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FGF and BMP signaling are required for specifying pre-chondrogenic identity in neural crest derived mesenchyme and initiating the chondrogenic program

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Summary

The pharyngeal endoderm is hypothesized as the source of local signals that specify the identity of neural crest-derived mesenchyme in the arches. *Sox9* is induced and maintained in pre-chondrogenic cells during condensation formation and endochondral ossification. Using explant culture we determined that pharyngeal endoderm was sufficient, but not necessary for specifying pre-chondrogenic identity, as surrounding tissues including the otic vesicle can compensate for signals from the pharyngeal endoderm. Multiple *Fgf* genes are expressed specifically in the pharyngeal endoderm subjacent to the neural crest-derived mesenchyme. FGF signaling is both sufficient and required for specification of *Sox9* expression and specification of pre-chondrogenic identity, as demonstrated by the addition of recombinant FGF protein or the FGF receptor inhibitor (SU5402) to explanted tissue, respectively. However, FGF signaling cannot maintain *Sox9* expression or initiate the chondrogenic program as indicated by the absence of *Col2a1* transcripts. BMP4 signaling can induce and maintain *Sox9* expression in isolated mesenchyme, but only in combination with FGF signaling induce *Col2a1* expression, and thus, chondrogenesis. Given the spatio-temporal expression patterns of FGFs and BMPs in the pharyngeal arches, we suggest that this may represent a general mechanism of local signals specifying pre-chondrogenic identity and initiation of the chondrogenic program.

Keywords

BMP4; *Col2a1*; columella; condensation; chondrogenesis; development; endochondral; FGF; pharyngeal arch; *Sox9*; SU5402

Introduction

The mechanism involved in induction and patterning of neural crest (NC)-derived Hox-positive mesenchyme in the pharyngeal arches is an area of intense interest (Creuzet et al., 2005). Mesenchyme of the 2nd pharyngeal arch gives rise to several skeletal elements, including the retroarticular process, columella, posterior basihyoid and ceratobranchial elements of the tongue skeleton (Ruhin et al., 2003; Kulesa et al., 2004). NC migrates from the chick midbrain region at Hamburger and Hamilton (HH)9 (Hamburger and Hamilton, 1951) in an anterior to posterior wave, with the hyoid stream beginning its migrating from rhombomere 4 (r4) at approximately HH11 (Couly et al., 1992). Small r3 and r5 contributions, which do not undergo apoptosis, add to the anterior and posterior hyoid stream, respectively (Kulesa and Fraser, 2000; Graham et al., 2004; Kulesa et al., 2004).

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Cranial NC migration is completed by HH14 with proximal and distal populations forming medial to more lateral hyoid elements (Kontges and Lumsden, 1996).

Avian development of the hyoid derived cartilage depends on signals that arise from pharyngeal endoderm (Ruhin et al., 2003; Creuzet et al., 2004a). Removal of anterior to progressively more posterior stripes of medial, or lateral foregut endoderm at premigratory stages, demonstrates that the pharyngeal endoderm is required for patterning of specific skeletal elements. At the level of the 2nd arch, these include elements such as the basihyoid and ceratobranchial (Ruhin et al., 2003). Turning mesenchyme into cartilage condensations necessitates that epithelial-mesenchymal interactions perform several functions. Migrating skeletogenic mesenchyme is first localized, with local epithelial-mesenchymal interactions influencing the size of the progenitor population for each cranial skeletal element, providing condensation initiation signals, and then permitting differentiation of chondrocytes and, finally, ossification (Hall, 2008). Previous studies looked at the presence or absence of cartilage elements, but did not examine the intervening steps to determine when the failure took place (Creuzet et al., 2005). Mesenchyme localization, specification of pre-chondrogenic identity or the chondrogenic program might each be affected.

The columella arises from NC mesenchyme that migrates to the proximal 2nd arch, apposing the pharyngeal endoderm (Kontges and Lumsden, 1996). The single chick middle ear bone, the columella, is a composite structure consisting of persistent and replacement cartilage elements. The proximal bony columella inserts into the oval window of the cochlear duct and the more distal extracolumella cartilage inserts into the tympanic membrane, spanning the width of the middle ear cavity (Jaskoll and Maderson, 1978; Wood et al., 2010). The effect of signals from the subjacent pharyngeal endoderm on the proximally localized mesenchyme of the columella was not analyzed in previous studies (B. Ruhin and N. le Douarin, personal communication). We wanted to know if the pharyngeal endoderm provided signals sufficient and necessary for specifying the putative columella mesenchyme to a chondrogenic fate. The positioning of the columella mesenchyme and endoderm make an ideal tissue to investigate the intervening time points between migration and chondrogenesis.

We hypothesize that signals required for specifying pre-chondrogenic identity and the onset of the chondrogenic program in this mesenchymal population are localized to the subjacent pharyngeal endoderm. To examine this question extirpated tissues from the middle ear region were grown in collagen gel culture. *Sox9,* a member of the high mobility group (HMG) domain containing transcription factors, is implicated in NC specification, with *Sox9* expression in mesenchyme a marker of pre-chondrogenic identity (Cheung and Briscoe, 2003; Wood et al., 2010). The onset of *Col2a1* expression indicates the initiation of the chondrogenic program at E7.5 in chick (Zhao et al., 1997; Eames et al., 2004; Betancur et al., 2010). Through our tissue recombination experiments we show that the pharyngeal endoderm adjacent to the post-migratory mesenchyme, giving rise to the putative columella condensation, is sufficient, but not required to induce *Sox9* expression and, thus, pre-chondrogenic identity.

Endodermal signals arising in the caudal part of the foregut influence the Hox-positive mesenchyme in the 2nd and more posterior arches, inducing pre-chondrogenic identity (Ruhin et al., 2003). Reciprocal signaling between the Fgf8 expressing endoderm and NC cells in the facial region is required for condensation formation (Creuzet et al., 2004b). Numerous FGF genes with spatially restricted expression patterns are present in the region (Ohyama et al., 2007; Schimmang, 2007), potentially mediating these epithelial-mesenchymal interactions (Shigetani et al., 2000). Therefore, we surveyed known FGF family members to identify *Fgf* genes expressed in the pharyngeal endoderm. Embryos were

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analyzed at the migratory and post-NC migratory stages HH14 and HH18/19 when mesenchymal *Sox9* expression is first detected.

