Mutation in the type IB bone morphogenetic protein receptor *alk6b* impairs germ-cell differentiation and causes germ-cell tumors in zebrafish

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Germ-cell tumors (GCTs), which arise from pluripotent embryonic germ cells, exhibit a wide range of histologic differentiation states with varying clinical behaviors. Although testicular GCT is the most common cancer of young men, the genes controlling the development and differentiation of GCTs remain largely unknown. Through a forward genetic screen, we previously identified a zebrafish mutant line, *tgct*, which develops spontaneous GCTs consisting of undifferentiated germ cells [Neumann JC, et al. (2009) *Zebrafish* 6:319–327]. Using positional cloning we have identified an inactivating mutation in *alk6b*, a type IB bone morphogenetic protein (BMP) receptor, as the cause of the zebrafish GCT phenotype. Alk6b is expressed in spermatogonia and early oocytes, and *alk6b* mutant gonads display impaired BMP signal transduction, altered expression of BMP target genes, and abnormal germ-cell differentiation. We find a similar absence of BMP signaling in undifferentiated human GCTs, such as seminomas and embryonal carcinoma, but not in normal testis or in differentiated GCTs. These results indicate a germ-cell–autonomous role for BMP signal transduction in germ-cell differentiation, and highlight the importance of the BMP pathway in human GCTs.

non-seminoma | SMAD | teleost | haplotype

Development of the germ line in multicellular organisms requires a series of carefully regulated events, including specification of primordial germ cells, patterning of the gonad, and the initiation and maintenance of gametogenesis (1–3). Improper germ-cell development, regulation, and gametogenesis are associated with infertility, inherited chromosomal abnormalities, and germ-cell tumors (GCTs) (4–7). Several embryonic signaling pathways have been implicated in the development of germ cells, somatic gonadal cells, or both, including wnt, Hedgehog, Notch, FGF, and TGF-β/bone morphogenetic protein (BMP) (4–8). The wnt/β-catenin pathway is differentially activated in subsets of childhood GCTs (9), and the wnt pathway regulator, APC, is subject to regulation by epigenetic mechanisms or loss-of-heterozygosity in childhood GCTs (10).

BMP signaling is required for proper germ-line development and regulation of proliferation and differentiation of germ cells during spermatogenesis (5, 7). In vitro, exogenous BMP4 appears to drive the differentiation of spermatogonial stem cells from enriched germ-cell cultures (11, 12). BMP2 and glial-derived neurotrophic factor (GDNF), also a TGF-β family ligand, have likewise been implicated in the regulation of spermatogonial stem cell self-renewal (13, 14). In mouse models, inactivation of BMP8b led to reduced or delayed germ-cell proliferation and differentiation during the first wave of spermatogenesis at puberty, and increased apoptosis of pachytene spermatocytes, leading to reduced fertility in the adult (15). Targeted inactivation of BMP8a resulted in increased meiotic cell apoptosis and germ-cell degeneration and inactivation of BMP7 in the BMP8a-deficient background exacerbated the phenotype (15, 16). Taken together, these studies have established the BMP signaling pathway as an important regulator of germ-line specification and the initiation and maintenance of spermatogenesis. However, the multiplicity of ligands, receptors, and cell types involved has made elucidation of specific signaling events difficult. We previously described a zebrafish mutant that develops highly penetrant, dominantly inherited testicular GCTs (*tgct*) (17). Here we report that a mutation in activin receptor-like kinase 6b (*alk6b*), the zebrafish homolog to human Bmpr1b, is responsible for the TGCT phenotype. A premature termination codon that truncates the kinase domain results in the abrogation of active BMP signaling in the testis and leads to impaired differentiation of spermatogonial stem cells. Further understanding of the role of the BMP/TGF-β signaling pathway in germ-cell differentiation may provide insight to the development of human GCTs.

Results

Testicular Tumors of Premeiotic Germ Cells Develop in *tgct* Mutants. Previously, we described the identification of a zebrafish model of heritable testicular GCT during a forward genetic screen to identify cancer susceptibility genes (17). Adult homozygous and heterozygous males from the *testicular germ-cell tumor* (*tgct*) mutant line develop gonadal tumors consisting of large, primitive germ cells (Fig. 1 *B*, *D*, and *F*). Homozygous *tgct* females exhibit a profound oocyte maturation defect and are infertile because of impaired oocyte differentiation (Fig. 1*H*). The large tumor cells that make up the majority of the tumor testis do not express phosphorylated histone H2AX, a marker of meiosis (18, 19), suggesting that the tumor cells are premeiotic germ cells (Fig. 2*B*). The tumor cells uniformly express *ziwi*, a marker enriched in zebrafish spermatogonia (Fig. 2 *C* and *D*) (20). To further delineate the lack of differentiation of the cells, we removed the testes from wild-type and mutant males, cultured the germ cells, and carried out flow cytometric DNA content profiling (FACS). Unlike mammalian testis, the zebrafish testis is capable of undergoing transmeiotic differentiation to mature, functional sperm in vitro (21, 22). FACS analysis of the testicular explants showed that, after 8 d in culture, the wild-type spermatogonia differentiated to haploid (1N) cells but the *tgct* cells remained diploid and did not undergo meiosis (Fig. 2*F*). Taken together, these results

