Exploration and Analysis of Jagged/Notch Signaling in Lamprey Pharyngeal Arches

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Defended March 21, 2013

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Abstract

The development of the jaw was an important event in vertebrate evolution that led to their success as top predators, but little is known about the molecular mechanisms behind its development. In trying to narrow down important molecules for jaw formation we studied lamprey, a jawless vertebrate representing the most basal vertebrate. While skeletal morphology of lamprey differs greatly from jawed vertebrates, expression patterns of several genes important for skull formation indicate a similar (conserved) dorsoventral (D/V) axis in both species, despite the different physical appearances. Previous findings showed that a signaling pathway involving the ligand Jagged and receptor Notch (Jagged/Notch) is important for forming the D/V axis in fish, suggesting the possibility that these factors could be key components in the pathway that led to the development of the jaw. Furthermore, Jagged is the gene that causes Alagille syndrome, a developmental disorder in humans characterized by abnormal craniofacial structure. Using in situ hybridization, we compared the expression patterns of Jagged/Notch in lamprey and zebrafish and found that they share similar D/V expression patterns, with a few small differences. This finding indicates that this patterning is likely an ancestral trait, and that changes somewhere genetically downstream of the pathway were more important for the evolution of the jaw. Where exactly these changes occurred remains unknown, but the evidence that downstream factors of Jagged/Notch are important in axis formation puts us one step closer to understanding the developmental mechanisms behind the evolution of the jaw.
Acknowledgements

First and foremost I would like to thank my primary thesis advisor, Daniel Meulemans Medeiros, for the opportunity to work in his lab. It has been an honor to work with him and it has been an amazing experience, exposing me to new information sources and techniques that have helped both to broaden and hone my skills as a scientist. The opportunity he has given me will provide valuable experience for my future in graduate school and in the professional scientific community.

I would also like to thank Dr. Maria Cattell, without whom I would have been utterly lost on the journey that has been this thesis project. It was under her guidance that I learned the techniques necessary for successful work. Her skill and patience allowed me not only to thoroughly understand my work, but also taught me how the relationship between teacher and student in a scientific setting should be. Her influence will contribute greatly to my success in the future.

Finally, I would like to thank Dr. David Jandzik for his expert assistance in sectioning embryos as well as the additional members of my thesis committee, Dr. Barbara Demmig-Adams, Dr. Jennifer Knight, and Dr. John Agnew for supporting me in my undergraduate career. The wealth of knowledge I have obtained from each of them has proved invaluable, as is their assistance in this project, and I will never forget the inspiration they have given me to pursue and succeed in a scientific career.
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INTRODUCTION

The development of the jaw was a major step in vertebrate evolution that led to their success as major predators. Strong diversification of vertebrates occurred after the jaw evolved, but what remains unknown is exactly how and when in the evolutionary timeline the jaw arose. To narrow down important molecules for jaw formation we utilize the lamprey, a modern jawless vertebrate. Along with hagfish, lamprey represent the most basal group of all modern vertebrates and possess a craniofacial structure similar to that of some of the oldest fossil vertebrates. While skeletal morphology of lamprey differs greatly from that of jawed vertebrates, several genes important for craniofacial formation are expressed in similar patterns during early development. This similarity suggests distinct dorsal and ventral precursors are present in both groups, albeit with different physical appearances.

Recent findings (Zuniga, et al., 2010) showed that genes coding for the ligand Jagged and the receptor Notch are involved in a signaling network (Jagged/Notch) that is important for forming the D/V axis in the fish head skeleton, indicating that it may also be involved in the pathway that led to the development of the jaw. Furthermore, when mutated, Jagged is the gene that causes Alagille syndrome, a developmental disorder in humans characterized by abnormal craniofacial structure (Li, et al., 1997; Oda, et al., 1997). The present thesis assesses the expression patterns of Jagged/Notch in lamprey and compares it to that of zebrafish in the search for insight into the developmental regulations involved in vertebrate jaw formation. We found that lamprey and zebrafish share similar D/V expression patterns of Jagged/Notch, with a few small differences. This common patterning is likely an ancestral trait, suggesting changes downstream of the pathway were likely more important for jaw evolution. While these changes have yet to be elucidated, the evidence that downstream factors of
Jagged/Notch are important in axis formation advances the understanding of the developmental mechanisms behind the jaw.