Multistep crosstalk is required between the endoderm and NC-derived mesenchyme, leading to cartilage formation (Creuzet et al., 2005). Our functional studies demonstrate that FGF signaling is both sufficient and necessary for induction of pre-chondrogenic identity, but cannot maintain *Sox9* expression to initiate the chondrogenic program. Expression of the chondrocyte marker *Col2a1* is, however, elicited with pharyngeal endoderm present, indicating the requirement for a second signal. BMP4 signaling alone is able to induce and maintain *Sox9* expression, but it is the combination of BMP and FGF signaling that is required for induction of the chondrogenic program. We show that BMP4 in combination with FGF8 is sufficient to initiate the chondrogenic program and suggest that this may be a general patterning mechanism within the more posterior arches, which all express combinations of these secreted signaling molecules.

Results

Pharyngeal endoderm is sufficient to induce *Sox9* expression

Ablation of caudal endoderm results in loss of ceratobranchial and epibranchial cartilage elements (Ruhin et al., 2003); however, the timing of the mechanism involved has not been closely examined. We hypothesize that signals from pharyngeal endoderm are sufficient to specify pre-chondrogenic identity, which is required for condensation and chondrogenesis to occur. Pharyngeal endoderm and the proximal mesenchyme of the 2nd arch are directly apposed (Fig. 1). It is likely that local signals, specifying the NC-derived mesenchyme pre-chondrogenic fate, arise from the endoderm. Neural crest cells located in the dorsal neural tube have *Sox9* expression at premigratory stages HH9-12, which is down regulated at the onset of migration (Cheung and Briscoe, 2003). The NC migrates to the second arch and is located proximally at the entrance to the arch and distally within the arch pouch by HH14. The putative skeletogenic mesenchyme does not regain *Sox9* expression until HH18 (Cheung and Briscoe, 2003)(our unpublished results).

To test the sufficiency of pharyngeal endoderm to induce *Sox9* expression in mesenchyme we used an explant culture system. Mesenchyme from the proximal 2nd arch region, including cells fated to form the columella (the single middle ear bone of the chick) (Wood et al., 2010; Chapman, 2011) were explanted with or without the subjacent endoderm from HH14, HH19 and HH24 embryos into collagen gel culture (Exp. 1-3, Table 1). The more distal mesenchyme within the 2nd arch pouch was excluded (Fig. 1, see Experimental Procedures for a detailed description).

Mesenchymal tissue explanted at HH14 was grown to embryonic day (E)6 and analyzed for the presence of *Sox9* transcripts (Exp. 1, Fig. 2A). Mesenchyme with endoderm present expressed *Sox9* in all cases (n=36/36, Fig. 2), whereas explants of mesenchyme alone did not (n=4/26). Tissue sections of zero hour explants show mesenchyme with endoderm or mesenchyme alone as expected (not shown). We also confirmed that transplanted mesenchyme does not express *Sox9* at HH14 (zero hour explants, not shown). The small number of explants with *Sox9* expression likely included remnants of pharyngeal endoderm. Cleanly separating the thin endodermal layer is challenging without the use of enzymes. To avoid disrupting secreted signaling factors we avoided the use of enzymes to separate these tissues. This result indicates that signals from pharyngeal endoderm are sufficient for induction and maintenance of *Sox9* expression.

From HH18, mesenchyme fated to form skeletal condensations expresses *Sox9* (not shown). We wanted to know if the expression of *Sox9* in this tissue indicated that it was specified to

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a pre-chondrogenic identity and would thus maintain *Sox9* expression when cultured in isolation. Mesenchyme was explanted from HH19 and HH24 embryos and grown to E6 with or without pharyngeal endoderm present (Exp. 2 and 3, Fig. 2). When cultured with pharyngeal endoderm, HH19 and HH24 explants maintained *Sox9* expression at E6 (n=3/4 and 8/8, respectively, Table 1). When cultured alone, in the absence of pharyngeal endoderm, mesenchyme also maintained *Sox9* expression (n=3/3 and 8/8, respectively, Fig. 2). This experiment indicates that mesenchyme was able to maintained *Sox9* expression in the absence of any signals from the pharyngeal endoderm and the cells were thus specified as pre-chondrogenic by HH19.

These results support our hypothesis that signals from the pharyngeal endoderm at post-migratory stages (HH14 onward) are sufficient to induce *Sox9*, specifying the NC-derived mesenchyme to a pre-chondrogenic fate, and that by HH19 these signals are no longer required to maintain *Sox9* expression. Moreover, this experiment confirms that the NC requires local epithelial derived signals and is not pre-patterned to a pre-chondrogenic fate. Furthermore, based on these results, the surface ectoderm subjacent to which these cells migrate does not appear to be required for the onset of *Sox9* expression.

Pharyngeal endoderm is not required for *Sox9* expression

Although sufficient, it is not clear if pharyngeal endoderm is required for *Sox9* expression. Ablation of subjacent pharyngeal endoderm in ovo results in the loss of the hyoid skeletal elements: the ceratobranchial and epibranchial cartilages. In these experiments, whole mount embryos were manipulated at HH9 were incubated until E8 and then stained for the presence of cartilage (Ruhin et al., 2003). In this context, pharyngeal endoderm is required for skeletogenesis. However, the requirement for signals from the pharyngeal endoderm in inducing and maintaining *Sox9* expression, and thus pre-chondrogenic identity was not addressed.

