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Coexpression of *Fgf8* and *Shh* yields Ectopic Cartilage in the Zebrafish Larval Head Skeleton

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Abstract

Deep homology is the use of similar genetic pathways in the development of structures that are not directly related by common ancestry. One such pathway is the Hedgehog (Hh) and Fibroblast growth factor (Fgf) feedback loop associated with the outgrowth of limbs, external genitalia and, as recently hypothesized, barbels in fishes. Barbels are sensory projections from the head found in a number of groups of fishes, with the most familiar example being the “whiskers” of catfishes. The present study tested whether two components of this loop, *Shh* and *Fgf8*, were sufficient to induce ectopic barbels in the zebrafish, a result that would strengthen the case for deep homology between barbels and other vertebrate appendages. Zebrafish were injected with DNA constructs for the heat-inducible expression of *Fgf8* and *Shh*. Larvae with induced overexpression of these genes were cleared and stained for skeletal structures and examined for barbel-like projections using light and scanning electron microscopy. Coexpression of *Fgf8* and *Shh* was found to be sufficient for inducing ectopic cartilages in the vicinity of the jaw, which were reminiscent of the supports of barbels in some fish species. At least one of these cartilages supported a projection from the face, but the possibility that this projection represents an ectopic jaw could not be ruled out. Regardless of the identity of the ectopic cartilages/projection, the results of this study provide further evidence for the conservation of a mechanism for driving appendage outgrowth in a wide variety of species and structures.

Introduction:

One of the most important discoveries of work in molecular and evolutionary developmental biology over the past thirty years is the prevalence of “deep homology” – the use of similar genetic pathways in the development of structures that are not directly related by common ancestry (Shubin et al. 1997; Carroll 2008). The pattern of deep homology suggests that novel structures of organisms need not evolve *de novo*, but instead may arise from the modification of pre-existing gene networks (Shubin et al. 2009). One of the earliest and most famous examples of deep homology was the finding that the transcription factor *Pax6* is associated with eye development throughout the animal kingdom, despite the extreme diversity of eye morphology among phyla (Callaerts et al. 1997). Another example is the finding of expression of *Dlx* transcription factors in appendages as diverse as mouthparts in insects, limbs in vertebrates, tube feet in echinoderms and siphons of tunicates (Panganiban et al. 1997).

Subsequent comparative studies of appendage development have revealed additional similarities between gene networks regulating insect and vertebrate limb development, but have identified phylum-specific features as well (Pueyo and Couso 2005). An example of the latter is a positive feedback loop required for vertebrate limb outgrowth that involves extracellular signaling molecules in the Hedgehog (Hh) and Fibroblast growth factor (Fgf) families (Zeller et al. 2009). Interestingly, this feedback loop may represent a deep homology within the appendages of vertebrates, as it likely plays a similar role in the outgrowth of the external genitalia of mammals (Cohn 2011; Lin et al. 2013) and cartilaginous projections from the gill arches of cartilaginous fishes (Gillis et al. 2009; 2011).

A vertebrate appendage type that is only beginning to be studied at the developmental genetic level is the barbel of fishes (LeClair and Topczewski 2010). Barbels have been defined as elongate projections from the head, innervated by branches of the cranial nerves and usually having sensory components such as taste buds (Fox 1999). The most well known example is probably the characteristic “whisker” of catfishes, but they are found in a number of other groups of fishes, including the zebrafish (*Danio rerio*), where they are believed to have arisen independently (Fox 1999; LeClair and Topczewski 2010). Hawkins (2011) provided evidence from gene expression studies and loss of function approaches that an Hh-Fgf positive feedback loop is involved in the outgrowth of barbels of the Channel catfish, *Ictalurus punctatus*. The aim of the present study was to investigate further the involvement of such a feedback loop in barbel development using gain of function approaches in the more experimentally-tractable zebrafish model system. The specific ligands *Shh* and *Fgf8* were ectopically expressed singly and in combination during zebrafish embryonic development and the fish examined at larval stages by skeletal staining and scanning electron microscopy (SEM) for the presence of ectopic barbels. The combined expression of *Shh* and *Fgf8* was found to be sufficient to produce ectopic cartilages on the head; significantly, the barbels of catfishes, but not those of zebrafish are supported by a cartilaginous rod (LeClair and Topczewski 2010; Hawkins 2011). These data are consistent with a deep homology of the barbels of fishes and other vertebrate appendages.

Background

Fibroblast growth factor (Fgf) and Hedgehog (Hh) signaling pathways

Paracrine factors, or small molecules that diffuse over short distances and induce intrinsic changes in neighboring cells, play a crucial role in organ initiation, morphogenesis, and differentiation during embryonic development. Many of these paracrine factors are members of a small number of families of signaling ligands, including the Bone morphogenetic protein (Bmp), Fibroblast growth factor (Fgf), Hedgehog (Hh), and Wnt families. The paracrine factors investigated in the present study, *Sonic hedgehog (Shh)*, and *Fibroblast growth factor 8 (Fgf8)* are both morphogens, meaning that their effects on cell differentiation can be different based on concentration as well as the exposure time of the ligand (Dessaud et al. 2007).