BACKGROUND

The Agnathan Skull

Early vertebrates had a relatively simple head skeleton consisting of unjointed rods made of cartilage (Cernya, et al., 2010). Molecular mechanisms driving the divergence of the gnathostomes (jawed vertebrates) have since produced modern species with a wide array of craniofacial skeletal structures, including the jaw. Lamprey, along with hagfish, is a modern jawless vertebrate representing the most basal group of vertebrates, and is a suitable model for understanding the early agnathan (jawless vertebrate) skeleton.

Lamprey have a pharyngeal skeleton that supports the gills made of neural crest-derived cellular cartilage, like all other vertebrates (Langille and Hall, 1988; McCauley and Bronner-Fraser, 2003; McCauley and Bronner-Fraser, 2006). During development of the fish, the cranial neural crest cells (CNCC) migrate ventrally from the anterior neural tube in three streams to the pharynx, where they eventually form the head skeleton. This neural crest developmental mechanism is the same in both lamprey and in jawed vertebrates, conserving an anterior/posterior axis formation of the pharyngeal arches (gill arches), (Cernya, et al., 2010).

Agnathans differ from jawed vertebrates in that this skeleton consists of cartilage bars fused to form an unjointed branchial basket supporting posterior arches, similar to that seen in fossil agnathans (Ges, et al., 2006; Shu, et al., 2003), and this morphology is thus thought to reflect the ancestral state. The lamprey skull is very much symmetrical, lacking a morphological D/V axis so that both sides are essentially mirror images of each other. Gnathostomes, however,
have a more asymmetrical skull that has obvious morphological differences in its dorsal and ventral aspects, creating a distinct upper and lower jaw. (Cernya, et al., 2010).

**The Gnathostome Skull**

In gnathostomes, the ancestral cartilaginous rods that made up the agnathan skull are modified to form a complex and asymmetrical structure with distinct dorsal and ventral cartilages. These cartilages ultimately formed the mandible (lower jaw) and its supporting structures from the first (mandibular) and second (hyoid) arches, respectively, as well as an intermediate, specialized joint tissue connecting them (Medeiros and Crump, 2012). Although recent genetic analyses of mouse, chick, and zebrafish (Depew, et al., 2002; Jeong, et al., 2008; Miller, et al., 2003; Talbot, et al., 2010; Zuniga, et al., 2010) embryos have identified several genes that interact to form these skeletal elements, the exact mechanism underlying the original modification is still a mystery.

Just like that of lamprey, the pharyngeal skeleton of gnathostomes is derived from cranial neural crest cells. Once in the pharynx these cells receive signals that regulate their proliferation and pattern the head skeleton (Clouthier and Schilling, 2004; Gans and Northcutt, 1983). Neural crest cells (NCC) of the mandibular arch are separated into dorsal and ventral components, each with their own facial structure derivatives. The ventral mandibular NCCs develop into Meckel’s Cartilage as the foundation for mandible formation while the dorsal mandibular NCCs become the palatoquadrate (a joint connecting the mandible to the skull). The hyoid arch is also separated into dorsal and ventral components, with the ventral hyoid NCC becoming jaw support structures such as the ceratohyal, symplectic, and branchiostegal rays and the dorsal hyoid NCC becoming the hyomandibular and opercle (gill covering) (Crump, et al., 2006; Clouthier and
Dorsal cartilages generally have a plate-like structure, in basal gnathostomes like ray-finned fish, while ventral cartilages retain the ancestral rod-like morphology seen in lamprey (Zuniga, et al., 2010). The aforementioned structures are all products forming or related to the lower jaw. The maxilla (upper jaw) is formed from the maxillary process, a structure derived from the NCCs anterior to the mandibular arch, and lack any known D/V axis forming patterns.