A transverse slice of the head at the level of the 2nd arch (Fig. 1A) was explanted from HH14 embryos and cultured to E6 (Exp. 4, Fig. 2B). The slice included all the tissues at this level; neural tube, notochord, otic vesicle, mesenchyme, surface ectoderm, pharyngeal endoderm and blood vessels. The slice was hemisected into left and right sides. The intact left side slice acted as a controlwith all tissues left intact, whereas the pharyngeal endoderm was removed from the right hand slice, leaving all other tissues undisturbed (Fig. 2B). In both control and experimental tissues *Sox9* was expressed in all cases (n=10/10), demonstrating that although sufficient, pharyngeal endoderm is not required for *Sox9* expression and the specification of pre-chondrogenic identity. Signals from surrounding tissues must, therefore, compensate for the loss of the pharyngeal endoderm.

Signals from other tissues are sufficient to induce *Sox9* expression

It must be noted that our explant experiments likely include mesenchymal cells fated to become other skeletogenic structures, not just the proximal 2nd arch NC mesenchyme fated to become the columella. Some of the lateral otic capsule population is likely included when we extirpate the mesenchyme. The origin of otic capsule mesenchyme is more complex than for the proximally situated columella, with contributions from the first somite, cephalic mesoderm and NC (Couly et al., 1993), our unpublished data). For the otic capsule, endogenous inductive epithelial-mesenchymal signals originate from the placodal ectoderm of the otic epithelium (Hall, 2008). *Sox9* is a common marker of pre-chondrogenic cells and, thus, will label both otic capsule and columella populations. The only marker to distinguish between these populations is Peanut Agglutinin Lectin (PNA). Otic capsule mesenchyme does not label with PNA unless pre-treated with neuraminidase to remove the sialic acid residues present in this population, allowing us to distinguish 2nd arch mesenchyme from

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otic capsule (Wood et al., 2010). Thus, we could distinguish which pre-cartilaginous populations were present in our explants (Exp. 5, n=13/13, Fig. 2C). Explants with *Sox9* positive cells were incubated with PNA-HRP. Some *Sox9* cells were labeled with PNA, whereas others were not labeled. This result leads us to conclude that both NC-derived mesenchyme and otic capsule cell populations were included in our explants.

Next we determined which other tissues in the region have signals capable of inducing *Sox9* (Exp. 6). When otic vesicle was included with mesenchyme, *Sox9* was expressed in all cases (n=16/16, Fig. 2). As the otic epithelium is adjacent to the otic capsule mesenchyme, signals from the otic epithelium would be expected to normally induce and maintain *Sox9* expression in the otic capsule mesenchyme {Hall, 2008 #8}. Signaling molecules diffuse away from their source into the mesenchyme. It is likely that inducer molecules from the otic epithelium are able to substitute for signals from the pharyngeal endoderm and induce *Sox9* in the NC-derived mesenchyme. Of interest, removal of the otic placode does not affect development of the columella, indicating that although sufficient, signals from the otic epithelium are not required for columella development (Reagan, 1917). We also found that neural tube and notochord are sufficient to induce *Sox9* expression, although the endothelium of blood vessels was not (not shown).

A long-standing question is whether the surface ectoderm at these stages in the chick has the potential to specify the mesenchyme (Exp. 7). Our explants of pharyngeal endoderm and mesenchyme demonstrate that the surface ectoderm, past which NC migrates, is not required to induce *Sox9* expression (Exp. 1). However, ectoderm at the level of the pharyngeal groove, when combined with the adjacent post-migratory mesenchyme, resulted in 5/20 (25%) cases positive for *Sox9* expression. There are two possible explanations for this result. Firstly, the explant was contaminated with pharyngeal endoderm, giving a similar result to the baseline results for mesenchyme alone. Or secondly, that the surface ectoderm of the 2nd arch expresses an inducer, whereas the more dorsolateral surface ectoderm encountered during migration does not (Fig. 1B). Pharyngeal endoderm and surface ectoderm at this level both express *Fgf8*, a known inducer of *Sox9* (Monsoro-Burq et al., 2003). Thus, in some of our surface ectoderm and mesenchyme explants we may have inadvertently included the *Fgf8* expressing ventral ectoderm, leading to induction of *Sox9* expression. These results lead us to survey the spatio-temporal expression patterns of *Fgf* genes in the surrounding tissues.

Spatiotemporal analysis of *Fgf* expression in the ear-forming region

Neural crest migrates into a proximal position in the 2nd pharyngeal arch, undergoes condensation and by endochondral ossification forms the single middle ear bone of the chick, the columella (Wood et al., 2010). We hypothesize that FGF signals are required for induction of *Sox9* and specifying pre-chondrogenic identity. Ectopic hyoid (tongue) cartilage is evident when additional endoderm or FGF8 beads are grafted into the 2nd arch region (Ruhin et al., 2003; Creuzet et al., 2004a). However, the effect on the columella was not analyzed (B. Ruhin and N. le Douarin, personal communication). We analyzed *Fgf* expression patterns to identify the spatio-temporal expression of FGF family members in the region. Cranial NC migration begins at HH9 in the midbrain and progresses in an anterior to posterior direction, migrating at HH11 from rhombomere 4 toward the 2nd arch. We, therefore, chose to analyze *Fgf* expression using in situ hybridization at HH9 (pre-migration), HH14 (post-migration) and at HH19 when PNA labeling and *Sox9* expression indicates the first signs of NC differentiation into pre-chondrogenic mesenchyme (Wood et al., 2010). *Sox9* expression is maintained in chondrogenic cells of the columella up to E8, when it is down regulated in anticipation of ossification, as marked by the onset of Ihh expression at E10 (Wood et al., 2010).

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At pre-migration stages *Fgf3, 4, 8* and *19* are expressed in the endoderm fold adjacent to where NC-derived mesenchyme will migrate (Karabagli et al., 2002; Paxton et al., 2010). Following completion of NC migration at HH14, expression of *Fgf3, 4, 8* and *19* are maintained in the fold of the pharyngeal endoderm (Fig. 2A-D). *Fgf*3 and *Fgf*4 are weakly expressed (Fig. 2a, b), whereas *Fgf8* and *Fgf19* have strong expression (Fig. 2c, d), with *Fgf8* also being expressed in the surface ectoderm at the groove of the arch (asterisk).

