BONE MORPHOGENETIC PROTEIN SIGNALING REGULATES GERM CELL

PLURIPOTENCY, SEXUAL DIFFERENTIATION

AND CANCER SUSCEPTIBILITY

APPROVED BY SUPERVISORY COMMITTEE

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James F. Amatruda, M.D., Ph.D.

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Diego Castrillon, M.D., Ph.D.

_____

Melanie Cobb, Ph.D.

_____

Ondine Cleaver, Ph.D.

DEDICATION

This work would not have been accomplished without the unwavering support of my family through good and bad times. Those that are blood and those that have made lasting impressions in my life one way or another. This work is dedicated to all of them for they each contributed to its completion in one way or another.

I'd like to dedicate this to my mom and sister who has been instrumental in the accomplishment of this large feat. Mom it is your sacrifices, your strength, your courage, and your intellect that guides me EVERY SINGLE DAY. You never let us believe that coming from a single parent home living in poverty was anything to stand in our way from becoming successful. You guided us and pushed us to excel way passed any limit we ever set for ourselves and never allowed us to quit. Meli your unconditional love was instrumental in this achievement. Your artistic abilities are so inspiring and drive me to think outside the box in order to better visualize my science. Thank you for pushing me in the right direction when it was needed and for your everlasting support. I know I can call you anytime and you will be by my side.

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Lupita you were the aunt I never had. Thank you for the adventures and showering Meli and I with love despite us not being related. At times you were like our second mom and were unbelievable understanding about everything. I know I can count on you any time. A fragment of this work is also dedicated to you.

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BONE MORPHOGENETIC PROTEIN SIGNALING REGULATES GERM CELL PLURIPOTENCY, SEXUAL DIFFERENTIATION AND CANCER SUSCEPTIBILITY

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ANGELICA SANCHEZ

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BMP SIGNALING REGULATES GERM CELL PLURIPOTENCY, SEXUAL DIFFERENTIATION AND CANCER SUCEPTIBILITY

Angelica Sanchez, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2016

James F. Amatruda, M.D., Ph.D.

Testicular germ cell tumors are the most common malignancy found in young men between the ages of 14-40. While these tumors are highly curable with cisplatin based combined chemotherapies, the treatments come with very detrimental side effects and ultimately fail in up to 15% of patients. These patients have no other avenues of treatment and often times succumb to the disease. Very little is currently known about the biology of the tumors but risk factors highlight possible mechanisms of action. For example, patients with Disorders of Sex Development (DSD) have increased risk for developing malignant germ cell tumors. DSDs manifest at birth and present with atypical gonadal or anatomical sex as well as chromosomal

aberrations[1, 2]. Some cases are explained by the presence of chromosomal aberrations, but the cause of many others remains unknown. Such syndromes thus highlight the potential links between germ cell pluripotency, sexual differentiation and cancer susceptibility. A recent GWAS study [3] identified association of BMP7 with testicular dysgenesis syndrome; however the molecular mechanisms behind this association remain unknown. Previously, we described the development of testicular germ cell tumors in zebrafish carrying a mutation in *bmpr1bb*, a BMP family receptor, and demonstrated that human GCTs have defects in BMP signaling. Here I use this model and next-generation sequencing analysis in a cross-species comparative oncology approach to identify genes and pathways with fundamental importance to the development of the human disease. I further defined the role of specific BMP ligands in mediating germ cell differentiation and identified reciprocal somatic- and germ cell BMP signaling events that regulate germ cell differentiation and maturation. Using genetic crosses to further impair BMP signaling, I found that zebrafish doubly heterozygous for mutations in *bmpr1bb* and *bmp2b*, *bmp7a* or *smad5* have profoundly impaired gonadogenesis and altered male:female sex ratios. Affected fish also exhibit markedly abnormal gonadal differentiation, including the presence of undifferentiated gonadal tissue and the occurrence of biphenotypic gonads, as well as greatly increased germ cell tumor susceptibility. Our findings implicate defective BMP pathway signaling as a potential factor in DSDs and GCT susceptibility. Our goals are to identify biological mechanisms that govern germ cell differentiation and to understand how defects in this process cause human disease.

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PRIOR PUBLICATIONS

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Dranow D.B., Hu K., Bird A.M., Lawry T., Adams M.T., Sanchez A., Amatruda J.F. and Draper B.W. Bmp15 is an oocyte-produced signal required for maintenance of the adult female sexual phenotype in zebrafish. PLoS Genetics *in press* (2016).

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LIST OF ABBREVIATIONS

BMP- Bone morphogenetic protein

DSD- Disorders of sex development

GWAS- Genome wide association study

GCT- Germ cell tumor

NGS- Next generation sequencing

PGC- Primordial germ cell

Sdf1a- Stromal-derived factor 1a

DND- Dead end

DAZL- Deleted in azoospermia-like

Stra8- Stimulated by retinoic acid gene 8

DMRT1- Doublesex and mab-3 related transcription factor

CNS- Central nervous system

YST- Yolk sac tumor

TGCT- Testicular germ cell tumor

ENU- N-ethyl-N-nitrosourea

TGF-β- Transforming growth factor beta

FACS- Fluorescence activated cell sorting

DPF- Days post fertilization

ITGCN- Intratubular germ cell neoplasia unclassified

CIS- Carcinoma *in situ*

AD- Allelic discrimination

PFA- Paraformaldehyde

GFP- Green fluorescent protein

PCR- Polymerase chain reaction

RIN- RNA integrity number

RMA- Robust multiarray average

PCA- Principal component analysis

TCGA- The Cancer Genome Atlas

LOH- Loss of heterozygosity

RT-PCR- Reverse transcription polymerase chain reaction

H&E- Hematoxylin and eosin

ZEBRAFISH GERM CELL TUMORS
Introduction

GERMLINE DEVELOPMENT IN FISH, MOUSE AND HUMAN

Primordial Germ Cell (PGC) Specification

The earliest cells of the germline lineage are known as Primordial Germ Cells (PGCs). PGC specification follows two distinct mechanisms, either preformation or induction, depending on the organism. Shortly after fertilization, zebrafish embryos, similar to *C. elegans*, *Drosophila* and *Xenopus*, contain preformed maternal RNA and proteins that are prepackaged in a cytoplasmic nuage, known as a germ plasm [4-7]. The germ plasm selectively and transcriptionally silences the expression of somatic genes while keeping the PGCs in a primitive pluripotent state. In flies and worms, RNA binding proteins including Pumilio and Nanos contribute to this repression of somatic fate [8, 9]. In zebrafish, *nanos* is essential for the development of PGCs [8].

The identification of the zebrafish *vasa* ortholog greatly facilitated efforts to understand the development of the zebrafish germline [10-13]. Vasa is an RNA helicase that was previously shown to be a component of the germline in nematodes, flies and frogs [14-17]. In zebrafish, maternally produced *vasa* mRNA is localized to electron-dense material at the cleavage planes of embryos at the 4-cell stage. By the 4000-cell stage, four *vasa*-positive PGCs can be identified. These will undergo several rounds of proliferation to produce a population of 25-50 migrating PGCs that will form the gonad [10, 11, 13, 18].

In contrast to organisms with preformed germline components, in mice and humans PGCs are produced in the epiblast through a process known as induction. In response to signals produced in the extraembryonic ectoderm and visceral endoderm, including Bone Morphogenetic Proteins (BMP) 2, 4 and 8b [19-22], some of the epiblast cells begin to express the marker *Fragilis* (also known as *Mil-1/ Ifitm3*), signaling their competence to become germ cells [21, 23-29]. A few of these *Fragilis*-expressing cells will begin to express the transcriptional repressor *Prdm1* (PR-domain-containing protein 1), also known as *Blimp1* (B-lymphocyte-induced maturation protein 1) [30]. *Blimp1,* along with *Blimp14,* acts to repress expression of somatic genes and maintain expression of pluripotency genes such as *Stella, Oct3/4* and *Nanog* [23, 31-39].

PGC Migration

In nearly all metazoans, PGCs arise at sites distant from the future gonad and must migrate during embryogenesis to reach the proper location for eventual gonadogenesis [40-43]. In contrast to other organisms, zebrafish PGCs arise in four random locations with respect to the developmental axis of the embryo. The PGCs migrate to a series of intermediate targets, converging into bilateral clusters in the presomitic mesoderm, where the gonad will form [6, 13, 18, 44]. This guided migration is orchestrated by an attractant gradient established by somatic expression of Stromal-derived factor 1 (sdf1a), also known as CXCL12 [45, 46]. The sdf1a ligand interacts with the chemokine receptor cxcr4b expressed on the surface of PGCs [45, 46]. This homing signal is further fine-tuned by the cxcr7 receptor on somatic cells, which acts as a repulsive guidance cue [47].

In mice and other mammals, the newly specified PGCs must travel from the primitive streak and localize to the endoderm around stage E7.5 [48, 49]. From E8 to E9.5 the PGCs migrate through the dorsal mesentery of the hindgut. Upon reaching the level of the gonadal ridges, the PGCs exit the hindgut in bilateral streams to populate the gonadal mesoderm [40, 49]. The c-Kit receptor and its ligand, KitL, are critical for the migration and survival of PGCs in mice [50-53].

Germ cell differentiation

Irrespective of the future sex of the animal, germ cells in zebrafish initially begin to differentiate into oocytes and are in a state known as juvenile hermaphroditism [54]. Upon the start of male germ cell differentiation, oocytes in male zebrafish undergo apoptosis as the mature male germline emerges [55]. Characterizations of male sex determination factors have identified several genes that are specific to the male gonads, such as anti- Müllerian hormone (*amh*) and *sox9a* [56-58]. Furthermore, *sox9a* was shown to control juvenile hermaphroditism and regulate the transition to male fate producing mature sperm [59].

During gonadogenesis, the number and persistence of germ cells makes important contributions to sex determination [60, 61]. Several studies have revealed that lower PGC numbers promote male differentiation in zebrafish. Loss of *dead end* (*dnd*), an RNA-binding protein that is essential for PGC survival [62], leads to loss of all germ cells and male phenotypic differentiation [60, 63, 64]. Similarly, loss of function *ziwi*, the zebrafish *piwi* homolog, leads to reduced numbers of germ cells and male phenotypic development [65]. Remarkably, Dranow

and Draper found that adult female zebrafish depleted of germ cells due to loss-of-function mutations in *nanos3* convert to a male phenotype [61]. They concluded that a germ cell-derived signal acts on the somatic gonad to promote female development directly or indirectly by repressing male-specific gene expression.

In mice and other mammals, licensing is the initial step in which germ cells begin to differentiate into their respective sex cells dependent on the sex chromosomes present in the organism. This licensing event is initiated by the RNA-binding protein known as Deleted in azoospermia-like (DAZL); *Dazl* mutants no longer express sex specific markers and do not begin meiosis [66, 67]. During this transition, the germ cells are released from their transcriptional repression [68]. In prenatal males, presence of the SRY gene on the Y chromosome regulates expression of Sox9 and is necessary for proper male gonadal development and spermatogenesis [69-71]. Humans with a heterozygous mutation in *SOX9* develop a bone disorder known as campomelic dysplasia that is accompanied by gonadal sex reversal, while XY mice with homozygous mutations exhibit ovarian development [72-74]. These studies emphasized the importance of proper Sox9 expression in testis development. To maintain male identity prenatally, Stra8 (Stimulated by retinoic acid gene 8), an enzyme required for meiosis initiation, must be persistently repressed by Cyp26B1 [75-77]. Postnatally, repression of Cyp26B1 releases repression of Stra8 and allows germ cells to respond to retinoic acid, which initiates meiosis [75-77]. The germ cells are now able to exit G1/G0 and can then proceed to make mature sperm.

As in the fish, gonadal sex in mammals appears not to be completely fixed. The transcription factor DMRT1 is required in both somatic cells and germ cells for normal male gonadal differentiation [78, 79]. Loss of Dmrt1 in adult Sertoli cells of the testis results in transformation of the cells to granulosa cells and transition of the gonad to a more ovarian-like phenotype [80]. Furthermore, forced expression of DMRT1 in the ovary reprograms granulosa cells to Sertoli cells, leading to the development of structures resembling seminiferous tubules [81].

HUMAN GERM CELL TUMORS

In humans**,** GCTs occur in infants, children and young adults [82] (Figure 1.1). Both childhood and adolescent/adult GCTs are thought to originate from Primordial Germ Cells (PGCs). Owing to the pluripotent nature of these cells, GCTs can take on a variety of different histologic fates [83]. GCTs in which the PGCs retain pluripotency and do not differentiate are known as seminomas (also called ‘dysgerminomas’ in females and ‘germinomas’ when occurring in the CNS). In contrast, GCTs in which the cells take on a variety of differentiation states are designated non-seminomas, of which embryonal carcinoma is thought to represent the stem cell component. GCTs differentiated to somatic cell lineages (endoderm, mesoderm, and ectoderm) are known as teratomas. Finally, GCTs may take on extraembryonic differentiation resembling the fetal yolk sac (yolk sac tumors) or the placenta (choriocarcinoma).

The histology of GCTs is similar in both males and females, whether occurring in the testis, ovary or extragonadal sites, implying origin from a common precursor cell [82]. However, there are some epidemiologic differences in the incidence of different types of GCT. In males

there are two peaks of testicular GCT incidence, one in early childhood at age 3-4, and a second, much larger peak that begins at puberty and is maximal at around age 30. In females, there is an early peak from age 0-2 representing the incidence of sacrococcygeal teratoma, an extragonadal GCT, in newborns and infants. Beginning at age 5-6, the incidence of ovarian GCT increases with age, becoming maximal at age 20-25 [84].

While the overall incidence of GCT (about 12,500 cases/year in the USA) is lower than that of common epithelial cancers such as lung, breast and prostate cancer, testicular GCT is the most common cancer and the leading cause of cancer death in young men [82]. The incidence of GCT is increasing around the world, for unknown reasons [84]. There are several known risk factors that increase the risk of developing testicular germ cell tumors. They include but are not limited to disorders of sexual development (DSD), gonadal dysgenesis, cryptorchidism (undescended testis), familial background, environmental exposure, and genetic association. Familial risk can increase the risk of developing GCT 4-fold in a male with a father who had GCT or up to 9-fold in a male whose brother had GCTs. The incidence of GCT varies widely in different geographic regions [85, 86], leading to the idea that the environment may strongly influence risk for testicular GCT.

There are also important age-dependent differences in GCT histologic spectrum. Type I GCTs occur in infants and children and consist of teratomas and yolk sac tumors (YSTs). Type II tumors in adolescents and adults have more diverse histology, and include seminomas, non-seminomas or mixed tumors containing both seminomatous and non-seminomatous elements.

Based on differing epidemiology, clinical outcome and histologic spectrum, GCTs in young children may be biologically distinct from GCTs occurring in older (post-pubertal) populations.

This idea is increasingly supported by molecular evidence. Whereas Type I tumors show variable Loss of Imprinting (LOI) at loci such as IGF2 and H19, adult-type GCTs tend to show complete erasure of imprinting [87, 88]. This result is interesting, because it implies that Type I GCTs may arise at an earlier stage of PGC development, since PGCs undergo erasure of imprinting during early development, and this erasure is largely completed by the end of PGC migration. Cytogenetic data consistently show loss of Chromosome 1p and 6q in Type I tumors, while Type II tumors also commonly exhibit amplification of Chromosome 12p. More recently, studies directly comparing the gene expression patterns of pediatric and adult GCTs have demonstrated distinct transcriptional profiles in tumors of similar histology arising in different age groups (for example, in yolk sac tumors of children vs. yolk sac tumors of adolescent/adults) [89]. Taken together, these studies support the notion that different biological mechanisms may drive childhood and adolescent/adult germ cell tumorigenesis**.**

Current cisplatin-based therapy for GCTs cures most patients [84, 90, 91], but major challenges remain. 15% of patients have cisplatin-resistant tumors and will eventually die of their disease [92]. Furthermore, there is significant therapy-related toxicity in patients treated for GCTs. Late effects in patients treated with cisplatin, etoposide and bleomycin (the current first-line regimen for GCT) include pulmonary fibrosis [93], renal insufficiency and salt-wasting [94-96], infertility and hormonal changes [97-101], hyperlipidemia [102, 103], Raynaud's

phenomenon [94, 101, 104, 105], obesity [102, 103, 106-108] and neuropathy [109]. Ototoxicity results in significant hearing loss, as assessed by audiogram, in nearly 80% of patients [94, 100]. Survivors of GCT treatment have twice the risk of early onset cardiovascular disease [99] and second malignancies [110, 111].

Compared to many other solid malignancies, relatively few somatic mutations have been described in GCTs. This lack of knowledge inhibits the development of targeted therapy that could provide an alternative or adjunct to standard chemotherapy. Amplification of Chromosome 12p is a pathognomonic feature of adolescent/adult GCTs, but no genes in this region have definitively been linked to germ cell tumorigenesis [112]. The most commonly reported mutated gene is *KIT*, a tyrosine kinase growth factor receptor that plays important roles in germ cell development [113-118]. Mutations have also been reported in *NRAS* and *KRAS*, signaling components of the MAP kinase pathway that act downstream of *KIT* [119-122]. Central Nervous System GCTs exhibit *KIT* and *RAS* mutations [123]. Somatic mutations in *BRAF*, another MAP kinase pathway member, have been associated with cisplatin resistance in adult TGCTs [124]. More recently, exome sequencing of TGCT identified somatic mutations in several genes including *CIITA*, *NEB, PDGFRA*, *WHSC1* and *SUPT6H* [125]. A larger study of 42 adult TGCTs identified somatic mutations in *CDC27* and mutations in *XRCC2* associated with cisplatin resistance [126]. To date, the mutation spectrum of Type I GCTs has not been reported.

A number of genome-wide association studies (GWAS) have been conducted in men with TGCT, resulting in the identification of a large number of novel germ cell tumor

susceptibility loci. In 2009, two groups reported strong linkage of GCT susceptibility to loci on chromosomes 5, 6 and 12, suggesting roles for *SPRY4* and *KITLG*, both components of receptor tyrosine kinase signaling, as well as for the pro-apoptotic *BAK1* gene [127, 128]. A follow-up study by Turnbull and co-workers identified additional susceptibility loci near the *DMRT1*, *TERT* and *ATF7IP* genes [129]. The studies have subsequently been replicated in other patient populations [130-132]. More recently, further GWAS studies have identified new candidate GCT susceptibility loci, including genes with potential roles in telomere regulation, such as *PITX1*, or germ cell development, such as *TEX14, RAD51C, PRDM14* and *DAZL* [133, 134].