**Dorsoventral Expression Pattern in Lamprey and Zebrafish**

Despite the lack of D/V symmetry in the lamprey skull morphology, within the pharyngeal arches are sites where genes important for skull formation are expressed in a pattern surprisingly gnathostome-like, with distinct dorsal and ventral components. Hand, Msx, and Dlx paralogs (gene duplicates within a genome) are downstream elements of Edn1, a ventralizing factor, that are present in the facial region during skull formation. Combinatorial expression of these elements break down into four domains that correspond to the four distinct cell types aligned at different levels along the D/V axis in both species (Fig. 1, 9,10), (Cernya, et al., 2010; Gaskell, 1908; Kuraku, et al., 2010; Martin, et al., 2009; Miller, et al., 2003; Morrison, et al., 2000; Schaffer, 1896; Talbot, et al., 2010; Wright and Youson, 1982). While the morphology differs, this expression is conserved pre-patterning, suggesting that critical changes resulting in jaw divergence occurred downstream of these factors. Although it is important to note that while the patterns closely resemble each other, there are a few slight differences whose impact, while not yet indicative of jaw formation, is not understood. Specifically, the dorsal most domain in lamprey expresses 3 Dlx paralogs along with Msx, while in gnathostomes, only 2 Dlx paralogs marks this spot (Fig. 1).
Figure 1: Modified from Cerney, et al. (2010). D/V gene expression domains in zebrafish arches (A) showing Hand, Msx, and Dlx paralog distribution as well as Jag1 and Hey1 in dorsal structures. Lamprey arch expression (B) domains showing Hand, Msx, and Dlx paralog distribution.

Jagged/Notch Signaling in the Facial Skeleton

The Notch signaling pathway plays a role in many developmental processes and is involved in the determination of a variety of cell fates, including neuron differentiation and cartilage formation (Gilbert, 2010). Notch signaling is triggered when transmembrane ligands of the Delta and Jagged/Serrate families bind Notch receptors located on adjacent cells. The binding triggers cleavage and release of a Notch intracellular domain that translocates to the nucleus and acts as a gene regulatory (transcription) factor for genes in the Hey, Her, and Hes classes in a process called lateral inhibition, where the cell containing the ligand affects the fates of its neighboring cells (Gilbert, 2010; Wang, 2011; Zuniga, et al., 2010).

In Drosophila, Notch is responsible for patterning fields of cells in organ precursors (Diaz-Benjumea and Cohen, 1995) and in vertebrates has been implicated in the development of several organs such as the ear (Brooker, et al., 2006; Kiernan, et al., 2006), liver (Geisler, et al., 2008; Lozier, et al., 2008), pancreas (Golson, et al.,2009), and cardiovascular system (High, et al., 2008) as well as craniofacial structure formation (Engin and Lee, 2010). Several components
of the Jagged/Notch pathway, such as zebrafish Jag1b (Zecchin, et al., 2005), mouse and human Jag1/JAG1 (Kamath, et al., 2002a and 2002b; Mitsiadis, et al., 1997), and zebrafish and mouse Jag2/Jag2 (Jiang, et al., 1998; Zecchin, et al., 2005), and mouse Notch2 (Higuchi, et al., 1995; Mitsiadis, et al., 1997) are expressed in facial skeletal precursors. The exact role of Jagged/Notch signaling in craniofacial development, however, is uncertain. Interestingly, heterozygous mutations in human JAG1 or NOTCH2 have been linked to Alagille syndrome, a genetic disorder characterized by craniofacial abnormalities (Li, et al., 1997; Oda, et al., 1997).