At HH19, *Fgf*3 is expressed in the pharyngeal endoderm between the first and second arch (Fig. 3A). *Fgf8* and *Fgf19* are expressed in the pharyngeal endoderm at the anterior edge of the 2nd arch (Fig. 3B, C). *Fgf8* is no longer expressed in the surface ectoderm. *Fgf*4 expression has strengthened, but is limited to the posterior of the 2nd arch (Fig. 3D). We find no other *Fgf*s expressed in the pharyngeal endoderm.

These expression patterns indicate that *Fgf8* and *Fgf19* are consistently expressed in the pharyngeal endoderm pre and post migration stages, with weaker early *Fgf3* and *Fgf4* expression (HH14). *Fgf4* expression may play a role in induction, but not maintenance, as it is down regulated by HH19 in the endoderm at the anterior of the arch where the columella will form.

Although FGF19 has a known role in inner ear induction (Ladher et al., 2005), as candidate inducer of *Sox9,* a more detailed analysis of *Fgf19's* spatio-temporal expression pattern was needed. We analyzed expression in an extended stage series (HH8-14) by in situ hybridization. Expression is initially detected in the mesenchyme and weakly in the ventral hindbrain at the level of the ear-forming region (Fig. 4A). By the six somite stage pharyngeal endoderm begins to express *Fgf19* (Fig. 4B, C). Folding of the anterior intestinal portal forms the pharyngeal endoderm lip (Fig. 4D, arrowed). This coincides with the onset of NC migration. Low levels of *Fgf19* expression in hindbrain continue until around HH10 with pharyngeal endoderm showing increased numbers of transcripts (Fig.4E). At HH12, expression is visible at the level of 2nd arch and a new domain of expression appears in the endoderm of the 4th arch (Fig. 4G, g). By HH14, *Fgf19* expression in pharyngeal endoderm is directly subjacent to the NC-derived columella mesenchyme (Fig. 4H). Given the spatio-temporal *Fgf* expression patterns, we next tested if FGF signaling was sufficient and necessary for *Sox9* expression.

FGF signaling is required and sufficient for induction of *Sox9* expression

To test if FGF signaling was required to induce *Sox9* expression we turned again to our culture system (Exp. 8, Table 2). A bead soaked in 10 mM SU5402 *Fgf*-receptor inhibitor or PBS (control) was cultured with a HH14 explant of mesenchyme with pharyngeal endoderm. Explants cultured for 24 hours with the FGF-receptor inhibitor failed to express *Sox9* (n=2/24, Fig. 6A), while explanted tissue cultured with control PBS soaked beads did induce expression of *Sox9* (n=10/12). These results indicate that FGF signaling is required to induce *Sox9* expression and thus, specification of NC mesenchyme to a pre-chondrogenic fate.

In the converse experiment, we tested if addition of recombinant FGF protein was sufficient to induce *Sox9* expression (Exp. 9). We added a bead soaked in recombinant FGF8 protein to HH14 explants with only mesenchyme present, resulting in *Sox9* expression in all cases (n=5/5) after 24 hours in culture (Fig. 6B). Similarly, FGF19 soaked beads induced *Sox9* when added to mesenchyme explants (n=6/8). In controls, mesenchyme only explants placed together with PBS soaked control beads showed no induction of *Sox9* expression (n=1/7).

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FGF signaling can induce, but not maintain *Sox9* expression

FGF signals are able to induce *Sox9* expression during a 24 hour culture period, but we wanted to know if this one signal was sufficient to maintain *Sox9* over time (Exp. 10, Table 3). Mesenchyme explants cultured with an FGF8 soaked bead to E6 could not maintain *Sox9* expression (n=1/8, Fig. 7A). Neither could FGF19 soaked beads (Table 3). However, we have shown that signals from the pharyngeal endoderm are able to maintain *Sox9*, suggesting that a second secreted signaling molecule is required in addition to FGF.

Bmp2, 4 and *7* are expressed in restricted temporospatial patterns in the pharyngeal arch epithelia and mesenchyme (Shigetani et al., 2000; Bell et al., 2004; Darnell et al., 2007; Wood et al., 2010). We confirmed that Bmp4 is expressed in both the pharyngeal endoderm and mesenchyme of HH14 explants (not shown). When we applied BMP4 soaked beads alone (n=8/10), or together with FGF8 soaked beads (n=4/6), explants were able to induce and maintain *Sox9* expression to E6(Fig. 7A).

Together these data demonstrate that FGF signaling is sufficient and required for induction of *Sox9* expression and, thus, specification of pre-chondrogenic identity in mesenchyme tissue. However, FGF signals are unable to maintain this identity. Somewhat unexpectedly, even in the absence of FGF signaling, recombinant BMP protein was able to not only induce, but also maintain *Sox9* expression in long-term cultures. We know from experiment 8 that FGF signals are required for induction of *Sox9,* when mesenchyme and pharyngeal endoderm are the only tissues present. It will be interesting to determine if BMP signaling could induce *Sox9* in mesenchyme alone when FGF signals were being blocked by SU5402, indicating an important interaction between the two signaling pathways.

Next we examined the role of pharyngeal endoderm and secreted signaling factors in initiating the chondrogenic program, leading to over differentiation and cartilage formation.

Pharyngeal endoderm signals sets mesenchymal cells on a pathway to chondrogenesis

Col2a1 expression is normally detected in mesenchyme cells from E7.5 with the onset of overt chondrogenesis (Wood et al., 2010). To determine if mesenchyme was able to express the chondrogenic marker *Col2a1*, HH14 explants were grown to E8 (Exp. 11, Table 3). Mesenchyme in combination with pharyngeal endoderm strongly expressed *Col2a1* (n=12/12, Fig. 7B), with the endoderm remaining negative for *Col2a1* transcripts. HH14 mesenchyme cultured alone did not express *Col2a1* (n=0/6), indicating that pharyngeal endoderm contains all the signals necessary for chondrogenesis to proceed. We next examined to role of FGF8 and BMP4 in this process.