Because of the many unresolved issues concerning the causes and biological mechanisms of GCTs, and the urgent need to develop and test new treatments, animal models of GCT are badly needed. However, to date relatively few models have been available. The 129Sv inbred mouse strain was found more than 50 years ago to be susceptible to testicular teratoma formation, with spontaneous tumors forming in 1-5% of animals [135]. Mutations in several genes have been shown to increase the teratoma incidence in 129Sv mice, including *Dead end* [136] and *Dmrt1*. In fact, *Dmrt1*-mutant 129Sv mice develop testicular teratomas at nearly 100% incidence [137, 138]. The teratomas that develop in the 129Sv background strongly resemble the Type I testicular teratomas occurring in young boys. However, these teratomas are benign tumors of relatively little clinical consequence and mouse models of seminomas or of non-seminomas other than Type I teratomas have not been described. Recent progress suggests that zebrafish may be a useful addition to GCT models, at least for seminomas.

ZEBRAFISH GERM CELL TUMOR MODELS

During the past several years, several fish models have been described with abnormal germline development that recapitulates certain aspects of human germ cell tumors. Three of these models were identified using the zebrafish, while one was identified in Medaka. All of these models demonstrate an inability to undergo proper germ cell differentiation and as a result exhibit immature, enlarged testes. Three of the models were identified through forward genetic N-ethyl-N-nitrosourea (ENU)-based mutagenesis screens while one is a transgenic line. These models can serve to provide further insight into human seminomas with the possibility to contribute to our understanding of the human disease.

The first fish model described was identified in Medaka, following an ENU mutagenesis screen to uncover genes that are important for germ cell and gonadal development [139]. Morinaga el al identified the *hotei* "hot" mutant fish, which was initially characterized as having extreme gonadal hypertrophy. The gonads were further examined and found to have increased numbers of germ cells that resided in disrupted testicular architecture, as well as decreased ability to produce mature sperm as compared with their wild-type siblings. The loss of function mutation was mapped, by positional cloning, to reside in the anti-Müllerian hormone receptor II (*amhrII*). This receptor belongs to the TGF-β/BMP superfamily and in humans is known to play a role in Müllerian duct regression in males. The mutation, in exon 9 of *amhrII*, changes a tyrosine residue in the highly conserved kinase domain to cysteine.

Neumann and co-workers used ENU mutagenesis and high-throughput histologic screening to identify a line of zebrafish that developed testicular germ cell tumors (GCTs) with

high penetrance (>80% incidence in homozygotes by 4 months of age) (Figure 2). They showed that the mutation is dominantly inherited, and causes the development of tumors of primitive, undifferentiated testicular GCTs resembling human seminoma (Figure 3). Like human seminomas, the zebrafish seminomas were sensitive to radiation therapy and underwent apoptosis in response to DNA damage [140]. To identify the molecular nature of the mutation, these investigators developed a haplotype-mapping panel and, using this technique coupled with further high-resolution meiotic mapping, identified a mutation in the Type IB BMP receptor, *alk6b/bmpr1bb*, as the cause of the zebrafish GCTs [141]. BMPs (Bone Morphogenetic Proteins), members of the TGF-β superfamily, play important roles in development and differentiation, but had not been linked to GCTs. Neumann *et al.* showed that the mutation causes loss of BMP signal transduction, and that BMP signaling is required for germ cell differentiation. This work represents the first identification through positional cloning of a cancer predisposition mutation in the zebrafish.

To understand the relevance of BMP signaling to human GCTs, Fustino *et al*. profiled the state of the BMP pathway in clinical GCT specimens and showed that undifferentiated human GCTs (seminomas and embryonal carcinomas) also lack BMP signaling [142]. This work also identified the miR-200 family of microRNAs as modulators of the BMP pathway, providing an explanation for differential BMP signaling activity in seminomas vs. yolk sac tumors. Collectively these studies identified BMP signaling as a key node in GCT differentiation and a promising target for novel therapies.

The first, and to date only, transgenic model of GCTs was identified by Gill *et al*. In this model, the Simian Virus 40 (SV40) T-antigen (TAg) is driven by the lymphocyte specific tyrosine kinase (lck) promoter from *Fugu*. Originally intended as a T-cell leukemia model, these fish did not manifest thymic malignancies, but rather testicular GCT. By 36 months, about 20% of the offspring developed tumors [143]. When comparing the tumors with wild type testis, the GCTs were clearly enlarged and lacked organized seminiferous tubules. Furthermore, the testicular GCTs were predominantly composed of undifferentiated spermatogonial-like germ cells determined by both sorting the cells by size (FACs) and histological analysis (Figure 3) [143]. Expression patterns of human seminoma specific genes were unchanged as compared to zebrafish wildtype testis. Various *RAS* genes were also sequenced in the zebrafish to identify any de novo mutations that could explain the TGCTs in the fish but none were found.

Finally, the most recent GCT model was identified through a forward genetic ENU screen. The causative mutation is a nonsense mutation in the leucine-rich repeat (LRR)-containing protein *lrrc50* [144]. Initially, this forward genetic screen was performed to identify genes involved in ciliary motility. *lrrc50* mutants develop a phenotype resembling that of human primary ciliary dyskinesia (PCD). Early in development, the *lrrc50* homozygous mutants develop a curved body axis, dilated pronephric ducts, kidney cysts and severe edema, which ultimately results in embryonic death at 8 days post-fertilization (dpf) [144]. Interestingly, heterozygous *lrrc50* mutants display increased incidence of testicular GCT, suggesting a novel tumor suppressor role for lrrc50.

lrrc50 heterozygous mutants display a 90% GCT penetrance in their 2nd and 3rd years of life [145]. Histological analysis of the tumors found that there were severely reduced numbers of differentiated germ cells, and most of the cells morphologically resembled early spermatogonial cells (Figure 3). These cells also stained positive for phospho-histone H3 (a mitotic marker) in single cells unlike the wild type testis, which showed reduced staining in clusters. 44.4% of the tumors examined showed a loss of the *lrrc50* wildtype allele, consistent with a role for *lrrc50* as a tumor suppressor.

This model is, to date, the only one of a gene with documented mutations in the respective gene in human seminomas [145]. Basten *et al*. identified *LRRC50* nonsense mutations in two pedigrees with family history of seminomas. In a separate analysis of 38 patients with sporadic seminomas, 5 demonstrated heterozygosity for different germline *LRRC50* mutations. The mutations were shown to be functional nulls by their inability to complement the lethal larval phenotype of *lrrc50* mutants. These results establish *LRRC50* as the first gene specifically linked to seminoma predisposition in humans. These results prompted an investigation of the possible roles of cilia in germ cell development. In a novel finding, the investigators demonstrated the presence of primary cilia in normal spermatogonia and showed that LRRC50 colocalizes with the axoneme in spermatogonial stem cells, that its expression is cell cycle-regulated and that it colocalizes with condensed chromosomes [145]. These results shed new light on the possible importance of ciliary signaling in germ cell development and germ cell tumorigenesis.

SUMMARY

Germ cell tumors are among the most common cancers of young people, and the leading cause of cancer death in young men. The wide age range of incidence and histologic diversity of the tumors creates challenges in GCT biology and treatment. While some genomic data are now beginning to point to possible molecular origins of these tumors, the paucity of representative animal models is an impediment to further functional genomic and translational studies.

To date the zebrafish has proved to be a powerful model for understanding the biology of PGC development and migration. While there are important differences between human and fish germ cells, a number of conserved mechanisms have been identified, suggesting that studies in zebrafish can contribute importantly to our understanding of human germ cell biology.

Zebrafish models of human germ cell tumors have also begun to make important contributions. The studies described above have identified several signaling pathways, such as the TGF-β/BMP pathway and cilia-mediated signaling, that are directly relevant to human seminomas. The development of seminoma-like tumors in fish transgenic for the known oncogene SV40 large T, and the response of GCTs to DNA-damaging agents, suggests that these models recapitulate important aspects of the oncogenic phenotype of human GCTs.

There are, however, several important caveats that apply to the current models. There is no evidence that these models of GCTs become invasive or malignant, as do some examples of

human seminomas. To date, true zebrafish models of ovarian GCT (as opposed to models of impaired maturation of post-meiotic oocytes) have not been described. Reverse genetic models that use gain or loss-of-function approaches and germ cell-specific promoters to test the role of specific GCT candidate genes, at specific developmental stages, remain to be developed. This is a particularly significant gap in current approaches, because it is likely that the ability of germ cells to develop along somatic lineages, as seen in non-seminomas, is limited to specific stages of development in which germ cells retain some pluripotency. In humans, non-seminomas are more resistant to treatment and more likely to metastasize than are seminomas, and are responsible for the majority of deaths from GCT, emphasizing the need for such models. However, there is reason to be optimistic. The great recent progress in the field, with the availability of new promoters, new genome editing techniques and increasing information about the genomic landscape of human GCT, means that the next few years promise to see great advances in our understanding of GCT, and better options for GCT patients.

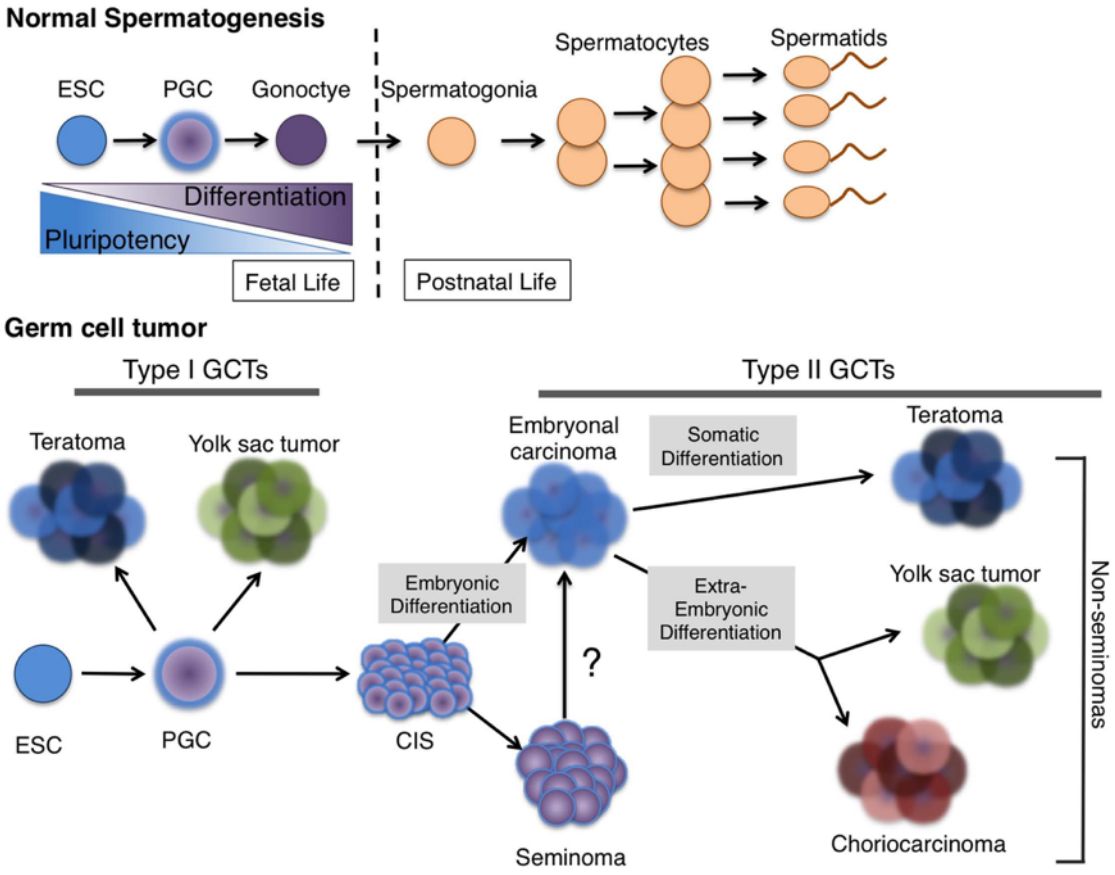


Figure 1. 1. Mammalian spermatogenesis and human germ cell tumor (GCT) histological classification. The transition of primordial germ cells from embryonic stem cells to gonocytes is facilitated by increased expression of germ cell specific genes and decreased expression of embryonic pluripotency genes. In humans, sex chromosomes dictate the fate of a gonocyte; in the testis self-renewing spermatogonial stem cells give rise to progeny that undergo meiosis to produce mature sperm. Germ cell tumors arise from primordial germ cells (PGCs). Type I GCTs are generally seen in neonates and young children and include Teratomas and Yolk Sac Tumors. Type II GCTs occur in adolescents and adults and may proceed through a carcinoma *in situ* (CIS) precursor (also known as Intratubular Germ Cell Neoplasia). CIS cells can give rise to both Seminoma and Non-Seminoma, the stem cell component of which is Embryonal Carcinoma (EC). EC may undergo somatic differentiation into Teratomas, containing derivatives of ectoderm, mesoderm and endoderm. Alternatively, EC cells may differentiate into GCTs resembling extraembryonic tissues, such as Yolk Sac Tumor and Choriocarcinoma. Mixed tumors containing both seminoma and non-seminoma components also occur.

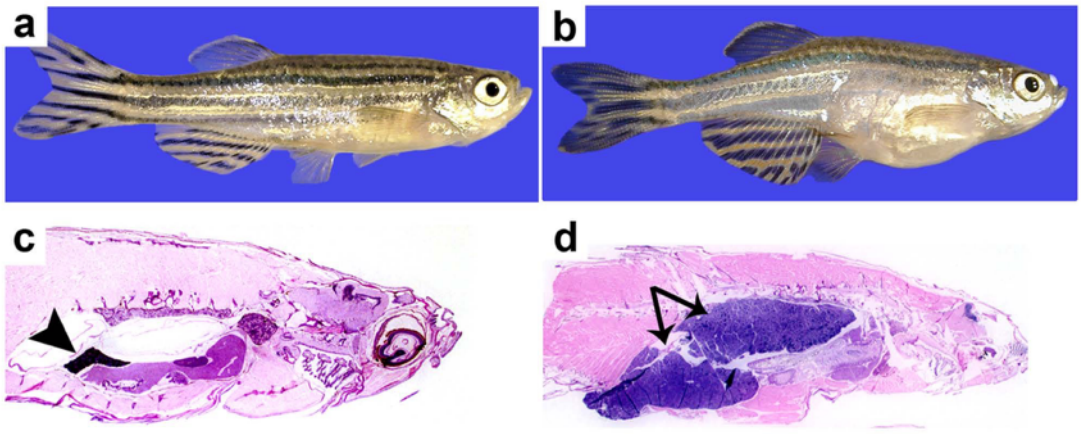


Figure 1.2. Testicular GCT in *bmpr1bb* mutant zebrafish. Mutants develop GCTs because of impaired germ-cell differentiation. Compared with adult wild-type males (a and c) (arrowhead: normal testis), adult *bmpr1bb* males display abdominal distension and marked testicular enlargement (b and d) (arrow: testicular tumor). Modified with permission from Neumann *et al.*, 2011.

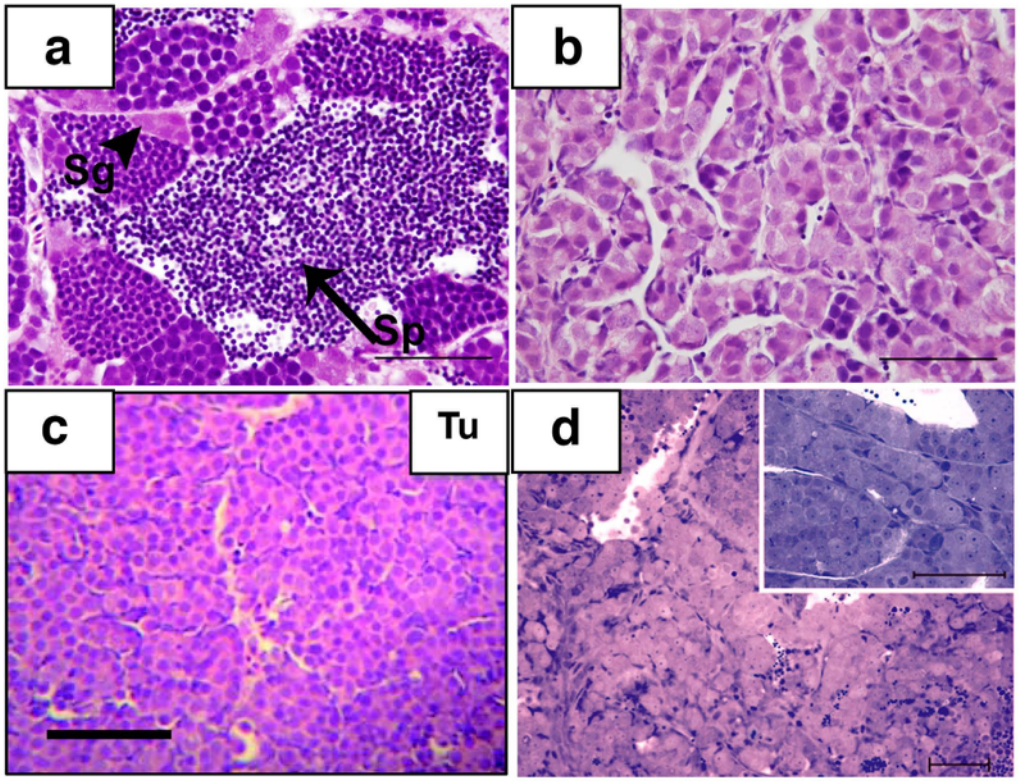


Figure 1.3. Zebrafish germ cell tumor models exhibit histologic similarity. a, normal testis. The testis consists of cysts or lobules of spermatogenic cells surrounded by a basement membrane and somatic cells. Small clusters of spermatogonia are seen adjacent to the basement membrane (arrowheads). Successive stages of differentiation are also evident, including primary and secondary spermatocytes, spermatids and mature spermatozoa. b, Germ cell tumor in a *bmpr1bb* mutant testis, showing loss of differentiation and an excess of primitive, spermatogonial-like cells. c, Testicular germ cell tumor from the Tg*(flck:TAg)* T24 line. Tu: tumor. d, testicular GCT in a *lrrc50*^{H255h} fish. Two magnifications of the same tumor shown (largest in insert), with loss of differentiated germ cells in the tumor. Scale bars: 50 microns. (a, b: modified with permission from Neumann *et al.*, 2011. c, modified with permission from Gill *et al.*, 2010. d, modified from Basten *et al*., 2013.

CHAPTER TWO
Zebrafish germ cell tumor model characterization reveals commonalities with human germ cell tumors

Introduction

Despite highly successful cisplatin based combined chemotherapies, testicular cancer remains the most common type of malignancy found in young men between the ages of 14-40 [82]. The incidence has been steadily increasing worldwide nearly doubling in the past several decades, and marked geographical variations in incidence could be indicative of an environmental risk factor but is more likely genetic as suggested by recent GWAS studies [146-148]. Testicular cancer belongs to the family of tumors known as germ cell tumors (GCTs) which are neoplasms arising from primordial germ cells. GCTs can develop in the testis, ovary, or extragonadal locations along the midline correlating with the path in which germ cells migrate to the site of gonadogenesis, the presumptive gonad. A commonly accepted model suggest that most of these tumors descend from a common precursor legion known as carcinoma *in situ* or intratubular germ cell neoplasia unclassified (ITGCN) which is supported by patient data in which patients with CIS go on to later develop testicular cancer [149-152].