Fgf8 is a member of the Fgf family, which comprises 22 members in mammals (Itoh and Ornitz 2011). Of these, fifteen, including *Fgf8*, function as paracrine factors, binding to tyrosine kinase Fgf receptors (Fgfrs), which are encoded by four genes in mammals. The binding of Fgfs to Fgfrs induces receptor dimerization, phosphorylation and activation of a variety of downstream pathways including RAS-RAF-MAPK, PI3K-AKT, STAT and PLC γ (Itoh and Ornitz 2011). *Fgf8* knockout mice die at embryonic day 8 as a result of defective cell migration during gastrulation (Sun et al. 1999). Conditional knockouts and generation of hypomorphic alleles have revealed requirements for *Fgf8* in the development of branchial arches, teeth, limbs, heart, and brain (Trumpp et al. 1999; Lewandoski et al. 2000; Moon and Capecchi 2000; Frank et al. 2002; Chi et al. 2003; Mariani et al. 2008). In addition, *Fgf8* is alternatively spliced (Crossley and Martin 1995), and spliceform-specific

knockouts have revealed different functions for these spliceforms (Guo 2010). In humans, mutations in *Fgf8* are associated with hypogonadism and facial clefting (Krejci et al. 2009). *Fgf8* has also been of interest in treating osteoarthritis by the pharmaceutical company Merck Serono, investigating the degeneration of cartilaginous tissue (Beenken, 2009).

Shh is one of three mammalian genes homologous to *Drosophila Hedgehog* (Ingham et al. 2011). The secreted N-terminal fragments of these proteins bind to the transmembrane proteins Patched-1 (PTC1) or Patched-2 (PTC2), relieving their inhibition of another transmembrane protein, Smoothened (SMO). Activated SMO leads to the accumulation of full-length GLI proteins, which function as transcriptional activators; GLI is otherwise cleaved into a form that functions as a transcriptional repressor (Ingham et al. 2011).

Knockouts of *Shh* or *Smo* in mice lead to cyclopia and defects in the brain, spinal cord, vertebrae, ribs, limbs, genitalia, hair and teeth (Chiang et al. 1996; 2001; St-Jacques et al. 1998; Perriton et al. 2002; Gritli-Linde et al. 2002). *Shh* additionally functions in the development of blood vessels, smooth muscles, heart, lung, kidneys, bladder, inner ear, and taste buds (Varjosalo and Taipale 2008). In humans, mutations in *Shh* are associated with cyclopia, holoprosencephaly (failure of the forebrain to divide into left and right halves), hypotelorism (closely spaced eyes), polydactyly (extra digits), and syndactyly (fused digits) (Roessler et al. 1996; Anderson et al. 2012). Aberrant *Shh* signaling is linked to a variety of cancers (Varjosalo and Taipale 2008). Interestingly, increased expression of *Shh* is associated with eye loss and increase in taste bud number in the evolution of blind cavefish (Yamamoto et al. 2004; 2009).

Fgfs and Shh in Appendage Development

The tetrapod limb is the best-characterized model for the genetic control of vertebrate appendage development. The limb is formed from the lateral plate mesoderm and becomes a bud with ectodermal cells surrounding mesenchymal ones (Duboc and Logan 2009; Zeller et al. 2009). Two signaling centers known to regulate patterning and outgrowth of the limb are the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA) (Laufer et al. 1994 Kimmel et al. 2004). The AER is a thickening of the epithelium that runs along the distal margin of the limb bud and maintains a zone of proliferating, undifferentiated mesenchymal cells underneath it (Fernandez and Ros 2008; Duboc and Logan 2009). Removal of the AER leads to truncation of the limb, suggesting that it is necessary for maintenance of outgrowth and patterning along the proximo-distal (PD) axis (Duboc and Logan 2009). The ZPA is a domain of mesenchymal cells in the distal, posterior region of the limb bud, which when transplanted anteriorly, leads to mirror image digit duplications (Zeller et al. 2009). This phenotype indicates the importance of the ZPA for patterning the limb along the anterior-posterior (AP) axis.

The activity of the AER is mediated by Fgfs (Mariani et al 2008). *Fgf4*, *Fgf8*, *Fgf9*, and *Fgf17* are expressed in the AER (Sun et al. 2000), and Fgfs are sufficient to replace the AER in maintaining limb outgrowth and patterning (Niswander et al. 1993). Compound knockouts in mice showed that *Fgf8* was sufficient among AER-expressed Fgfs to maintain normal limb structure (Mariani et al. 2008). Conditional inactivation of *Fgf8* in the AER resulted in shortened limbs, lacking some skeletal elements (Lewandoski et al. 2000; Moon and Capechhi 2000). Inactivation of additional AER-expressed Fgfs resulted in increasing severity of the skeletal phenotype.

The activity of the ZPA is mediated by *Shh*. Expression of this gene in the limb is localized to the ZPA and transplantation of *Shh*-expressing cells to the anterior region of a chick limb buds results in ZPA-like digit duplications (Riddle et al. 1993). Interestingly, *Shh* is important for patterning the limb along the PD axis as well as the AP axis. The limbs of *Shh* knockout mice exhibit PD truncations as well as loss of elements along the AP axis (Chiang et al. 1996; 2001).

The coordinated effects on AP and PD patterning of both Fgfs and *Shh* may be explained by a positive feedback loop between them, in which Fgf expression in the AER is necessary for the maintenance of *Shh* expression in the ZPA and *vice versa* (Niswander et al. 1994; Laufer et al. 1994; Sun et al. 2000). While this feedback loop applies to *Fgf4*, *Fgf9*, and *Fgf17* in the AER, *Fgf8* expression in this location appears to be maintained by a signal other than *Shh*. The Fgf-*Shh* feedback loop has been shown to be involved in maintaining the outgrowth of other appendages, including external genitalia (Haraguchi et al. 2000; Perriton et al. 2002; Cohn 2011; Lin et al. 2013), the gill rays of cartilaginous fishes (Gillis et al. 2009), and notably for the present work, the barbels of fishes (Hawkins 2011). In all three of these examples, *Fgf8* expression appears to be regulated by *Shh*, suggesting that there may be differences in the Fgfs comprising the positive feedback loop compared with the limb.

