset) into genomic DNA. E. *β-actin-alk6b*^{wt} Tol2 transgene injected *alk6b*$^{tgct-/-}$ female with restored oocyte maturation (*) and incorporation of the transgene into genomic DNA (inset). JN23 and JN10 refers to the sample designation.

Sequence analysis of the coding exons of *alk6b* revealed a G-to-A transition mutation in *tgct* mutant zebrafish. The mutation introduces a premature termination codon and is predicted to truncate the protein after residue 256 (figure 6). The mutation was not present in wildtypes of the AB strain, or in the original *wik* strain used for mutagenesis (not shown). To confirm that the $alk6b^{tgct}$ allele is responsible for the impaired germ cell differentiation, we generated a Tol2 transposon to assess the ability of wildtype *alk6b* cDNA under the control of a ubiquitous β-actin promoter to restore germ cell differentiation in $alk6b^{tgct}$ mutants. At 6 months of age, we sacrificed confirmed $alk6b^{tgct-/-}$ mutants to examine transverse gonadal sections by H&E staining for restoration of germ cell differentiation. We found that 26.6% of the homozygous mutant females had rescue of the oocyte maturation defect (figure 6d, e). We conducted PCR analysis on genomic DNA isolated from paraffin-embedded ovarian tissue and confirmed that the rescued females tested positive for the injected transgene. In agreement with our hypothesis, these results indicate that the $alk6b^{tgct}$ mutant allele leads to impaired germ cell differentiation.

Alk6/BMPR1B is a member of the TGF-β/BMP receptor superfamily. In this pathway, Bone Morphogenetic Protein (BMP) ligands bind a heterotetrameric complex of two Type II BMP receptors and two Type I BMP Receptors. Ligand binding causes the Type II receptors to phosphorylate the Type I receptors, stimulating the Serine/Threonine kinase activity of the Type I receptors. The principal downstream targets of Type I BMP receptors are SMADs 1, 5 and 8, which in turn bind coSMAD4. This phospho-SMAD/coSMAD complex enters the nucleus and mediates expression or repression of BMP target genes (Miyazono et al 2005; Nishimura et al 2003; Schmierer & Hill 2007).

The mouse and human genomes encode two Type I receptors (BMPR1A, also known as ALK3, and BMPR1B/ALK6). These genes are duplicated in zebrafish; the ALK3 orthologs are designated *alk3a* and *alk3b*, and the ALK6 orthologs are designated *alk6a* and *alk6b* (Little & Mullins 2009).

The mutation in Alk6b results in loss of function.

The *tgct* mutation is predicted to truncate Alk6B in the N-terminus of the serine/threonine kinase domain that is responsible for phosphorylation and activation of rSMAD 1/5/8 in response to binding of BMP ligands. Therefore we predicted that *alk6b*^{tgct} would be a loss-of-function allele. To test this hypothesis, we performed morpholino knockdown of zebrafish BMP pathway receptors in embryos and assessed the ability of the mutant *alk6b* allele to rescue the knockdown phenotype (Figure 7). In zebrafish, the embryonic expression of different BMP receptor family members and paralogs create functional redundancy in the pathway, making it necessary to knock down multiple pathway members to generate a phenotype (Little & Mullins 2009). Knockdown of both BMPR1A orthologs, *alk3a* and *alk3b*, causes a subtle defect in ventral expression of the BMP target gene *gata2* at the 75% epiboly stage (Figure 7b). In agreement with previous results (Little & Mullins 2009), we find that simultaneous knockdown of *alk3a*, *alk3b* and *alk6b* (triple knockdown) abrogates ventral *gata2* expression. Coinjection of a full-length *alk6b* mRNA that was engineered to be resistant to morpholino knockdown into the triple knockdown embryos restored *gata2* expression. In contrast, injection of an *alk6b* mRNA containing the G256A mutation was unable to

fully rescue *gata2* expression in triple knockdown embryos. The residual BMP-mediated *gata2* expression in the presence of the mutant *alk6b* allele suggests that the mutant allele is either a hypomorphic allele, or a complete loss-of-function allele that maintains some ability to stabilize BMP receptor complexes containing residual maternally-expressed wild-type Alk6b protein.