Zuniga et al., (2010) showed that Jag 1b is expressed in the dorsal mandibular and hyoid arches of zebrafish. When mutated, dorsal derivatives of the hyoid (hyomandibular, and opercle) take on the characteristics of ventral hyoid derivatives (rod shape and branchiostegalar ray morphology) and the dorsal derivatives of the mandibular arch (palatoquadrate) is truncated. Although wild type Notch is more evenly distributed throughout the dorsoventral axis, mutants showed similar morphological changes as Jag1b mutants. Human JAG1 misexpression resulted in ventral hyoid derivatives (ceratohyal and branchiostegalar ray) taking on more dorsal characteristics, while the ventral symplectic was lost entirely. Ventral mandibular derivatives (Meckel’s cartilage) also took on a more dorsal morphology (similar to the palatoquadrate).

With this data, Zuniga et al., (2010) concluded that Jagged/Notch signaling is necessary and sufficient for dorsal skeletal morphology in the hyoid and mandibular arches of zebrafish.

Because of the important role of this signaling in dorsalization of the head skeleton in this zebrafish model of gnathostomes, Jagged/Notch is considered a gene of great interest for jaw development and evolution. Comparing the expression patterns of this pathway to that in agnathans may provide important information about critical changes that took place during vertebrate evolution that lead to jaw formation. If the expression patterns of Jagged/Notch differ,
it could implicate this pathway as a key part in that change. Conversely, similar patterns may suggest that despite phenotypic differences, the molecular mechanisms for skull development in jawed and jawless vertebrates are very similar, with a relatively small change downstream of Jagged/Notch signaling being critical. A better understanding of these mechanisms will give insight to the general principles by which the head skeleton is formed as well as helping to identify the developmental controls that have been modified to produce the impressively vast array of vertebrate head skeletons.

**METHODS**

Genes for Notch, Jagged1, and Jagged1b were identified and cloned using PCR and TA cloning procedures and used to create riboprobes. Embryos were then subjected to in situ hybridization to visualize expression patterns.

**Embryos**

Embryos in 24 (9-11dpf) and 26.5 (13-15dpf) stages of development were harvested and preserved in 4% Formaldehyde overnight, rinsed and stored in methanol.

**Gene Identification**

Notch exons were PCR amplified from adult lamprey genomic DNA according to standard methods. Jagged 1 and Jagged 1b were amplified from cDNA using standard rtPCR technique and cloning methods.

**Primer Specifications**

Primer sequences were designed by Dr. Maria Cattell at the University of Colorado, Boulder to measure mRNA species that identify Notch, Jagged1, and Jagged1b.
<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Direction</th>
<th>Oligo Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch</td>
<td>Forward</td>
<td>CCGCTGTATAATGCCTAC</td>
</tr>
<tr>
<td>Notch</td>
<td>Reverse</td>
<td>ATTTAGGTGACACTATAGAGCGGATAGAGCGGCTCCA</td>
</tr>
<tr>
<td>Jagged 1</td>
<td>Forward</td>
<td>GGCACCTTCGTGATGATG</td>
</tr>
<tr>
<td>Jagged 1</td>
<td>Reverse</td>
<td>CCTTGCACCTCACCTCGAC</td>
</tr>
<tr>
<td>Jagged 1b</td>
<td>Forward</td>
<td>GTGGTGCGGAACAAGCTCTG</td>
</tr>
<tr>
<td>Jagged 1b</td>
<td>Reverse</td>
<td>GGACTTCACGCATTCCACTGCA</td>
</tr>
</tbody>
</table>

**Polymerase Chain Reaction**

A PCR reaction mixture containing the following were exposed to PCR cycling conditions of hot-start activation of GoTaq DNA polymerase at 94°C for 2 min and 30 cycles of denaturation at 94°C for 30s, annealing 55°C for 30s, and elongation at 72°C for 60s:

39.0uL diH2O  
5.0uL 10X PCR reaction buffer  
1.0uL dNTPs  
0.5uL Forward Primer  
0.5uL Reverse Primer  
2.5uL DMSO  
1.0uL Super C template DNA  
0.5uL DNA polymerase GoTaq

**Gel Electrophoresis and Purification of PCR Products**

PCR products were run on a 1% agarose gel to confirm product formation and extracted using the Wizard Gel Extraction Kit and vacuum protocol.