Both FGF and BMP signaling are required to initiate the chondrogenic program

When HH14 mesenchyme was cultured with either an FGF8 (n=0/6) or FGF19 (n=0/5) soaked bead (Exp. 12), none of the explants expressed *Col2a1* at E8. FGF signaling induces, but cannot maintain *Sox9* expression and is also insufficient for induction of the chondrogenic program as indicated by the absence of *Col2a1* transcripts at E8. It is possible that this was because FGF signals that are required to induce *Col2a1* expression must be sustained for an interval that cannot be achieved using the bead culture system. We repeated the experiment, replacing the media each day with recombinant FGF8 protein included in the media (1 mg/ml) to supply the tissue constantly over the incubation period, but were unsuccessful in inducing *Col2a1* expression (n=0/8, not shown). Taken together, these results suggest that another signaling pathway is required to facilitate induction of the chondrogenic program.

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BMP4 recombinant protein soaked beads were added to mesenchyme explants (Exp. 13), with or without addition of FGF8 beads (Fig. 7C). Explants were analyzed for *Col2a1* expression at E8. BMP4 alone was only able to induce *Col2a1* expression in the mesenchyme in 20% of cases (n=2/10). However, FGF8 together with BMP4 soaked beads, induced *Col2a1* expression in the majority of cases (n=5/7). This result suggests that the pharyngeal endoderm uses the combination of BMP and FGF signals to induce pre-chondrogenic identity and initiate the chondrogenic program.

To test if FGF and BMP signals were required following specification of pre-chondrogenic identity, *Sox9* expressing mesenchyme explanted from HH19 embryos was incubated to E8 (Exp. 14). Zero hour explants all expressed *Sox9* (not shown). Isolated mesenchyme explants cultured to E8 all strongly expressed *Col2a1* (n=7/8, Fig. 7D). Combining explants with either FGF8 (n=6/7) or BMP4 (n=7/7) soaked beads had did not change the *Col2a1* expression. These results suggest that the chondrogenic program is able to proceed autonomously in skeletogenic mesenchyme from HH19, without further input following specification of pre-chondrogenic identity. Mesenchyme explants extirpated at HH24 all showed *Col2a1* expression at E8 (n=6/8).

In conclusion, these results demonstrate that FGF signaling induced, but could not maintain *Sox9* expression, whereas BMP4 signaling was sufficient to both induce and maintain *Sox9* expression. Together with our FGF receptor inhibitor experiment demonstrating that FGF signaling is required for *Sox9* expression we conclude that FGF and BMP signaling together specify pre-chondrogenic identity. Moreover, combined FGF8 and BMP4 signaling was needed to initiate the chondrogenic program as indicated by *Col2a1* expression.

Discussion

A refined model of cartilage formation in the neural crest-derived mesenchyme

We present a model in which pharyngeal endoderm is sufficient, but not required, to specify pre-chondrogenic identity and initiate the chondrogenic program in skeletogenic mesenchyme from HH14 embryos, as defined by the presence of *Sox9* and *Col2a1* transcripts respectively. Specification of pre-chondrogenic identity and initiation of the autonomous chondrogenic program is complete by HH19 in the presence of signals from the pharyngeal endoderm. Mesenchyme isolated at HH19 and HH24 expresses *Sox9* and *Col2a1* during long-term culture.

Furthermore, our gain- and loss-of-function studies using HH14 explants of isolated mesenchyme demonstrate that FGF signaling is both sufficient and required for induction of *Sox9*, but cannot maintain this expression long term. BMP4 recombinant protein can, however, both induce and maintain *Sox9* expression, although by itself it is a weak initiator of the chondrogenic program. FGF and BMP signaling together are required for overt differentiation and expression of the chondrogenic marker, *Col2a1*.

As mesenchyme shares a common ground pattern (Minoux et al., 2009), with multiple FGF and BMP signaling molecules temporospatially restricted to the pharyngeal endoderm of all the arches {Creuzet, 2004 #13}, this is likely a general patterning mechanism, although this remains to be tested.

Pharyngeal endoderm is sufficient to induce and maintain *Sox9* expression, but not required

Ablating pharyngeal endoderm, leaving all other tissues in the region intact elicits *Sox9* expression. As demonstrated by our recombination experiments, signals from the otic epithelium act as an inducer of *Sox9* in the NC-derived mesenchyme. Similarly, neural tube,

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notochord and 2nd arch *Fgf8* expressing surface ectoderm can acts as inducers, but endothelial vessels, such as the dorsal aorta, cannot. Although other tissues are sufficient, given the distance from the putative columella in vivo, it is likely that the pharyngeal endoderm acts as the endogenous inducer.

In order to chondrify, premigratory cranial NC cells in mammals must interact with the cranial ectoderm in an inducer/responder relationship. In chick, signals from the surface ectoderm of the face are required to pattern the beak at later stages, but not other skeletal elements (Helms and Schneider, 2003; Schneider and Helms, 2003). In lower vertebrates, such as amphibians, post-migratory NC interacts with the branchial endoderm (Hall, 2008). Our results demonstrate that in chick, the non-FGF expressing dorsolateral surface ectoderm is unable to specify pre-chondrogenic identity. However, *Fgf8*-expressing surface ectoderm at the level of the pharyngeal groove is sufficient in this regard. We note that *Fgf8* expression in this tissue is short-lived, appearing and then disappearing between HH8 and HH15. Thus, ectodermal expression might attract or prime NC cells as they migrate past the groove to reach the distal portion of each pouch.

FGF signaling is sufficient and required to induce, but cannot maintain, *Sox9* expression

Specifying cells that follow the skeletogenic pathway requires exposure to FGF signaling, with FGF8 signaling being a potent inducer of pre-chondrogenic identity (Ruhin et al., 2003; Creuzet et al., 2004a; Minoux et al., 2009). The role of FGF8 in specification of skeletogenic mesenchyme is undisputed, but interestingly we show that FGF8 is unable to maintain *Sox9* expression in isolated mesenchyme.