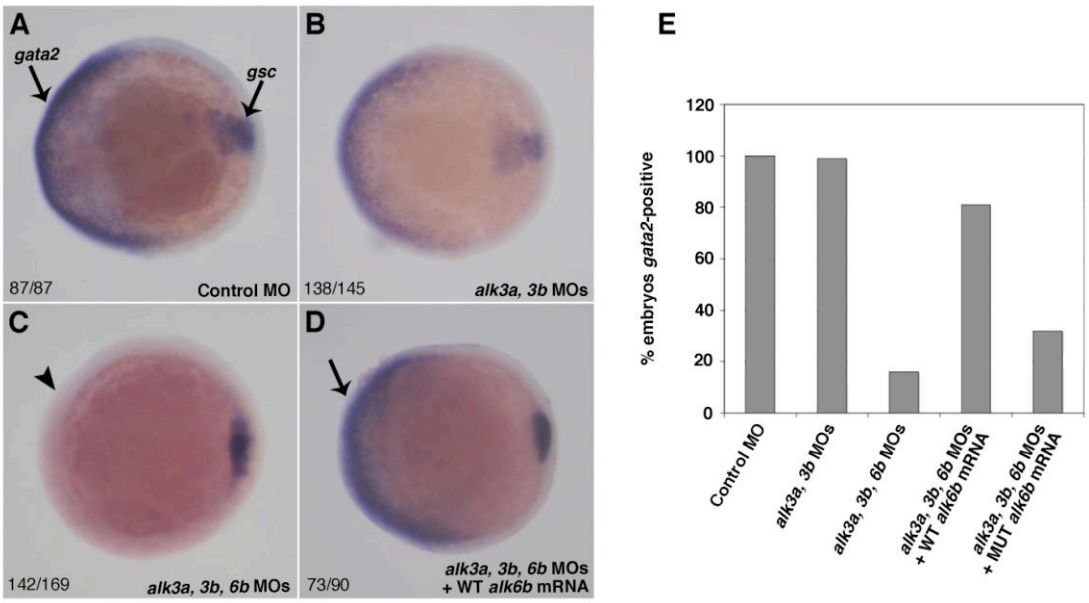


Figure 7: *alk6b*^{tgct} is a loss-of-function allele. (A-D) Whole-mount *in situ* hybridization demonstrating expression of the BMP target gene, *gata2* in control and morpholino knockdown embryos. *goosecoid (gsc)* expression in the prechordal plate marks the presumptive dorsal side of the embryo A. Control morpholino injected embryos at 75% epiboly showing normal ventral expression of *gata2*. B. Morpholino knockdown of the type IA BMP Receptors *alk3a* and *alk3b* maintains *gata2* expression. C. Triple knockdown of *alk3a*, *alk3*b and *alk6b* leads to complete loss *gata2* expression.. D. Injection of wildtype *alk6b* mRNA into triple knockdown embryos rescues *gata2* expression. E. Quantification of percentage of embryos expressing *gata2* after morpholino knockdown and rescue with wildtype or *alk6b*^{tgct} mRNA.

Bmpr1b is highly expressed in spermatogonia and in immature oocytes.

To understand the mechanisms by which inactivating mutations in *alk6b* could impair germ cell differentiation and lead to germ cell tumors, we examined the expression pattern of zebrafish *alk6b* by RT-PCR, immunohistochemistry and in situ hybridization. *alk6b* is expressed most prominently in the ovary, testis, kidney, and skin with some expression seen in other tissues including brain, intestine, and skeletal muscle (figure 8). Most tissues express two alternative splice forms, which differ in the presence

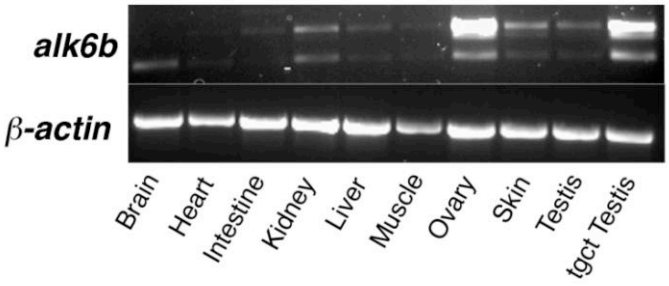


Figure 8: *alk6b* organ expression. RT-PCR indicates that *alk6b* is expressed in most organs with increased expression in the ovary, testis, skin, and kidney.

or absence of exon 4. In the ovary and testis the longer splice form is most prominent. Testicular germ cell tumors arising in *alk6b* mutants consistently express high levels of *alk6b* mRNA, suggesting that the majority of mutant *alk6b* mRNA is not subject to nonsense-mediated mRNA decay. We used in situ hybridization to examine *Alk6b* expression in wildtype and *alk6b-/-* mutant ovaries (figure 9). In wildtype ovaries, we

saw prominent expression in stage I and II oocytes with decreasing expression in more mature oocytes. *alk6b-/-* ovaries have impaired oocyte maturation and display consistent *alk6b* expression in the accumulated immature oocytes, again reinforcing that mutant *alk6b* mRNA is not subject to nonsense-mediated degradation.

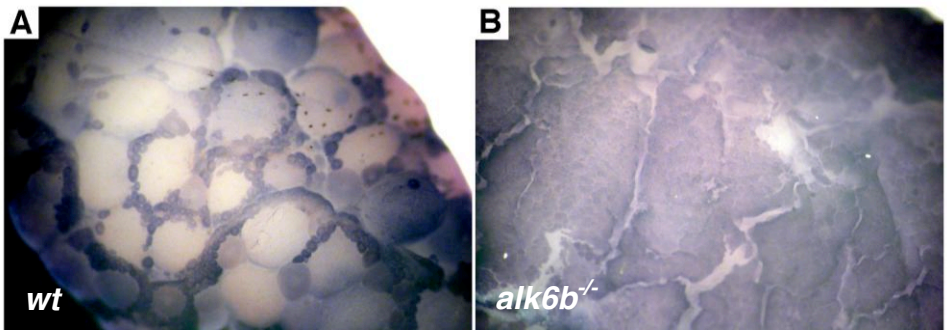


Figure 9: *alk6b* is expressed in immature occytes. (A) *in situ* hybridization indicates that *alk6b* is predominantly expressed in immature occytes (stage I & II) in the wildtype ovary. (B) *alk6b*$^{-/-}$ mutant ovaries have an accumulation of *alk6b* expressing immature occytes with an absence of mature oocytes.

We prepared an antibody directed against the N-terminus of zebrafish *alk6b* and carried out immunohistochemistry on sections of wildtype and alk6b mutant testis (figure 10). In wildtype testis, Alk6b protein is present in a small number of large, peripherally located germ cells in each lobule, consistent with expression in spermatogonia (Figure 10A). In sections of testicular germ cell tumors from *alk6b* mutants, the great majority of the spermatogonial-like tumor cells exhibit Alk6b protein expression. This result is consistent with the RT-PCR expression data showing increased *alk6b* mRNA expression in germ cell tumors.

TGCTs have loss of BMP signaling activity and altered BMP target gene expression.