In addition to their role in maintaining appendage outgrowth, a variety of results suggest that *Shh* and *Fgf8* may be involved in appendage induction and initiation.

Expression of *Fgf8* has been found in intermediate mesoderm in mouse and chick before limb bud initiation and can be found when limb bud formation is first detected in the ventral limb ectoderm (Crossley et al. 1996; Boulet et al. 2004). Beads soaked in FGF8

protein are sufficient to induce ectopic limbs in chick embryos (Crossley et al. 1996). Similarly, beads soaked in SHH protein are capable of inducing ectopic beaks in the chick (Hu and Helms 1999; Brito et al. 2008) and gill rays in cartilaginous fishes (Gillis et al. 2009).

In addition to their ability to induce appendages when expressed singly, *Fgf8* and *Shh* may synergize in inducing appendage outgrowth (Abzhanov and Tabin 2004). *Fgf8* and *Shh* have been found to be expressed in the frontonasal primordium (FNP) and the mandibular primordium (MNP) in the rostral-ventral epithelium in the chick (Hu et al. 2003). The FNP gives rise to the mid and upper face, including a boundary that is believed to give rise to frontonasal outgrowth. *Fgf8* was found to be in the most dorsal region of the FNP and *Shh* in the ventral portion, creating the frontonasal ectodermal zone (FEZ). It was found that the FEZ induced could “override” a prepatter in neural crest cells and lead to FNP outgrowth in chick embryos. (Hu et al. 2003). When ectopically expressed via viral vectors, *Fgf8* and *Shh* yielded cartilaginous outgrowths from the head of chick embryos (Abzhanov and Tabin 2004).

While *Fgf8* and *Shh* have been found to be necessary for barbel outgrowth in the channel catfish (Hawkins 2011), whether they are sufficient to induce barbels remains unknown. To investigate this question, I turned to a more experimentally tractable fish model system, the zebrafish (*Danio rerio*).

The zebrafish as a model for barbel development

The zebrafish is a widely used model organism in biomedical studies. Advantages of this species include the ease of embryo production, the transparency and external

development of embryos, their short generation time (3 months), and the variety of tools developed for manipulating their development, including transgenesis (Nüsslein-Volhard and Dahm 2002).

The embryonic development of the zebrafish has been described in detail (Kimmel et al. 1995). The cleavage period lasts for about one and a half hours, during which the embryo divides to the 64 cell stage. Shortly afterward, the blastula stage begins and lasts for three hours. This is the period shortly before gastrulation and is also the time when the midblastula transition occurs and the yolk syncytial layer forms. Gastrulation occurs 5 to 10 hours post fertilization (hpf). This phase allows cell movements and changes in shape, producing the primary germ layers and the embryonic axis. During the segmentation period (10-24 hpf), the primary organs are visible, the somites have developed and the embryo elongates. It is during this time that the first cells differentiate morphologically. The pharyngula period extends from 24-48 hpf and during this time the branchial arches, which give rise to the jaws and gills are prominent. The pharyngula period is followed by hatching at 48-72 hpf, at which time the fish is considered to be a larva.

Head cartilages first appear at 45 to 48 hpf and are mostly derived from neural crest cells that migrate ventrally from the hind- and midbrain (Schilling et al. 1996). By 5 dpf, the cartilaginous head skeleton consists of the neurocranium, which surrounds the brain, and the viscerocranium, comprising the jaws and gill arches, collectively referred to as pharyngeal arches (Piotrowski et al. 1996). The main component of the neurocranium examined in the present study is the ethmoid plate (ep), underlying the brain at the anterior of the head (Fig 1A). The first pharyngeal, or mandibular, arch will become the jaws, with Meckel's cartilage (mc) representing the lower jaw and the palatoquadrate (pq)

the upper jaw. The second or hyoid arch includes the ventral midline basihyal (bh), ventro-lateral ceratohyals (ch) and dorsal hyosymplectics (hs). The mandibular and hyoid arches are followed by five gill arches, consisting at this stage of median basibranchials (bb) and lateral ceratobranchials (cb1-5).

The barbels of the zebrafish have recently been characterized as a model for studying tissue growth and repair (LeClair and Topczewski 2010). They consist in adults of a large maxillary pair and a smaller nasal pair, both of which start to bud at 30-40 days post fertilization (dpf). Zebrafish barbels are covered with taste buds innervated by extensions of the facial nerves. In addition to blood vessels, they contain a central rod of connective tissue that is likely collagen, keratin, or elastin, but not cartilage or bone (LeClair and Topczewski 2010).

Another recently developed model for barbel development is the Channel catfish, *Ictalurus punctatus* (Hawkins 2011). This species has four pairs of barbels that appear much earlier in development than those of the zebrafish, beginning at approximately 4 dpf (Northcutt 2005; Hawkins 2011). An additional difference with the barbels of the zebrafish is that Channel catfish barbels have a central rod made of cartilage (Joyce and Chapman 1978; Hawkins 2011).

