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Zebrafish as a Future Model for the Study of Human Chordomas

by

Kristen Errico

Andreas Fritz, Ph.D.
Adviser

Department of Biology

Andreas Fritz, Ph.D.

Adviser

Patrick Cafferty, Ph.D.

Committee Member

Jenna Debs

Committee Member

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Chordomas are rare malignant bone tumors of the spine that arise during human development. Current treatment options are of limited success and overall prognosis is poor. Discovery of the ability to generate chordoma-like tumors in zebrafish embryos has shown promise for an *in vivo* model to study chordoma causes, formation, and treatment options by using zebrafish for high throughput drug screens. Characterization of the notochordal tumors in zebrafish is necessary to confirm that they are biologically representative of human chordomas. Using *in situ* analysis, we have attempted to determine when and where TGF-β2,3 and DRAKR1b1,10 are expressed in developing zebrafish embryos to better understand the mechanism for tumor formation and the characteristic features of the tumors. TGF-ß is believed to play a role in tumor formation, as zebrafish tumor induction is dependent on a drug blocking TGF-ß receptors. DRAKR is believed to be analogous to the characteristic expression of AKR in chordomas. Due to unforeseen difficulties obtaining embryos to test in the laboratory, the limited data obtained is supplemented with a literature-based review of chordoma research. Research indicates that zebrafish remain a useful tool to study chordomas. With further characterization of chordoma-like tumors in zebrafish, we expect to discover possible novel drug targets including, but not limited to, TGF-β and AKR.

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I also thank my family and friends for their love, support, and motivation. I want to dedicate this project to Mark, Stacey, Ryan, and Shaina Errico in the hopes that I can make them all as proud as they have made me.

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Chapter 1: Literature-Based Background

Chordomas:

Chordomas are rare malignant tumors that arise in bones of the spine in humans (Figure 1). There are approximately 300 new cases per year worldwide (McMaster et al. 2001). Current treatment plans involve radiation and chemotherapy. Overall, treatment is fairly unsuccessful (York et al. 1999). Prognosis is poor as patients rarely survive past 10 years post-diagnosis. Very little is known about the cause and mechanism of chordoma formation.

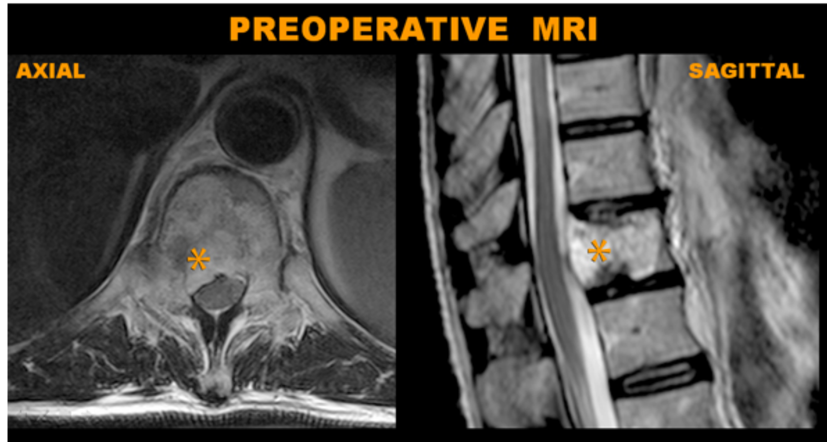


Figure 1: The chordoma (marked with *) in the vertebra is compressing the spinal cord.

(Indiana University Health)

Chordomas are believed to arise from notochoral cells during human development. The notochord is a feature of development in many species that is a key structure in embryogenesis. During embryonic development, notochord cells express key markers such as *Brachyury* and *sonic hedgehog*. These genes are shut off as development progresses and cells mature and differentiate to their final fate. It is believed these notochord cells are chordoma precursors because the cells of the tumor re-express these genes that are characteristic of the embryonic stage. Indeed, expression of *Brachyury* is a defining hallmark of human chordomas (Shalaby et al. 2009).

Notochord Background:

The notochord is present transiently in all vertebrates including humans and zebrafish during embryonic development. Notochord cells are the embryonic precursors to *nuclei pulposi* (NP) cells and mature intervertebral discs (Romeo and Hogendoorn 2006). Immature NP cells are also found to express both Brachyury mRNA and protein (Vujovic et al. 2006) (Tang et al. 2012).

The notochord secretes signals that pattern surrounding tissues by providing position and fate information. The notochord plays a key role in the formation of the central nervous system. It also plays a key structural role as a cartilage-like support structure until other skeletal elements form. The notochord forms from the dorsal organizer, which in turn becomes part of the tail bud at the end of gastrulation. The tail bud contains stem cell-like cells, which are required for axis elongation in vertebrate embryos, and form the chordamesoderm. Chordamesoderm cells are rapidly dividing notochord precursor cells and require nodal signaling (Hagos and Dougan 2007) for their formation. They become distinct from other mesoderm as they rearrange by intercalation and convergence toward the dorsal midline. This elongated patch of cells become rod-like in appearance characteristic of the notochord as osmotic pressure within an internal vacuole pushes against a thick extracellular sheath (Stemple 2005).