Testicular germ cell tumors are classified by their age of onset and their distinct histopathology which, depending on the level of differentiation, correlates with their clinical behavior [82]. Seminomas resemble more undifferentiated germ cells and respond well to current therapies such as radiation and cisplatin-based chemotherapy. On the other hand, non-seminomas (embryonal carcinomas, yolk sac tumors, choriocarcinomas, teratomas and mixed

tumors) have varying levels of differentiation are clinically more aggressive than seminomas. While the current therapies work well for most patients they come with some very burdensome side effects such as increased risk for cardiovascular disease, impaired fertility and even second malignancies [99, 110, 111, 153]. Patients with either subtype of TGCTs can relapse but those who have non-seminomas tend to have the highest rate of relapse. Furthermore, patients with refractory disease have very limited treatment options and poor survival rates.

The exact cause of these tumors is currently unknown but current risk factors are highlighting potential insights to the biological causes. Familial risk underscores the strong genetic cancer susceptibility as brothers of patients have a four to tenfold increased risk of developing TGCTs. Furthermore, genome wide association studies (GWAS) have identified several loci implicating biological pathways in TGCT susceptibility, such as KIT-KITLG signaling (KITLG, BAK1, SPRY4, and GAB2), germ cell development (DAZL, PRDM14, and HPGDS), DNA repair (RAD51C and RFWD3), and sex determination (ZFPM1, and DMRT1) among others [127, 128, 130-134]. The most frequent chromosomal abnormality, present in nearly all TGCTs, is the gain of small arm of chromosome, forming an isochromosome of 12 (i12p) [112, 154, 155]. However, the causative gene or genes on 12p are not known. Taken together these risk factors are beginning to unravel potential biological pathways of interest in tumor development.

Currently, animal models spanning the diverse subtype spectrum of testicular germ cell tumors are lacking. All of the existing animal models have limitations and none completely recapitulate all aspects of the human disease. The 129Sv mouse model, first identified in the

1950s by Leroy Stevens [156] [157] is a model of juvenile testicular teratomas that occur in young children. Mutations in several genes including Dead end (*Dnd1*) and *Dmrt1* were shown to drastically increase the teratoma incidence in the 129Sv background, suggesting a role of these genes in tumor biology [135-138]. Two zebrafish (*Danio rerio*) models resembling seminomas have been identified. As described in Chapter 1, one of these models arose from mutations in the *lrrc50* gene, which has been shown to be important in human germ cell tumor predisposition [145]. The other zebrafish germ cell tumor model highlights the importance of the Bone Morphogenetic Pathway (BMP) in germ cell development and recapitulates the histological differentiation pattern of seminomas [140, 142, 158]. Here we aim to use the latter model in combination with human germ cell tumor sequencing data to identify novel biological pathways that could implicate germ cell tumorigenesis in humans and better our understanding of this disease.

Methods

Zebrafish Care and Maintenance

Zebrafish were kept on a 10-hour light/14-hour dark cycle and maintained with standard procedures [159]. All protocols were approved by the Institutional Animal Care and Use Committees at UT Southwestern Medical Center, an AALAC-accredited institution.

Tissue, RNA, and DNA isolation

For tail genomic DNA extraction, fish were anesthetized using 0.02% tricaine and a small portion of their fin was clipped. Tail DNA lysis was performed using the HotSHOT method as previously described [160]. For tumor RNA and DNA isolation, fish were euthanized using

tricaine and tumors were dissected and placed into TRIzol (Invitrogen). Simultaneous tumor genomic DNA and total RNA extraction was performed following the manufacturer's protocol.

RT-PCR, Loss-of-Heterozygosity genotyping

Reverse transcription of 150ng of total RNA was performed using RT^2 First Strand Kit (Qiagen). PCR amplification (see Table 1 for primers) of the *bmpr1bb* locus from either tail DNA lysis, tumor genomic DNA, or tumor cDNA was performed prior to genotyping using GoTaq Flexi DNA polymerase (Promega) using respective primers. A custom allelic discrimination (AD) assay (Applied Biosystems) was used to genotype as previously described [158]. Sanger sequencing (Applied Biosystems, ABI) was used to confirm sample genotypes following PCR clean up using ExoSAP-IT (Affymetrix).

Morpholino microinjections

Dnd MO [62] or control MO (5'CCTCTTACCTCAGTTACAATTTATA-3') were obtained from Gene Tools (Philomath, OR) and injected into *bmpr1bb*$^{+/-}$ incross embryos at the 1-cell stage. Injected progeny were raised until they were 3 months old.

In Situ Hybridization

A *vasa*-specific riboprobe was created by PCR amplification of zebrafish *vasa* cDNA (Open Biosystems) using primers containing T3 and T7 polymerase sites (Table 5). The antisense strand was *in vitro* transcribed using T7 polymerase (Promega) and DIG RNA labeling kit (Roche). Lithium chloride extraction was used to precipitate the RNA probe. The *in situ*

hybridization procedure was followed as previously described [161] for 5dpf aged zebrafish embryos.

Zebrafish histology scoring

Fish were euthanized at 3 months and 6 months using tricaine and fixed in 4% paraformaldehyde (PFA)/1X phosphate-buffered saline for 48 hours at 4ºC. Following fixation, fish were dehydrated and paraffin embedded. A hematoxylin and eosin-stained transverse section was scored for gonadal morphology by two blinded, independent observers.

Generation of transgenic line for gonadal visualization

The ziwi promoter-GFP plasmid was a kind gift of Bruce Draper (UC Davis, California). Using the Tol2 Gateway cloning system [162], fish were injected with 100ng/uL Tol2 transposase mRNA and 50ng/uL of plasmid containing ziwi-GFP construct. Potential transgenic zebrafish were screened at 30dpf for GFP expression and a line was established from transgenic founders. The F3 population was outcrossed into the *bmpr1bb*$^{+/W256X}$ background. F4 populations were genotyped for *bmpr1bb* as described above and also for the ziwi-GFP transgene using PCR amplification (Table 1).

Tissue isolation for microarray analysis

Zebrafish were genotyped at 50dpf as described above for both *bmpr1bb* and the *ziwi-GFP* transgene. One cohort of fish was sacrificed at 60dpf and a separate cohort at 10.5 months of age. Zebrafish were euthanized with Tricaine and dissected under an epifluorescence stereomicroscope. The posterior end of the gonad was fixed and saved for histological analysis,

and remaining gonad was preserved in RNAlater (Ambion). Total RNA extraction was performed using RNeasy Micro Kit (Qiagen) following the manufacturer's protocol. RNA quality was assessed using an Aligent Bioanalyzer and only samples with RNA integrity numbers (RIN) of 7 or above were used.

Microarray

Microarray analysis was performed using the Affymetrix GeneAtlas WT Expression platform. 100ng of total RNA was used to synthesize double stranded cDNA (Ambion, WT expression Kit). Fragmentation of 5.5ug of single stranded cDNA and subsequent labeling was performed using the GeneChip WT terminal labeling Kit (Affymetrix, Santa Clara, Ca). Labeled samples were hybridized (GeneAtlas Hybridization, Wash, and Stain Kit for WT Array Strips) to a Zebrafish Gene 1.1 ST array strip and were read using the Gene Atlas imaging station after washing (Affymetrix).

Microarray analysis

Raw data directly from the array was processed by an in-house computational pipeline written by PERL and R to conduct background correction, normalization, summarization, differential expression analysis and pathway enrichment analysis. Gene expression data was normalized using the robust multiarray average (RMA) algorithm. Differentially expressed genes were examined by two-tailed student's t test. For all statistical analyses, $P<0.05$ was considered to be statistically significant.

Zebrafish RNA sequencing

RNA samples from zebrafish germ cell tumors and normal testis tissues were sequenced on the HiSeq2500 (Illumina) with 2 X 100 read length. For the raw paired-end sequencing data, adapter removal and quality-filtering were conducted by Cutadapt (1). Alignment to the reference zebrafish genome, build GRCz10, was performed by Bowtie2 (2). TopHat2 (3) and Cufflinks (4) were used for alignment, de novo assembly of reads into transcripts (including mRNA, lincRNA, and many other RNA species) and differential expression analysis.

Comparison of gene expression data between human cancers and zebrafish tumors

Previously, we conducted RNA sequencing for 32 human germ cell tumors (GCTs) and normal testis and ovary tissues. We used results from this sequencing for a comparison analysis between human and zebrafish GCTs. To minimize bias, we used the same computational pipeline as mentioned above for zebrafish GCTs to process RNA-seq data of human GCTs, and using human reference genome hg19 instead of GRCz10 in the alignment and de novo assembly when analyzing human data. We also performed principal component analysis (PCA) using human and zebrafish gene expression data by an in-house computational pipeline with PERL and R scripts.

To further confirm that zebrafish GCTs resemble human GCTs in the characteristics of gene expression, we downloaded four human cancer RNA sequencing datasets from The Cancer Genome Atlas (TCGA) database, including lung adenocarcinoma (LUAD), breast invasive carcinoma (BRCA), prostate adenocarcinoma (PRAD), bladder carcinoma (BLCA). To minimize bias, we used the same computational pipeline as mentioned above for human GCTs to process

RNA-seq data of these four human cancers. Binomial test was used to compare human cancers and zebrafish tumors. $P<0.05$ was considered to be statistically significant.

Results

Germ cells are required to form testicular tumors in zebrafish

Several features of the gonadal tumors arising in *bmpr1bb* mutants suggest that the tumors are of germ cell origin, including localization to the gonads, morphology of the tumors and expression of markers such as *piwil1* [140, 141]. Nevertheless, owing to the presence of non-germ cell somatic cells in the developing gonad, it remained possible that the tumors arise from another cell population. To confirm the germ cell origin of zebrafish testicular germ cell tumors, we knocked down expression of *dnd* using an antisense morpholino. *dnd* is an RNA-binding protein that is essential for germ cell development [62]. To confirm the efficacy of the knockdown, we performed *in situ* hybridization against vasa, a germ cell specific marker, on 5dpf wildtype embryos that had been injected with either a control morpholino or a *dnd* morpholino that was previously shown to eliminate germ cells [62] (Figure 1). We then performed incrosses of *bmpr1bb*$^{+/-}$ heterozygotes, injected the embryos with control or *dnd* morpholino, and raised the fish to 3 months of age. We sacrificed the fish, determined the *bmpr1bb* genotype, and prepared histologic sections for analysis of gonadal morphology. All *bmpr1bb*$^{+/+}$ fish injected with control MO exhibited normal testicular gonadal development (Figure 1 C, G). *bmpr1bb*$^{-/-}$ embryos injected with the control MO developed germ cell tumors, as expected (Figure 1 D, H). Injection of the *dnd* morpholino resulted in adult fish with gonadal sacs comprised of somatic cells and lacking germ cells in both *bmpr1bb*$^{+/+}$ (Figure 1E, I) and

bmpr1bb$^{-/-}$ gonads (Figure 1 F, J). Thus tumors arise in *bmpr1bb* mutants only in animals harboring germ cells, indicating a germ cell origin for these tumors.

Haploinsufficiency of *bmpr1bb* results in zebrafish germ cell tumors

We previously reported that germ cell tumors arise in both *bmpr1bb* heterozygous and homozygous mutants [140, 141]. Tumors occur in *bmpr1bb*$^{+/-}$ heterozygotes at lower penetrance (~80% compared to 100% for *bmpr1b*$^{-/-}$ homozygous mutants) and increased latency (median age of onset 8 months *vs.* 3 months for homozygous mutants). These results could be explained by a loss-of-heterozygosity mechanism whereby the wild-type allele of *bmpr1bb* was lost over time, leading to a complete loss of *bmpr1bb* function and tumor development. Alternatively, heterozygotes may have levels of signaling activity too low to prevent tumor formation (haploinsufficiency). To distinguish these possibilities, we genotyped the *bmpr1bb* locus in 10 GCTs along with accompanying normal tailfin DNA. We also prepared cDNA from tumor RNA and sequenced the region of the transcript including the W256X mutation. Figure 2 shows representative results from 4 tumor-normal pairs. In all cases, the wildtype allele was retained and expressed in the tumor. Therefore, tumors in *bmpr1bb*$^{+/-}$ heterozygotes appear to arise from haploinsufficiency of *bmpr1bb*.

Zebrafish germ cell tumors resemble human germ cell tumors with shared gene expression profiles

All zebrafish germ cell tumor models to date exhibit a similar histologic patterns with tumors consisting of masses of undifferentiated germ cells most resembling human seminomas [163]. However, the gene expression profile of the zebrafish tumors has not been directly

compared to that of different subtypes of human GCTs. For this comparison, we focused on the three most common human malignant germ cell tumor subtypes: germinomas, embryonal carcinomas, and yolk sac tumors ("germinoma" is a generic term to refer to biologically identical tumors that are designated "seminomas" in males and "dysgerminomas" in females).

Initially, we hypothesized that the gene expression profile of zebrafish germ cell tumors would most closely resemble that of human germinomas because of the histologic similarity of zebrafish GCTs to human germinomas. To test this hypothesis, we performed either human microarray analysis or RNASeq of human GCTs (15 embryonal carcinomas, 22 germinomas, and 28 yolk sac tumors or 5 germinoma, and 23 yolk sack tumor respectively) as well as normal testis and normal ovary. In parallel we isolated GCTs from 10 month old *bmpr1bb* homozygous mutants for RNASeq. We quantified RNA expression as FPKM and used cufflinks to identify differentially expressed genes in each dataset. We then compared the zebrafish GCT differentially expressed genes with each of the histologic subtypes of human GCT (Figure 2.3). Surprisingly, the zebrafish germ cell tumor gene expression profiles showed similar degrees of overlap with all three subtypes of human GCT. There was a core set of 146 genes with similar expression profile in all three human GCT subtypes and in zebrafish GCTs. Each of the subtypes also expressed certain genes in common with the zebrafish GCTs, however no subtype appeared more similar to the zebrafish tumors than any other (Figure 2.3C). We obtained a similar result using Principal Component Analysis to compare global gene expression patterns in zebrafish and human GCTs. This analysis showed that zebrafish tumors form a cluster equally distinct from both germinomas and non-germinomas (in this instance, yolk sac tumors) (Figure 2.3B).

We considered the possibility that the comparative analysis had simply uncovered a core set of genes associated with neoplastic transformation, that was present in cancers from both fish and humans. To investigate this possibility, we used publicly available data of gene expression from human lung, breast, prostate and bladder adenocarcinomas to identify the differentially expressed genes in each of these cancer types. We then asked whether the fish GCTs or the different human cancer types were more similar to specific human GCT subtypes. In every case, we found that the zebrafish tumors had the highest statistically significant overlap with the three human GCT subtypes (Fig. 2.3C). To confirm that the percent overlap was greater than what could occur by chance, we performed random simulation analysis of the RNA-Seq data. This was carried out by randomly selecting 10,000 genes that were upregulated or down regulated in all three human germ cell tumor cancer subtypes. The random simulation was performed 10,000 times and then the expected random chance overlap for each human germ cell tumor subtype was averaged to be between 15-16% (solid line in figure 2.3C), regardless of subtype. This result was further validated when we compared the 60-day old zebrafish tumor precursor lesion (data not shown).

Comparative genomic analysis of zebrafish and human germ cell tumors highlights shared biological pathways

The overlap between the zebrafish GCTs and the three human GCT subtypes prompted us to look further into the genes that had the same directional gene expression pattern in both human GCTs and the zebrafish GCTs. All three human germ cell tumor subtypes shared 146 genes that had the same directional change in gene expression pattern as seen in the zebrafish GCTs (Figure 2.3A). KEGG pathway analysis highlighted several biological pathways that may

play crucial roles to human germ cell tumors such as ribosome biogenesis, pathways in cancer, regulation of actin cytoskeleton, and the MAPK signaling pathway amongst several others (Figure 2.3D).

Discussion

Over the past several decades, treatments for TGCTs have remained the same despite the advances in next generation sequencing. There is still ample room for improvement as 15% of patients relapse from cisplatin based therapies and generally do not have alternative treatment options leaving these patients end up dying from their disease [92]. Animal models for TGCTs are also limited and do no not always recapitulate the malignant characteristics of the disease [163]. In our study we aimed to further characterize the zebrafish TGCT model and identify overlapping characteristics with human germ cell tumors in order to exploit the zebrafish TGCT model to help produce a more accurate representation of the human disease.

Oftentimes, loss of heterozygosity of the wild type allele is seen in inherited cancer syndromes such as germinal retinoblastoma and breast cancer. Surprisingly, we found no evidence for loss of heterozygosity in the *bmpr1bb*$^{+/W256X}$ tumors we sequenced. This is interesting because not only do many cancers exhibit LOH of tumor-suppressor genes, but the most recently identified zebrafish GCT model found LOH in the *lrrc50* gene which contributed to the tumor phenotype and was further shown to be associated with human GCTs [144, 145]. As our data point to haploinsufficiency, rather than LOH, as the mechanism of GCT, this result could suggest that certain threshold level of BMP signaling is required for proper regulation of germ cell pluripotency and differentiation. However, this does not rule out the possibility that

GCTs arise in *bmpr1bb* mutants due to acquisition of other somatic mutations that contribute to tumorigenicity.

The gonadal niche is composed of both somatic cells and germ cells. There is intricate communication between these two cell types that promotes proper germ cell proliferation, differentiation and maturation. We have previously described that the zebrafish GCTs were comprised of germ cells by their strong ziwi expression which is one of very few established markers [141]. To circumvent this issue, we eliminated all the germ cells in the organism and found that germ cell tumors no longer developed in the *bmpr1bb*$^{W256X/W256X}$ background. These results suggest that tumors in *bmpr1bb*$^{W256X/W256X}$ mutants are of germ cell origin and it further strengthens our zebrafish tumor model as a model of germ cell tumors.

Using the zebrafish GCT model we sought to determine the particular human germ cell tumor subtype that it most resembled by using RNA sequencing data of both zebrafish GCTs and seminomas, yolk sac tumors, and embryonal carcinoma human subtypes. We hypothesized that the fish tumors would most likely resemble seminomas since their pathology seemed to be most similar. However, we found that the *bmpr1bb* model shares commonalties with all three subtypes at similar rates. This unique signature could support the currently accepted theory for germ cell tumors that states that each of these tumors is derived from a precursor germ cell that has become malignant early in development. Our results are also intriguing because many GWAS studies found no genes implicated in cancer susceptibility that stratified by GCT subtype which further supports the idea that there really are shared characteristics between all the different human GCT subtypes resulting from a common primitive lesion [128, 129, 134].