Next, we tested whether the *alk6b* mutation leads to loss of functional BMP signaling activity in the testis, by performing immunohistochemical staining of phospho-SMAD1/5/8 on sections of wild-type and *alk6b* mutant testis (figure 10 C,D). In wildtype testis, a limited number of cells with nuclear phosphoSMAD1/5/8 are identifiable at the periphery of each testis lobule (Figure 10 C). In contrast, phospho-SMAD staining is absent in the testis tumors from *alk6b* mutants' testis. Thus, there is loss of active BMP signaling in the *tgct* mutant testis. To corroborate this

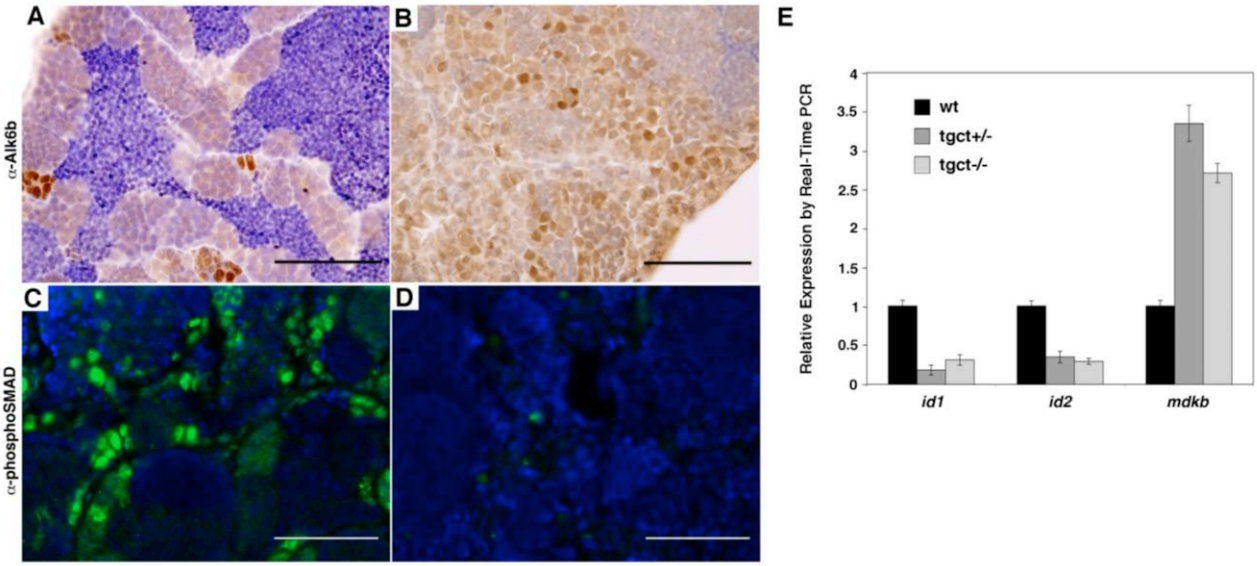


Figure 10: Germ cell-specific Alk6b expression and loss of BMP signaling activity in *alk6b*$^{sw\#}$ mutants. A. immunohistochemistry for Alk6b shows that protein expression is primarily limited to the spermatogonia in the wildtype testis. B. the greater number of undifferentiated spermatogonial-like cells leads to an overall increase of Alk6b in TGCTs. C, D. immunohistochemistry for phospho-SMAD of wildtype and mutant testis. In wildtype testis (C), BMP signaling is active as indicated by phospho-SMAD staining, whereas the mutant testis (D) has an absence of staining. E. Quantification of BMP target gene expression of *id1, id2,* and, *midkine-B (mdkb)* by real-time RT-PCR in *alk6b*$^{sw\#}$ mutants relative to wildtype.. *id1* and *id2* in the mutant testis are downregulated relative to wildtype testis while *mdkb* is upregulated in the mutant testis.

finding, we conducted quantitative Real Time RT-PCR on *id1*, *id2*, and *midkine-B* (*mdkb*), three well-characterized BMP target genes. Consistent with the phospho-SMAD immunohistochemistry results, germ cells tumors from both heterozygote and homozygote *alk6b* mutants display downregulation of *id* gene expression and upregulation of *mdkb* (figure 10E). Thus, despite a relative overexpression of the mutant Alk6b protein in the testicular tumors, the BMP pathway is nonfunctional and BMP target gene expression is altered.

Differential BMP signaling activity is evident in histologically distinct human germ cell tumors.

In the past decade, there has been an increase of studies on potential roles for BMP signaling in tumorigenesis. Evidence indicates that the BMP pathway may play a role in tumor cell differentiation in subsets of prostate, breast, and glioblastomas. In our zebrafish model, loss of BMP signaling is associated with impaired germ cell differentiation. Based on a potential role for BMPs in tumor cell differentiation in cancers and the data resulting from our zebrafish model, we wanted to know whether BMP signaling plays a role in the differentiation of human germ cell tumors. We conducted immunohistochemical staining of phospho-SMAD 1/5/8 comparing 26 clinically-annotated human germ cell tumors displaying different histologies. Results indicate variable BMP signaling activity in histologically distinct GCTs (table 2). The most striking variation is between germinomas and yolk sac tumors. Only 2 of 8

germinomas, an undifferentiated GCT type, displayed weak (<5%) phospho-SMAD 1/5/8 staining. In contrast, 9 of 10 well-differentiated yolk sac tumors displayed focal or diffuse nuclear phospho-SMAD staining, indicating high BMP signaling activity. These results suggest a possible role for BMP signaling in the differentiation of GCT tumors.