1.) Gel containing PCR product was melted and a ratio of 10uL of Membrane Binding Solution to 10mg agarose gel slice was added and allowed to incubate at room temperature for 10 minutes.  
2.) Gel solution was added to an SV minicolumn attached to vacuum and run through. Membrane Wash Solution diluted with 95% ethanol was then added and run through first with 700uL and then a second time with 500uL.  
3.) Transfer of the minicolumn to a collection tube for centrifugation at 16,000g for 5 minutes removed any remaining solution.
4.) DNA was eluted by adding 50uL of nuclease-free water to the minicolumn and centrifuged at 16,000g for 1 min in a fresh collection tube.

**TA cloning**

Cloning of PCR products was done using the PJET cloning kit. A mixture of 5.0 uL of 2x Reaction Buffer, 3.5uL PCR product and 0.5ul DNA Blunting Enzyme were heated to 70°C for 5 minutes and immediately placed on ice. 0.5uL PJET and 0.5uL of T4 Ligase were added and allowed to incubate at room temperature for 15 minutes.

**Transformation**

1.) 3.0uL of the TA cloning reaction were added to a 1.5mL tube of chemically competent bacterial cells and set on ice for 30 minutes.
2.) The tube was then placed in a 42°C water bath for 1 minute to heat shock the cells and placed immediately back onto ice.
3.) 500uL of LB nutrient broth was added and they were incubated on a 37°C shaker for 1 hour.
4.) Following incubation the cells were spun down for 1 minute at 13,000g, decanted, and resuspended in 100uL LB before being plated on to LB agar plates with carbenicillan. Plates were incubated at 37 degrees overnight.

**Prepping Liquid Cultures of Bacteria**

A pipette tip containing a single colony from the bacterial plate was added to a culture tube containing 2mL of LB and 2uL of 100mg/ml ampicillin so that there were up to 10 tubes, each containing one tip.

**Mini-Prepping Liquid Cultures**

Protocol from the Omega plasmid mini-prep kit were used for mini preps. All solutions provided in kit.

1.) Cells were spun down at 10,000g for 1 min and decanted to be resuspended in 250uL Solution I/RNase A Solution.
2.) 250uL of Solution II was added and allowed to incubate at room temperature for 2 minutes.
3.) 350uL Solution III was added and following the appearance of white flocculence, the cells were centrifuged at 13,000g for 10 minutes.
4.) 100uL of Equilibration Buffer was added to the minicolumn attached to a vacuum source and run through.
5.) Supernatant from centrifugation was added to the minicolumn and run through.
6.) 300ul HB Buffer and 700uL DNA Wash Buffer (X2) were sequentially added to and run through the minicolumn.
7.) DNA was then centrifuged at 13,000g for 2 minutes for complete drying before being eluted with 50uL Elution Buffer.
**Restriction Digest**

A standard 20uL restriction digest was performed in a 37°C water bath for 1 hour to confirm cloned plasmid with insert

13.0uL water
2.0uL 10x BSA
2.0uL DNA
0.5uL xbal enzyme
0.5uL xhoI enzyme

**Sequencing**

100-200ng plasmid DNA in total volume of 10uL was sent to ACGT for sequencing.

**Riboprobe Synthesis**

1.) Digestion of 1-2ug of plasmid DNA overnight.
2.) Phenyl-chloroform extraction of the digest was performed twice with 200uL Phenyl-chloroform.
3.) Precipitation and resuspension of digest was done using 20uL NaOAC, 500ul ethanol, 1uL glycogen and 11uL RNAse free water.
4.) Riboprobe synthesis reaction using 4uL of 5X Transcription Buffer, 2uL of 100mM DTT, 2uL DIG labeled nucleotides, 11uL linearized DNA, 1.0uL SP6 RNA Polymerase, and 0.25uL RNAse inhibitor.
5.) Synthesis was left incubate overnight at 37°C
6.) The next morning 1.0uL DNAse was added incubated for 15 minutes at 37°C
7.) Unincorporated nucleotides were removed with a G-50 column.
8.) The next step was a precipitation that involved adding 10ul of 8M LiCl, 1.0uL glycogen, RNAse-free water to bring to total volume of 100uL, and finally adding 200uL of 100% Ethanol.