Given the fact that the *bmpr1bb* zebrafish GCT model does not have loss of heterozygosity, that the tumors are of germ cell origin and encompasses characteristics of 3 different human GCT subtypes, this model could be a valuable platform for functional genomic validation of potential subtype-specific GCT driver genes going forward. It is possible that the signature from our sequencing effort could highlight a core set of misregulated genes found in the lesion of origin ITGCN (CIS) prior to becoming a full blown tumor. Building on these early features, there could be an independent set of genes that drive these precursor GCTs into becoming more yolk sac tumor, seminoma, or embryonal carcinoma-like. Further work is needed to determine the similarities of our model to ITGCN by performing comparative sequencing analysis between the human and zebrafish lesions but this model could give us the foundation needed to drive tumorigenesis of different subtypes and further our understanding of the human disease with the potential of identifying novel therapies.

Primers used to amplify a PCR product for genotyping

Gene	Forward	Reverse	Amplicon(bp)
bmpr1bb	tgcagaggaccatcgccaa	tcgtgtctcatcaggacggtc	181
ziwi-GFP	gcatttgtaaagcaccggtc	cgccgtaggtcagggtggtc	250

Primers used for amplifying product for AD Assay genotyping

Gene	Forward	Reverse	Amplicon(bp)
bmpr1bb	tcgcccctccagcgg	cgcccctcagcgg	107

Table2.1. Primer sequences for the various steps of genotyping. Different parts of the genotyping process were required for *bmpr1bb* genotyping. Genotyping for ziwi-GFP was performed by PCR and product was visualized on a 1% agarose gel.

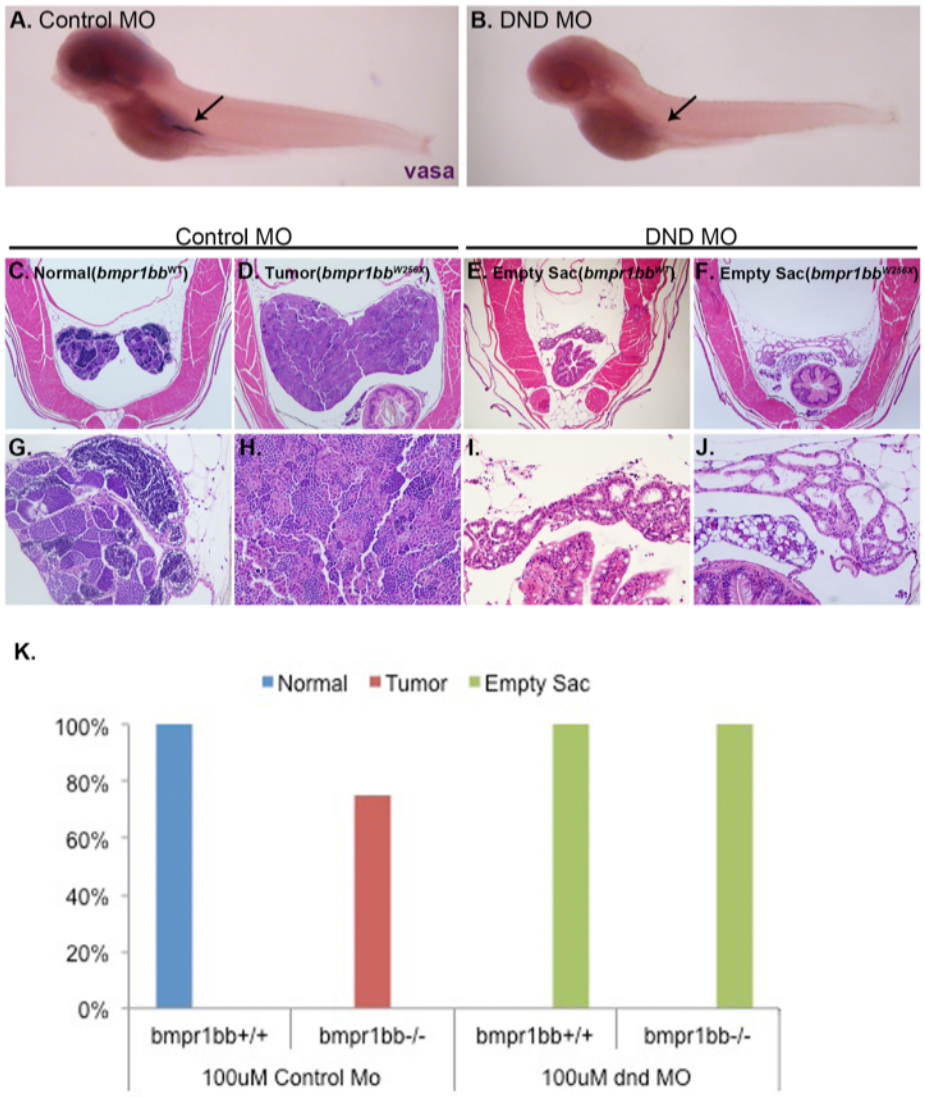


Figure 2.1. Tumor phenotype is lost in *bmpr1bb*+/- fish injected with DND morpholino in order to ablate germ cells. (A) At 5dpf, control morpholino (MO) injected embryos retain their germ cells as shown by *in situ* hybridization against vasa. (B) Dead end morpholino injected embryos lack germ cells as seen by the absence of vasa staining. (C), (G) 10X and 40X, respectively, Hemotoxylin and eosin staining of control MO injected wildtype testis. (D), (H) 10X and 40X, respectively, H&E images of control injected *bmpr1bb*-/- gonad with tumor phenotype. (E), (I) 10X and 40X, respectively, H&E images of DND morpholino injected *bmpr1bb*-/- gonad consisting of empty somatic cell sacs instead of the tumor phenotype. (I) Quantification of tumor and empty sac phenotype in both wildtype and *bmpr1bb*-/- gonads for control and dead end morpholino injected embryos.

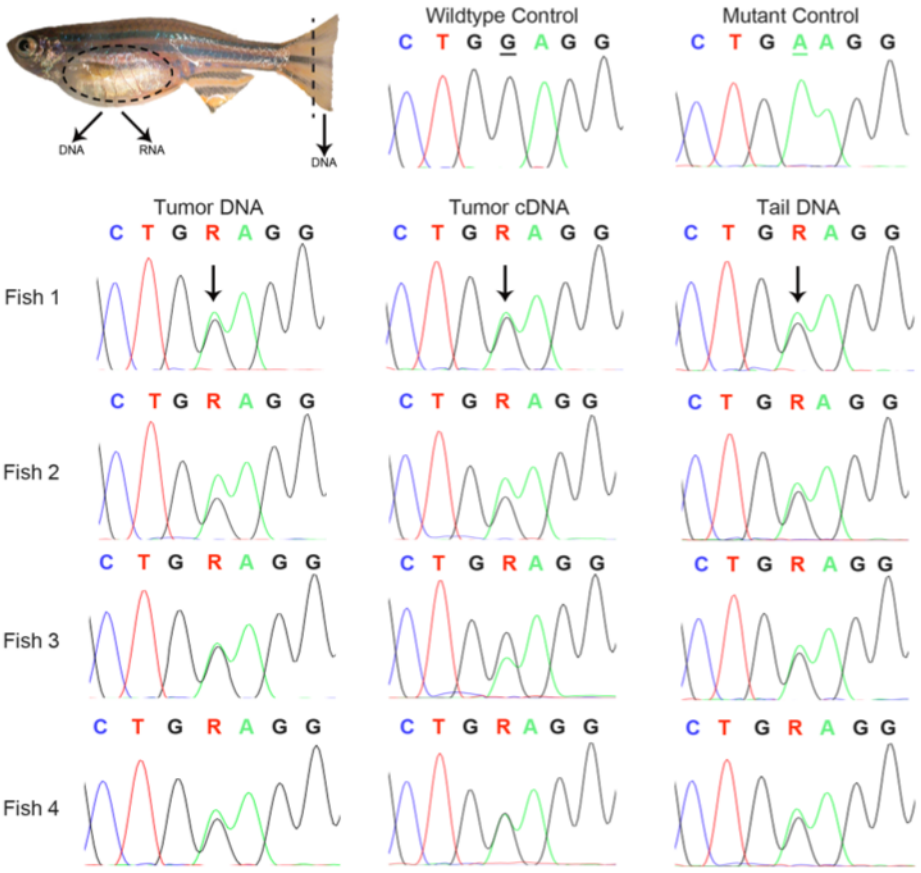
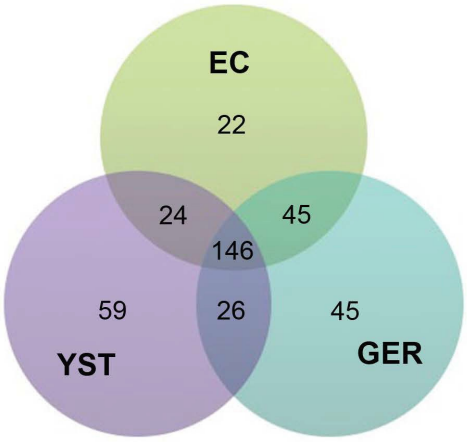


Figure 2.2. *bmpr1bb*+/- tumors do not exhibit loss of heterozygosity in analysis of RNA or DNA. Tail genomic DNA and Tumor genomic DNA and RNA were extracted from *bmpr1bb*+/- fish. Wildtype and *bmpr1bb* homozygous mutant tail genomic DNA were used as controls and show the single nucleotide alteration (TGG>TGA) that causes a W256X amino acid change. Matched samples (N=3) of tail genomic DNA, tumor genomic DNA and tumor cDNA were genotyped by both AD Assay and Sanger sequencing at this locus (arrows). Representative histograms for four fish are shown as examples and reveal that tumors remain heterozygous at the *bmpr1bb* locus.

A.



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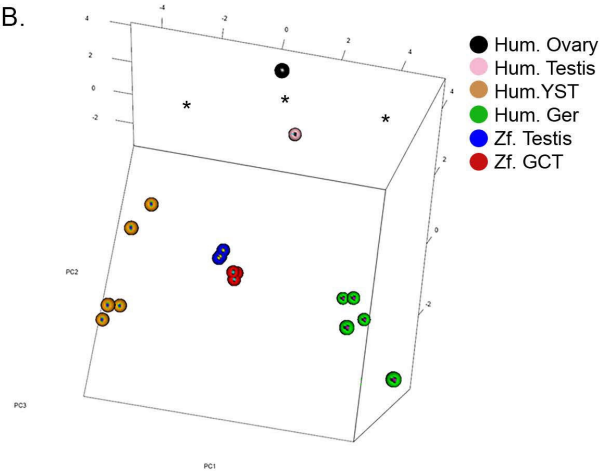
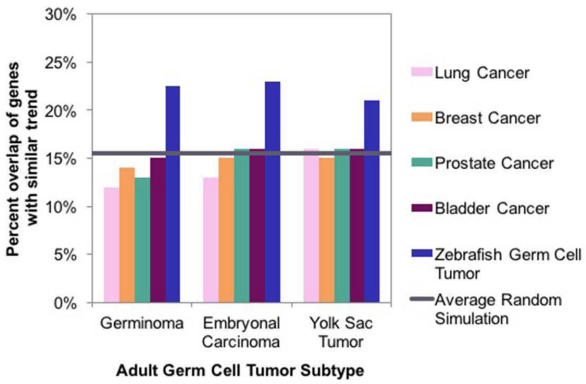


C.



D.

KEGG Biological Pathway Description	Genes	FDR q-value
Ribosome	FAU, RPL5, RPL11, RPL15, RPL18A, RPL19, RPL21, RPL22, RPL27, RPL28, RPS4X, RPS8, RPS9, RPS11, RPS15A, RPS19, RPS21, RPS26, RPS29, RPL23	2.31E-21
Pathways in cancer	RAF1, HRAS, RAC2, RAC3, NRAS, PDGFRA, FGF13, FGF18, PLCG2, MYC, NFKB2, LAMB2, PTGS2, BAX, CASP8, HDAC1, WNT5A, WNT2B, FZD10, BMP2, GLI2, JUP, SPI1, RASSF1, FADD, TRAF1, TRAF4	2.42E-17
Regulation of actin cytoskeleton	RAF1, HRAS, RAC2, RAC3, NRAS, PDGFRA, FGF13, FGF18, PPP1CA, ITGB7, ITGA5, ITGB2, LIMK1, ARPC4, GSN, MSN, ARHGEF1, FGD1	1.34E-11
Fc epsilon RI signaling pathway	RAF1, HRAS, RAC2, RAC3, NRAS, PLCG2, MAPK14, BTK, INPP5D, LCP2, FCER1G	1.88E-09
MAPK signaling pathway	RAF1, HRAS, RAC2, RAC3, NRAS, PDGFRA, FGF13, FGF18, MYC, NFKB2, MAPK14, RAP1A, MAP3K7, RPS6KA1, CACNA2D2, NR4A1, SRF	2.74E-09
Focal adhesion	RAF1, HRAS, RAC2, RAC3, PDGFRA, LAMB2, PPP1CA, ITGB7, ITGA5, RAP1A, VASP, ILK, VWF, COL5A3	3.43E-08
B cell receptor signaling pathway	RAF1, HRAS, RAC2, RAC3, NRAS, PLCG2, BTK, INPP5D, PTPN6	2.51E-07
Primary immunodeficiency	BTK, LCK, PTPRC, ADA, IL2RG, IL7R, RFXANK	2.52E-07
Natural killer cell mediated cytotoxicity	RAF1, HRAS, RAC2, RAC3, NRAS, PLCG2, ITGB2, LCP2, FCER1G, PTPN6, LCK	3.24E-07
T cell receptor signaling pathway	RAF1, HRAS, NRAS, MAPK14, LCP2, MAP3K7, PTPN6, LCK, PTPRC, CD28	3.36E-07

Figure 2.3. Zebrafish GCTs (10 month old) have similar transcriptional profile to all human germ cell tumor subtypes. (A) Venn diagram depicting the overlap of the number of genes that contain similar trends as zebrafish germ cell tumors found in the different human germ cell tumors. (B) Principal component analysis where each dot represents a different sample

as depicted in the legend. (C) Comparison of the overlap of genes with similar trends of several different human cancer types and zebrafish germ cell tumor with adult germ cell tumor subtypes. (D) KEGG pathway analysis of the differentially expressed genes with similar trends shared between human and zebrafish germ cell tumors identified in Figure A.

CHAPTER THREE
Impaired BMP signaling causes derepression of foxl3 leading to DSD-like gonadal phenotypes and increases testicular cancer susceptibility in *bmpr1bb*$^{W256X/W256X}$ mutants

Introduction

Patients with disorders of sex development (DSDs) are at a higher risk for developing germ cell tumors of the seminoma or non-seminoma subtypes. DSDs generally manifest at birth and present with atypical gonadal or anatomical sex as well as chromosomal aberrations [1, 2]. DSDs encompass several syndromes, many previously known as intersex syndromes, including gonadal dysgenesis, cryptorchidism, Turner syndrome, and Klinefelter's syndrome among many others [2].

DSDs account for approximately 7.5% of all birth defects[164]. Testicular tumor risk for patients with DSDs ranges anywhere between 15-30% depending on the particular syndrome [165]. The cause of DSDs in a majority of the patients is unknown as only 20% of have a molecular diagnosis [1]. Since these DSDs arise at birth, a commonly accepted theory as to their cause is the effect of the fetal and or the gonadal microenvironment [2, 151]. This theory is further supported by many animal studies which found that exposure to either estrogens or anti-androgens caused a variety of DSDs [2, 166-169].

From mammals we have learned that the gonadal niche is comprised of somatic cells known as Sertoli or granulosa cells in males and females respectively. These cells provide signals to guide proper sex determination of the germ cells. These niches are also made up of steroidogenic cells known as Leydig in males or theca cells in females [170, 171]. Several

components important in either sex determination or sex differentiation implicated with these cell types are often mutated or overexpressed in DSDs further solidifying the idea that the microenvironment of the gonad is extremely important. Interestingly, there is an overlap in genes identified in animal model studies to be essential for gonadal formation and those that were identified through sequencing efforts of patients with DSDs such as *amh*, *foxl2* (Forkhead box L2), *dmrt1*, and *sox9a* [79, 172-180].

In this study we have found that zebrafish *bmpr1bb*$^{W256X/W256X}$ mutants display gonadal phenotypes similar to that of human DSDs and are also highly susceptible to developing testicular germ cell tumors as compared to the *bmpr1bb* haploinsufficeny model previously described [141]. Here, we more fully characterize this model and examine the expression levels and patterns of both male sex specific somatic markers *amh, dmrt1*, and *sox9a* as well as female specific markers *foxl2, foxl3*, and *cyp19a1a*. *foxl3*, a duplicated copy of *foxl2* previously known as *foxl2b*, was previously identified in Medaka (*Oryzias latipes*) as a gene necessary for the suppression of spermatogenesis in the ovary [181]. This model highlights the importance of the BMP signaling pathway in sex determination and cancer susceptibility and could further our understanding of the link between sex determination and testicular cancer susceptibility.

Methods

Zebrafish Care and Maintenance

Zebrafish were kept on a 10-hour light/14-hour dark cycle and maintained with standard procedures [159]. All protocols were approved by the Institutional Animal Care and Use Committees at UT Southwestern Medical Center, an AALAC-accredited institution.

Generating double and triple *bmpr1bb*$^{W256X/W256X}$ mutants

BMP signaling heterozygous zebrafish mutants, *bmp2b*, *bmp7a* and *smad5*, were obtained from the Zebrafish International Resource Center (ZIRC) and have been described previously [182-184]. Fish were created by crossing BMP heterozygous mutants into our tumor background as depicted in Figure 3.1.

Genotyping of zebrafish

Fish were anesthetized using 0.02% tricaine and a small portion of their fin was clipped. Tail DNA lysis was performed using the HotSHOT method [160]. Lysed tail DNA was used to amplify genomic DNA by PCR using GoTaq Flexi DNA polymerase (Promega) for *bmpr1bb*, *bmp2b*, *bmp7a* or *smad5* locus' using respective primers (see table 2.1 and 3.1 for primers). A custom allelic discrimination (AD) assay (Applied Biosystems) was used to genotype, as previously described [158], for *bmpr1bb*, *bmp2b* and *bmp7a*. For *smad5*, *pgy*^{dty40} genotyping was performed using restriction enzyme digestion reaction as previously described [182].

Generation of transgenic line for gonadal visualization of young zebrafish

The ziwi promoter-GFP plasmid was a kind gift of Bruce Draper (UC Davis, California). Using the Tol2 Gateway cloning system [162], fish were injected with 100ng/uL Tol2 transposase mRNA and 50ng/uL of plasmid containing ziwi-GFP construct. The transgenic F3 population was outcrossed into the *bmpr1bb*$^{+/W256X}$ background. Genotyping of F3 populations for *bmpr1bb* as described above and for the ziwi-GFP transgene using PCR amplification and agarose gel for visualization (Table 2.1).