The notochord secretes many signals including nodal and nodal-related proteins. Nodal expression forms a gradient leading to different mesodermal and axial mesendodermal fates. The nodal ligand is a member of the TGF-β family of signaling molecules.

Another role of the notochord in the patterning of the embryo is the formation of the neural tube by the hedgehog proteins. Specifically, sonic hedgehog (SHH) is secreted in a graded fashion to induce a range of ventral spinal cord fates. It simultaneously suppresses the expression of characteristic dorsal genes that would induce dorsal cell fates (Placzek et al. 1993; Yamada et al. 1993; Yamada et al. 1991). SHH also patterns the somites that run parallel to the notochord along both sides in the trunk and tail. These somites will later develop into muscle and vertebrae (Cunnliffe et al. 1999).

In our model of interest, the zebrafish, at 12 hours post-fertilization (hpf) the chordamesoderm expresses a variety of marker genes that are characteristic of an undifferentiated state. As development continues, the pressure in the internal vacuole builds, the cells undergo a drastic increase in diameter. As these cells terminally differentiate, these early embryonic marker genes begin to shut off.

The Misregulation of Brachyury as a Marker for Chordomas:

The nuclear transcription factor, brachyury, is characteristically expressed in chordomas and is often used as a diagnostic marker (Figure 2). Other features of chordomas, such as their immunoreactivity for S-100, do not differentiate the chordoma from related bone tumors such as chondrosarcomas (Nibu et al. 2013). Chondroitin sulfate proteoglycan 4 (CSPG4) is a marker specific to chordomas, however, it is only expressed in roughly 62% of the tumors. This discounts its value as a marker because it could lead to a false negative diagnosis (Schwab et al. 2009).

Brachyury is a valuable diagnostic tool because it differentiates a true chordoma from other related bone tumors such as chondrosarcomas. At the transcription level,

chordomas resemble a broad range of cartilaginous neoplasms, however, only chordomas express brachyury (Vujovic et al. 2006). It has been shown that brachyury is present in over 90% of the chordomas biopsied (Jambhekar et al. 2010). Research has shown that of 181 sporadic chordoma samples, 7% showed amplification of the *Brachyury* locus, 39% were polysomic for chromosome 6, and 4.5% showed a minor allelic gain of *Brachyury* (Presneau et al. 2011). The duplicated copies of *Brachyury* can become paralogs and act synergistically in controlling shared target genes (Nibu et al. 2013). Abnormalities relating to brachyury are present in both hereditary and sporadic chordomas (Nibu et al. 2013).

The transcription factor, brachyury, is a member of the T-box gene family. Brachyury is mainly responsible for posterior mesoderm formation and axial development. Its expression is ultimately restricted to the notochord and the tail bud (Showell et al. 2004). It is believed misregulation of some of the genes controlled by Brachyury may be the underlying mechanism for chordoma formation (Nibu et al. 2013).

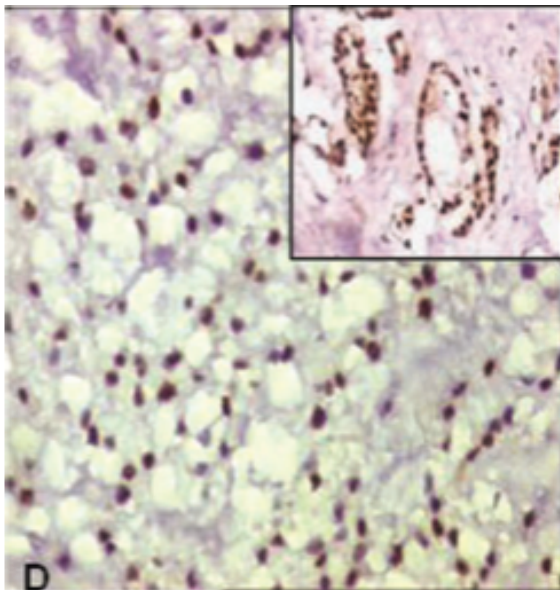


Figure 2: Classical chordoma cells stained positive for brachyury (Jambhekar et al., 2010)

It has been shown that the T-box binding motif is identical in humans and zebrafish (among other organisms) and that brachyury acts as an activator of transcription. Brachyury serves as a master regulator of an elaborate oncogenic transcriptional network (Nelson et al. 2012). This may in part explain why brachyury is a marker of chordomas.

Similarly, zebrafish require *no tail* (ntl), the zebrafish ortholog of brachyury, for notochord differentiation. Its expression stops when the cells become differentiated as development progresses. Without functional ntl, the notochord and posterior mesoderm fail to form. As the name suggests, embryos without functioning ntl have no tail (Halpern et al. 1993). The somite myotomes of ntl mutants are disorganized and fail to take their chevron-like shape characteristic in wild-type embryos. The cells in ntl mutants exhibit defective convergence. Convergence movements affect the shaping and the movement of somites. The axial mesoderm cells become scattered along the midline rather than forming a distinct notochord precursor (Halpern et al. 1993). An experiment using chimeras further illustrates the harmful impact on *ntl* mutants on the forming notochord. A chimera is an organism containing genetically distinct cells. In chimeras formed from transplanting *ntl* mutant donor cells into a wild-type host, the *ntl* mutant cells do not become part of the differentiated notochord. However, in the converse experiment, where wild-type donor cells are transplanted into host embryos expressing *ntl* mutant cells, the wild-type donor cells differentiated as notochord despite the surrounding mutant cells (Halpern et al. 1993).