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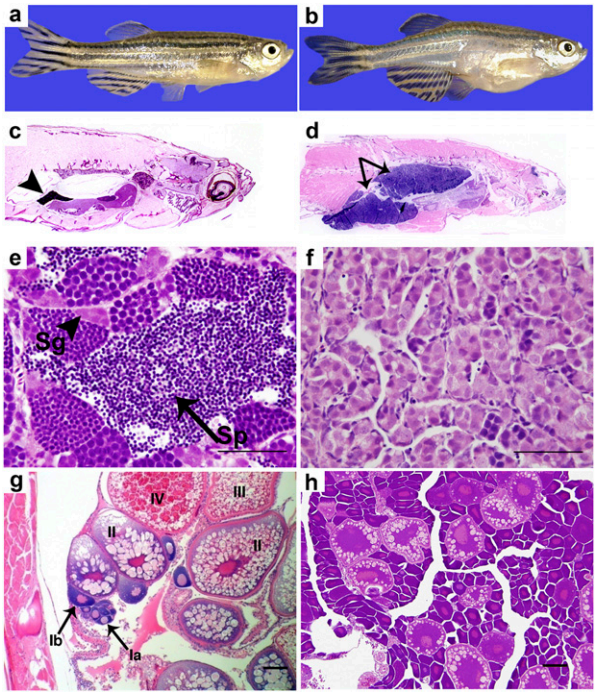

Fig. 1. The *tgct* mutants develop GCTs because of impaired germ-cell differentiation. Compared with adult wild-type males (*A* and *C*) (arrowhead: normal testis), adult *tgct* males display marked testicular enlargement (*B* and *D*) (arrow: testicular tumor). Wild-type testis (*E*) exhibits normal spermatogenesis (arrowhead: spermatogonia; arrow: spermatozoa). The *tgct* mutant testis (*F*) accumulates primitive germ cells. Although wild-type ovary (*G*) shows all stages of oocyte maturation up to mature stage IV oocytes, oocyte maturation is impaired in *tgct* homozygous females (*H*). [Scale bars: 100 μm (*E* and *F*); 50 μm (*G* and *H*).]

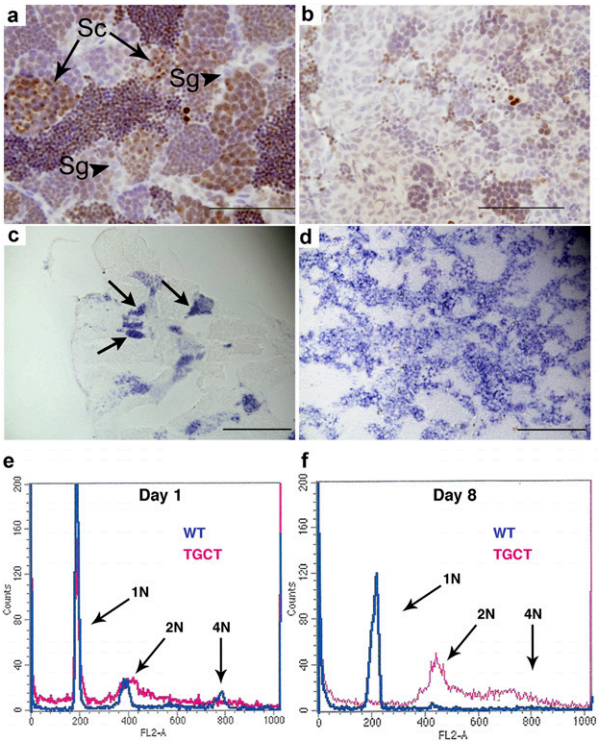

Fig. 2. The *tgct* mutants exhibit impaired differentiation and reduced meiosis in vitro and in vivo. In wild-type testis (*A*), phosphohistone H2Ax marks clusters of primary spermatocytes undergoing meiosis (arrows). Spermatogonia (arrowheads) are phosphohistone H2AX-negative. The *tgct* mutant testis (*B*) exhibits severely reduced clusters of meiotic spermatocytes. The tumor cells are phosphohistone H2AX-negative. (*C* and *D*) In situ hybridization for the germ-cell–specific gene *ziwi*. In wild-type testis (*C*), *ziwi* expression is strong in clusters of spermatogonia (arrows) and rapidly declines as cells enter meiosis. The *tgct* testis tumors (*D*) show uniformly high expression of *ziwi*. (*E*) FACS analysis for DNA content of cultured wild-type and *tgct* testis on day 1. (*F*) FACS analysis on day 8 after culturing. Wild-type spermatogonia have completed meiosis to become haploid spermatocytes and spermatozoa (1N), but *tgct* tumor cells remain diploid (2N) and do not undergo meiosis. [Scale bars: 100 μm (*A* and *B*); 200 μm (*C* and *D*).]