GCT Histology	#	Negative	Focal Cytoplasmic	Diffuse Cytoplasmic	Focal Nuclear	Diffuse Nuclear
Germinoma	3 (5)	2 (4)	-	-	1* (1)*	-
Immature Teratoma	7 (1)	-	5 (1)	-	3 (1)	-
Mature Teratoma	1 (3)	(1)	1 (2)	-	-	-
Choriocarcinoma	0 (1)	-	(1)	-	-	-
Embryonal Carcinoma	2 (2)	(1)	1 (1)	1	1	1
Yolk Sac Tumor	6 (4)	-	-	6 (4)	3	3 (3)

Table 2: phospho-SMAD 1/5/8 staining in human germ cell tumors of distinct histologies. Germ cell tumors of distinct histologies exhibit differential phospho-SMAD activity. The most significant distinction was seen between germinomas and yolk sac tumors; 6 of 8 germinomas were negative for phospho-SMAD staining, whereas only 1 of 10 yolk sac tumors was negative.
* <5% cells stained positive, ( ) histologies were part of a mixed germ cell tumor

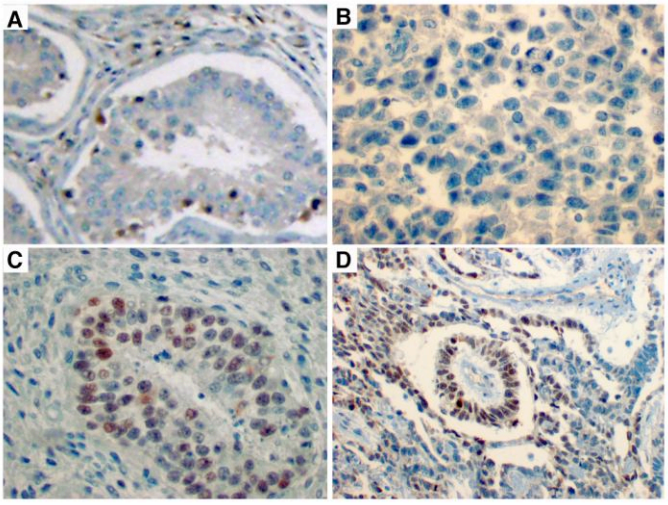


Figure 11: Differential phospho-SMAD activity in human germ cell tumors. Phospho-SMAD staining in human germ cell tumors of distinct histologies indicates differential BMP signaling activity in germ cell tumors. Normal testis exhibits positive staining in spermatogonia (A). Germinomas have an absence of phospho-SMAD positive cells (B). Immature teratomas (C) and yolk sac tumors (D) both display positive phospho-SMAD staining.

Discussion

Proper development and regulation of germ cells is vital to ensure the well-being of the individual and for proper transmission of genetic material to the next generation. Several developmental pathways have been implicated as having crucial roles in this process including wnt, Hedgehog, and Notch (Cook & Capel 2010). More recently, the BMP pathway has also been shown to play significant roles in germline development and maintenance. Specifically, the BMP pathway plays key roles in germ cell specification and the initiation and maintenance of spermatogenesis (Zhao 2003).

We identified a nonsense mutation in BMP receptor, *alk6b*, the zebrafish homolog to human *bmpr1b*, which impairs germ cell differentiation and causes testicular germ cell tumors in zebrafish. Affected adult male fish develop bilateral TGCTs that can grow to occupy the entire abdominal cavity. The tumors are notable for impaired spermatocytic differentiation that leads to the accumulation of undifferentiated spermatogonial-like cells that disrupt normal testicular architecture. Homozygous mutant females display an oocyte maturation defect that affects fertility and causes an increase of stage I and II immature oocytes.

The nonsense mutation truncates the Alk6b kinase domain while keeping the extracellular ligand binding domain, transmembrane domain, and GS box activation domain intact. Although the results from our morpholino knockdown assay and the lack of phenotype in heterozygous females indicate that the nonsense mutation causes loss-of-function, we cannot rule out the possibility that the truncated Alk6b receptor has dominant-negative function. There is precedence for such a model. In *Xenopus*, a

similarly truncated BMP receptor acts as a dominant-negative protein to abrogate BMP signaling in developing embryos (Suzuki et al 1994). Furthermore, Bambi, a naturally occurring mock BMP receptor that lacks a kinase domain, acts in a dominant-negative manner to inhibit BMP signaling by forming a nonfunctional complex with BMPs and type II BMP receptors (Miyazono 2000; Onichtchouk et al 1999). The generation of transgenic wildtype zebrafish lines that express the mutant *alk6b* allele through a ubiquitous or germ cell specific promoter, now ongoing, may help to clarify whether the truncated protein functions as a dominant-negative receptor.

We show that zebrafish Alk6b is normally expressed in the early germ cells in the testis and ovary. The accumulation of spermatogonial-like cells in TGCTs and immature oocytes in the ovary leads to an overall increase in mutant *alk6b* mRNA suggesting that it is not subject to nonsense-mediated mRNA decay. Despite the increase of *alk6b* in the testis, we demonstrate that the truncation of *alk6b* causes a loss of BMP signaling activity in TGCTs. Results of this study indicate a novel germ cell autonomous role for zebrafish *alk6b* in germ cell differentiation.

The BMP pathway in spermatogenesis.

Targeted inactivation of BMPs in mice and Drosophila has revealed essential roles for the BMP pathway in spermatogenesis. BMP expression studies have demonstrated that both germ cells and supporting Sertoli cells express specific BMP pathway components that interact to regulate germ cell proliferation and differentiation.

However, a prior study suggested that Bmpr1b is not expressed in the mouse testis and thus has not been included in some subsequent studies (Puglisi et al 2004; ten Dijke et al 1994). This may suggest that Bmpr1b is not required in mice for spermatogenesis or it is also possible that Bmpr1b may indirectly affect in spermatogenesis from outside of the testis. Here we show that zebrafish Alk6b is expressed in spermatogonial cells and the loss of Alk6b function in the testis causes testicular tumors consisting primarily of premeiotic spermatogonial-like cells that are unable to undergo spermatocytic differentiation. This indicates a specific requirement for BMP signal transduction through Alk6b for germ cell differentiation in zebrafish. Increasing numbers of tumor cells in TGCTs result in enlarged, distended abdomens in affected males and indicates a potential role for Alk6b in the inhibition of spermatogonial cell proliferation.