**In Situ Hybridization**

Embryos were subjected to ISH using the following week long protocol:

- Day 1: Treat embryos with Proteinase K, an enzyme that partially digests the embryo, to allow the RNA probe to get inside the tissues. Then incubate in hybridization solution first without the probe, and then with 1uL, and let it sit overnight.

- Day 2: Wash out the excess unhybridized probe several times with a hot washing solution, so only probe that has hybridized to target mRNA is left stuck to the embryo. Then get the embryo ready for incubation with anti-DIG antibody.

- Day 3: Wash embryos 3-4 times to dislodge any antibody that may be weakly bound in the embryo, and leave the embryos in one last wash overnight.

- Day 4: Wash embryos with AP buffer, add substrates and incubate.
-Day 5 – removing first antibody
1) Wash in PBST 3X 10min.
2) Wash in 0.1% glycine-HCl (pH 2.2) + 0.1% tween at RT for 10min. to remove 1st antibody
3) Wash in PBST 4X 5min. at RT
4) Rinse one time with MAB-T
5) Wash 1X 10min. with MAB-T
6) Wash in blocking soln. for 1hr. @ RT
7) Replace blocking soln. with block + second antibody (1:3000 DIG or 1:6000fluor.) rotate at 4°C ON.

-Day 6 – Wash out extra antibody
1) Repeat day 3 MABT washes

-Day 7 – develop second antibody
1) repeat day 4, rinse and store in methanol

**Sectioning of Embryos**

Embryos were mounted in gelatin and sliced at 20uM using a cryostat.
RESULTS

Figure 2: Lamprey embryo whole mounts showing ISH expression of Notch in stage 24 lateral view (A), stage 24 ventral view (B), stage 27 lateral view (C) and stage 27 ventral view (D). Expression is shown in ventral (black arrow) and dorsal mandibular arch (red arrow).

Figure 3: Lamprey embryo whole mounts showing ISH expression of Jag1 in stage 24 lateral view (A), stage 24 ventral view (B), stage 26.5 lateral view (C) and stage 26.5 ventral view (D). Expression is shown in dorsal mandibular arch (arrow).

Figure 4: Lamprey embryo whole mounts showing ISH expression of Jag1b in stage 24 lateral view (A), stage 24 ventral view (B), stage 26.5 lateral view (C) and stage 26.5 ventral view (D). Expression is shown in dorsal mandibular arch (arrow).
Figure 5:
Transverse sections of lamprey embryos showing ISH expression of Notch in stage 24 (A, B) and stage 27 (C, D). Expression of Notch is evenly distributed throughout the D/V axis of arches.

Figure 6:
Transverse sections of lamprey embryos showing ISH expression of Jag1 in stage 24 (A, B) and stage 26.5 (C, D). Uneven distribution of Jag1 along the D/V axis shows higher expression in the dorsal half and less in the ventral half of the pharyngeal arch. Jag1 expression was also observed in endoderm (c).

Figure 7:
Transverse sections of lamprey embryos showing ISH expression of Jag1b in stage 23 (A, B) and stage 26.5 (C, D). Stage 23 embryos show little expression of around pharyngeal arch while stage 26.5 show expression similar to that of Jag1 with some in the dorsal half of the arch and significantly less in the ventral half.

* For Figures 5-7: Outlined in red is the neural crest derived mesenchyme of the first pharyngeal arch with designations of dorsal (d) and ventral (v) localizations. Any differences in mesenchyme shape and size can be attributed to alternation between pharyngeal arch and pouch along the pharynx.
FIGURE 8:

Figure 8: Transverse section of Jagged1b (A) and Jagged1 (B) ISH broken down in to 4 domains: dorsal (d), dorsal intermediate (di), ventral intermediate (vi) and ventral (v). Highlighted in yellow are areas of concentrated Jagged.