indicate that the testicular tumor cells in the *tgct* mutant are premeiotic germ cells that are defective in differentiation.

Positional Cloning of the *tgct* Mutation. To identify the molecular defect in the *tgct* strain, we carried out positional cloning, first using interval haplotype analysis (23, 24) to assign the *tgct* mutation to zebrafish chromosome 10 (Table S1). To generate more markers for recombination analysis, we made a high-resolution meiotic map of chromosome 10 using 493 F1 embryos from a hybrid backcross to place novel microsatellites on the interval (Tables S2 and S3). Using these microsatellite markers on a panel of fish that developed tumors, representing 449 mutant meioses, we localized the *tgct* mutation to an interval containing three genes: the netrin receptor *unc5c*, the type IB BMP receptor *alk6b*, and the LIM domain protein *pdlim5* (Fig. 3*A*). A similar syntenic arrangement of these three genes is also found in human and mouse. Intragenic recombinants were present in both *unc5c* and *pdlim5*, limiting the critical interval to 0.82 cM. Sequence analysis of the coding exons of *alk6b* revealed a G-to-A transition mutation in the mutants that introduces a premature termination codon and is predicted to truncate the protein after residue 256 (W256X) (Fig. 3 *B* and *C*). To confirm that the *alk6b*^{W256X} allele is responsible for the impaired germ-cell differentiation, we restored wild-type *alk6b* expression in *alk6b*$^{W256X/W256X}$ mutants by using a transposon to generate mosaic insertions of a wild-type *alk6b* transgene under the control of a *β-actin* promoter. Of the transposon-injected mutant females, 26.6% had rescue of the oocyte maturation defect (Fig. 3 *D* and *E*). Thus, *tgct* encodes *alk6b*.

Alk6/BMPR1B is a member of the TGF-β/BMP receptor superfamily. In this pathway, BMP ligands bind a heterotetrameric complex of two type II BMP receptors and two type I BMP receptors, stimulating the serine/threonine kinase activity of the type I receptors. Phosphorylation of SMADs 1, 5, and 8 by type I receptors allows SMAD1/5/8 to bind SMAD4, forming an active transcription factor (25–27). Based on the loss of the kinase domain, we predicted that *alk6b*^{W256X} would be a loss-of-function allele. To test this hypothesis, we performed morpholino knockdown of zebrafish BMP pathway receptors in embryos and assessed the ability of the wild-type and W256X mutant *alk6b* alleles to rescue the knockdown phenotype (Fig. 4). In zebrafish, the embryonic expression of different BMP receptor family members and paralogs creates functional redundancy in the pathway, making it necessary to knock down multiple pathway members to generate a phenotype (28). Simultaneous knockdown of BMPR1A orthologs *alk3a* and *alk3b*, along with *alk6b* (triple knockdown), abolished ventral expression of the BMP target, *gata2* (Fig. 4*C*). Coinjection of a wild-type *alk6b* mRNA into triple knockdown embryos restored *gata2* expression (Fig. 4*D*). In contrast, injection of *alk6b*^{W256X} mRNA was unable to rescue *gata2* expression in triple knockdown embryos (Fig. 4*E*). The small amount of BMP-mediated *gata2* expression in the presence of the *alk6b*^{W256X} mRNA suggests that the mutant allele may retain some ability to stabilize BMP receptor complexes containing residual maternally-expressed wild-type Alk6b protein.