Ultimately, Brachyury is characteristic of the embryonic stage. It should be shut off in the mature organism, but it is re-expressed in chordoma cells. This is indicative of

misregulation of brachyury. It is still unclear if and how healthy Brachyury-expressing notochord cells become the Brachyury-expressing cells of chordomas (Nibu et al. 2013).

Research has shown that chordomas contain cancer stem-like cells because the chordoma cells expressed stem cell surface markers (Aydemir et al. 2012). This could present an avenue where Brachyury may be used as a therapeutic target. A study using adenoid cystic carcinomal cell lines showed that cancer stem cell characteristics can be reversed by the knockdown of *Brachyury* (Shimoda et al. 2012). The targets of Brachyury also represent possible therapeutic targets (Nibu et al. 2013).

The Misregulation of the mTOR Pathway:

In addition to Brachyury, the mammalian target of rapamycin (mTOR) pathway is misregulated in a variety of cancers including chordomas. Various components of the pathway are misregulated in different types of cancers (Dazert and Hall 2011). One such component of interest, PTEN (phosphatase and tensin homolog), a negative regulator of this pathway, is typically absent or highly downregulated in chordomas. Hyperexpression of both mTORC1 and Akt, other components of the mTOR pathway, are believed to stem from the lack of PTEN expression. This inappropriate activation of Akt in combination with mTORC1 are believed to play a role in the formation of chordomas (Han et al. 2009).

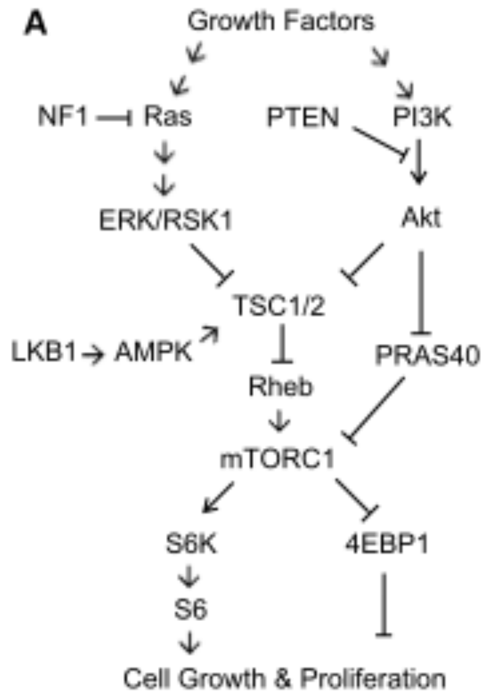


Figure 3: mTOR pathway schematic- decreased PTEN expression leads to decreased downregulation of Akt. Hyperactivity of Akt and mTORC1 result in upregulation of S6 leading to increased cell growth and proliferation (Han et al., 2009)

One target of the mTOR pathway is the p70-S6 kinase. This kinase phosphorylates the S6 ribosomal protein which leads to an increase in protein synthesis and cell proliferation (Figure 3). Phosphorylated S6 is a characteristic of chordomas. This pathway can be inhibited by rapamycin. Inhibition by rapamycin has been shown to decrease proliferation in chordomas (Figure 4) (Han et al. 2009).

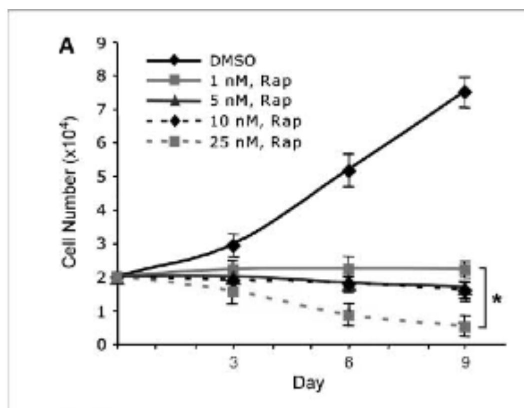


Figure 4: (A) Increased concentrations of the drug rapamycin are correlated with decreased cell proliferation (Han et al., 2009)

The Zebrafish Model System and Formation of Chordoma-Like Tumors

Zebrafish are an ideal model to study vertebrate development. The zebrafish is a small freshwater fish native to rivers of the Himalayan region. It is a popular model to study embryonic development because it is easily cultured and the embryos develop rapidly in eggs outside of the mother and are relatively large, robust, and transparent (Patterson 2004).