Both *alk6b* homozygous and heterozygous mutant males exhibit normal spermatogenesis prior to tumor development. This may reflect a specific requirement for Alk6b in the maintenance of spermatogenesis but not necessarily in the induction of spermatogenesis upon reaching sexual maturity. Immunohistochemistry staining for phospho-SMAD in testes sections of young *alk6b* homozygous mutants or heterozygotes with early tumors, show evidence of phospho-SMAD staining in areas of normal spermatogenesis and lack of staining in sites of tumor development (data not shown). Results from a preliminary real-time PCR study which assays BMP target gene expression in aging wildtype males may further reinforce the idea that Alk6b is required for maintenance but not in induction of spermatogenesis (appendix A). In this experiment, testis RNA from wildtype males of increasing age were assayed for *id1* and *id2* expression levels. At 3 months of age, there was low *id1* and *id2* expression which

significantly increased by 8 months of age and then gradually declined at 1 year and > 2 years age. Despite positive phospho-SMAD staining in wildtype testes at 3 months of age, the onset of sexual maturity, we observe low expression of BMP target genes, *id1* and *id2*, mRNA. This suggests that alternate BMP receptors, that are not upstream receptors of *id1* and *id2,* may be involved during the induction of spermatogenesis while Alk6b is required for germ cell differentiation after induction has occurred.

In mice, BMP2, BMP4, BMP7, BMP8a and BMP8b, are all expressed in the testis and have various roles during spermatogenesis (Zhao 2003). We are currently crossing the *alk6b* mutant line with other known zebrafish BMP mutant lines including BMP2b, BMP4, and BMP7, which may help to elucidate specific BMP ligands that are required for germ cell differentiation in zebrafish.

The role of zebrafish Alk6b during development.

BMPs are powerful components with vital roles during various phases of development such as PGC specification, dorsoventral patterning, left-right asymmetry, and organogenesis. In mice, Bmpr1b is involved in early development of the appendicular skeleton and is required for chondrocyte differentiation (Yi et al 2000). In zebrafish, BMP signaling during embryogenesis is required for dorsoventral patterning and left-right asymmetry (Kishimoto et al 1997; Monteiro et al 2008). However, we did not detect a visible embryonic phenotype in our *alk6b* mutant line. There are several possibilities for these results. Similar to other pathways, various BMP pathway

components, including *alk6*, have undergone gene duplication in zebrafish. The functional redundancy of the BMP pathway may help to compensate for the loss of *alk6b* during development. Also, the presence of maternally provided wildtype Alk6b protein may counteract any loss of BMP signaling due to the mutation. There is also a possibility that despite embryonic expression, *alk6b* is not required during embryogenesis and that the *alk6a* paralog may be one of the essential components during embryogenesis. In fact, *alk6a* is expressed during embryogenesis and early limb formation (Nikaido et al 1999). However, we do not rule out subtle embryonic defects that are not readily seen and are currently investigating potential roles for *alk6b* during embryogenesis.

The BMP pathway in germ cell tumors.

Most cancers of the testes originate in the germ cells and include seminomas, nonseminomas, and spermatocytic seminomas. Seminomas, the most prevalent cancer in men aged 20 to 40 years, originate in the primordial germ cells (PGCs) or gonocytes and are found primarily in the testes, although they can be located in different parts of the body. Seminomas are malignant and have additional copies of chromosome 12p (Oosterhuis & Looijenga 2005). In the US, approximately 8000 cases of type II TGCTs are diagnosed each year resulting in 300-400 deaths and the incidence of seminomas is steadily rising for unknown reasons (Carver & Sheinfeld 2005). The nonseminomas of adults include embryonal carcinomas and the more differentiated forms of teratomas, yolk sac tumors, and choriocarcinomas. Spermatocytic seminomas are found only in

adult male testes and are generally benign (Oosterhuis & Looijenga 2005). It is not known what causes germ cells to develop into tumors and the underlying genes and molecular pathways responsible for TGCTs have not yet been identified due to the lack of a suitable animal model. The accumulation of primitive, undifferentiated germ cells in tumors from *alk6b* mutants resemble the histology seen in human seminomas. Other shared features with seminomas include impaired germ cell differentiation and sensitivity to radiation therapy (Neumann et al 2009). However, the noninvasive, benign nature of the *alk6b* tumors along with bilateral tumor occurrence is more reminiscent of spermatocytic seminomas. The zebrafish model may be an invaluable tool for increasing our understanding of human germ cell tumors.

BMP signal transduction may play a role in the differentiation and development of human germ cell tumors. Inactivating mutations in co-Smad4 were identified in 2 of 20 seminomas (Bouras et al 2000). BMP target genes, Id1 and Id2, were found to be differentially expressed between seminomas and yolk sac tumors. High expression of Id1 and Id2 was seen in well differentiated yolk sac tumors, suggesting a potential upregulation of BMP signaling, whereas the undifferentiated seminomas had low Id1 and Id2 expression (Albanese et al 2001). More recently, comparative genome expression comparing type I and type II germ cell tumors, revealed that BMP2 and BMP4 are highly expressed in the well differentiated yolk sac tumors (Palmer et al 2008). Based on these reports and the results from our zebrafish model, we examined BMP signaling activity in human germ cell tumors of distinct histologies and discovered differential BMP signaling activity between the different tumor types. In agreement with our zebrafish model, the undifferentiated germinomas exhibited absence of BMP activity whereas the well-

differentiated yolk sac tumors consistently maintained BMP signaling activity. Currently, we are conducting further examinations of the potential roles of BMP signaling in germ cell tumor development, including quantitative real-time PCR comparing expression of TGF-β/BMP members between germinomas and yolk sac tumors. Consistent with the phospho-SMAD staining, preliminary data indicates that yolk sac tumors display higher expression of BMP components relative to the germinomas (N. Fustino and J.F. Amatruda, in progress).