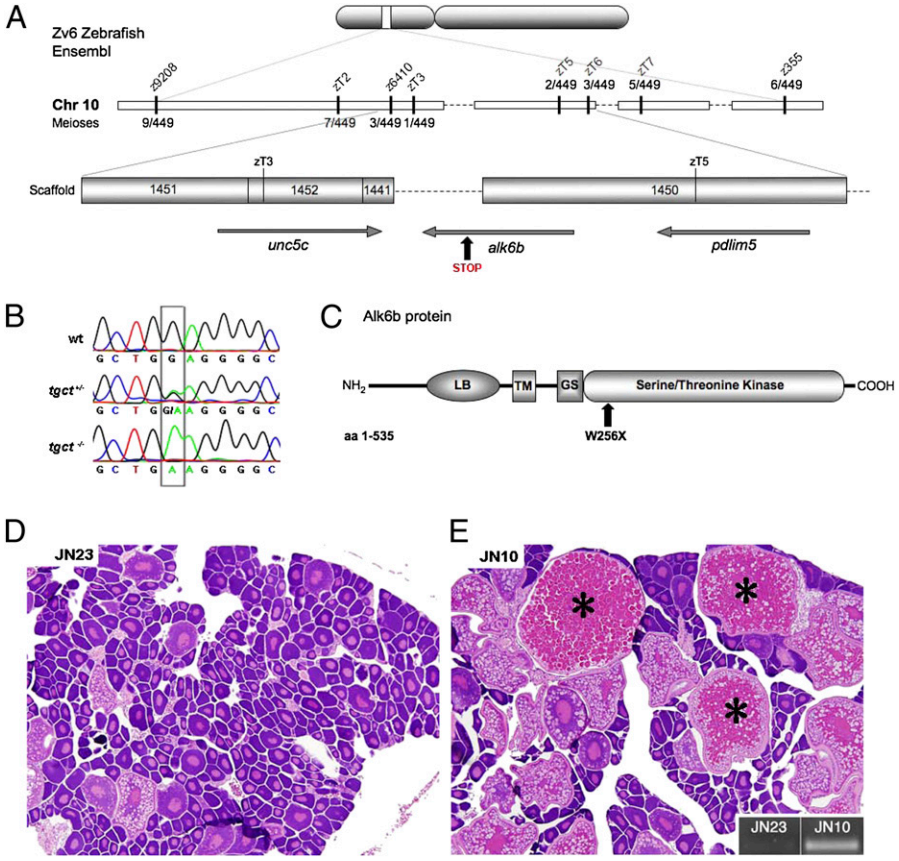

Fig. 3. Positional cloning of the *tgct* mutant locus. (*A*) Position of the *tgct* mutation on chromosome 10 between novel SSLP markers zT3 and zT5, located on Zv6 Ensembl scaffolds 1452 and 1450, respectively. (*B*) Chromatogram showing the G to A transition in *alk6b*^{W256X} mutants. (*C*) Schematic of Alk6b protein illustrating the Activin-like receptor (AcvR) ligand binding (LB), transmembrane (TM), GS box (GS), and serine/threonine kinase domains. The *tgct* mutation is located in the kinase domain at amino acid 256. (*D*) A *β-actin-alk6b*^{wt} transgene injected *alk6b*^{W256X} female showing no rescue of oocyte maturation and no transgene incorporation (*E*, *Inset*) into genomic DNA. (*E*) A *β-actin-alk6b*^{wt} transgene injected *alk6b*^{W256X} female with restored oocyte maturation (*) and incorporation of the transgene into genomic DNA (*Inset*). (*D* and *E*: magnification 50×.)

***Alk6b* Is Expressed in Germ Cells of the Testis and Ovary.** To understand the mechanism by which inactivating mutations in *alk6b* could lead to GCTs in *alk6b*^{W256X} mutants, we prepared an antibody directed against the N terminus of zebrafish *alk6b* and carried out immunohistochemistry on sections of wild-type and *alk6b*^{W256X} mutant testis (Fig. 5). In wild-type testis, Alk6b protein is present in a small number of large, peripherally located germ cells in each lobule, consistent with highest expression in spermatogonia (Fig. 5*A*); some spermatocytes also display lower levels of Alk6b expression. In sections of testicular GCTs from *alk6b*^{W256X} mutants, the great majority of the tumor cells exhibit Alk6b protein expression. This result is consistent with the RT-PCR expression data showing increased *alk6b* mRNA expression in GCTs (Fig. S1), and suggests that *alk6b*^{W256X} mRNA is not subject to nonsense-mediated mRNA decay. We also detected abundant expression of *alk6b* in early (stage I/II oocytes), but not in somatic cells of the ovary (Fig. S2).