There are other Fgf genes in the region, with FGF8 a known regulator of *Fgf19* expression (Ladher et al., 2005; Gimeno and Martinez, 2007). In our explants the effect of FGF19 on mesenchymal cells appears less robust than FGF8, with a smaller number of cells expressing *Sox9*. Although we did not quantify this, it is tempting to speculate that the endogenous role of FGF19 may be specification or patterning of only a subset of mesenchymal cells. Given the proximity of the columella cells to pharyngeal endoderm the columella mesenchyme may specifically require FGF19, although this remains to be tested. *Fgf19* expression is detected only in distal portions of the 2nd and 4th arches, lending credence to this idea. The weaker, spatio-temporally restricted expression of *Fgf3* and *Fgf4* in 2nd arch endoderm will also be the subject of future examination. Zebrafish *fgf3* endoderm expression is required for formation of arches 1-4 (David et al., 2002). Interestingly, these authors also suggest that signals in addition to FGFs are required in endoderm to pattern the skeletal elements arising from the arches.

Pharyngeal endoderm is required for induction of the chondrogenic program

Explants from HH14 embryos cultured with pharyngeal endoderm were able to express *Col2a1* at E8. Once the mesenchyme population is specified, by HH19, chondrogenesis can proceed even in the absence of pharyngeal endoderm. Thus, pharyngeal endoderm is not only sufficient to specify pre-chondrogenic identity, but must be the source of a multiple signaling molecules, acting to initiate chondrogenesis (Creuzet et al., 2005). We show that BMP4 is sufficient in this regard.

BMP signaling has a role in promoting compaction of pre-chondrogenic cells leading to formation of the cartilage anlagen (the pre-chondrogenic condensation) and is an obvious candidate as an additional signal. BMP4 is expressed in a suitable temporospatial pattern (Wood et al., 2010). Loss of BMP signaling leads to failure of condensation formation and failure to differentiate into chondrocytes, even in condensed cells (Duprez et al., 1996; Yoon et al., 2005; Bandyopadhyay et al., 2006; Barna and Niswander, 2007; Karamboulas et al.,

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2010). Furthermore, BMP4 is a known inducer of *Sox9*, and our results support this finding, showing that BMP4 also maintains *Sox9* expression over the longer term. BMP4 can also act as a direct transcriptional activator of *Col2a1* expression (Semba et al., 2000). In our mesenchyme explants, FGF8 or BMP4 signaling alone are able to induce, but only BMP4 signaling can maintain *Sox9* expression. Neither signal alone leads to *Col2a1* expression, it is only when FGF and BMP signaling is combine that *Col2a1* transcripts are detected at E8.

Reconciling models of hyoid skeletal formation

The results presented here add new insight into our understanding of the tissue and signaling mechanism required to induce and pattern NC-derived mesenchyme. NC-derived pharyngeal arch cells appear to share a common ground pattern (Minoux et al., 2009), with earlier transplantation experiments demonstrating the sufficiency and requirement for pharyngeal endoderm in imparting regional identity (Ruhin et al., 2003; Creuzet et al., 2004a). Supporting this, ablation of the adjacent pharyngeal endoderm in ovo resulted in the loss of the basihyoid and ceratobranchial skeletal elements (Ruhin et al., 2003). However, the mechanism resulting in failure was not evaluated in these studies. There are several possible stages at which skeletal elements that might be affected: NC migration, specification of pre-chondrogenic identity, condensation formation or chondrogenesis.

FGF signals act as an attractant for migrating NC cells (Creuzet et al., 2005). Genetic ablation of endoderm, in zebrafish *van gogh*, *cas* and *bon* mutants results in failure to form cartilage (Piotrowski and Nusslein-Volhard, 2000; David et al., 2002; Piotrowski et al., 2003). In the *cas* and *bon* mutants particularly, the lack of endoderm formation leads to failure of branchial arch formation. The NC cells migrate, but fail to enter the branchial arches, clustering instead on the surface of the yolk in disorganized masses, losing the markers of pre-chondrogenic mesenchyme. We suggest that a similar mechanism acts in chick, where removal of endoderm at the 2nd arch level during pre-migratory stages (Ruhin et al., 2003; Creuzet et al., 2004a), results in failure of the NC to become correctly localized and/or specified as pre-chondrogenic (David et al., 2002). Addition of an FGF8 source in place of the ablated endoderm rescues this phenotype by attracting the migrating cells and specifying pre-chondrogenic identity. Furthermore, our results show that FGF signals from other inducer tissues in the region can act to induce pre-chondrogenic identity, but would not localize the NC to the correct position. Thus, our results indicate that pharyngeal endoderm is sufficient, but not required for imparting pre-chondrogenic identity.

Due to the experimental paradigm used in earlier studies this distinction was not apparent. Failure of the NC cells to migrate to the correct location and thus be induced and maintain pre-chondrogenic identity would account for loss of specific skeletal elements. Thus, our experimental paradigm benefits from using NC cells that are post-migratory, avoiding the complications of localization. Thus, it appears to be the case that FGF signals arising from the pharyngeal endoderm are required to localize the migrating NC. Both FGF and BMP signals can induce pre-chondrogenic identity, although only BMP can maintain this state. In our longer-term cultures neither FGF, nor BMP signals alone are sufficient to induce the chondrogenic program. In conclusion, it is only when both signaling factors are combined that the chondrogenic program induced, as marked by the onset of *Col2a1* expression.

Experimental Procedures

Embryos

Fertilized Bovan Brown x Rhode Island Red chicken eggs (Morgan Poultry Center, Clemson University) were incubated at 38.5°C in a rocking incubator to the desired stage. Whole mount in situ hybridization was performed as previously described (Chapman et al., 2002).