FIGURE 9:

Figure 9: Cerney et al., (2010). Transverse sections and ISH expression of MsxB, Hand, and Dlx genes in the larval lamprey head broken down into four domains: dorsal (d), dorsal intermediate (di), ventral intermediate (vi), and ventral (v).

Expression of Notch Throughout Pharyngeal Arches

Notch expression was seen throughout the dorsoventral axis of the pharyngeal arches in later stage lamprey embryos (Fig. 2C), with no obvious localization in either domain of the pharyngeal arch mesenchyme (Fig. 5). This pattern is similar to that see in zebrafish (Zuniga et al., 2010), which indicates that any differences in downstream activity of the Jagged activated pathway is not due to absence of the receptor or Notch intracellular domain specific activity.
Expression of Jagged in Dorsal and Intermediate Pharyngeal Arches

Localized expression of Jag1 in the dorsal pharyngeal arches was observed in early and late stage lamprey embryos (Fig. 3C) and closer inspection of transverse sections confirms the expression as restricted to the dorsal domain, although it seems to be concentrated in the dorsal intermediate opposed to dorsal most mesenchyme of the pharyngeal arch (Fig. 6, 8). This pattern varies slightly from the expression seen in zebrafish, and while the difference may be insignificant, it could also indicate a different ancestral function of Jagged in skull formation. Expression of Jag1b was also patterned this way, although to a lesser extent.

DISCUSSION

Jagged Localized to Dorsal Arch

The localized expression of Jagged1 and Jagged1b to the dorsal side of the pharyngeal arches mirrors the expression seen in zebrafish. While it cannot be excluded that lamprey have a different Notch receptor patterning obscured by weak probe specification, it is unlikely due to its importance for CNS and cartilage formation during embryonic development. The possibility will likely be excluded upon future analysis of embryos with longer hybridization time for stronger staining and clarification. Thus, the conserved signaling of Jagged from lamprey to gnathostome present from this work suggests that ancestral agnathans already possessed these patterns as well. The drastic morphological abnormalities seen in zebrafish with mutant Jagged/Notch (Zuniga, et al., 2010), indicates this signaling is crucial to the development of dorsal aspects of the jaw, but the similar patterning between zebrafish and lamprey we observed suggests that it is downstream factors of this pathway that underwent critical changes to eventually drive its development.
In zebrafish, the transcription factor and Jagged/Notch signaling product Hey1 was observed to be present in dorsal domains of the facial skeleton (Zuniga, et al., 2010). If post transcriptional inhibition of Jagged or Notch mRNA occurred, then no functional protein would have been produced to activate Hey1. Its presence indicates that the Jagged/Notch signaling pathway was active and uninterrupted in these areas. Although Jagged is continuously dorsally restricted, its expression expands to a point in older embryos where fewer Dlx genes are expressed at a location just ventral to it, compared to the intermediate domains. This area, just ventral to Jagged expression, also expresses Hey1, (Fig. 1, 8) as expected if Jagged was activating Notch on adjacent cells (Zuniga, et al., 2010) and raises the possibility that Hey1 may interact with or inhibit some Dlx activity. Interestingly, while the extension of Jagged is also seen in lamprey, the domains it reaches have more, not fewer Dlx genes expressed in the same region.

**FIGURE 10:**

![Figure 10](image)

**Figure 10:** Modified from Cerney, et al. (2010). D/V gene expression domains in zebrafish arches (A) showing Hand, Msx, and Dlx paralog distribution as well as Jag1 and Hey1 in dorsal structures (circled). Lamprey arch expression (B) domains showing new Jag1 in dorsal intermediate domain (circled) in addition to Hand, Msx, and Dlx paralog distribution.