Hypothesis

In addition to investigating further the deep homology of barbels and other vertebrate appendages, the present study is intended to test the hypothesis that the differences in the timing of barbel appearance between the zebrafish and the channel catfish result from the heterochronic (different developmental time) deployment of *Shh*

and *Fgf8* induction signals. This hypothesis is based on the requirement for *Shh* and *Fgf8* for channel catfish barbel development (Hawkins 2011) and the ability of *Shh* and *Fgf8* singly and in combination to induce other appendages (Crossley et al. 1996; Abzhanov and Tabin 2004; Brito et al. 2008; Gillis et al. 2009). A prediction of this hypothesis is that early activation of *Fgf8* and/or *Shh* signaling in the zebrafish will induce barbel initiation during early larval development. To test the hypothesis, transgenic methods were used to ectopically and heterochronically express *Fgf8* and *Shh* in the zebrafish.

Materials and Methods

Animals

Wild type zebrafish embryos were obtained from natural spawnings of adults from a commercially available strain, as well as the inbred Tübingen (TU) line (Nüsslein-Volhard and Dahm 2002). Embryos were reared at 28.5 °C in 30% percent Danieau solution (58 mM NaCl, 0.7 KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂), to which 0.03% 1-phenyl-2-thiourea was added to inhibit pigmentation. All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the University of Colorado.

Transient transgenic overexpression of zebrafish proteins

DNA constructs for the heat-inducible overexpression of *Shh* and *Fgf8* have been described previously (Yamamoto et al. 2009; Jackman et al. 2013). These constructs, designated *hsp70:fgf8-gfp* and *hsp70:shh-gfp*, contained the cDNA coding region of the zebrafish gene of interest fused at its 3' end to *Green fluorescent protein (Gfp)*. The fusion

proteins were regulated by the heat-inducible zebrafish *hsp70* promoter, allowing ubiquitous expression following heat shock (Halloran et al. 2000). The plasmid constructs additionally contained I-Sce I meganuclease restriction sites for enhancing chromosomal integration of the transgenes (Rembold et al. 2006). A similarly constructed plasmid containing *Gfp* as the only coding sequence (*hsp70:gfp*) was used as a control for the effects of DNA injection.

Plasmid DNA (40 ng/ μ l in I-Sce I buffer with 0.2% phenol red dye) was incubated with I-Sce I meganuclease, following Rembold et al. (2006). 1 nl of this mixture (containing 40 pg DNA) was injected into the blastomere of one cell stage embryos. Induction of transgene expression was accomplished by incubation at 40 °C for 1 hr at 12 hpf, 24 hpf, or 36 hpf. Larvae were sacrificed at 4 dpf by tricaine methanesulfonate overdose. This age was chosen because of the potentially lethal effects of Fgf overexpression by later ages (Jackman et al. 2013), the lack of dependence of larvae at this age on feeding, (Kimmel et al. 1995), and the observation that Channel catfish of a similar developmental stage have already initiated barbel development (Hawkins 2011). Following euthanasia, larvae were fixed overnight at 4 °C with 4% formaldehyde in phosphate-buffered saline (PBS) pH 7.4. They were then rinsed in PBS and transferred to 100% ethanol for storage.

Clearing and Staining

In order to visualize the relationship between any ectopic barbels obtained and the head skeleton, fixed larvae were cleared and stained for cartilage and bone using alcian blue and alizarin red dyes following Walker and Kimmel (2007). This technique was

expected to facilitate the identification of barbels if they included a cartilaginous rod, as seen in the early-developing barbels of the Channel catfish (Hawkins 2011).

Alcian blue stains the proteoglycans associated with the extracellular matrix of cartilage cells (chondrocytes), while alizarin red stains calcium found in bones and the dentine of teeth. Incorporation of $MgCl_2$ in the staining process instead of an acid prevented decalcification of bones and also allowed differentiation of different types of mucosubstances (Walker and Kimmel 2007). Such differentiation is possible because Mg^{2+} competes with the alcian dye for negative charges in the polysaccharides in matrix.

Light Microscopy

Cleared and stained larvae were examined for head skeletal abnormalities and ectopic barbels (head projections with or without cartilage supports) using a stereomicroscope (Leica MZ12.5). Selected specimens were imaged with a Zeiss AxioCam digital camera mounted on an inverted compound microscope (Zeiss Axiovert 135).

Scanning electron microscopy

Two larvae with potential ectopic barbels and two wild type controls were subjected to scanning electron microscopy (SEM) to increase contrast and depth of field and thereby obtain a better idea of the shape of head projections. Glycerol was rinsed from the cleared and stained, DNA-injected specimens with increasing amounts of PBS and the specimens were then dehydrated in increasing concentrations of ethanol. The wild type controls were not subjected to clearing and staining, but instead had been fixed and stored in methanol. Specimens in 100% ethanol were then placed in porous containers and dried

in a Tousimis Autosamdri 815 critical point dryer. The dried specimens were then mounted on aluminum stubs with nail polish. After overnight drying, the specimens were sputter coated with gold using a Cressington Model 108 sputter coater. The gold-coated specimens were then imaged with a JEOL JSM-6480 scanning electron microscope.

Results:

Control DNA injections resulted in a small percentage of larvae with cartilage abnormalities

In order to determine the effects of DNA injection itself on larval morphology, embryos were injected with the *hsp70:gfp* construct and heat shocked at 12 or 24 hpf. 78 of 89 injected larvae (88%) exhibited a phenotype indistinguishable from the wild type (Fig. 1A, B). The remaining 11 larvae exhibited cartilage defects, consisting mostly of reduction of element size or staining (Fig 1C). Pericardial edema (fluid filled swelling around the heart) was also observed. No *hsp70:gfp*-injected larvae exhibited ectopic cartilages or outgrowths of the head.