Loss of BMP Signaling Activity in *alk6b*^{W256X} Mutants and Human GCTs. We tested whether the *alk6b* mutation leads to loss of functional BMP signaling activity in the testis by performing immunofluorescence detection of phospho-SMAD1/5/8 (pSMAD1/5/8) on sections of wild-type and *alk6b* mutant testis (Fig. 5 *C* and *D*). In wild-type testis, a limited number of large cells with nuclear pSMAD1/5/8 are identifiable at the periphery of each testis lobule, consistent with highest BMP signaling in spermatogonia (Fig. 5*C*). Fainter signal is also visible in other germ cells, likely representing some degree of continued BMP signaling as cells differentiate to spermatocytes. Our results, however, do not rule out the presence of BMP signaling in somatic cells. In contrast, pSMAD1/5/8 staining is absent in the testis tumors from *alk6b* mutant testis (Fig. 5*D*). We also used quantitative RT-PCR to confirm down-regulation of the BMP target genes *id1*, *id2*, and up-regulation of *midkine-B* (*mdkb*), normally a BMP-repressed gene, in *alk6b*^{W256X} mutant testes (Fig. 5*E*). Thus, despite a relative overexpression of the mutant alk6b protein in the testicular tumors, the BMP pathway is nonfunctional and BMP target gene expression is altered. Taken together, these results indicate a germ-cell–specific requirement for BMP signaling mediated by Alk6b during germ-cell differentiation.

Based on these results, we hypothesized that misregulation of BMP signaling contributes to the pathogenesis of human GCTs, and may determine the state of differentiation of the germ cells in the tumors. Human GCTs occur as germinomas (called seminomas in males and dysgerminomas in females), which consist of primitive, embryonic germ cells, and nonseminomas, which may be undifferentiated (embryonal carcinoma) or differentiated (teratoma, yolk-sac tumor, or choriocarcinoma) (4, 5). The undifferentiated tumors occurring in zebrafish *alk6b* mutants most resemble human seminomas. We used antiphospho-SMAD1/5/8 staining to assess the state of BMP signaling in a tissue microarray containing 206 human GCT specimens of different histologic types. Consistent with earlier results (12, 29), nuclear phosphoSMAD1/5/8 is present in spermatogonia in normal testis

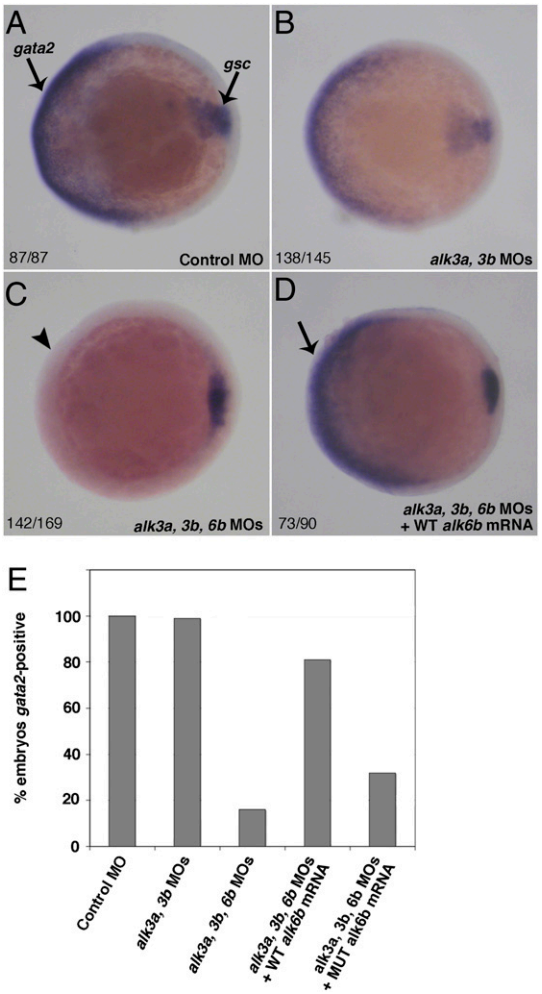

Fig. 4. *alk6b*^{W256X} is a loss-of-function allele. (*A–D*) Whole-mount in situ hybridization demonstrating ventral expression of the BMP target gene *gata2* in control and morpholino knockdown embryos. *Goosecoid* (*gsc*) expression in the prechordal plate marks the presumptive dorsal side of the embryo. (*A*) Control morpholino injected embryos at 75% epiboly showing normal ventral expression of *gata2*. (*B*) Morpholino knockdown of the type IA BMP receptors *alk3a* and *alk3b* slightly reduces *gata2* expression. (*C*) Triple knockdown of *alk3a*, *alk3*b, and *alk6b* leads to complete loss *gata2* expression. (*D*) Injection of wild-type *alk6b* mRNA into triple knockdown embryos rescues *gata2* expression. (*E*) Quantification of percentage of embryos expressing *gata2* after morpholino knockdown and rescue with wild-type or *alk6b*^{W256X} mRNA.