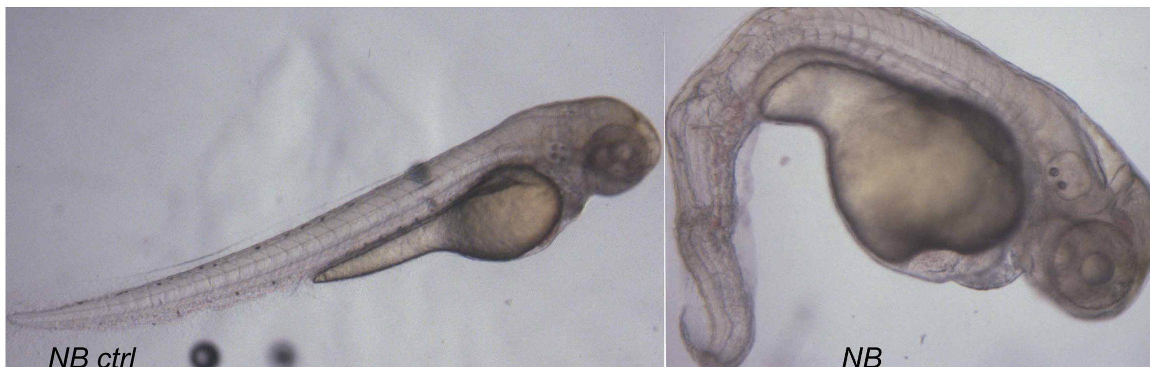


Figure 5: (A) control with DMSO, no chordoma-like tumors
(B) Treated with 25μM SB-505124, two chordoma-like tumors of the notochord

It is possible to generate chordoma-like tumors in zebrafish. These tumors are similar to chordomas in that they express the analogous marker, *No tail* (ntl) (Figure 6). Ntl is the zebrafish ortholog of *Brachyury*. The drug SB-505124 is responsible for the induction of these tumors in zebrafish (Figure 5). Embryos at the mid-somitogenesis stage (14-18 somite stage) of development treated overnight with 25μM SB-505124 show signs of chordoma-like tumor formation. SB-505124 competitively inhibits the ALK4, 5, 6, and 7 receptors (Byfield et al. 2003) (Derynck and Zhang 2003) (Hagos and Dougan 2007) that mediate signaling by the TGF-β, nodal, and activin ligands, which are all members of the TGF-β superfamily. Upon receptor activation, the transcription factors Smad2 and Smad3 are phosphorylated. Smad2, 3, and 4 together regulate target genes.

The nodal/ TGF-β pathway regulates a wide variety of processes and therefore induces a wide variety of cellular responses (Schier 2009). TGF-β/nodal signaling has been associated with tumor progression. TGF-β sometimes serves as a tumor suppressor; in the absence of functioning TGF-β, the unsuppressed tumors may progress (Bachman and Park, 2005). As mentioned, these signals are key in notochord development in healthy zebrafish and other vertebrate embryos. It is unsure exactly how the blockage of these signals results in notochord tumor formation.

Figure 6: in situ expression of ntl and shh (A) and (B) ntl expression, (C) shh expression

Chapter 2: Laboratory Research

Purpose:

Due to the lack of viable treatment options and the malignancy of the chordoma, a rare type of bone tumor, it is imperative to research improved treatment options. Zebrafish show potential as an in vivo model to study chordomas. We can generate chordoma-like tumors in zebrafish embryos that have exhibited traits that are characteristic to chordomas. Exposure of zebrafish embryos to the drug SB-505124 yields the expression of chordoma-like tumors. A variety of markers that characterize chordomas have been found as orthologs in chordoma-like tumors. These markers include *Brachyury*, *sonic hedgehog*, and the expression of other genes that are typically characteristic of developing cells, but are reexpressed in chordomas. The goal of our current research is to validate this zebrafish model, so that it may someday be used as a tool for drug discovery research. Zebrafish have been shown to be an ideal organism for small molecule studies because the use of the entire organism allows combination of animal testing and small molecule screens (Murphey and Zon 2006). This model would be ideal for high-throughput drug screens due to the nature of the embryos. They are relatively inexpensive, small, and abundant relative to other models, such as mice. Mice have proven to be potentially less ideal than zebrafish because they are more expensive, the model requires a mature organism (approximately 2 years of age), and there has been some difficulty generating chordoma-like tumors in mice. Even when chordoma-like tumors are generated in mice, the organism dies shortly after, limiting its value as a drug screen (Harfe 2010). Currently, there are drug screens being performed on in vitro chordoma cell lines, but it is unsure if this is representative of in vivo conditions or *in situ*

chordoma behavior. For this reason, an in vivo model would be a more effective drug-screening tool.

The ultimate goal, which extends beyond the scope of this study, is to develop a line of transgenic zebrafish that will be used for high-throughput drug screens that will identify novel drug targets and therapeutic compounds to improve the treatment of chordomas by either preventing tumor formation or eliminating tumors upon formation. To accomplish this goal, we must first determine how similar chordoma-like tumors in zebrafish are to chordomas. This will validate or invalidate the use of chordoma-like tumors as a model for chordomas. We also must determine what ligand the drug SB-505124 is blocking to cause tumor formation. It is known to block several ligands of the nodal/TGF-β pathway, but it's role in tumor formation remains unknown.

Research Aim:

The primary focus is to evaluate the similarities between chordomas and chordoma-like tumors and to continue to characterize the chordoma-like tumors in zebrafish so we may better understand their mechanism of formation in addition to identifying possible drug targets. Specifically, the genes TGF-β2, TGF-β3, DRAKR1b1, and DRAKR1b10 were investigated. The roles and expression of these gene products were studied via *in situ* hybridization. Due to difficulties obtaining embryos, less laboratory work was completed than expected. For this reason, this thesis is a combination of laboratory and literature-based research.