Ectopic Fgf8 expression resulted in abnormalities of the head skeleton but no structures resembling barbels

The sufficiency of *Fgf8* alone for inducing ectopic cartilage was tested with injection of the *hsp70:fgf8-gfp* construct followed by heat shock at 12 or 24 hpf and clearing and staining at 4 dpf. Abnormal phenotypes were not quantified, but included reduction in size or absence of cartilages, as well as severe disorganization of the cartilage pattern (Fig. 1D). In some cases, eyes were absent as well (Fig. 1D). Of the 125 embryos injected with this construct, none had unambiguous ectopic cartilages or outgrowths of the head.

Ectopic Shh expression resulted in abnormalities of the head skeleton but no structures resembling barbels

Injection of the *hsp70:shh-gfp* construct alone followed by heat shock at 12 or 24 hpf and clearing and staining at 4 dpf resulted in abnormal phenotypes resembling those obtained for *Fgf8* overexpression, including missing or disorganized cartilages (Fig 1E, F) and missing eyes (Fig. 1F). While not quantified, the severity of abnormal phenotypes appeared to be less for the *Shh* construct than for the *Fgf8* construct. Of the 83 embryos injected with *hsp70:shh-gfp*, none had unambiguous ectopic cartilages or outgrowths of the head.

Ectopic coexpression of Shh and Fgf8 yielded a large range of abnormalities of the head skeleton, including ectopic cartilages and a projection from the lower jaw

187 of 387 (48%) of larvae co-injected with the constructs *hsp70:fgf8-gfp* and *hsp70:shh-gfp* and heat shocked at 12, 24 or 36 hpf exhibited cartilage anomalies (Fig 2, 3). As with single injections of the constructs, these anomalies included reduced or missing elements, as well as severe disorganization of the skeletal pattern. Unlike the case for single injections, ectopic cartilages were seen in 13 individuals (e.g. Fig 2B, C; Fig 3B-F). Locations of such cartilages included anterior to the ethmoid plate (Fig. 2B-C), as well as projecting from or anterior to Meckel's cartilage (Fig. 3B-F).

Because of the resemblance of ectopic cartilages in the vicinity of the jaws to the barbels of the Channel catfish, two specimens with such cartilages were investigated further with SEM to determine whether they were associated with projections from the

head resembling barbels. One specimen (Fig 3B-C) had a broad-based projection from the face inferred to be supported by the ectopic cartilage and located lateral to a mouth opening of reduced size (Fig 4D-E). All of the cells visible on this projection exhibited the morphology characteristic of fish epithelial cells (including microridges – Elliot 2000) and none that of taste buds (Fig 4F). The other fish examined by SEM possessed a loop in one half of Meckel's cartilage and an ectopic cartilage anterior to the loop (Fig. 3D). SEM revealed a continuous bar of tissue, inferred to be supported by the ectopic cartilage, and connecting the upper and lower jaws (Fig. 4G-H). This bar separated two openings of unequal size, the larger of which appears to be a mouth of fairly normal morphology, and the smaller of which may represent an aberrantly shaped ectopic mouth (Fig. 4I). This ectopic mouth is underlain by the looped half of Meckel's cartilage.

Discussion

*Specificity of the morphological anomalies seen with *Fgf8* and *Shh* overexpression*

To determine whether the injection process itself or the amount of DNA injected was causing the aberrant phenotypes observed following *Fgf8* and *Shh* overexpression, embryos were injected with an equivalent concentration of *hsp70:gfp*. 12% of these control embryos exhibited altered morphology of the cartilaginous head skeleton. In contrast, 48% of the embryos co-injected with *hsp70:fgf8-gfp* and *hsp70:shh-gfp* exhibited such a phenotype. The number of affected individuals is significantly higher for this treatment compared to the control ($p=8 \times 10^{-11}$, Fisher's Exact Test), suggesting that overexpression of *Fgf8* and/or *Shh* was influencing cartilage morphology. The altered cartilage morphology

seen in some of the control injections may be the result of damage from the injection needle or the toxicity of high concentrations of DNA (Rembold et al. 2006).

Additional support for the specificity of the results obtained is provided by the nature of the phenotypes observed. For example, eye loss, which was seen following overexpression of *Fgf8* and of *Shh*, has been also been described following *Fgf8* overexpression in another fish species, the Japanese medaka (Bajoghli et al. 2004), and overexpression of *Shh* in the zebrafish (MacDonald et al. 1995). Increased expression of *Shh* has also been proposed as part of the mechanism for evolutionary loss of eyes in a blind cavefish (Yamamoto et al. 2004). Manipulation of *Shh* or *Fgf8* expression has been shown to influence the pattern of both the neurocranial and the viscerocranial cartilages in the zebrafish (Walshe and Mason 2003; Crump et al. 2004; Wada et al. 2005; Eberhart et al. 2006; Schwend and Ahlgren 2009; Swartz et al. 2012). The reduction and loss of elements we observed in some cases are actually characteristic of loss of function of these genes, however, suggesting that there may be upper and lower thresholds for expression levels that are compatible with cartilage element formation.