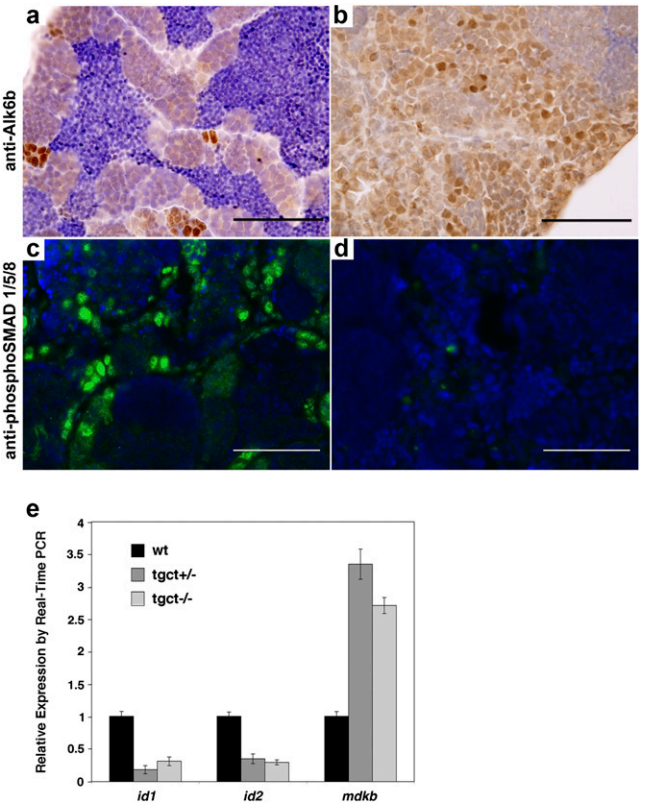

Fig. 5. Alk6b is expressed in spermatogonia and is required for BMP signaling activity in the zebrafish testis. (*A*) Immunohistochemistry for Alk6b shows that protein expression is primarily limited to the spermatogonia in the wild-type testis, with faint staining in some spermatocytes. (*B*) Alk6b is highly expressed in zebrafish GCTs. (*C* and *D*) immunofluorescent detection of phospho-SMAD1/5/8 on cryosections of wild-type and mutant testis. Nuclear p-SMAD1/5/8 is present in wild-type testis (*C*), indicating active BMP signaling. BMP signaling is absent in *alk6b*^{W256X} mutant testis (*D*). (*E*) Quantitative Real-Time PCR of BMP target genes in wild-type and tumor testis. The BMP targets *id1* and *id2* are expressed at lower levels in tumors, whereas *mdkb* expression is derepressed. (Scale bars, 100 μm.)

(Fig. 6*A*). However, only 10% to 22% of seminoma and embryonal carcinoma (undifferentiated tumors) were positive for pSMAD1/58, whereas 60% to 90% of immature teratomas and yolk sac tumors (which are differentiated) were pSMAD1/5/8 positive (Fig. S3). Representative examples are shown in Fig. 6. Thus, absence of BMP signaling activity correlates with impaired differentiation in zebrafish spermatogenesis and in human GCTs.

Discussion

Complex, reciprocal signaling between germ cells and somatic cells underlies the differentiation of germ cells during formation of the embryonic gonad and during gametogenesis in the adult gonad. Multiple ligands in the TGF-β superfamily are thought to play a role in this process, including TGF-β, BMPs, anti-Mullerian hormone (AMH), and GDNF. BMP signaling is required for the specification of primordial germ cells during embryogenesis (30–32), and contributes, along with GDNF signaling, to the self-renewal of spermatogonial stem cells (13, 14) and the maintenance of spermatogenesis (16). Here we present evidence that alk6/BMPR1B is cell-autonomously required for germ-cell differentiation. In zebrafish, *alk6b* is expressed in early germ cells of the ovary and testis but not in somatic cells of the gonad. The *alk6b*^{W256X} mutation abrogates the receptor's function and leads to a loss of BMP signaling activity in the testis. Mutant testes exhibit profoundly impaired germ-cell differentiation in vitro and in vivo, consistent with a specific requirement for BMP signal transduction activity within differentiating germ cells. In *alk6b* mutant males, the gonads are greatly enlarged, further suggesting that BMP signaling not only governs differentiation but may also serve to restrict germ-line stem cell number or proliferation rate. We find a similar correlation between lack of BMP signaling and impaired differentiation in human seminomas. We sequenced the BMPR1B coding sequence in 100 human seminomas; one tumor contained a previously reported snp in exon 9 (rs35973133 encoding a missense Arg225His mutation) and the other tumors had no coding-sequence mutations, suggesting different mechanisms account for lack of BMP signaling in the human and zebrafish GCTs.