A similar pattern of differential BMP activity is also seen in other cancers. Various prostate and breast cancer studies have demonstrated increased expression of BMP components and BMP activity in well differentiated tumors and early bone metastasis. In contrast, downregulation of BMP components and loss of BMP signaling activity is also associated with highly malignant, poor prognosis, undifferentiated prostate and breast tumors (Alarmo & Kallioniemi 2010; Kim et al 2000). Taken together with the results from our study of BMP activity in GCTs, a dual role for BMP signaling may be indicated in germ cell tumor suppression and germ cell tumorigenesis.

Conclusion & Future Directions

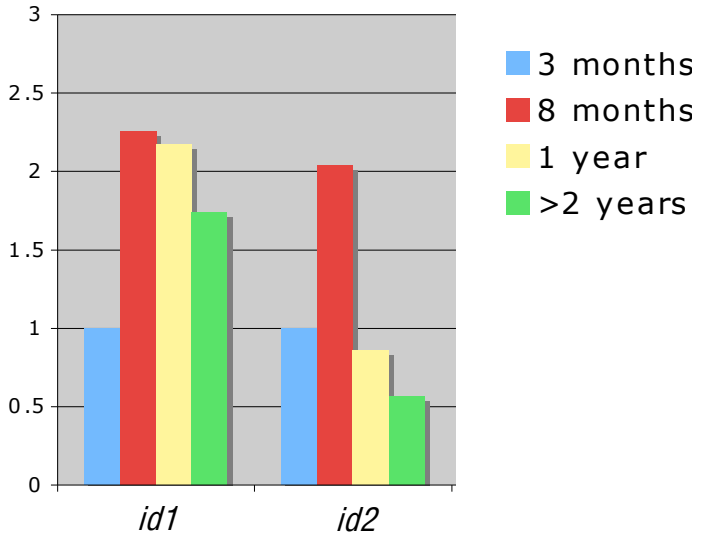
These data have demonstrated the importance of BMP signaling and *alk6b* in zebrafish germ cell differentiation and has indicated a potential role for BMP signaling in human germ cell tumor differentiation. This report highlights the importance of continuous study of the BMP pathway in germ cell regulation and the impact that

aberrant signaling may have on the development and differentiation of human germ cell tumors.

In the zebrafish model, in order to gain a better understanding of the mechanism of germ cell tumor development, there is a need to identify *alk6b* upstream and downstream components. Currently, we are looking at tumor incidence and latency in *alk6b* mutants crossed with one of the known zebrafish BMP mutants (BMP2b, BMP4, and BMP7) to assess any impact to the *alk6b* phenotype. We will also generate transgenic zebrafish lines that will attempt to rescue or phenocopy the *alk6b* phenotype. We will initially focus on expressing *id1* and *id2* in *alk6b* mutants to assess the ability for these genes to rescue the TGCT phenotype. We will also overexpress *mdkb* in a wildtype line and examine the testes for GCTs. CHIP-seq may also be used to identify direct Smad targets in the testis. We can also use the *alk6b* line to conduct small molecule and drug screens to potentially identify alternate therapies for GCTs.

Several studies in the human germ cell tumors are now ongoing to determine potential roles for BMP signaling in germ cell tumor development. These studies include examining TGF-β/BMP signaling pathway mRNA and protein expression in germinomas and yolk sac tumors, microRNA array analysis to identify potential microRNAs that affect BMP signaling, high-resolution CGH arrays, and methylation analysis of human *Bmpr1b* in seminoma genomic DNA samples.

APPENDIX A



Supplementary Figure 1: BMP signaling declines in testis as male zebrafish age. Relative expression of *id1* and *id2* in zebrafish testis is initially low at 3 months of age. There is a striking increase of both genes at 8 months of age, followed by a decline in expression as the males age.

CHAPTER FOUR
Methods

Fish Maintenance:

Zebrafish were maintained according to standard procedures (Detrich et al 1999) on a 14 hour light/10 hour dark cycle in a recirculating system (Aquaneering, Inc., San Diego, CA). Zebrafish embryos were obtained by natural spawning of adults and staged according to (Kimmel et al 1995). Tricaine methanesulfonate was used as an anesthetic for all procedures and for euthanasia. All work with zebrafish was carried out under protocols approved by the Institutional Animal Care and Use Committees at UT Southwestern Medical Center, an AAALAC-accredited institution.

Genetic mapping, Linkage analysis, Interval haplotype analysis:

For interval haplotype analysis, we first identified polymorphic markers that distinguished wik and AB strains at the centromere and telomeres of each zebrafish chromosome, using known SSLPs (simple sequence length polymorphisms) from the MGH genetic map (Shimoda et al 1999). This analysis identified 50 haplotypes covering the 25 zebrafish chromosomes (Supplementary Table 1). Next, a known tgct carrier female of the wik strain was mated to an AB strain male followed by a backcross to AB. We selected 15 affected males with testicular germ cell tumors from this F2 backcross, and genotyped with the centromeric and telomeric markers. Probable linked haplotypes were identified based on the exclusion of intervals with a large number of non-recombinant haplotypes from the unaffected parent, using the program MaxX2.V1 (kindly provided by David Beier, Harvard Medical School, Boston, MA) (Supplemental Table 2).