The Purpose of In situ Hybridization

Different cells express different genes. Which genes are expressed in any cell depends on the stage of development. To better characterize the chordoma-like tumors and to understand how they are formed, it is important to know when and where genes are expressed.

The Purpose of Studying TGF-β2,3

It is not known exactly how SB-505124 interacts with the nodal/TGF-β pathway to cause chordoma-like tumors. This drug is known to inhibit this pathway which is used by several ligands including nodal and TGF-β2,3, however, if it not known which of these ligands is being blocked at the time of the drug administration that results in tumor formation. The purpose of my research this year has been to better characterize the formations of these tumors so we may understand potential drug targets. Nodal is not expressed when the drug is administered, therefore it is likely not what is being blocked to cause tumor formation. As mentioned, nodal is required for formation of the chordamesoderm, thus early treatment of embryos with SB-505124 leads to a loss of these notochord precursor cells. However, drug treatment at a later stage of development, at 15-16 hpf, leads to chordoma-like tumor formation. Nodal, one of the ligands blocked by the drug, is not active at this time. We have looked at the ZFin database to examine other ligands that are expressed in the correct timeframe. TGF-β2,3 are active at this time, therefore, it is believed chordoma-like tumor formation is a result of the blockage of TGF-β, not nodal. It is likely TGF-β signaling is required for terminal differentiation of the notochord, similar to the anti-growth role TGF-β plays in epithelial cells (Seoane 2006). For this reason, TGF-β2,3 are RNA targets of interest for *in situ* hybridization.

If *in situ* expression patterns in chordoma-like tumors support the theory that one or both of these ligands are being blocked by SB-505124 to cause tumor formation, more testing using morpholinos must be completed to confirm this theory. We would block TGF-β2,3 activity using morpholinos. If blockage of these ligands by SB-505124 is responsible for tumor formation, we would expect blockage of their activity by morpholinos to mimic the effects of the drug. We would expect to see chordoma-like tumor formation or an increased sensitivity of the embryos to SB-505124. This sensitivity would be marked by chordoma-like tumor formation from a lower dose of the drug after exposure to morpholinos. Definitive confirmation that the blockage of these ligands causes tumor formation represents an increased understanding of the mechanism of formation, thus presenting potential drug targets.

The Purpose of Studying DRAKR1b1,1b10

We plan to use DRAKR expression as a tool to evaluate how similar chordoma-like tumors are to chordomas. Much like brachyury, AKR has been found to be highly activated in chordomas. Though there is no evidence suggesting AKR is a cause of tumor formation, it is believed to be a secondary consequence of another misregulated pathway. If chordomas and chordoma-like tumors are similar, we would expect to see highly activated DRAKR in chordoma-like tumors. Determining how similar chordoma-like tumors are to chordomas is essential in evaluating how valuable chordoma-like tumors are as a tool for drug screens.

The AKR1B10 locus of the chromosomal region 7q33 is frequently deleted or amplified in human chordomas (Nibu et al. 2013). Several cases of familial chordoma have been documented. Though rare, one such ten-member family underwent a genome-

wide analysis to investigate linkage. The chromosomal linkage was found to be 7q33 (Bhadra et al. 2006). Later research on the same family showed that one individual did not inherit the 7q33 haplotype. The search for an additional susceptibility loci revealed that a duplicated area shared by all individuals contained only one locus, Brachyury (Yang et al. 2009). Nonetheless, 7q33 remains an area of interest. DRAKR1b1,1b10 are the are the zebrafish orthologs of this region of interest.

There remain difficulties in comparing chordomas to chordoma-like tumors. Chordomas are often N-stage cancer by the time they are discovered and researched. Many pathways and anti-mitogenic mechanisms have been affected by this point. Chordomas have progressed to a much later stage compared to chordoma-like tumors in zebrafish. The chordoma-like tumors would be more similar to early stage chordomas in humans, but early stage tumors have never been found because chordomas are not discovered until they manifest themselves as other physical problems.

Hypothesis:

Characterization of the expression of TGF-β2,3 and DRAKR1b1,10 in chordoma-like tumors in zebrafish via RNA *in situ* hybridization will contribute to the general characterization of chordoma-like tumors in zebrafish so that zebrafish may serve as a model for human chordomas in the future. Expression of DRAKR1b1,10 will serve as an additional marker of similarity or dissimilarity between chordomas and chordoma-like tumors to evaluate the value of the tumors as potential drug screens. Expression patterns of TGF-β2,3 will help to determine if these are the ligands being blocked by SB-505124,

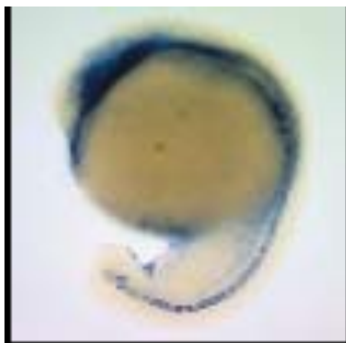
ultimately improving our understanding of the mechanism of formation of chordoma-like tumors.

Results:

Note: Some pictures in the following figures are from outside research. They are present for comparative purposes to verify the success of the designed probes.