Overexpression of Fgf8 and Shh induces ectopic cartilage formation

3% of the larvae co-injected with *hsp70:fgf8-gfp* and *hsp70:shh-gfp* developed ectopic cartilages. Whether this phenotype requires overexpression of both genes remains unclear, however. While no ectopic cartilages were seen with single injection of *hsp70:fgf8-gfp* or *hsp70:shh-gfp*, only for the former construct are the numbers significantly different from those obtained for co-injection ($p = 0.045$ and 0.138 , respectively, Fisher's Exact Test). In the chick, ectopic expression of *Fgf8* and *Shh* synergizes to produce ectopic head

cartilages (Abzhanov and Tabin 2004). While *Fgf8* expression alone was capable of inducing cartilage nodules and expression of *Shh* induced outgrowths from the head that lacked cartilage, co-expression was required to induce cartilaginous outgrowths. In contrast to the results obtained by Abzhanov and Tabin (2004), other studies showed that *Shh* overexpression alone was capable of inducing ectopic cartilage outgrowths in the chick (Haworth et al. 2007; Brito et al. 2008). It is possible that ectopic *Shh* expression was being induced in the vicinity of endogenous *Fgf8* expression in the latter studies, making it difficult to disentangle the effects of *Fgf8* and *Shh* in studies such as the present one.

Are Shh and Fgf8 overexpression sufficient to induce barbels?

The structure most resembling a barbel induced by overexpression of *Fgf8* and *Shh* in the present study was an ectopic branch from Meckel's cartilage that supported a broad-based projection from the lower jaw (Fig. 3B, C; 4D-F). This structure is more reminiscent of a catfish barbel than a zebrafish barbel, as only the former has a cartilage support (LeClair and Topczewski 2010; Hawkins 2011). Interestingly, catfish barbels are formed early in development, as was the structure induced by *Fgf8* and *Shh* in the zebrafish. Components common to all teleost fish barbels include an outer epithelium, dermal connective tissue, blood vessels, and facial nerves that reach to taste buds (Fox 1999; LeClair and Topczewski 2010). Of these, only the outer epithelium was obvious in the injected fish and the cells resembled those of the surrounding face. Further development of the larva would have been required to determine whether taste buds were formed and the presence of blood vessels and nerves was not assayed.

The cartilage-supported projection induced in the present study by *Shh* and *Fgf8* overexpression most closely resembles the branches from Meckel's cartilage induced by *Shh* overexpression in the chick (Haworth et al. 2007; Brito et al. 2008). Such a branch was observed in an additional fish (Fig 3E-F) and the looped Meckel's cartilage of another fish (Fig 3D) may result from similar abilities of *Shh* and *Fgf8* to modify the direction of growth of this cartilage. While the similarity of the ectopic zebrafish cartilages to ectopic beaks in the chick might argue against the former being barbels, it is also possible that the conserved ability to induce ectopic projections from the jaw was co-opted in the origin of fish barbels.

Future directions

The transgenic experiments of the present study were designed to take advantage of the mosaic incorporation of DNA into the chromosomes of injected fish (Nüsslein-Volhard and Dahm 2002). Such mosaicism is the result of transgene integration at a stage later than the one cell stage at which embryos are injected. Normal and ectopic beaks in the chick are thought to arise at the boundaries between *Shh* and *Fgf8* expressing cells (Abzhanov and Tabin 2004). Such boundaries would not occur in transgenic lines of zebrafish uniformly expressing the two genes under the control of the *hsp70* promoter, but might occur if random integration of the *Fgf8* and *Shh* occurred independently in adjacent cells. Whether such incorporations in adjacent cells or patches of cells occurred was not directly assayed in the present study, and it remains possible that more obvious barbels could be induced in a larger sample of injected individuals. Additional experimentation with the timing of heat shock would also be valuable.

Potential future experiments with more predictable localization of SHH and FGF8 proteins include implantation of beads soaked in FGF8 and SHH or cells expressing these genes into the head of either zebrafish or catfish larvae. Such experiments could be combined with more specific assays for barbel characters, such as immunostaining for taste buds.

Conclusion

The present study demonstrates that *Fgf8* and/or *Shh* are sufficient to induce cartilage-supported outgrowths from the jaw region during early zebrafish development. While such outgrowths do not possess all of the defining features of barbels, these results, along with evidence of the necessity of *Shh* and *Fgf8* for barbel development (Hawkins 2011), suggest that regulation of their expression is a plausible mechanism for the evolutionary origin of barbels. Additional support for such a mechanism would bolster the case for the deep homology of barbels and other vertebrate appendages.

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Figure Legends

Figure 1. Cartilage phenotypes produced by single injections of *hsp70:gfp*; *hsp70:fgf8-gfp*, and *hsp70:shh-gfp*. All embryos were cleared and stained for cartilage with alcian blue at 4 dpf; A) Uninjected wild type. B) *hsp70:gfp* injected with wild type phenotype. C) *hsp70:gfp* injected with reduced staining of gill arches and severe edema. Meckel's cartilage and ceratohyal reduced in size. Eyes present but not in focus. D) *hsp70:fgf8-gfp* injected with unilateral loss of eye, severe edema and extremely disorganized cartilages. White asterisks indicate potential unfused halves of Meckel's cartilage and black asterisk potential ethmoid plate. Possible ectopic cartilages anterior to Meckel's cartilage, but abnormally shaped endogenous cartilages cannot be ruled out. E) *hsp70:shh-gfp* injected with reduced gill arch skeleton and potentially missing Meckel's cartilage. F) *hsp70:shh-gfp* injected missing eyes with severe edema and disorganized cartilages precluding identification of homologies. Abbreviations: bb, basibranchial; bh, basihyal; cb1-4 = 1st-4th ceratobranchials; ch, ceratohyal; ep, ethmoid plate; he, heart; hs, hyosymplectic; mc, Meckel's cartilage; pq, palatoquadrate.