Comparing Hey1 expression patterns between lamprey and gnathostomes would indicate if it is the molecule whose changes in expression drove the evolution of the jaw. If the patterns
differ, possible interactions between Hey family genes and Dlx genes could provide an explanation for differences in Dlx paralogs distribution, and provide further insight to the molecular mechanisms behind jaw development. Similarly, comparing other Notch activated factors such as those in the Her, and Hes families may be just as insightful. Any differences expression of these factors between the two species could indicate a change in regulatory elements that control the location of gene expression, and sequence analysis of these areas would indicate where these changes occurred. Further research and analysis of these downstream elements is required if we are to further narrow down and understand what underlying mechanisms drove the evolution of the jaw.

**Gain of Jagged in Dorsal Most Pharyngeal Arch**

Close inspection of Jagged expression in lamprey pharyngeal arches reveals a concentration in the dorsal intermediate domain with incomplete extension into both the dorsal most, and ventral intermediate domains (Fig. 8 and Fig. 9 for comparison to other genes expressed in both dorsal and ventral domains). While the localization of this expression is focused in the dorsal hemisphere, similar to patterns seen in zebrafish, the lack of Jagged in the very dorsal most domain differs notably. Whether or not this difference plays a large role in downstream signaling and axis formation remains to be seen.

Expression patterns of the Dlx, Msx, and Hand genes responsible for characterizing cells into four different types along the pharyngeal D/V axis appear to have been impressively conserved in gnathostome mandibular and hyoid arches, with a few small exceptions (Cernya, *et al.*, 2010; Gaskell, 1908; Kuraku, *et al.*, 2010; Martin, *et al.*, 2009; Miller, *et al.*, 2003; Morrison, *et al.*, 2000; Schaffer, 1896; Talbot, *et al.*, 2010; Wright and Youson, 1982). In
zebrafish Jagged signaling has been shown to inhibit some of the ventralizing genes activated by Edn1, including Dlx 3, 5, 6, Msx, and bapx1 in the dorsal domain (Zuniga, et al., 2010). It is therefore possible that the lack of Jagged in the dorsal most domain of lamprey is responsible for the presence of Msx and Dlx B,C,D, observed, while the same region in zebrafish co expresses only paralogs independent of Jagged/Notch signaling, Dlx1/2 (Fig. 1,10). Furthermore it may be interesting to take a closer look at lamprey Edn1 orthologs (a Jagged inhibitor) to determine if its presence in the domain explains the lack of Jagged expression in the dorsal most domain in lamprey.

The possibility that gnathostomes gained Jagged in their dorsal most domains may indicate that the original function of the pathway in ancestral agnathans was to specify intermediate tissue, and that dorsal expansion combined with different expression of other domain specifying genes created a novel dorsal phenotype. Future experiments in lamprey should focus on Notch signaling inhibitors such as DAPT, or Jagged overexpression to provide data for a more complete hypothesis. We know that blocking Jagged/Notch in zebrafish results in the transformation of dorsal structures to ventral structures (Zuniga, et al., 2010), but since lamprey lack dorsal morphology, any effect observed from blocking the pathway would provide valuable information about its original function in skull formation. Over expression experiments of Jagged in lamprey would provide information about the importance of dorsal most Jagged in the zebrafish jaw. If expansion into this domain has no effect on lamprey skull morphology, the small differences observed between the two species is insignificant. Conversely, the overexpression might promote a more dorsal morphology, indicating that this domain expression is of great importance to the development of the jaw.
The intertwining of Jagged/Notch, Edn1 and other important signaling factors in craniofacial development, paired with the combinatorial expression of domain specifying genes, (Cernya, et al., 2010; Gaskell, 1908; Kuraku, et al., 2010; Martin, et al., 2009; Miller, et al., 2003; Morrison, et al., 2000; Schaffer, 1896; Talbot, et al., 2010; Wright and Youson, 1982). produces a network of pathways whose detailed mechanisms remain largely unknown. Uncertain related functionality (orthology) in lamprey and gnathostome Dlx genes adds to difficulties in relating the two conditions (Kuraku, et al., 2009). However, the new evidence presented here points towards important downstream changes in Jagged/Notch as the signaling mechanisms that ultimately led to the evolution and development of the vertebrate jaw.