Figure 7: TGF-β2 expression *in situ*, (A) WT 21s (Chen et al. 2003), (B) WT 16-18s, (C) WT 30hpf

(A)



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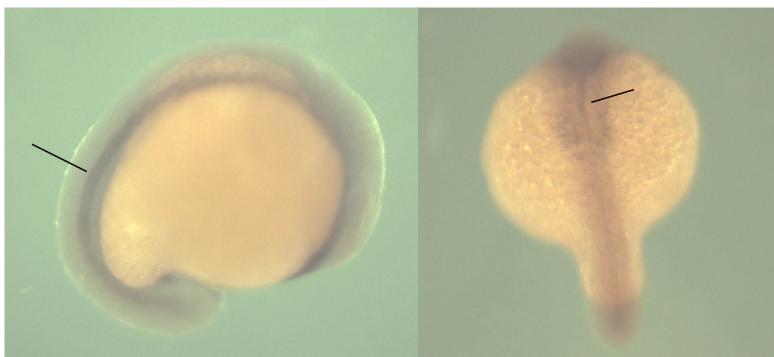

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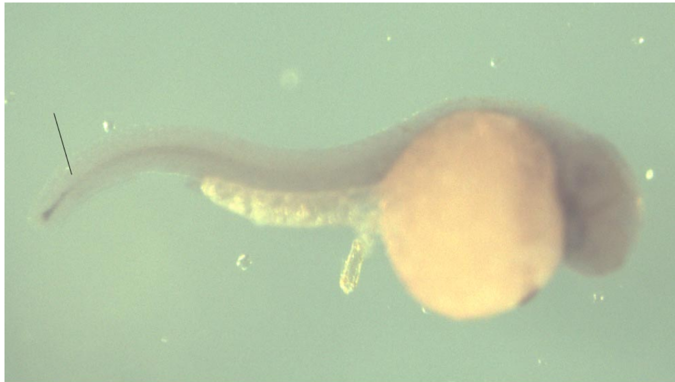
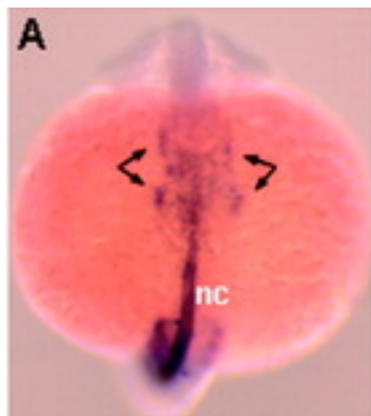
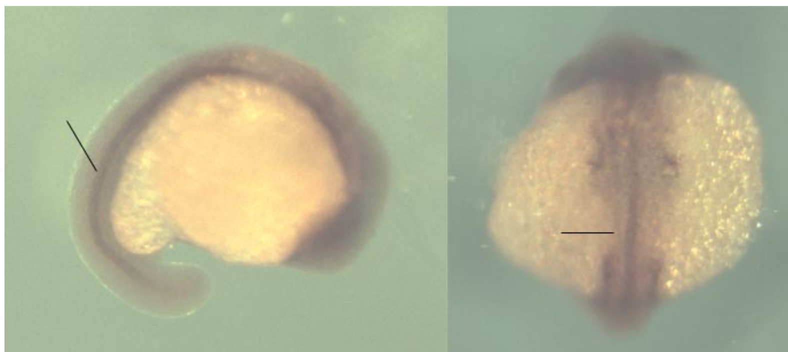


Figure 8: TGF-β3 expression *in situ*, (A) WT 14-19s (Cheah et al. 2005), (B) WT 16-18s, (C) WT 30hpf

(A)



(B)



(C)

Figure 9: drakar1b1 expression *in situ*. Left: WT 2dpf in DMSO (control), Right: drug-treated 2dpf in 25 μM SB-505124

Figure 10: drakr1b10 expression *in situ*. Left: WT 2dpf in DMSO (control), Right: drug-treated 2dpf in 25 μM SB-505124

TGF-β2 Expression

There is similarity in the experimental TGF-β2 expression and published data (Figure 7). There is clear staining of the notochord in embryos of all stages. There is no published data in the ZFin database for TGF-β2 expression at the specific phases of interest.

TGF-β3 Expression

There is similarity in the experimental TGF-β3 expression and published data (Figure 8). There is clear staining of the notochord in embryos of all stages. There is not published data in the ZFin database for TGF-β3 expression at the all phases of interest.

DRAKR1b1 Expression

There is no significant difference in expression between the control and drug treated embryos (Figure 9). There is no published data in the ZFin database for DRAKR1b1 expression.

DRAKR1b10 expression

There is no significant difference in expression between the control and drug treated embryos (Figure 10). There is no published data in the ZFin database for DRAKR1b10 expression.

Materials and Methods:

DRAKR 1b1,1b10 and TGF-β2,3 were stained via *in situ* hybridization in WT embryos at the 16-18 somite stage, 30 hpf, and 2dpf.