Figure 2. Representative phenotypes produced by coinjection of *hsp70:fgf8-gfp* and *hsp70:shh-gfp*. All embryos were cleared and stained for cartilage with alcian blue at 4 dpf; A) Uninjected wild type. B-F) *hsp70:fgf8-gfp*; *hsp70:shh-gfp* coinjected. B-C) Low and high magnification views of fish with ectopic cartilage (asterisk) lateral to ethmoid plate. D) Meckel's cartilage severely reduced on one side (asterisk) and absent on the other. E) Meckel's cartilage missing on at least one side. Identity of cartilage indicated by asterisk unknown. F) Eyes missing, severely disorganized cartilages. Meckel's cartilage likely absent. Possible misshapen ethmoid plate indicated by asterisk. Abbreviations: cb1-4 = 1st-4th ceratobranchials; ch, ceratohyal; ep, ethmoid plate; hs, hyosymplectic; mc, Meckel's cartilage; nc, notochord; ot, otolith; pq, palatoquadrate.

Figure 3. Ectopic cartilages potentially resembling barbels produced by coinjection of *hsp70:fgf8-gfp* and *hsp70:shh-gfp*. All embryos were cleared and stained for cartilage with alcian blue at 4 dpf; A) Uninjected wild type. B-F) *hsp70:fgf8-gfp*; *hsp70:shh-gfp* coinjected. B-C) Low and high magnification views of fish with ectopic cartilaginous projection from Meckel's cartilage (asterisk). D) Ectopic cartilage (asterisk) anterior to Meckel's cartilage. One half of Meckel's cartilage appears to be bent into a loop. E-F) Low and high magnification views of fish with ectopic cartilage (asterisk) intercalated between the two halves of Meckel's cartilage. Abbreviations: cb1-4 = 1st-4th ceratobranchials; ch, ceratohyal; ep, ethmoid plate; he, heart; hs, hyosymplectic; mc, Meckel's cartilage; pq, palatoquadrate.

Figure 4. SEM of zebrafish larvae. A) Uninjected wild type at 72 hpf, 200x. B-C) Uninjected wild type at 96 hpf. Mouth has migrated more anteriorly compared to 72 hpf, 190X and 370X. D-F) *hsp:fgf8-gfp* and *hsp70:shh-gfp* coinjected and heat shocked at 36 hpf, 230X, 700X, 950X. D) Head of the coinjected embryo with the projection from Meckel's cartilage projection seen in alcian stains indicated by white asterisk. E) Mouth of zebrafish smaller than wildtype. Possibly interrupted by ectopic projection. F) Ectopic projection on Meckel's cartilage supports a broad anterior projection lateral to a reduced mouth opening. G-I)

hsp70:fgf8-gfp and *hsp70:shh-gfp* coinjected and heat shocked at 12 hpf, 330X 650X, 2700X. G) Head with misshapen mouth and possible ectopic mouth. H) Higher magnification of the two mouths separated by epithelium. Ectopic cartilage anterior to Meckel's cartilage indicated by white asterisk and the looped Meckel's cartilage indicated by black asterisk. I) Ectopic mouth formation. Ectopic mouth is supported by the ectopic cartilage, previously observed with alcian stains, with a connection to the upper jaw, separating the two mouths. Abbreviations: e, eye; l, lens m, mouth; mc, Meckel's cartilage; n, nose.