Zebrafish histology scoring

Fish were euthanized at 60dpf, 70dpf and 90dpf using tricaine and head and tail portion were removed prior to being fixed in 4% paraformaldehyde (PFA)/1X phosphate-buffered saline for 48 hours at 4ºC. Following fixation, fish were dehydrated and paraffin embedded. A hematoxylin and eosin-stained transverse section was scored for gonadal morphology.

Tissue and RNA Isolation

Prior to fixing fish for histological analysis, a portion of the gonad was retained and placed into TRIzol (Invitrogen). RNA extraction was performed using manufactures protocol. Following extraction, RNA was purified using the RNAeasy Mini Kit (Qiagen). Quantification was achieved by using a NanoDrop.

Expression of somatic sex markers by RT-PCR

Two to four independent samples were used for either wildtype or *bmpr1bb*$^{W256X/W256X}$ gonads for each time point (60, 70 or 90dpf). Reverse transcription of either 100ng of total ovary RNA or 200ng of total testis RNA was performed using RT^2 First Strand Kit (Qiagen). PCR amplification using GoTaq Flexi DNA polymerase (Promega) of *amh, cyp19a1a, dmrt1, foxl2, foxl3, sox9a, and rpl13a* as an endogenous control (see Table 3 for primers) cDNA was performed and products were visualized on a 1% agarose gel.

Whole organ dissection and fixation for in situ hybridization

Wildtype or *bmpr1bb*$^{W256X/W256X}$ zebrafish aged 6 to 7 months were euthanized in tricaine. The head was removed and an incision was made along the bottom of the abdomen to create an open cavity before fixing the remain fish in 4%PFA overnight at 4ºC. Ovaries and testis of wildtype and *bmpr1bb*$^{W256X/W256X}$ were dissected after overnight fixation and placed into 100% methanol and stored at 4ºC overnight before proceeding to in situ hybridization.

Whole mount in situ hybridization

For each gene of interest, the cDNA sequences (obtained from Ensembl) of *amh, bmpr1bb, cyp19a1a, dmrt1, foxl2,* and *foxl3* were PCR amplified (See Table4 for primers used for cloning) and subsequently cloned into a TA vector (See Table 5 for vectors used). In the case of *vasa*, we obtained a plasmid from Open Biosystems. PCR amplification of the cDNA was used to create the riboprobe template for each gene and either a T7, SP6, or T3 polymerase sequence was used as a primer sequence except for vasa (See Table5 for template primers). The antisense strand was *in vitro* transcribed using the respective polymerase (see Table 5 for specific polymerase) (Promega) and DIG RNA labeling kit (Roche). Lithium chloride extraction was used to precipitate the RNA probe. The *in situ* hybridization procedure was followed as previously described [161] on wildtype or *bmpr1bb*-/- gonads.

Visualization of somatic cells

In order to visualize and characterize the somatic and germ cell interaction within our tumor line we obtained the Tg(*gsdf*:mcherry) transgenic line from Bruce Draper (UC Davis). PCR amplification of the transgene (see Table 1 for primers) was used for genotyping by tail fin clip as described above. These fish were crossed into our Tg(*ziwi*:GFP);*bmpr1bb*$^{+/W256X}$ line.

Several crosses were performed to create Tg(*ziwi*:GFP);Tg(*gsdf*:mcherry);*bmpr1bb*$^{W256X/W256X}$ transgenic zebrafish.

CRISPR/cas9 targeting of *foxl3*

Target sgRNA guides against foxl3 were identified using the website CRISPRscan.org [185]. Template guide oligos were chosen based on their high predicted score and low off-target matches. Oligos were annealed, filled in using T4 polymerase and in vitro transcribed as previously described [186]. Embryos from a *bmpr1bb*$^{+/W256X}$ incross were injected at the 1-cell stage. Injection mix consisted of ~1ug sgRNA, ~1ug cas9 recombinant protein and phenol red. The T7 Endonuclease surveyor assay was used to assess efficiency of our guides [187]. Genomic DNA was extracted from pools of 10 embryos at 24hpf using the method described above for tail lysing. PCR amplification was performed using primers (Forward-cctgaagcacacctggacac and Reverse-cctgctgcagacagtac) that spanned the targeted locus. 200ng of purified PCR product (Invitrogen PCR Purification Kit) were incubated at 37ºC for 30 min with T7 Endonuclease I enzyme (NEB) and digested products were visualized on an agarose gel. Remaining embryos were raised up to 3 months of aged and screened for altered GFP expression. Fish with altered GFP expression were sacrificed and histological analysis was performed as described above.

Results

Loss of BMP signaling results in biphenotypic gonads and female bias in *bmpr1bb*$^{W256X/W256X}$ mutants

Since the BMP signaling pathway has been implicated previously in germ cell tumors we were interested in utilizing our model to further test the role of specific pathway components in

the development of GCTs [142, 158]. To do so, we incorporated well-characterized zebrafish BMP mutants with mutations in either *bmp2b*, *bmp7a* and *smad5* into our tumor background in order to understand their biological contribution to germ cell tumorigenesis as shown in figure 3.1 [182-184]. Using *bmpr1bb*$^{W256X/W256X}$ mutants, double mutants (any BMP pathway heterozygote in combination with *bmpr1bb*$^{W256X/W256X}$) and a triple mutant (*bmp2b*+/-;*bmp7a*+/-;*bmpr1bb*$^{W256X/W256X}$) we observed a female bias in all genotypes as compared to wildtype zebrafish at 70dpf (Figure 3.2D). Furthermore, we found that fish homozygous for the *bmpr1bb* mutation exhibited a new gonadal phenotype. This phenotype is characterized as a biophentypic gonad in which a single zebrafish contains developing ovary and testis features (Figure 3.2C). Imposing more perturbations on the BMP signaling pathway, through the introduction of further mutant BMP pathway alleles, in the increased the incidence of biphenotypic gonads (for example, 15% biphenotypic gonads in *bmp7a*+/-;*bmpr1bb*$^{W256X/W256X}$ mutants; Figure 3.2D. In the absence of other BMP pathway alleles, *bmpr1bb*$^{W256X/W256X}$ mutants exhibit a low incidence of biphenotypic gonads, and the expected testicular tumors in males or oocyte maturation defects in females, at higher penetrance than what is seen in heterozygotes. Interestingly, increases in tumor incidence were not seen in double or triple mutants (Figure 3.2E and data not shown).

Younger *bmpr1bb*$^{W256X/W256X}$ mutants have severely delayed germ cell maturation and persistence of primitive germ cells

The unusual biphenotypic gonad phenotype prompted us to look at the gonads at an even earlier time point of 60dpf. At this developmental time point, wildtype fish have nearly completely mature testis or ovary (Figure 3.3A, B). In contrast, *bmpr1bb*$^{W256X/W256X}$ homozygous mutants displayed persistence of early primitive germ cells. Clusters of such cells can rarely be found in

the immature ovary (Figure 3.3B). However, in *bmpr1bb*$^{W256X/W256X}$ homozygous mutants the clusters are larger in size (Figure 3.3 C, D) and also appear at significantly higher rates than in their wildtype counterparts (Figure 3.3E).

bmpr1bb$^{W256X/W256X}$ mutants have misregulation of several sex specific markers

The observation that a significant number of patients who have disorders of sexual development (DSD), gonadal dysgenesis or cryptorchidism (undescended testis) have a higher propensity to develop testicular germ cell tumors highlights the idea that an underlying mechanism exists that ties together defective gonadal development and tumorigenesis [2] . The bilateral biphenotypic phenotype and persistence of primitive cells displayed by the *bmpr1bb*$^{W256X/W256X}$ mutants at early stages prompted us to look at several somatic sex specific markers that have been implicated in germ cell differentiation and maturation since all of these homozygous mutants go on to develop testicular germ cell tumors.

After the migration of primordial germ cells to the site of the developing gonad, zebrafish develop a primitive ovary-like gonad that persists until about day 21 of life [158]. Subsequently, the male and female definitive gonads develop, marked by sex-specific expression of certain genes. We examined the expression levels of *amh*, *dmrt1*, and *sox9a* which are well characterized to contribute to proper male gonadal development. Furthermore, we characterized the expression of *cyp19a1a*, *foxl2*, and *foxl3* which are known to contribute to female gonadal development. We chose to look at three different time points: 60dpf, 70dpf, and 90dpf (180dpf in the case of wildtype testis) which were time points in which we had seen primitive cell persistence, biphenotypic gonads and testicular tumors or maturation defects respectively.

Interestingly, we found that *bmpr1bb*$^{W256X/W256X}$ mutant males, regardless of time point, retained expression of female sex genes (Figure 3.3A). The most significant increases were seen in the expression of foxl2 and foxl3 for all time points. Male gene expression did not seem to change between the wild type and mutant counterparts. In contrast to males, female *bmpr1bb*$^{W256X/W256X}$ mutant gonads there did exhibit aberrant expression of male sex somatic genes at any time point (Figure3.3B).

bmpr1bb$^{W256X/W256X}$ mutants have altered spatial temporal gene expression patterns

Many studies over the years have characterized the intricate make-up of the gonadal niche. We've come to understand that both males and female germ cell require sex specific signals from somatic cells within these niches to properly specify and differentiate [188-191]. Since the *bmpr1bb*$^{W256X/W256X}$ mutants have altered somatic marker expression levels, we sought to explore whether spatial-temporal expression patterns were also altered using *in situ* hybridization. As shown in Figure 3.4B, in the females the expression patterns of somatic and germ cell-specific genes have minimal changes in their distribution; only *dmrt1* and *foxl2* appear to have higher expression in the ovaries with maturation defects. Note that individual ovaries exhibited slightly different proportions of immature oocytes in H&E sections (not shown), which may account for the small differences in gene expression seen within each time point for some genes in Figure 3.4B. These differences are reflected in the whole-mount in situ hybridization expression pattern (Figure 3.5, right side). Female *bmpr1bb*$^{W256X/W256X}$ mutant ovaries exhibit a higher proportion of immature oocytes, and thus they more densely express *amh, dmrt1* and *cyp19a1a* than do their wildtype counterparts (Figure 3.5). However, the relative somatic cell-germ cell patterning of expression appears relatively unaltered.

In contrast, in testicular tumors arising in *bmpr1bb*$^{W256X/W256X}$ mutant males, the somatic cell expression of markers such as *amh* is more compact compared to wildtype (Figure 3.6, left side). Based on the histologic appearance of these tumors, in which primitive germ cells accumulate but do not expand into replicating spermatocytes and sperm, we speculate that in the absence of BMP signaling the failure of germ cells to differentiate leads to an alteration of ratio of somatic cells to germ cells, accounting for the pattern of increased *amh* expression.

foxl3 appeared to have the most distinct and interesting expression pattern. Not only was *foxl3* expression elevated in both male and female *bmpr1bb*$^{W256X/W256X}$ mutant gonads, but the expression patterns were strikingly different than in their wildtype counterparts. *foxl3* is expressed in a restricted punctate pattern in wildtype females (Figure 3.6C) and even more sparse in males (Figure 3.6A). Interestingly, both male and female mutant gonads have a significantly higher cell *foxl3* positive population with a distinctive patterning (Figure 3.6B, D).

bmpr1bb displays a somatic cell expression pattern and loss of BMP signaling through the _bmpr1bb_ receptor results in altered somatic cell distribution.

Since the gonadal niche requires communication between somatic cells and germ cells we were interested in identifying whether the bmpr1bb receptor was expressed in the somatic supporting cells or the germ cells. In order to do so we used in situ hybridization to determine if *bmpr1bb* had an expression pattern that was more similar to the somatic marker amh (figure 3.A-D) or more similar to the germ cell marker vasa (figure 3.7I-L). As can be seen in figures 3.7E-H, the *bmpbr1bb* expression pattern more closely resembled the somatic marker *amh* suggesting

that it was being expressed in the somatic cells surrounding germ cell populations. We further confirmed this result by performing dissections of mature ovarian follicles to physically separate the somatic cell layer from the oocyte, followed by RT-PCR for germ cell and somatic cell markers. Expression of *bmpr1bb* was strongly enriched in the somatic cell layer (Dranow *et al.*, PLoS Genetics 2016, in press).

As previously mentioned, we hypothesized that the ratio of somatic cells to germ cells was altered in *bmpr1bb* mutants. To address this hypothesis, we used two reporter lines, Tg(*gsdf*:mCherry) to mark somatic cells and Tg(*ziwi*:GFP) to mark germ cells [192], crossed into the *bmp1bb* mutant tumor background, and observed any changes. In wildtype testis, somatic cells surround the testicular tubules comprised of germ cells undergoing different stages of spermatogenesis (Figure 3.7M). This patterning is exaggerated in the *bmpr1bb*$^{W256X/W256X}$ mutants as we observe more mCherry expression in an altered pattern. In the wildtype ovary, *gsdf*:mCherry expression is restricted to the intermediate-stage oocytes (stage II-III) and is less appreciable around the largest, most mature oocytes (figure 3.7O). On the other hand, in the *bmpr1bb*$^{W256X/W256X}$ mutant ovaries we find that most of the larger oocytes are surrounded by somatic cells as represented by the mCherry somatic-cell reporter. These observations support the idea that there is an increase in the number of somatic cells in both male and female mutant gonads.

Loss of *foxl3* in the *bmpr1bb* tumor background reverts sex skewing and introduces a new gonadal phenotype

Since *foxl3* appeared to be the most aberrantly expressed gene in the *bmpr1bb*$^{W256X/W256X}$ mutant gonads, and was recently implicated as a factor required for maintaining female gonadal fate, we hypothesized that aberrant expression of *foxl3* in *bmpr1bb*$^{W256X/W256X}$ mutants might account for the female sex bias in this background. Therefore, we tested whether introduction of mutations into *foxl3* in the tumor background using CRISPR/cas9 technology could ameliorate the sex bias. We designed two sgRNAs targeting zebrafish *foxl3* and demonstrated their effectiveness using a T7 surveyor assay (not shown). We then injected these sgRNAs along with Cas9 protein into single-cell stage zebrafish embryos from wildtype or *bmpr1bb*$^{W256X/W256X}$ mutant backgrounds, and raised the fish to adulthood. We scored the somatic sexual phenotype and took samples for histologic analysis at 3 months of age. As shown in figure 3.8A, injection of either sgRNA#1 and gRNA#2, in the *bmpr1bb*$^{W256X/W256X}$ mutants appears to normalize the female bias seen in uninjected *bmpr1bb*$^{W256X/W256X}$ mutants. Using the Tg(ziwi:GFP) germ cell specific reporter line in the mutant background, we observed that introduction of mutations into foxl3 alters the GFP expression pattern normally seen in *bmpr1bb*$^{W256X/W256X}$ males (Figure 3.8B-D) and females (Figure 3.8H-J). Both foxl3 guides seem to reduce the GFP expression pattern as compared to their *bmpr1bb*$^{W256X/W256X}$ mutant counterparts (Figure3.8D and J. Histological analysis, by H&E, of the gonads showed that the gonadal phenotypes were also changed in the *foxl3* CRISPR fish. A *bmpr1bb*$^{W256X/W256X}$ mutant gonad injected with foxl3 sgRNA#1 seemed to be predominately comprised of somatic cells in an abnormal pattern. This further highlights the importance of BMP signaling in maintaining proper somatic cell distribution via its effect on *foxl3* expression. In females injected with foxl3 gRNA#2, we found

that the ovary exhibited patches of spermatogenesis (figure 3.8L) as has been previously reported to occur due to *foxl3* deficiency in another fish model, Medaka (*Oryzias latipes*) [181].

Discussion

To date, no sex chromosomes have been identified in zebrafish, making the mechanism for sex determination obscure. While some genes have been shown to be expressed in a sex specific manner many other factors can contribute to sex determination including water temperature, tank crowding, food availability and even steroid exposure [193-197]. Using the *bmpr1bb*$^{W256X/W256X}$ mutant zebrafish GCT model we found that BMP signaling pathway plays a role not only in cancer susceptibility but also in the development of DSDs as loss of the BMP signaling pathway allowed mutants to develop a bipotential biphenotypic gonad and a female sex bias we previously had not seen in our heterozygous population. The involvement of the BMP signaling pathway in DSDs is supported by a recent GWAS study that has identified genetic variants in the BMP and TGFβ signaling pathways (bmp7 ligand and TGFβ3 receptor) that associate with testicular gonadal dysgenesis, a DSD, and cancer susceptibility [3]. Interestingly, *bmp7a*+/-;*bmpr1bb*$^{W256X/W256X}$ double mutants had the highest rate of biphenotypic gonads. The increased biophenotypic gonad phenotype and sex bias in the mutants establishes a role for BMP signaling in proper sex determination.

In mammals, the involvement of the BMP signaling pathway has been well characterized. BMP4 and BMP8 are known to specify embryonic stem cells into germ cells during embryogenesis [19-22]. However, we do not know the extent of the BMP signaling pathway involvement in maintaining the sex specific gonadal environment. Loss of BMP signaling in our

mutants led to a higher rate of early primitive cells persisting in much larger clusters, suggesting that there is a requirement of proper BMP signaling to drive these cells into either oogonial or spermatogonial fates. It is highly likely that since these primitive cells, which normally require proliferation and pluripotency signals, persist beyond their normal time point allow for a perfect microenvironment to maintain germ cell proliferation and pluripotency. The reciprocal signaling that is normally able to slow proliferation and drive germ cell differentiation away from pluripotency is overshadowed by the signals from the persistence of primitive germ cells, and eventually leading to the bipotential biphenotypic gonads described.

The expression pattern of the *bmpr1bb* receptor highlights its essential role within the somatic cells providing the proper microenvironment for the sex specific gonadal niche as is evidenced by the expansion and alteration of somatic and germ cells ratios in *bmpr1bb*$^{W256X/W256X}$ mutants. The improper somatic cell signal appears to contribute to the derepression of foxl3+ cell proliferation skewing the ratios of both somatic and germ cells which introduces more mixed signals creating an abnormal signaling environment within the gonadal niche and allowing for both ovaries and testis to develop within one gonad. Proper feedback loops are no longer in place to regulate proper sex determination and germ cell pluripotency regulated by BMP signaling. Further work is needed to properly evaluate the relationship between BMP signaling and *foxl3* as well as quantification of somatic:germ cell ratios for better understanding of this scenario.