Generation of probes for in situ hybridization:

Using the zebrafish gene bank, potential orthologs such as AKR were identified. The full sequences for the genes of interest were downloaded then a primer program determined which primers would be most effective for reverse transcription polymerase chain reaction. The following sequences were cloned into pCRII-TOPO cloning vectors (Invitrogen):

TGF-β2: final PCR product of 907 base pairs (bp)

5'→3'

GGCAGCGCAGCGAGGAGGAGTATTA

TTGCCGATGTAGTAGAGGATTGTGA

TGF-β3: final PCR product of 1128 bp

5'→3'

ATTCGGCAAGGACAGACACCAGAGC

AAAAGAGCGGACCGAACATTACACG

DRAKR1b1: final PCR product 773bp

5'→3'

CGGAGCCCACGTCTATGAAAAC

TCCAGCCCGAAAGCCCTAAAAT

DRAKR1b10: final PCR product 659 bp

5'→3'

ACTGAGGTCGGAGAGGGAATAAAA

TCTTGAATGCGCTGAGGTGTGAT

Generation of chordoma-like tumors:

SB-505124 (Sigma-Aldrich, catalog number S4696) is diluted with DMSO to form a solution with a concentration of 10mM. The solution is administered to embryos at 14-18s with a final drug concentration of 25μM. After 24 hours of exposure at 28.5°C, the drug is removed. Embryos are allowed to develop for another 24 hours before they are fixed in 4% PFA.

Discussion:

Intended method

The initial plan was to stain for DRAKR1b1,10 and TGF-β2,3 via *in situ* hybridization in both WT and SB-505124 drug treated embryos at a range of ages including 9s, 18s, 24hfp, 48hpf. This would enable comparison of gene expression in WT embryos and embryos with chordoma-like tumors. The wider age range would illustrate how the expression of these genes changes over time.

Due to difficulty obtaining healthy embryos, the full method could not be completely executed.

Accuracy of In situ Probes

Based on the data obtained with the limited embryo resources, it can be concluded that TGF-β2,3 is expressed at both the 16-18s stage and at 30hpf. Referencing some of the experimental data with published ZFin data has show the probes are working correctly.

The lack of expression of DRAKR1b1,10 in both WT and drug-treated embryos has show that either the probe is not working properly or that DRAKR1b1,10 is not characteristic of chordoma-like tumors, unlike the characteristic expression of AKR in

chordomas. There is no known positive control to verify the accuracy of the probes for these targets.

Future Experiments

A) Remake DRAKR1b1,10 probes in attempt to generate staining or find a positive control to verify correct expression. It is necessary to determine if these results are due to a nonfunctioning probe or if there is truly no expression of DRAKR1b1,10 in chordoma-like tumors.

B) Isolate drug treated notochords with chordoma-like tumors and WT notochords to perform microarray analysis. This would enable a broader understanding of what genes are misregulated in chordoma-like tumors in addition to the genes of interest.

C) Block TGF-β2,3 activity using morpholinos to test that these ligands are being blocked by SB-505124 to cause tumor formation. Identify chordoma-like tumor formation caused by morpholino exposure, or at least an increased sensitivity to SB-505124.

D) Administer drug treatment at later developmental phases to see if tumors continue to form. Older embryos that were 30-32hpf showed strong *in situ* expression of TGF-β2,3. It is known that drug administration at 16s yields tumor formation, but it may be possible to generate tumors embryos at 24-28hpf based on evidence that the ligand involved in formation is still actively expressed in this timeframe.

References

Aydemir, E., Bayrak, O., Sahin, F., *et al*. (2012). Characterization of cancer stem-like cells in chordoma. Journal of Neurosurgery *116*: 810–820.

Bachman, K., and Park, B. (2005). Duel nature of TGF-beta signaling: tumor suppressor vs. tumor promoter. Current opinion in oncology *17*: 49-54.

Bhadra, K. and Casey, A. (2006). Familial chordoma: A Report of Two Cases. J Bone Joint Surg Br *88-B*.

Byfield, S., Major, C., Laping, N., and Roberts A. (2003). SB-505124 is a selective inhibitor of transforming growth factor-β type I receptors ALK4, ALK 5, and ALK 7. Molecular Pharmacology *65*: 744-752.

Cheah, F., Wang Jabs, E., Chong, S. (2005). Genomic, cDNA, and embryonic expression analysis of zebrafish transforming growth factor beta 3 (*tgfβ3*). Developmental Dynamics *4*: 1021-1030.

Chen, S., and Kimelman, D. (2003). Submission and Curation of Gene Expression Data. ZFIN Direct Data Submission (http://zfin.org).

Cunliffe, V. and Ingham, P. (1999). Switching on the notochord. Genes & Dev. *13*: 1643-1646.

Dazert, R., and Hall, M. (2011). mTOR signaling in disease. Current opinion in cell biology *23*: 744-755.

Dooley, K., Zon, L. (2000). Zebrafish: a model system for the study of human disease. Curr Opin Genet Dev. *3*: 252-256.

Derynck, R., and Zhang, Y. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signaling. Nature *425*: 577-584.

Hagos, E., and Dougan, S. (2007). Time-dependent patterning of the mesoderm and endoderm by Nodal signals in zebrafish. BMC developmental biology *7*: 22.

Halpern M., Thisse C., Ho, R., Thisse, B., Riggleman, B., Trevarrow, B., Weinberg, E., Postlethwait, J., Kimmel, C. (1995). Cell-autonomous shift from axial to paraxial mesodermal development in zebrafish floating head mutants. Development *121*: 4257–4264.