The truncated form of *alk6b* produced by the *tgct* nonsense mutation contains the extracellular ligand-binding domain as well as the transmembrane domain and GS box. A similarly truncated BMP receptor functions as a dominant-negative modulator of

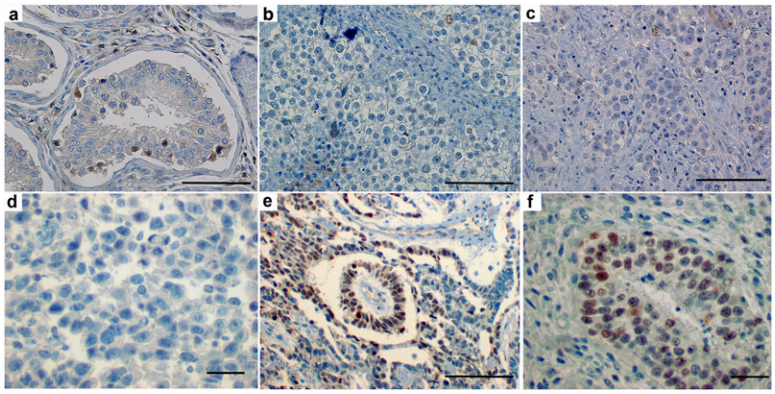

Fig. 6. BMP signaling is selectively absent in undifferentiated human germ cell tumors. Antiphospho-SMAD1/5/8 immunohistochemistry was performed on sections of human testis (*A*) and on human GCTs, including undifferentiated tumors seminoma (*B*), dysgerminoma (*C*) and embryonal carcinoma (*D*), and differentiated tumors yolk-sac tumor (*E*) and immature teratoma (*F*). (Scale bars, 100 μm.)

BMP signaling in developing *Xenopus* embryos (33). The occurrence of impaired germ-cell differentiation and GCTs in *alk6b*$^{+/W256X}$ heterozygous males could indicate a similar dominant-negative effect of the *alk6b*^{W256X} mutation. However, this model does not account for several features of the mutant line, including the decreased latency of the phenotype in homozygous males and the absence of a phenotype in heterozygous females, making haploinsufficiency of *alk6b* a more likely explanation for the tumor phenotype. Our data do not exclude an alternative loss of heterozygosity model, whereby tumors develop in heterozygous males because of loss of the wild-type *alk6b* allele.

BMP/SMAD signaling has previously been implicated in germline development in *Drosophila*, mice, and humans (12, 14, 29, 34, 35). BMP4 stimulates the proliferation and differentiation of spermatogonial stem cells in vitro (12). The embryonic lethality of Bmp4$^{-/-}$ mice precludes full analysis of a possible gonadal phenotype; however, Bmp4$^{+/-}$ mice exhibit germ-cell degeneration and reduced fertility. Expression of BMP family receptors in somatic cells to regulates differentiation of germ cells in *Drosophila* (36) and in the teleost, *Medaka* (37). In the *Medaka* fish mutant, *hotei*, a nonsense mutation in anti-Mullerian hormone receptor II (*AMHRII*) impairs gonadal development and results in a phenotype somewhat overlapping that of the zebrafish *alk6b* mutant. *Hotei* (*hot*) mutant fish develop enlarged gonads; males exhibit hypertrophic testes with disorganized spermatogenesis, whereas females display arrested follicular development. In contrast to *alk6b*, which is expressed specifically in germ cells, *AMHRII* is expressed in somatic cells. This difference may account for the more severe male germ-cell phenotype seen in the *alk6b* mutant, and also for other important differences between the models, such as the finding that one-half of homozygous *hot* genetic (XY) males undergo sex reversal and express female-specific genes in gonadal somatic cells. Further experiments need to be done to determine whether, for example, AMH or other factors acting on the somatic cells of the gonad control expression of BMP ligands that, in turn, act on the germ cells to mediate differentiation. Taken together with the gonadal phenotypes seen in mice with targeted mutations in BMP4, BMP7, and BMP8, these results emphasize the importance of BMP signaling in vertebrate gonadal development.

The *alk6b* homozygotes do not exhibit defects during embryogenesis, despite the importance of BMP signaling for embryonic development (38–40). This result may partly be explained by maternal expression of the wild-type *alk6b* allele, and partly by functional redundancy in this pathway. The ortholog *alk6b* is one of two BMPR1B orthologs in zebrafish. The related ortholog, *alk6a*, is also expressed during embryogenesis and early limb formation in zebrafish (41) and may be responsible for the majority of BMPR1B functionality in embryos, or may compensate for the loss of BMP signal transduction in *alk6b*$^{-/-}$ mutants. Thus, there appears to be functional divergence of the two *alk6* genes, with *alk6b* preferentially expressed in early germ cells of the gonad, where it mediates germ-cell differentiation. Indeed, in *alk6b* mutant testes, there is an absence of BMP signal transduction activity as measured by SMAD1/5/8 phosphorylation, emphasizing the lack of compensation by *alk6a* or other family members in this setting.