Here we have highlighted the requirement of appropriate BMP signaling that is necessary to establish and maintain germ cell pluripotency and differentiation by providing the correct

cellular signaling within the sex specific gonadal niche. Aberrant BMP signaling is shown cause misregulation of several somatic sex specific genes within the gonadal niche, eventually leading to biphenotypic gonads and cancer susceptibility. This intriguing connection between germ cell pluripotency, sexual development and cancer susceptibility highlights the familiar characteristics of DSDs. Our model can provide more insight into germ cell differentiation, sex determination, gonadal niche communication between germ and somatic cells, and the eventual steps leading to the tumorigenic events to developing a tumor. We can further leverage the model and together with DSD and GCT sequencing data to identify novel therapies and establish a more up to date molecular diagnosis.

Primers used to amplify a PCR product for genotyping

Gene	Forward	Reverse	Amplicon(bp)
bmp2b	CCTGGACGAGTACGAGAAGG	ATCTTCGAAGCCACTGGAAA	239
bmp7a	ACATGCTGGATCTGTATAACGCC	GGAGGATGCTTGTTGATTCAAAC	411
smad5	GCAACACCAAAAATGACTATTGATG	AAAACAAAGGCTTCCTCCCAGG	438
gsdf-mcherry	CGACTACTTGAAGCTGTCCTTCC	CAGCTTGATGTTGACGTTGTAGG	358

Primers used for amplifiying product for AD Assay genotyping

Gene	Forward	Reverse	Amplicon(bp)
bmp2b	TTGCCGATGAGAACAA	TGCCGATGGGAACAA	83
bmp7a	CAAAGCTCATCACCATGTC	AAGCTCATCCCCATGTC	69

Primers used for RT-PCR

Gene	Forward	Reverse	Amplicon(bp)
amh	CATCTGGTTCAGAGTGTG	CGTGTGGACTCAGAAATG	257
cyp19a1a	GCGTGTCTGGATCAATGG	CCTCTGAAGCCCTGGACC	223
dmrt1	GCCATCACCCGCCAGCAG	CTGAGGAGACACGTTATGGC	253
foxl2	CTGGGAAGGTTTGCGTGCG	CCGAGGCACCTTGATGAAGC	348
foxl3	CCACAGAGGATCTTCGCGTT	GATGCTGTTCTGCCATCCC	285
sox9a	GTCCATCTACGGTGTTCGCA	CCCTTCAGCACCTGGGAC	273
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	150

Primers used to clone gene for later use to make riboprobe

Gene	Forward	Reverse	Amplicon(bp)
amh	GCCATGGCTCAGGTCAAACAGC	GCGGCATTCGCACTTGGTCGC	528
bmpr1bb	CAAACAGCATCGAGACCTGA	CTCCGCCATAGTGAAGCAGT	600
cyp19a1a	GGAGTTGCAGGATGCCATCACAGC	GCTGGAAGAAACGACTCGGAAC	536
dmrt1	GCCATCACCCGCCAGCAG	ACTGGCAGCTGGAGATGGTC	506
foxl2	CTGGGAAGGTTTGCGTGCG	GGGCGGGGAGAGGTAACCG	531
foxl3	CCACAGAGGATCTTCGCGTT	GCACCGAGGTTTGCCATTAG	835

Primers used to make riboprobe template

Gene	Vector	Forward Primer	Reverse Primer	Polymerase
amh	pCRII-TOPO	TAATACGACTCACTATAGGG (T7)	ATTTAGGTGACACTATAGA (SP6)	SP6
cyp19a1a	pCRII-TOPO	TAATACGACTCACTATAGGG	ATTTAGGTGACACTATAGA	SP6
bmpr1bb	pCR4-TOPO	TAATACGACTCACTATAGGG	AATTAACCCTCACTAAAGGG (T3)	T7
dmrt1	pCR4-TOPO	TAATACGACTCACTATAGGG	AATTAACCCTCACTAAAGGG	T7
foxl2	pCR4-TOPO	TAATACGACTCACTATAGGG	AATTAACCCTCACTAAAGGG	T3
foxl3	pCR4-TOPO	TAATACGACTCACTATAGGG	AATTAACCCTCACTAAAGGG	T3
vasa	pME18S-FL3	aattaaccctcactaaagggaagtttgcatacgggacctg	taatacgactcactatagggctttctctcgctctcgctgt	T7

Table 3.1. Primers used for various experiments.

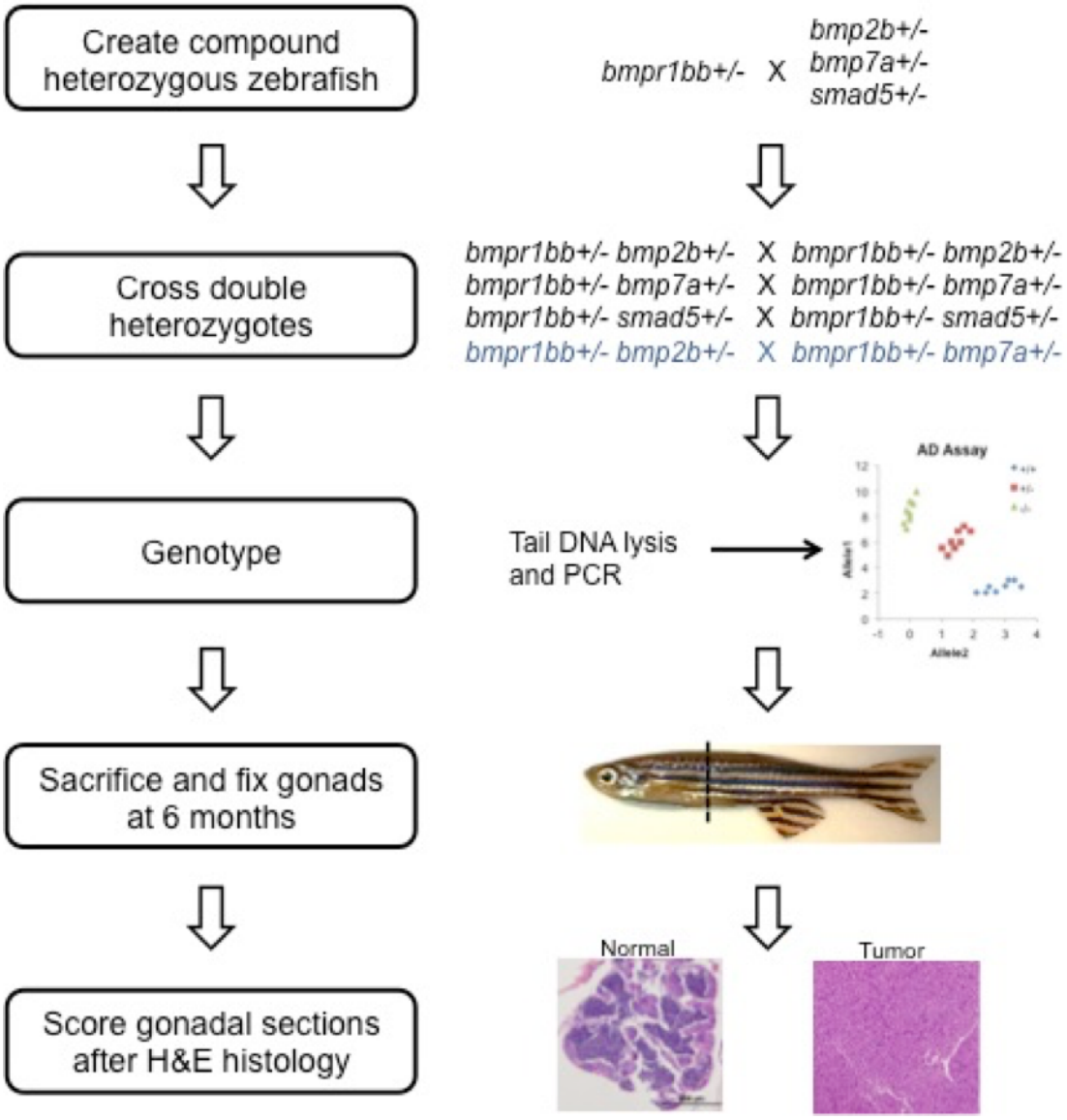


Figure 3.1. Schematic of experimental set up for creating double and triple BMP mutants. Zebrafish heterozygous for *bmp2b*, *bmp7a*, and *smad5* were crossed into tumor background line *bmpr1bb*+/-. Compound *bmpr1bb* homozygotes with haploinsufficiency of bmp ligand or *smad5* cellular transducer, as well as the triple mutant (*bmp2b*+/-;*bmp7a*+/-;*bmpr1bb*-/-) were created and maintained until they were 10 weeks of age. Zebrafish were genotyped prior to being sacrificed. Transverse paraffin embedded sections were made and subsequently stained with H&E. The sections were analyzed for sex distribution and gonadal phenotypes such as tumors or maturation defects.

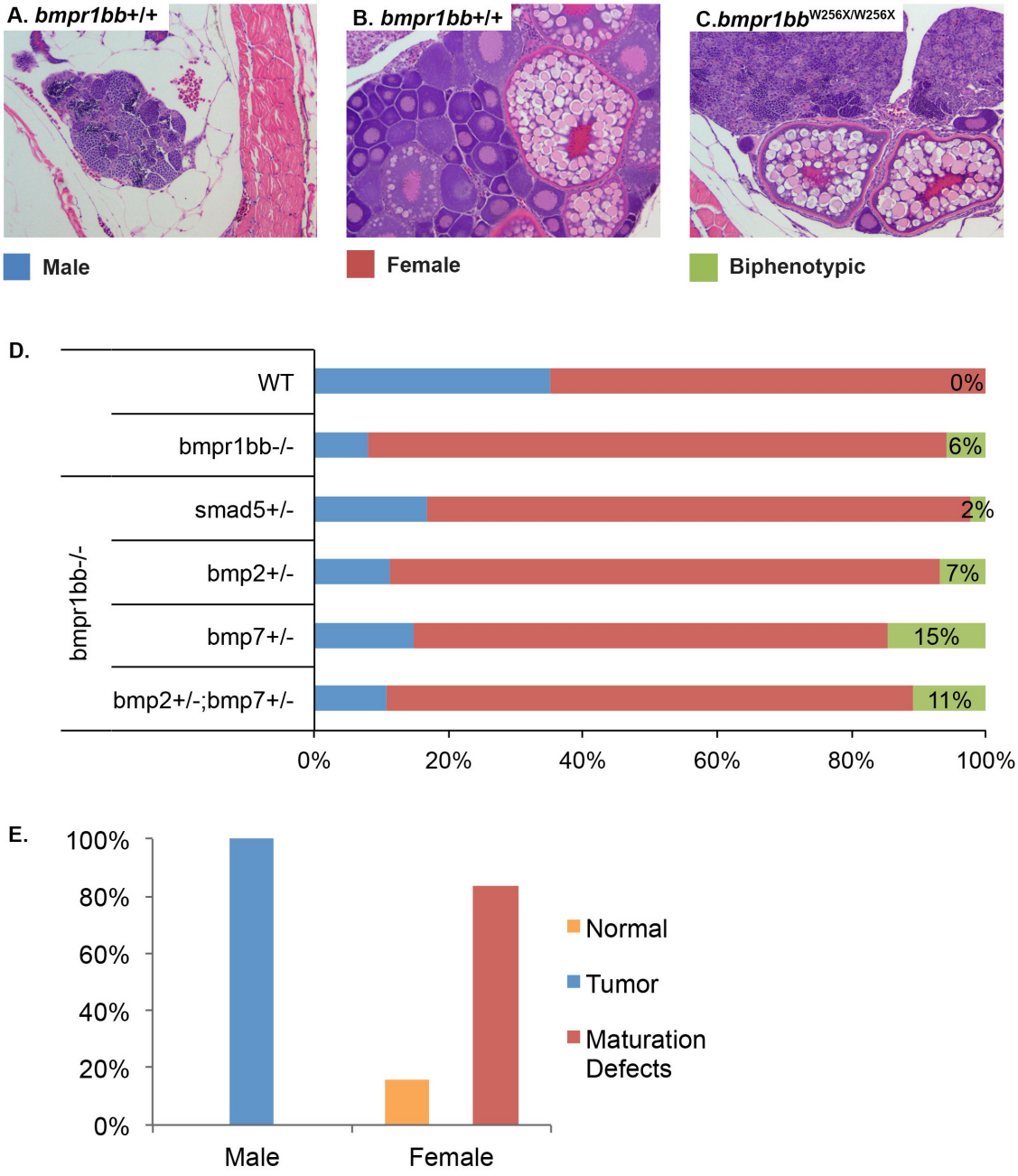


Figure3.2. *bmpr1bb*$^{W256X/W256X}$ mutants have bipotential biphenotypic gonads at 70dpf. Gonadal sex was determined by H&E sections from each fish. (A) At this time point *bmpr1bb*+/+ testis looked normal with all stages of spermatogenesis occurring. (B) Wildtype females were not quite mature as they had a higher percentage of stage I-III oocytes and very few stage IV. (C) An example of a biphenotypic gonad displaying both testicular tumor and ovarian tissue within the same gonad. (D) Quantification of the sex distribution among the different genotypes. WT N=37, *bmpr1bb*-/- N=101, *smad5*+/-;*bmpr1bb*-/- N=42, *bmp2b*+/-

;bmpr1bb-/- N=44, *bmpr7a;bmpr1bb-/-* N=41, *bmp2b;bmp7a;bmpr1bb-/-* N=37 (E) Quantification of the gonadal phenotypes observed in males and female *bmpr1bb*$^{W256X/W256X}$ mutants. Male N=8, Female N=87.

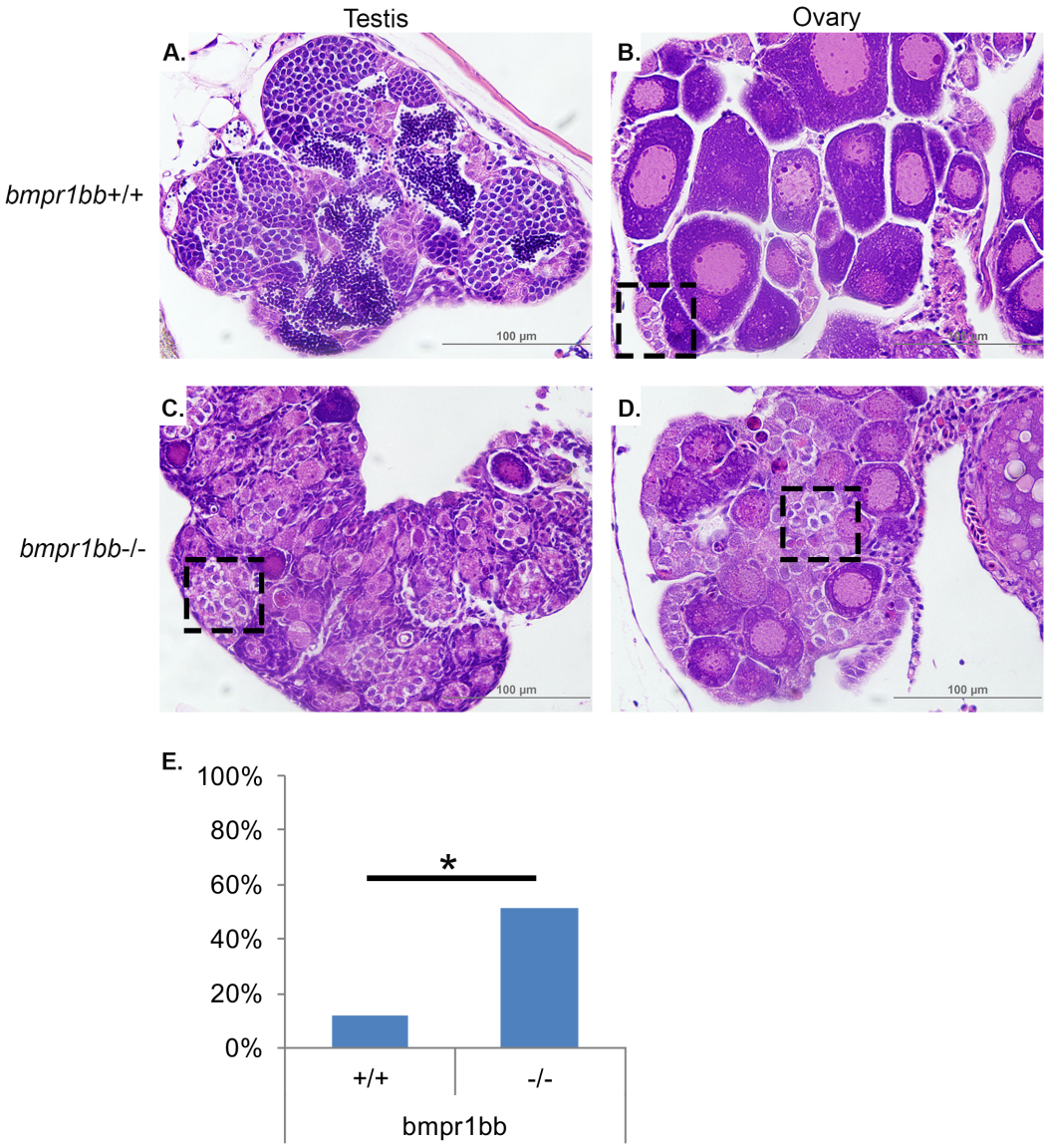


Figure 3.3. *bmpr1bb*$^{W256X/W256X}$ 60dpf gonads are severely delayed and have persistence of primitive cells. Histological analysis for persistence of primitive cells was performed by H&E staining of transverse sections. (A) Wildtype testis at this stage did not have any persistence of primitive cells and had all stages of spermatogenesis occurring. (B) Representative image of a wildtype immature ovary with mostly stage I-II oocytes. (C) *bmpr1bb*-/- gonad that appears to be resembling a testis. (D) *bmpr1bb*-/- gonad that appears to be trending towards a female with the amount of stage I-II oocytes present. (E) Quantification of the percent of gonads that contained persistence of primitive cells in wildtype (N=54) and *bmpr1bb*$^{W256X/W256X}$ mutant gonads (=43). Dashed box outlines persistent primitive cells in B-D. * Fisher exact test p≤0.0001.