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Peanut Agglutinin Lectin (PNA) labeling was performed according to our standard protocol (Wood et al., 2010). For section analysis embryos were mounted in 20% gelatin, fixed in 4% PFA (paraformaldehyde) and sectioned at 50 μm using a Leica VT1000S vibratome, or embedded in 30% Sucrose/PBS (phosphate buffered saline) and cryosectioned at 10 μm using a Leica CM3050.

Tissue explants

Morphology of the head at HH14 and a section of the 2nd arch region are shown in figure 1. To preserve the integrity of cell surface receptors no enzymatic treatments were used to separate tissues. Embryos were harvested, washed in saline and transferred to L15 media during the fine manipulation before being transferred to collagen gel culture. Whole mount embryos were harvested in saline, with the head transected at the level of the 1/2 pharyngeal arch boundary anteriorly and the 2/3 arch boundary posteriorly. The remaining tissue formed a slice at the level of the 2nd arch (Fig. 1A). The tissues needed for explants at HH14 (E2), HH19 (E3) or HH24 (E4) were dissected from the equivalent region at each stage. The same basic morphology is present at each of the stages used. The slice was laid flat on the base of the petri dish and a flame sharpened tungsten needle (0.125 mm, WPI, Sarasota, FL) was used to remove tissues not required. For example, mesenchyme alone required the removal of the surface ectoderm, pharyngeal endoderm, distal pharyngeal arch, otic vesicle, neural tube, notochord and blood vessels, leaving only the proximal mesenchyme (Fig. 1B and Fig. 2 schematics). Using this methodology ensured that the proximal mesenchyme was the only tissue explanted to collagen gel culture, whereas the more distal mesenchyme within the pharyngeal arch was excluded. The older the tissue, the easier the tissue was to extirpate, as the size of the embryo had increased and thus the mesenchyme was considerably larger. To ensure that we explanted only the desired tissues, explants at zero hours were embedded and sectioned to determine the tissues present.

Collagen gel culture

Isolates were cultured in 3.3 mg/ml rat tail collagen (Roche Diagnostics, Indianapolis, IN) re-suspended in sterile 0.2% acetic acid. Collagen was prepared on ice using 440 μl collagen, 80 μl DEPC-H_2O, 60 μl 10X DMEM and 20 μl 7.5% bicarbonate solution. Following collagen embedding carbonated Neurobasal medium supplemented with Glutamax and Penicillin/Streptomycin was added. Cultures were grown at 37°C in a 5% CO_2 incubator, with daily changes in media until the desired stage was reached. Following fixation, tissue was processed for in situ hybridization or immunocytochemistry.

Bead implantation

Affi-Gel Blue Beads (100-200 μm, Bio-Rad, Hercules, CA) were washed in PBS for 1 hour. Beads were then soaked in 10 mM SU5402 (Pfizer), or 1 mg/ml human recombinant FGF8 (423-F8/CF, R&D systems), FGF19 (100-32, Peprotech), or 100 ng/ml mouse recombinant BMP4 (314-BP/CF, R&D systems) for 1 hour. SU5402 is dissolved in DMSO and beads were washed three times in PBS before implanting. Control beads were similarly treated with DMSO and washed in PBS before implanting. For the recombinant protein experiment the FGF and BMP proteins were re-suspended in PBS and, thus, no further washing of beads was necessary. PBS washed control beads were used in these experiments. Beads were kept on ice and as each tissue sample was prepared a bead was inserted in the collagen gel adjacent to the tissue. For experiment requiring both FGF and BMP, two beads were added adjacent to the tissue.

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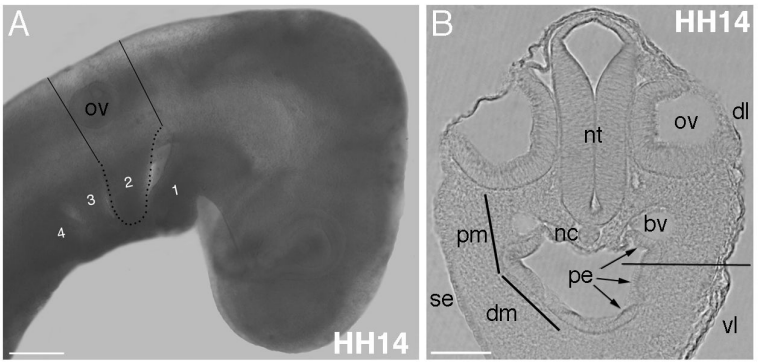

Figure 1. Location of tissues and anatomical landmarks for the explant cultures

(A) Lines on HH14 embryo show the level of cuts to remove a transverse slice of the head and 2nd arch region. Pharyngeal arches numbered 1-4, with 2nd arch outlined in black dots. (B) A section through the level of the otic vesicle and 2nd arch region showing the tissues present. Proximal to distal mesenchyme continuum indicated by lines on the left side. Pharyngeal endoderm lines the oral cavity (arrowed). Horizontal line through arch on right side indicates approximate level of cut to exclude ventral arch mesenchyme from the explants. Abbreviations: bv, blood vessel; dl, dorsolateral level; dm, distal mesenchyme; nc, notochord; nt, neural tube; ov, otic vesicle; pe, pharyngeal endoderm; pm, proximal mesenchyme; se, surface ectoderm; vl, ventrolateral level. Scale bars: A, 200 μm, B, 100 μm.