GCTs of the ovary and testis are among the most common malignancies in adolescents and young adults, but the molecular mechanisms underlying GCT are unknown. The pluripotent nature of the early germ cells from which these tumors arise leads to the development of a wide variety of differentiated and undifferentiated histologies in the tumors (42). Different GCT histologies may exhibit differential malignant potential and response to treatment, making it critical to understand the mechanisms governing germ-cell differentiation. Cisplatin-based chemotherapy is widely used in the treatment of GCTs. Although cisplatin therapy has been very effective in GCTs, it can cause severe side effects, including hearing loss, kidney damage, and elevated risk of cardiovascular disease and second malignancies (42). In addition, patients with poor-risk or cisplatin-resistant disease continue to have poor survival. For these reasons, alternative therapies for GCT are needed. In a recent analysis of childhood GCTs, expression of BMP2 and BMP4 was associated with differentiation state of the tumors (43). Together with our demonstration that BMP signaling directly mediates differentiation of germ cells in vivo, these results suggest that exploration of the BMP pathway and its effects on differentiation of GCTs may provide an alternative route to therapy of GCTs.

Experimental Procedures

Full methods are available in *SI Experimental Procedures*.

Interval Haplotype Analysis. Interval haplotype analysis was carried out with Simple Sequence Repeats from the Massachusetts General Hospital genetic map (44), using the program MaxX2.V1 (23) (Table S1). To resolve inconsistencies between the genetic map and the Zv6 Sanger Center Assembly, we generated a custom, high-resolution genetic map of the interval between z9208 and z7316, using a panel of 493 F2 embryos from an AB/wik hybrid backcross (Table S2).

In Vitro Differentiation Assay and FACS Sample Preparation. Testes were dissociated with dispase and cultured in modified DMEM/F12 on a gelatin layer for 1 to 8 d. FACS was performed as previously described (45).

Morpholino and mRNA Injections. To prevent nonspecific toxic effects, all morpholinos were all injected into p53-deficient tp53zdf1$^{/zdf1}$ embryos at the one-cell stage (28). A total volume of 2 nL morpholinos (MO) were injected

at the following concentrations: Gene-tools Standard Control MO (1.0 mM), Alk3a (0.25 mM), Alk3b MO1 (0.125 mM), Alk3b MO3 (0.125 mM), Alk6b-E4-MO1 (0.5 mM), alk6b-E3/5-MO2 (0.125 mM). All morpholinos used were purchased from Gene Tools. Morpholino sequences for Alk3a and Alk3b were as previously described (28). The sequences for the *alk6b* morpholinos were E4-MO1: TTTCGTTTCCCGTCCGTACCCGGAG and E3/5-MO2: AGCACGTTCGCTGTGTGTCACTCAG. *Alk6b* wild-type and mutant mRNA used in the rescue assays were generated using the T7 mMessage mMachine (Ambion) and coinjected with morpholinos at a concentration of 25 ng/mL.

In Situ Hybridizations. In situ hybridizations for *gata2* and *gsc* were performed as previously described (46). Embryos were mounted in 100% glycerol and images were taken at 8.0× magnification with a Leica MZ12.5 stereomicroscope equipped with a Nikon E4500 camera. The *ziwi* in situ hybridizations were performed on cryosections of wild-type and *alk6b* testis.

Quantitative Real-Time PCR. Testis RNA was prepared 500 μL TRIzol (Invitrogen) and purified using RNeasy mini kit (Qiagen). Primer sequences for Rpl13a and EF1a were as previously described (47). Primer sequences for *id1*, *id2*, and *mdkb* were designed using Genscript's online real-time primer design tool and span an exon-exon boundary with the exception of *mdkb*. All assays were performed on Applied Biosystems 7900 HT real-time PCR instrument and gene expression analysis was conducted by SDS 2.3 and RQ Manager 1.2 software.

Immunohistochemistry. Rabbits were immunized with a synthetic peptide CTAGRKETNGGS derived from Alk6b residues 44 to 54 (GenScript Corporation, an AAALAC-accredited organization). Immune serum was used for affinity purification against immobilized peptide. Zebrafish were prepared as previously described (48) and paraffin sections were subjected to antigen retrieval using Trilogy reagent (Cell Marque). Primary antibodies were phospho-SMAD1/5/8 (Cell Signaling) at 1:500, *N*-term Alk6b at 1:250, and γ-h2ax at 1:250. Slides were developed with the Immpress kit (Vector) and counterstained with hematoxylin. Human tissue microarrays were prepared using standard techniques and immunohistochemistry was performed on a Ventana Discovery automated immunostainer (Ventana) using standard immunoperoxidase techniques and hematoxylin counterstaining.

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