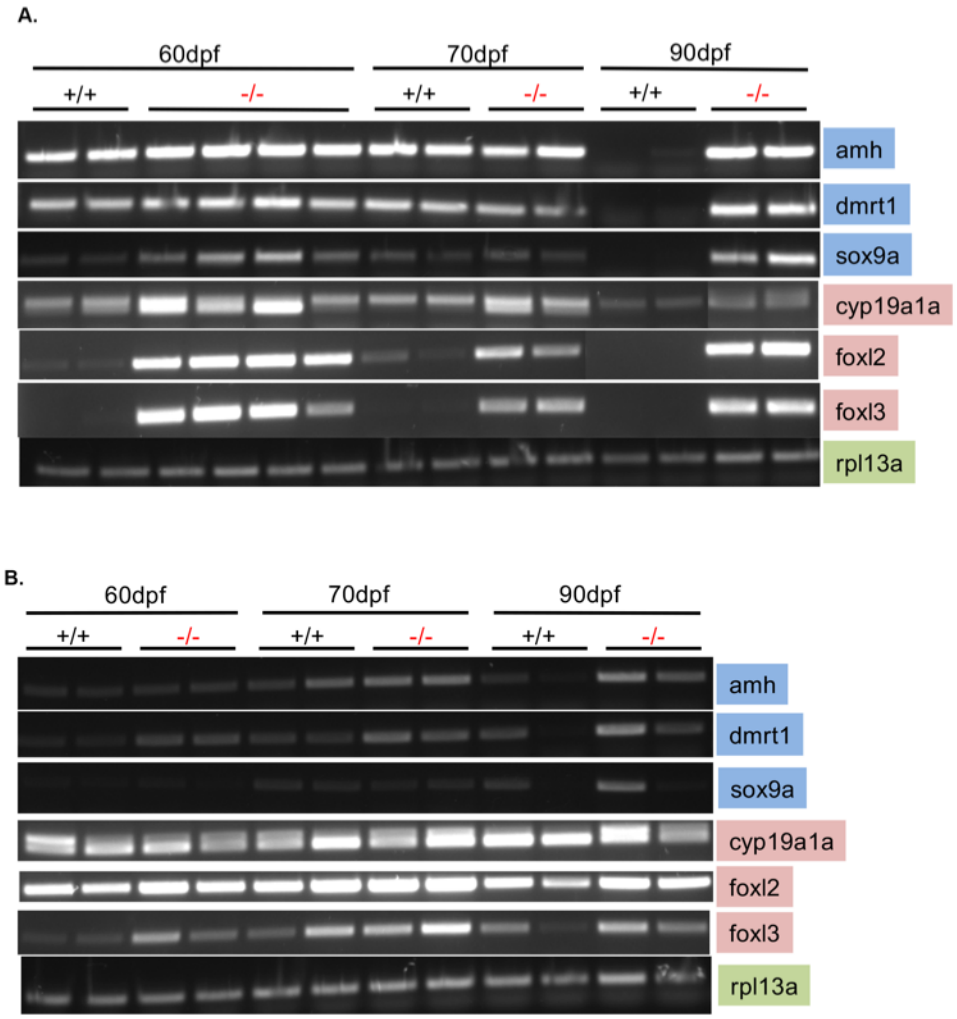


Figure 3.4 Testicular germ cell tumors express female sex specific genes normally not seen in wildtype testis. Expression of several somatic sex specific markers at 3 different time points (60dpf, 70dpf, and 90dpf) in *bmpr1bb*+/+ (+/+) and *bmpr1bb*$^{W256X/W256X}$ (-/-) male (A) and female gonads (B). Male specific genes are in blue, female specific genes are in pink and the general housekeeping gene is in green.

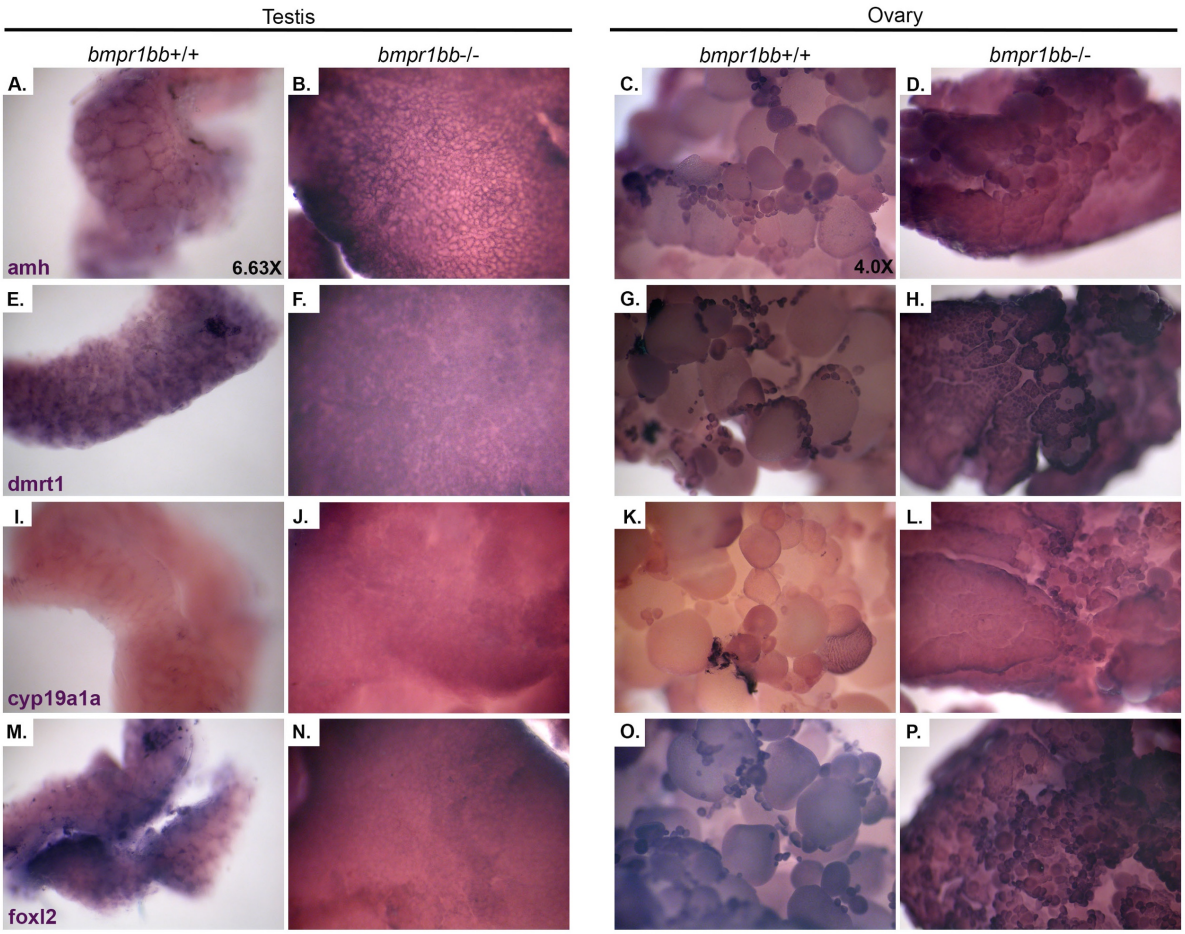


Figure 3.5. Spatial temporal expression patterns of several sex specific genes is distinct in *bmpr1bb*$^{W256X/W256X}$ mutant testis and ovary compared to their wildtype counterparts in gonads between 6-7 months of age. Depiction the expression pattern in wildtype testis (A, E, I, and M) or wildtype ovary (C, G, K, and O) of amh (A and C), dmrt1 (E and G), cyp19a1a (I and K), and foxl2 (M and O). Expression pattern in *bmpr1bb*$^{W256X/W256X}$ testicular tumors (B, F, J, and N) or ovaries with maturation defects (D, H, L, and P) of amh (B and D), dmrt1 (F and H), cyp19a1a (J and L), and foxl2 (N and P). All males were imaged using 6.0X magnification and females using 4.0X magnification.

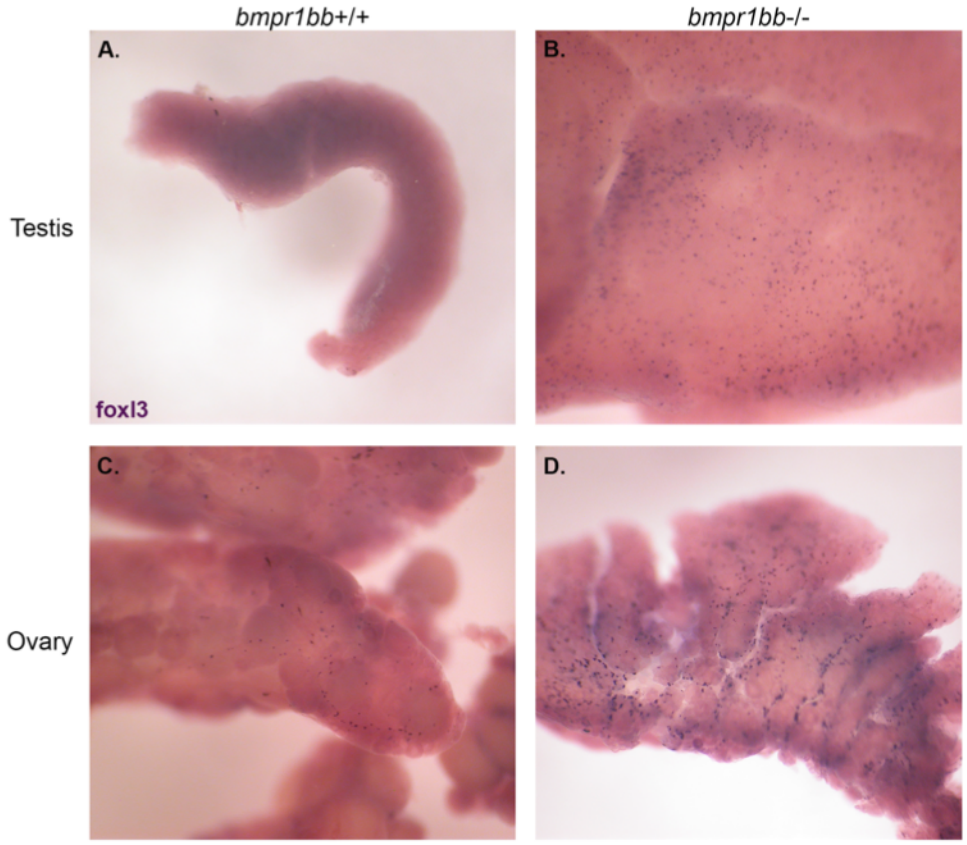


Figure 3.6. Expansion of foxl3+ cell population in *bmpr1bb*$^{W256X/W256X}$ mutant female and male gonads. (A) Wildtype testis with only a few foxl3+ cells. (B) *bmpr1bb*$^{W256X/W256X}$ testicular tumor with an enhanced number of foxl3 cells displayed in a punctate manner. (C) Wildtype ovary with a very restricted number and patterning of foxl3+ population of cells. (D) *bmpr1bb*$^{W256X/W256X}$ mutant ovary has increased number and expression of folx3 cells. All gonads were imaged under 4X magnification.

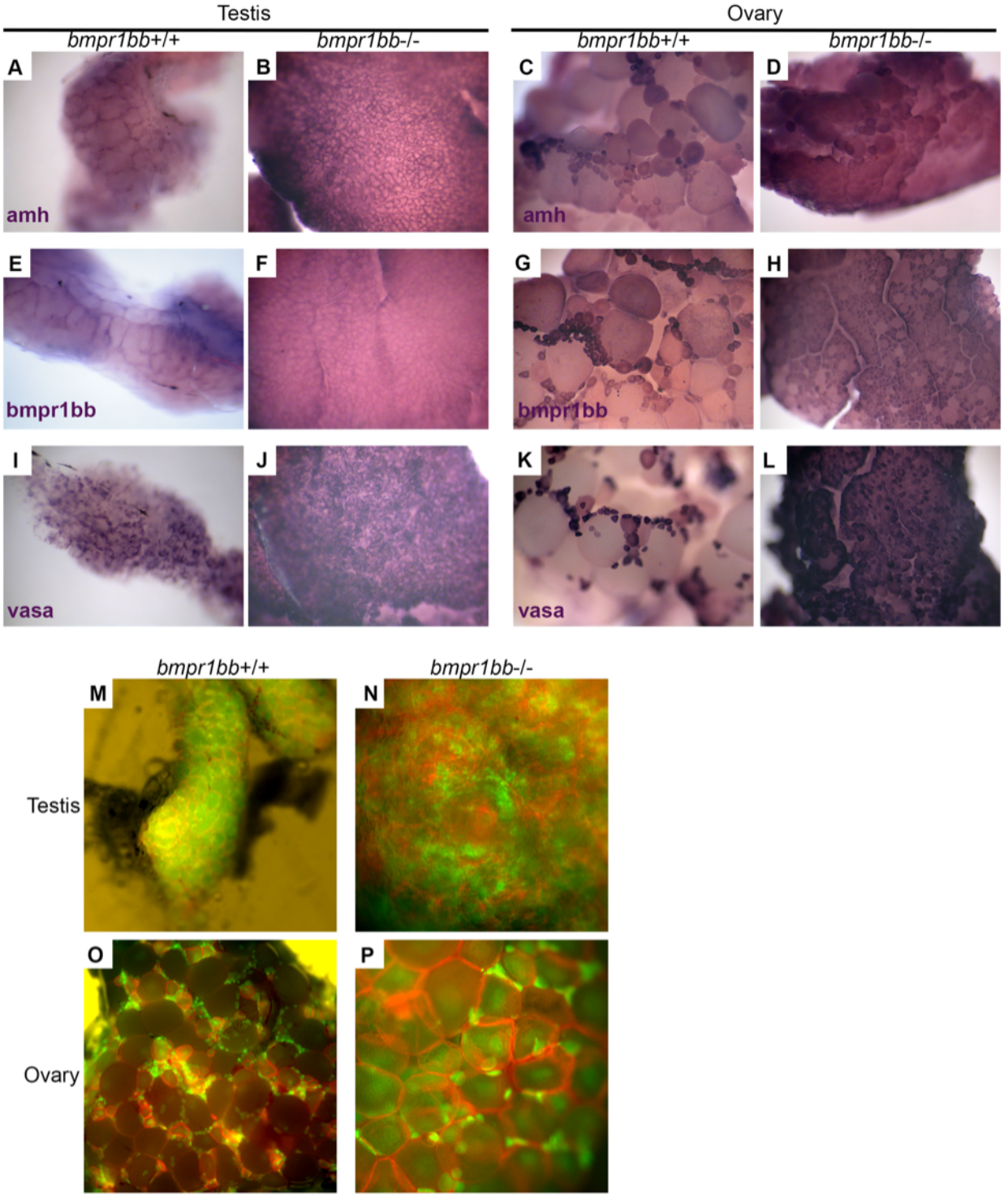


Figure 3.7. *bmpr1bb* receptor has somatic cell specific expression and mutants display an altered somatic to germ cell ratio at sexual maturity. The expression pattern of the *bmpr1bb* receptor in wildtype testis (E) and wildtype ovary (G) was compared to both a somatic marker, amh (A and C, and a germ cell marker, vasa (I and K) in males and females respectively. Mutant male (B, F, J) and female (D, H, and L) gonadal tissues were also stained with amh, *bmpr1bb*, or

vasa respectively. A reporter line was used to mark somatic cells in red (mCherry) and germ cells in green (GFP). (M) A wildtype testis that has somatic cells outlining the testicular tubules comprised with germ cells. (N) A mutant tumor that appears to have more somatic and germ cells in an abnormal pattern. (O) Wildtype ovary that displays very young small germ cells with the somatic cells surround the stage II-III staged oocytes. (P) *bmpr1bb*$^{W256X/W256X}$ mutant ovaries have expanded somatic and germ cells expression and nearly all oocytes have somatic cells surrounding them.

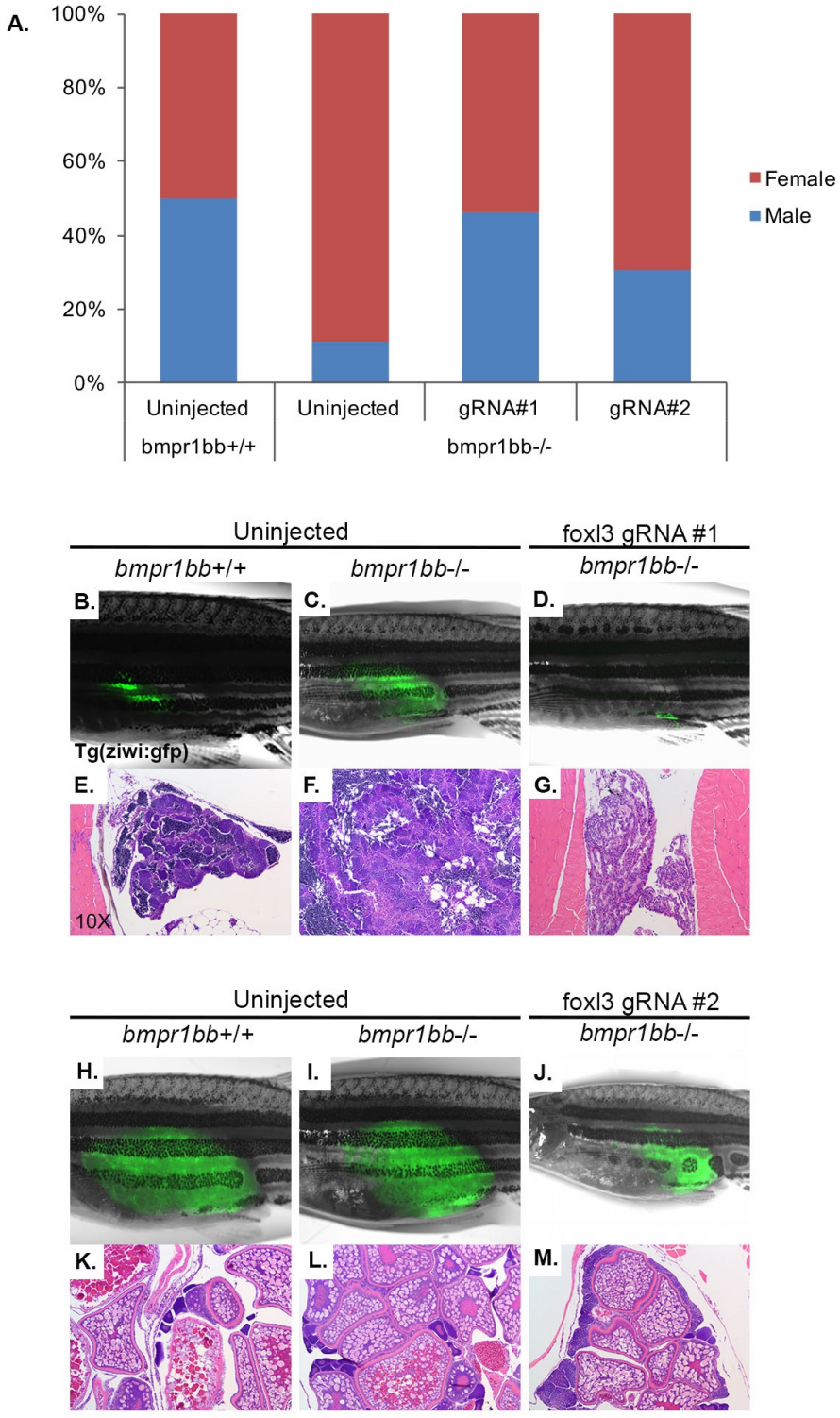


Figure 3.8. foxl3 CRISPR knockouts even out sex distribution and allow for a new gonadal phenotype to arise in the *bmpr1bb*$^{W256X/W256X}$ mutant background. (A) Quantification of the sex distribution between uninjected wildtype and *bmpr1bb*$^{W256X/W256X}$ mutant zebrafish

compared to fish that were injected with two different guide RNAs against f*oxl3*. (B) Wildtype male having restricted germ cell expression [Tg(ziwi:GFP)] and normal testicular architecture and spermatogenesis visualized histologically by H&E (E). (C) *bmpr1bb*$^{W256X/W256X}$ mutant with a testicular tumor has an expanded germ cell expression pattern and the architecture with varying spermatogenesis (F). (D) A *bmpr1bb*$^{W256X/W256X}$ mutant foxl3 gRNA injected male fish with abnormal GFP expression compared to both wildtype (B) and *bmpr1bb*$^{W256X/W256X}$ mutant (C) gonads. (G) Histological analysis revealed an interesting gonad comprised of somatic cells. (H) Wildtype female GFP expression pattern is much larger than wildtype males and the histological analysis shows a normally maturing ovary with stage I-IV oocytes present (K). (I) *bmpr1bb*$^{W256X/W256X}$ mutant ovary has a similar GFP expression pattern as the wildtype ovary. (L) Histological analysis reveals an immature ovary with no properly developed stage IV oocytes. (J) A *bmpr1bb*$^{W256X/W256X}$ mutant foxl3 gRNA injected female fish with reduced GFP expression compared to both wildtype (H) and *bmpr1bb*$^{W256X/W256X}$ mutant females (I). (M) Histology of the guide injected female fish reveals spontaneous spermatogenesis occurring within the ovary.