After initial assignment to chromosome 10, further high-resolution recombinational mapping was performed on a panel of 449 fish. In addition to existing SSLP markers (Shimoda et al 1999), novel candidate SSLP marker primer pairs for this work were generated using the Zebrafish SSR search website (Massachusetts General Hospital, Charlestown MA 02129; World Wide Web URL: http://danio.mgh.harvard.edu/markers/ssr.html). To resolve inconsistencies between the genetic map and the Zv6 Sanger Center Assembly (http://feb2007.archive.ensembl.org/Danio_rerio/index.html), we generated a custom, high-resolution genetic map of the interval between z9208 and z7316, using a panel of 493 F2 embryos from an AB/wik hybrid backcross (Supplementary Figure X).

Genotyping:

The 5' Nuclease Allelic Discrimination assay (Applied Biosystems) was used to genotype the alk6b mutant line. Sequences for the fluorogenic probes used were TCGCCCCTCCAGCGG (wildtype – VIC) and CGCCCCTTCAGCGG (alk6b mutant –

FAM). Primer sequences used for amplification were: AD assay alk6b F – 5' CAGATCCAGATGGTGAAGCAGAT 3' and AD assay alk6b R – 5' TCCTCTGTGGTGAAGAAGACCTT 3'.

In vitro differentiation assay and FACS sample preparation:

Testes were removed from euthanized fish, washed twice in 1X PBS with antibiotic/antimyotic (Gibco) and centrifuged at 1000 rpm for 5 minutes. PBS was removed after centrifugation and dispase (Becton Dickinson) added, followed by gentle agitation for 30 minutes at 32o C for tissue digestion. Digested tissues were resuspended and cultured in modified DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F12) (see medium recipe below) on a gelatin layer for 1-8 days. Cells were harvested and pelleted in 1 mL DMEM at 2400 rpm for 4 minutes. Supernatant was removed and pellet was resuspended in 500 µL 1X PBS. Samples were placed in FACS culture tubes and 1.5 mL Propidium Iodide (0.05 mg/mL)/ Sodium Citrate (0.1%)/Triton X-100 (0.0002%) mix was added, followed by 4 µL DNase-free RNase. DNA content of the samples were determined a Fluoresence-Activated Cell Sorting on a FACS Calibur flow cytometer (Becton-Dickinson).

For the modified DMEM/F12 medium used for culturing, the following reagents were added to 500 mL DMEM/F12 and then filter sterilized:
5 mL antibiotic/antimyotic, 5 mL MEM vitamins, 5 mL MEM non-essential amino acids, 5 mL 200mM L-Glutamine, 5% FBS (fetal bovine serum), and 5% trout serum.

Transgenic Line Generation:

Tol2 transposon transgenesis constructs were used to generate transgenic zebrafish lines for rescue (Kwan et al 2007).

The *Tol2* construct with wildtype *alk6b* driven by the ubiquitous promoter β-actin was generated following Invitrogen's Multisite Gateway Three-Fragment Vector Construction kit and protocol. The following clones were used:

5' Entry: p5E-*bactin2*
Middle Entry: pDONR 221
3' Entry: p3E-polyA
Destination vector: pDESTtol2pA2

The Phusion High-Fidelity PCR kit and protocol was used to add *att* sites to the ends of the wildtype *alk6b* DNA fragment (forward primer with *att* B1 site: 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTTCGTCTGCAGATTGAGGTG 3', reverse primer with *att* B2 site: 5' GGGGACCACTTTGTACAAGAAAGCTGGGTTCTGGAATACACCCCTGGAG 3'). The QIAquick Gel Extraction kit (Qiagen) was used to purify the DNA fragments with

attached *att* sites. BP and LR reactions were conducted as specified in the Gateway manual to generate the final *Tol2* expression construct.

Expression constructs (50ng/uL) with transposase RNA (40ng/ul) were injected into one-cell stage embryos from *alk6b*$^{+/tgct}$ incrosses. Injected embryos were raised to adulthood and screened for transgene incorporation. Adult *alk6b* homozygous mutant injected fish were sacrificed at 6 months of age. Transverse ovary and testis sections were paraffin-embedded and stained with H&E for examination for rescue.

DNA Isolation from Paraffin-Embedded Ovarian Sections:

Ovarian tissue was scraped from five 10 micron thick unstained paraffin-embedded sections and deparaffinized in 0.5mL Xylene for 15 minutes, followed by centrifugation at 13K rpm for 3.5 minutes. Supernatant was discarded and replaced with 0.5mL fresh Xylene for 30 minutes, followed by centrifugation (13K rpm, 3.5 minutes). Xylene was removed and pellet was washed with 100% Ethanol (15 minute incubation at room temperature, centrifugation at 13k rpm for 5 minutes), followed by washing with 70% ethanol following the above steps. After EtOH removal, 400 uL of Lysis/Proteinase K buffer (see below) was added to each sample and incubated overnight at 55^{o} Celsius. After incubation, 400 uL Phenol/Chloroform was added to sample and vortexed thoroughly to mix. Centrifuged at 13K, RT, for 7 minutes. Top aqueous phase was removed to new microcentrifuge tube. 1 volume chloroform was added to each sample, vortexed thoroughly, and centrifuged at 13K rpm, at RT, for 7 minutes. Top aqueous phase was removed to another new microcentrifuge tube. Added 1/10th volume 3M sodium acetate and 2 volumes 100% EtOH and incubated at -80^{o} C for 30 minutes. Centrifuged sample at 13K rpm, at 4^{o}C, for 30 minutes. Removed supernatant and washed pellet with 70% EtOH (centrifuged at 13K rpm, at 4^{o}C, for 5 minutes). Removed EtOH, air dried for 5 minutes, and resuspended DNA in 30 uL ddH_2O.