Figure 1

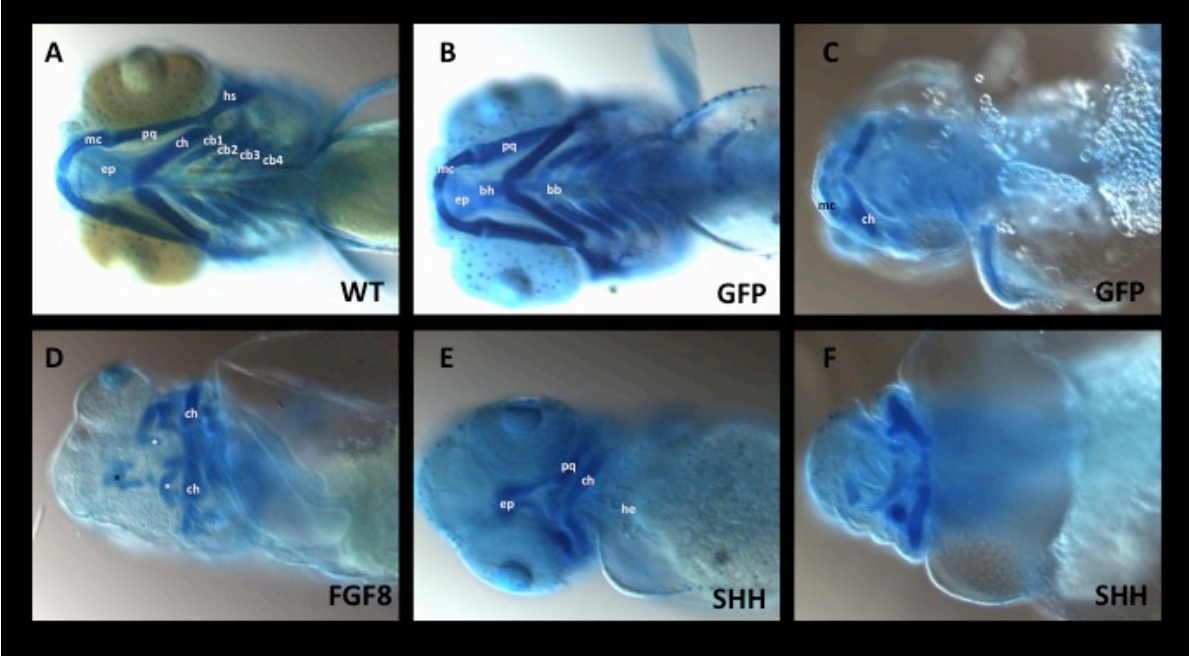


Figure 2

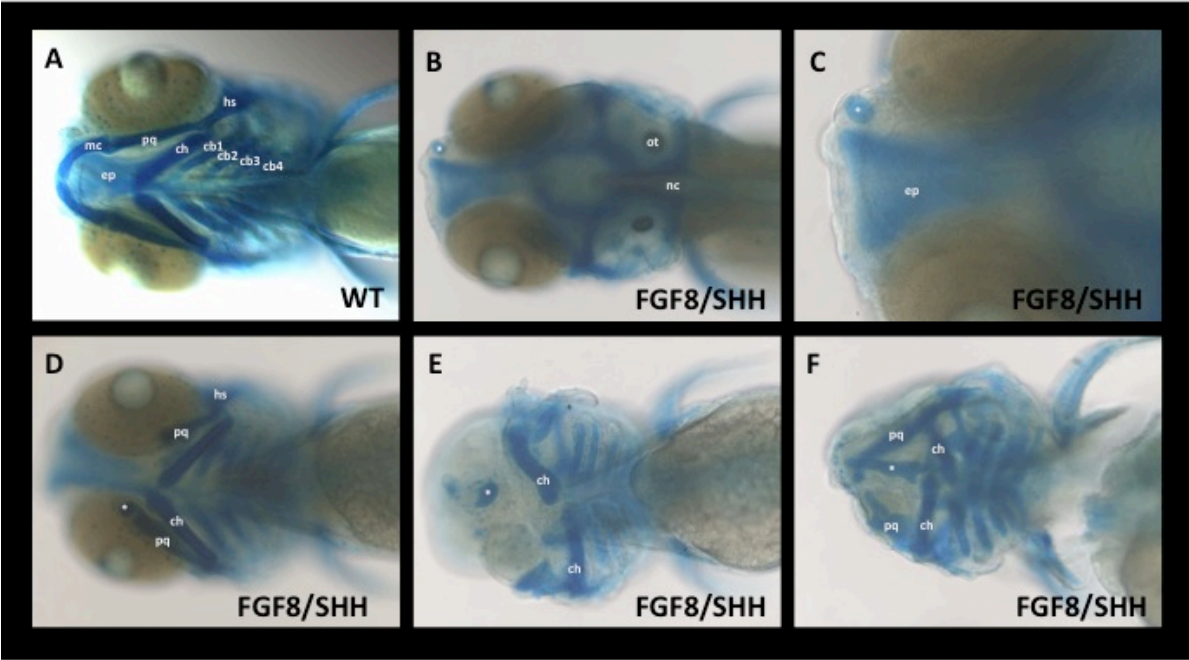


Figure 3

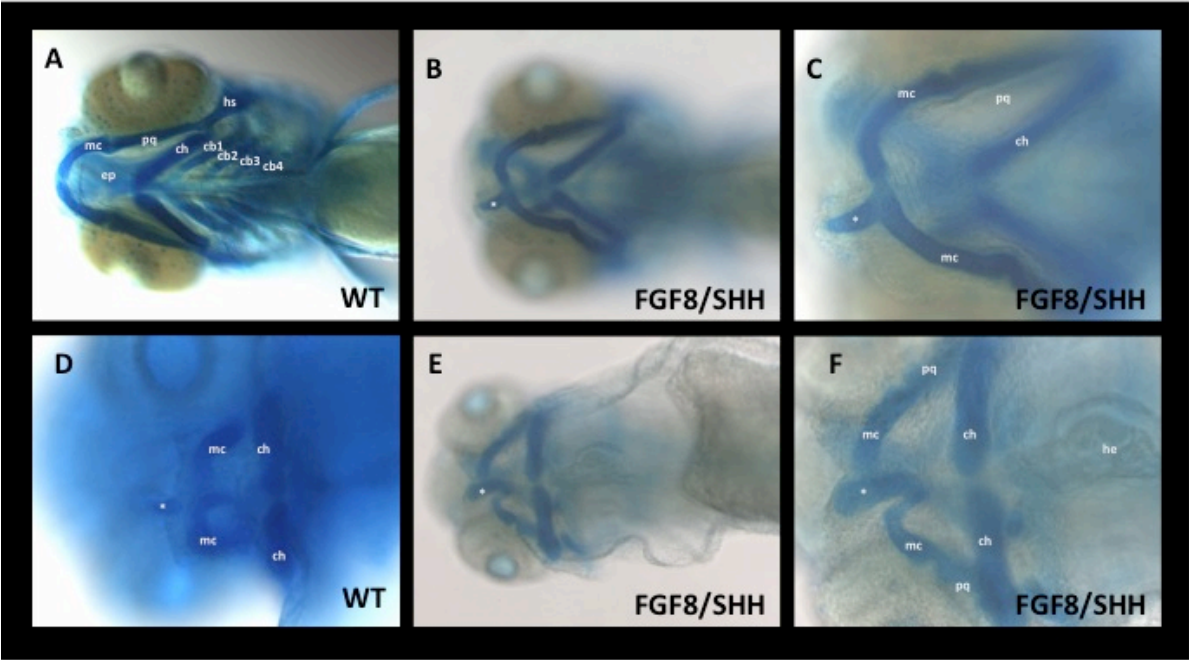


Figure 4