CHAPTER FOUR
Conclusion and Future Directions

While BMP signaling has been shown to be important in specification of embryonic stem cells into germ cells, little is known about the requirement of BMP signaling in sex differentiation and maintenance of sex specific gonadal niches. Here we describe how the heterozygous and homozygous *bmpr1bb* mutant tumor models reveal important and necessary roles of BMP signaling in germ cell pluripotency, sex determination, and cancer susceptibility (Figure 4.1). Specifically, we showed that loss of BMP signaling promotes germ cell tumorigenesis. Looking further, we found that complete loss of function receptors caused persistence of primitive cells. These primitive cells contributed to an altered gonadal niche exacerbated by misregulation of somatic and germ cell signals eventually leading to the establishment of bipotential biphenotypic gonads and to the development of germ cell tumors. Interestingly, the phenotypes seen in our tumor models overlaps with the known characteristics of DSDs and their elevated risk for developing GCTs potentially establishing a new DSD animal model.

We will be able to tackle several aspects of both DSDs and GCTs with one model. Since our findings showed that homozygous mutants captures aspects of all 3 human GCT subtypes, we can use it as a tool to identify either novel or cooperating genes identified through sequencing efforts of patient GCT samples by finding the biological importance of such genes. We have already begun these efforts and have sequenced many seminomas, yolk sac tumors and embryonal carcinomas to find somatic and germline mutations, copy number changes, and gene

expression alterations. Lin Xu, a bioinformaticist from our group, established a pipeline to compile the list of genes that were recurrently mutated, gained or lost in human GCTs and also had any association with germ cell biology in the literature. The genes are listed in figure 4.2. We focused on genes that had high copy number loss and high rates of mutations and identified 10 genes of interest to begin knocking them out via CRISPR/Cas9 technology in the zebrafish (Figure 4.3). One gene of interest to note is INSL3 (insulin like 3), which has been implicated in male germ cell differentiation, was both implicated in both the human data and the zebrafish RNAseq analysis (data not shown). This finding could potentially highlight how BMP more specifically regulates spermatogonial stem cell proliferation and differentiation which is summarized in figure 4.5. We have identified effective guide RNAs for targeting of several genes, and these fish are currently being raised for further characterization.

More experiments are required to determine if this approach will produce better models of GCTs that more accurately capture aspects of human GCTs. We may also be able to use the tumor background as a starting point to try to drive tumors into other subtypes and begin unraveling how DSDs promote germ cell tumorigenesis. In summary, our findings implicate defective BMP pathway signaling as a potential factor in mixed gonadal dysgenesis and GCT susceptibility tying together germ cell pluripotency, sex differentiation, and cancer susceptibility. We are hopeful that we can leverage our model to further understand the human disease and identify novel therapeutic alternatives.

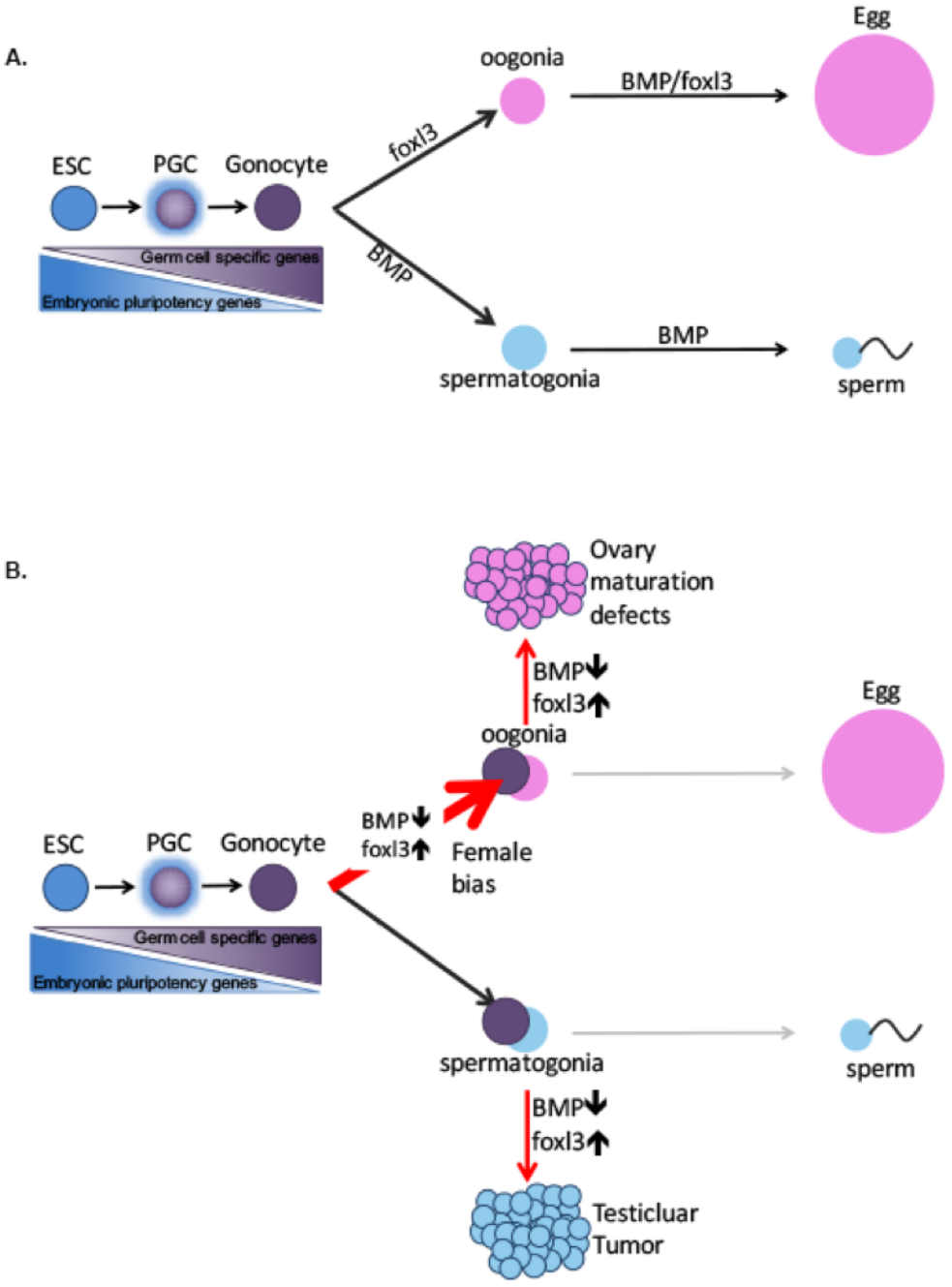


Figure 4.1. Working model describing how loss of BMP signaling and deregulation of foxl3 leads to ovarian maturation defects and testicular tumors. (A) Normal germ cell (purple circle) progression after specification from an embryonic cell. BMP signals are required to promote differentiate of a gonocyte into a spermatogonia in the male niche environment. BMP is also required to drive the spermatogonia to become mature sperm. Foxl3 is required early on for normal progression from a gonocyte to an oogonia. BMP signaling is necessary for proper oocyte maturation in the ovary. (B) When BMP signaling is lost, as in the *bmpr1bb*$^{W256X/W256X}$ mutants, we drive the system to become more female (large red arrow). Since we do not have

any BMP signaling the oocytes are unable to mature and immature oocytes began to accumulate in the ovary. Some gonocytes do go on to become spermatogonia however, with the loss of BMP we lose repression of foxl3 and the new signaling provides the perfect environment for testicular tumors to form.

	Mutation	Copy Number	Gene expression analysis									
	somatic and germline coding + noncoding mutations (freq<5% in the population)		female GCT	male GCT	GER	YST	OVARY	TESTIS		PGC rep1	PGC rep2	
CATSPER2	60	loss in 12% (18/148)	1.3	0.5	2.0	0.6	0.9	11.4		3.5	4.4	2.6
SOX30	39	loss in 24% (35/148)	0.1	0.0	0.3	0.0	0.3	4.5		1.4	0.7	2.1
AR	22	loss in 36% (54/148)	0.4	0.5	0.2	0.2	0.1	9.5		0.2	0.2	0.1
TAF7L	8	loss in 37% (55/148)	0.1	0.1	0.1	0.1	0.1	33.8		0.4	0.3	0.4
BMP8B	7	loss in 26% (38/148)	0.9	0.9	0.7	2.4	2.3	2.7		0.0	0	0
KDM1B	29	loss in 10% (15/148)	3.1	2.2	3.5	2.1	10.2	10.8		8.4	8.2	8.5
PHF7	28	loss in 9% (14/148)	6.5	1.7	7.7	1.3	1.9	81.2		3.1	3.1	3
SYCP1	27	loss in 11% (16/148)	0.1	0.1	0.1	0.0	0.0	15.5		0.1	0.1	0.1
TDRD1	21	loss in 11% (16/148)	0.3	0.1	0.5	0.0	0.0	4.1		0.4	0.4	0.3
TEX14	17	loss in 1% (2/148)	0.2	0.0	0.4	0.1	0.1	7.0		0.8	0.7	0.9
TEX15	24	loss in 4% (6/148)	0.2	0.1	0.1	0.0	0.0	6.2		0.0	0	0
WT1	16	loss in 8% (12/148)	0.5	1.7	0.5	2.4	0.1	37.6		0.6	0.7	0.4
DAZL	15	loss in 5% (7/148)	13.9	1.1	34.9	0.7	0.2	27.2		53.2	24.2	82.1
ESR2	15	loss in 0% (0/148)	0.6	0.4	0.8	0.5	0.2	3.8		1.1	0.9	1.2
FKBP6	14	loss in 3% (5/148)	0.3	0.2	0.8	0.4	0.3	12.8		0.5	0.1	0.8
DMRT1	13	loss in 13% (19/148)	4.2	0.1	11.3	0.1	0.0	21.3		9.2	6.8	11.6
NR5A1	13	loss in 11% (17/148)	0.9	0.5	0.8	1.4	0.5	17.1		0.2	0.3	0
PIWIL1	11	loss in 3% (4/148)	12.5	0.0	34.7	0.0	0.0	5.9		37.4	36.4	38.4
SOHLH1	10	loss in 3% (5/148)	0.0	0.0	0.0	0.0	0.0	5.1		0.1	0.2	0
DDX4/VASA	9	loss in 13% (19/148)	2.7	0.2	7.3	0.0	0.0	30.8		8.7	8.0	9.3
EZH1	8	loss in 11% (16/148)	4.9	2.2	11.0	4.7	12.9	19.7		22.5	21.2	23.8
INHA	7	loss in 4% (6/148)	0.8	0.7	0.6	1.9	1.3	108.2		0.5	0	0.9
GSTM1	5	loss in 14% (21/148)	9.7	2.0	25.8	6.7	0.6	141.0		1.5	1.2	1.8
TEX11	5	loss in 35% (54/148)	1.1	0.2	2.4	0.4	0.1	7.4		0.8	0.1	1.4
SOX2	4	loss in 4% (6/148)	3.9	0.4	0.0	0.4	17.5	4.7		0.0	0	0
SOX9	4	loss in 3% (4/148)	3.9	3.6	0.3	2.7	55.0	31.4		1.0	0.4	1.6
ESR1	4	loss in 29% (43/148)	0.6	0.6	0.5	0.3	0.2	1.7		0.3	0.4	0.1
BOLL	2	loss in 5% (8/148)	0.2	0.3	0.1	0.2	0.0	7.8		0.1	0.1	0.1
INSL3	2	loss in 24% (36/148)	3.5	0.1	0.7	0.2	0.4	94.2		0.1	0.1	0
KDM5D	2	loss in 29% (43/148)	0.6	9.5	0.0	6.2	4.0	33.9		12.0	10.6	13.3
NR0B1	2	loss in 33% (49/148)	0.1	0.2	0.3	1.0	0.0	12.4		0.3	0.2	0.4
DPPA3/STELLA	27	gain in 49% (73/148)	81.5	75.9	86.4	136.1	0.1	0.3		30.9	27.7	34.2
NANOG	9	gain in 48% (71/148)	62.2	0.2	148.2	4.2	0.1	0.2		139.1	114	164.2
SOX11	22	gain in 25% (37/148)	6.4	11.5	0.1	11.1	0.2	0.0		0.1	0.1	0.1
KIT	18	gain in 28% (41/148)	23.3	20.3	33.5	22.3	0.4	2.8		42.6	45.8	39.4
PRTG	27	gain in 8% (12/148)	13.8	20.3	1.1	21.5	0.1	1.7		2.6	3	2.2
ALPPL2	20	gain in 6% (9/148)	12.4	0.9	26.5	3.4	0.0	0.0		4.7	4.6	4.7
NANOS3	5	gain in 1% (2/148)	65.2	0.1	195.9	0.3	3.6	4.1		98.5	93.5	103.5
GDF3	4	gain in 48% (71/148)	32.4	55.7	16.9	134.6	0.2	0.1		0.9	1.7	0.1
LEFTY2	3	gain in 17% (25/148)	49.7	155.0	15.1	118.7	1.3	3.9		0.1	0	0.2
PRDM14	3	gain in 28% (41/148)	2.8	0.0	6.6	0.0	0.0	0.1		10.0	8.3	11.6
LIN28A	1	gain in 0% (0/148)	61.8	82.3	37.6	82.7	0.1	0.2		35.6	34.7	36.4

Figure 4.2. The initial list of genes that could be potential candidates to target using CRISPR/Cas9 technology as a way to establish novel GCT zebrafish models. Genes related to some aspect of germ cell pluripotency, determination or differentiation that have notable somatic and germline mutations, alterations in copy number, or gene expression in human germ cell tumors.

Gene
acrc (acidic repeat containing)
insl3 (insulin-like 3, Leydig cell)
bmp8a (bone morphogenic protein 8a)
ar (androgen receptor)
ezh1 (enhancer of zeste 1 polycomb rep
tfap2c (transcription factor ap-2 gamma)
prdm14 (pr domain 14)
nr5a1a (nuclear receptor subfamily 5 1)
nr5a1b (nuclear receptor subfamily 5 2)
jmjd1cb (jumonji domain containing 1b)

Figure 4.3 Final candidate list of genes to target via CRISPR/Cas9 in wildtype and *bmpr1bb*$^{W256X/W256X}$ mutant zebrafish background. The germ cell tumor team (Joshua Pierce, Lin Xu, James Amatruda, and myself) individually selected 10 genes. We complied all the gene list together to determine which genes were chosen most frequently and then those made the final list of 10 genes to begin targeting.

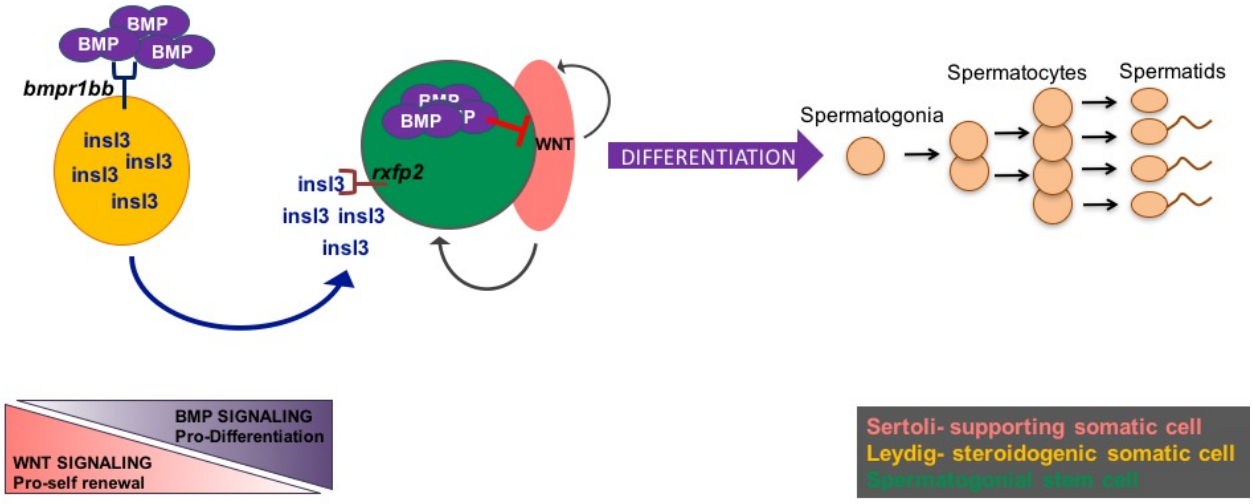


Figure 4.4 Working model depicting the role and source of BMP within the spermatogonial stem cell niche. The stem cell niche is composed of 3 major cell types which include Sertoli supporting somatic cells (light pink colored), Leydig steroidogenic somatic cells (yellow-orange colored), and the spermatogonial stem cells (green colored). Normally, spermatogonial stem cells (SSC) are maintained by WNT signaling. A BMP signal is derived from SSCs which represses the WNT signal coming from the Sertoli cells. The BMP signal then acts on the neighboring Leydig cells which produce insl3. Insl3 then acts on the SSCs through its receptor rxfp2 and instructs SSCs to begin to differentiate in spermatogonia. The spermatogonia will differentiate into spermatocytes and eventually functional spermatids.

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