Morpholino and mRNA injections:

To prevent non-specific toxic effects, all morpholinos were all injected into p53-deficient tp53zdf1/zdf1 embryos at the one-cell stage. 2 nL total volume of morpholinos were injected at the following concentrations: Gene-tools Standard Control MO (1.0 mM), Alk3a (0.25 mM), Alk3b MO1 (0.125 mM), Alk3b MO3 (0.125 mM), Alk6b-E4-MO1 (0.5 mM), alk6b-E3/5-MO2 (0.125mM). All morpholinos used were purchased from Gene Tools. Morpholino sequences for Alk3a and Alk3b were as described (Little & Mullins 2009). The sequences for the alk6b morpholinos were E4-MO1-TTTCGTTTCCCGTCCGTACCCGGAG and E3/5-MO2 – AGCACGTTCGCTGTGTGTCACTCAG. Alk6b wildtype and mutant mRNA used in the rescue assays were generated using the T7 mMessage mMachine (Ambion), linearized with XhoI (New England Biolabs) and co-injected with morpholinos at a concentration of 25 ng/uL. To make the alk6b rescue constructs resistant to morpholino

knockdown, the morpholino target sites in the alk6b mRNA sequence were altered as follows:

E4-MO1 target sequence:
Unaltered: ctccgggtacggacgggaaacgaaa, Altered: ctgcgcgttcgcaccggtaatgag

E3/5-MO2 target sequence:
Unaltered: ctgagtgacacacag/cgaacgtgct, Altered: ctcagagagactcag/cgtacctggt

in situ Hybridizations:

In situ hybridizations for gata2, gsc, and alk6b were performed as described (Thisse & Thisse 2008). Embryos were mounted in 100% glycerol and images were taken at 8.0X magnification with a Leica MZ12.5 stereomicroscope equipped with a Nikon E4500 camera.

RT-PCR:

Total RNA was extracted from wild-type and alk6b-/- testes using Trizol reagent (Invitrogen). Genomic DNA was eliminated with RNase-free DNase I (Invitrogen). To compare the amount of midkine-b (mdkb) mRNA in 1.0 ug of each RNA sample, semi-quantitative RT-PCR was conducted using the Qiagen One-Step RT-PCR kit. Primers for reverse transcription and mdkb cDNA amplification were mdkb-forward (5' GAGAGCTGAACGCAGACACA 3') and mdkb-reverse (5' AACAGCGCCTTCTTCAATGT 3').

Quantitative Real-Time PCR:

For RNA extraction, testes were dissected from adult wild-type and alk6b mutant fish and immediately homogenized in 500uL Trizol (Invitrogen) using the Tissuemiser homogenizer from Fisher Scientific and incubated at room temperature for 30 minutes. Chloroform (200uL) was added to each sample, vortexed for 15 seconds and incubated for 3 minutes at room temperature. Samples were then centrifuged at 13K rpm for 15 minutes at 4oC and the upper aqueous phase was removed to a new tube. One volume of 70% ethanol was added for RNA precipitation and samples were loaded onto a RNeasy mini kit (Qiagen) spin column and processed according to the RNeasy kit protocol. First strand cDNA from 1 ug total RNA was obtained using random hexamer primers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Primer sequences for Rpl13a and EF1a were as described Tang et al., 2007 (Validation of qPCR reference genes paper) Primer sequences (see list below) for id1, id2, and mdkb were designed using Genscript's online real-time primer design tool and span an exon-exon boundary with the exception of mdkb.

id1: Forward 5' GCATCTCTGTGGAGAACGG 3', Reverse 5' CGATGCTTCGATGATCTGTT 3'

id2: Forward 5' CCCTGACAACACTCAACACA 3', Reverse 5' AACCAAACACCTGATTAACGG 3'

mdkb: Forward 5' TAGCGGAGACTGTGGAAATG 3', Reverse 5' ATGGGACTTTGCACTTGGTT 3'

Standard dilution and disassociation curves were performed to test the validity of each primer pair prior to conducting the relative quantitative assay. All assays were performed on Applied Biosystems 7900 HT real-time PCR instrument and gene expression analysis was conducted by SDS 2.3 and RQ Manager 1.2 software.

Alk6b N-term Antibody:

Rabbits were immunized with a synthetic peptide CTAGRKETNGGS derived from Alk6b residues 44-54 (GenScript Corporation). Immune serum was used for affinity purification against immobilized peptide.

Immunohistochemistry:

For zebrafish immunohistochemistry: zebrafish were euthanized with tricaine, fixed 48 hrs., 4 ˚C. in 4% paraformaldehyde/1X phosphate-buffered saline, and decalcified 5 days in 0.5 M EDTA (Moore et al 2002). Fixed, decalcified specimens were dehydrated and paraffin-embedded, and 4 um transverse sections were prepared. For immunostaining, the slides were deparaffinized, placed in Trilogy reagent (Cell Marque) and subjected to antigen retrieval for 15 minutes in a pressure cooker. Endogenous peroxidase activity was quenched by incubation in 0.3% H2O2 for 30 minutes at room temperature, and non-specific binding sites were blocked in 2.5% horse serum for 30 minutes at room temperature. Slides were incubated overnight at 4^{o} C with the following antibodies: phospho-SMAD 1/5/8 (Cell Signaling) at 1:500, N-term Alk6b (GenScript) at 1:250, and gamma-h2ax at 1:250 (incubated at RT for 2 hours). Slides were washed extensively in PBS with 0.1% Tween-20 (PBST), incubated with anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Immpress kit, Vector) at RT for 30 minutes, washed in PBST and developed with diaminobenzidine/H2O2 (Immpress). Slides were counterstained with hematoxylin (Invitrogen), dehydrated and mounted with Depex.

Immunohistochemistry on the human germ cell tumors was conducted by Children's Medical Center of Dallas, Pathology Department. The phospho-SMAD 1/5/8 antibody from Cell Signaling was used.

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