Halpern, M., Ho, R., Walker, C., and Kimmel, C. (1993). Induction of muscle pioneers and floor plate distinguished by the zebrafish no tail mutation. Cell *75*: 99-111.

Han, S., Polizzano, C., Nielson, G., Hornicek, F., Rosenberg, A., and Ramesh, V. (2009). Aberrant hyperactivation of akt and mammalian target of rapamycin complex 1 signaling in sporadic chordomas. Clinical cancer research : an official journal of the American Association for Cancer Research *15*: 1940-1946.

Harfe, B. (2010). A Mouse Model of Chordoma. The Liddy Shriver Sarcoma Initiative. http://sarcomahelp.org/research/chordoma-mouse.html

Jambhekar, N., Rekhi, B., Thorat, K., Dikshit, R., Agrawal, M., and Puri, A. (2010). Revisiting chordoma with brachyury, a “new age” marker: analysis of a validation study on 51 cases. Archives of pathology & laboratory medicine *134*: 1181-1187.

McMaster, M., Goldstein, A., Bromley, C., Ishibe, N. ,and Parry, D. (2001). Chordoma: incidence and survival patterns in the United States, 1973–1995. Cancer Causes and Control *1*: 1–11

Murphey, R., and Zon, L. (2006). Small molecule screening in the zebrafish. Methods *39*: 255-261.

Nelson, A., Pillay, N., Henderon, S., Presneau, N., Tirabosco, R., Halai, D., Berisha, F., Flicek, P., Stemple, D., Stern, C., Wardle, F., Flanagan, A. (2012). An integrated functional genomics approach identifies the regulatory network directed by brachyury (T) in chordoma. J Pathol. *3*: 274-285.

"Treating spinal chordoma with a multidisciplinary approach for superior outcome." *Strength in Knowledge*. Indiana University Health, 16 April 2013. Web. 1 April 2014. <https://iuhealth.org:8443/knowledge/detail/treating-spinal-chordoma-with-a-multidisciplinary-approach-for-superior-out>

Nibu, Y., José-Edwards, D., Di Gregorio, A. (2013). From Notochord Formation to Hereditary Chordoma: The Many Roles of Brachyury. BioMed Research International *2013*.

Patterson, M. (2004). Zebrafish model human developent and disease. Public Library of Science.

Placzek, M., Jessell, T. M. and Dodd, J. (1993). Induction of floor plate differentiation by contact-dependent, homeogenetic signals. Development *117*: 205.

Presneau, N., Shalaby, A., Ye, H., *et al*. (2011). Role of the transcription factor T (Brachyury) in the pathogenesis of sporadic chordoma: a genetic and functional-based study. Journal of Pathology *223*: 327–335.

Rome, S. and Hogendoorn, P. (2006). *Brachyury* and chordoma: the chondroid-chordoid dilemma resolved?. Journal of Pathology *209*: 143–146.

Schier, A. (2009). Nodal morphogens. Cold Springs Harbor perspectives in biology *1*: a003459.

Schwab, J., Boland, P., Agaram, N., *et al*. (2009). Chordoma and chondrosarcoma gene profile: implications for immunotherapy. Cancer Immunology, Immunotherapy *58*: 339–349.

Seoane, J. (2006). Escaping from the TGFb anti-proliferative control. Carcinogenesis *11*: 2148-2156.

Shalaby A., Presneau N., Idowu B., *et al*. (2009). Analysis of the fibroblastic growth factor receptor-RAS/RAF/MEK/ERK-ETS2/bracyury signaling pathway in chordomas. Modern Pathology *22*: 996-1005.

Shimoda, M., Sugiura, T., Imajyo, I., Ishii, K., and Chigita, S. (2012). The T-box transcription factor Brachyury regulates epithelial-mesenchymal transition in association with cancer stem-like cells in adenoid cystic carcinoma cells. BMC Cancer *12*: article 377.

Showell, C., Binder, O., and Conlon, F. (2004). T-box genes in early embryogenesis. Developmental dynamics: an official publication of the American Association of Anatomists *229:* 201-218.

Stemple, D. (2005). Structure and function of the notochord: an essential organ for chordate development. Development *135*: 2503-2512.

Tang, X., Jing, L., Chen, J. (2012). Changes in the molecular phenotype of nucleus pulposus cells with intervertebral disc aging. Public Library of Science ONE: e52020.

Vujovic, S., Henderson, S., Presneau, N., *et al*. (2006). Brachyury, a crucial regulator of notochordal development, is a novel biomarker for chordomas. J Pathol. *209*: 157–165.

Yamada, T., Pfaff, S. L., Edlund, T. and Jessell, T. M. (1993). Control of cell pattern in

the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. Cell *73*: 673 -686.

Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M. (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. Cell *64*: 635 -647.

Yang, X., Ng, D., Alcorta, D., *et al*. (2009). T (Brachyury) gene duplication confers major susceptibility to familial chordoma. Nature Genetics *41*: 1176–1178.

York, J., Kaczaraj, A., Abi-Said, D., et al. (1999). Sacral chordoma: 40- Year experience at a major cancer center. Neurosurgery *1*:74–80.