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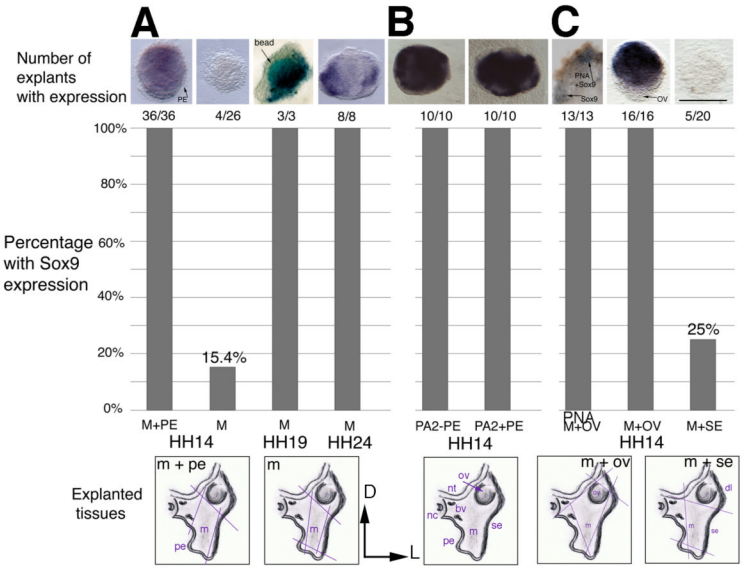

Figure 2. *Sox9* expression in explants from the proximal 2nd arch region

The schematics below the graph illustrate the tissues in the region. Orientation is dorsal to the top and lateral to the right. The colored lines indicate the cuts made to extirpate the tissue. The graph shows the number and percentage of explants that tested positive for *Sox9*. The tissues included in each graph are listed on the horizontal axis. (A) Explants were removed from chick embryos at HH14, HH19 or HH24 and grown in collagen gel culture to E6. Mesenchyme cultured with pharyngeal endoderm from HH14 tissues expresses *Sox9* at E6, whereas mesenchyme alone does not. At HH19 and HH24, *Sox9* is expressed in the mesenchyme at E6 in the absence of pharyngeal endoderm. (B) Transverse sections of the head at the level of the 2nd arch with or without pharyngeal endoderm present express *Sox9* in all cases. The schematic below is labeled with the tissues found in this region at the time of culture. (C) Extirpation of the otic vesicle with mesenchyme results in PNA and *Sox9* expression. Mesenchyme explanted with surface ectoderm expresses *Sox9* in a quarter of cases. Abbreviations: bv, blood vessel; D, dorsal; dl, dorsolateral; L, lateral; m/M, mesenchyme; nc, notochord; nt, neural tube; ov/OV, otic vesicle; PA2, 2nd pharyngeal arch level tissue; pe/PE, pharyngeal endoderm; PNA, Peanut Agglutinin Lectin; se/SE, surface ectoderm. Scale bar: 100 μm.

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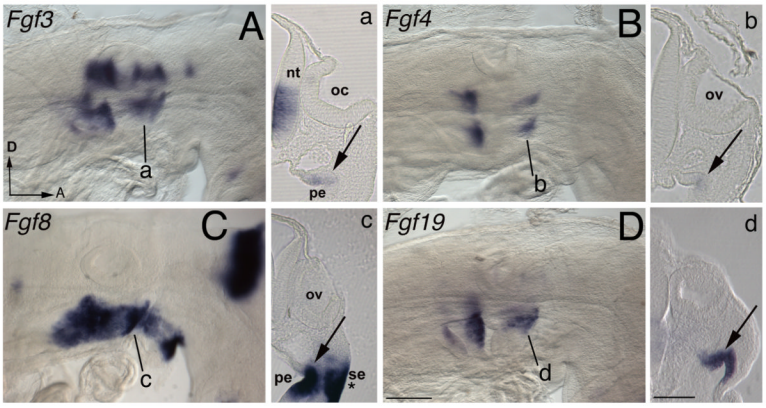

Figure 3. *Fgf* expression at the completion of NC migration

(A-D) Whole mount HH14 stage embryos with anterior facing to the right and dorsal to the top of the page. (a-d) Corresponding 50 μm gelatin sections are indicated by a black line on the whole mount images. (A, B) Weak *Fgf*3 and *Fgf*4 expression is detected in the pharyngeal endoderm lip (arrowed a, b), whereas strong *Fgf8* and *Fgf19* (C, D) endoderm expression is observed (arrowed, c, d), with *Fgf8* also expressed in the adjacent surface ectoderm at the groove of the pharyngeal arch (asterisk). Abbreviations: A, anterior; D, dorsal; nt, neural tube; pe, pharyngeal endoderm; oc, otic cup; ov, otic vesicle; se, surface ectoderm. Scale bars: (A-D) 100 μm, (a-d) 100 μm.

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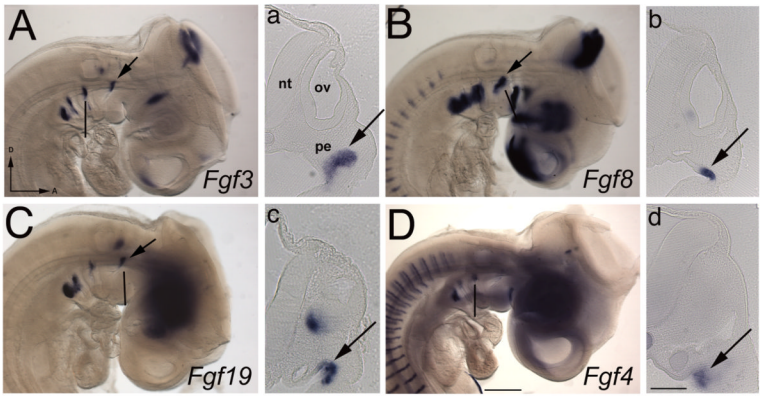

Figure 4. *Fgf* expression in during specification of pre-chondrogenic identity

(A-D) HH18/19 stage embryos, with the head to the right and dorsal towards the top of the page. The black line indicates the level of the corresponding transverse 50 μm gelatin sections (a-d). (A) *Fgf3* is expressed in the pharyngeal endoderm in the 1st pouch (arrowed). (a) The section is through the endoderm at the anterior of the 3rd arch. (B, b) *Fgf8* and (C, c) *Fgf19* are expressed in the proximal anterior region of the 2nd arch (arrowed) and the vestibuloacoustic ganglion. (D, d) *Fgf4* expression is limited to the posterior 2nd arch endoderm (arrowed). Scale bars: (A-D) 100 μm, (a-d) 100 μm.

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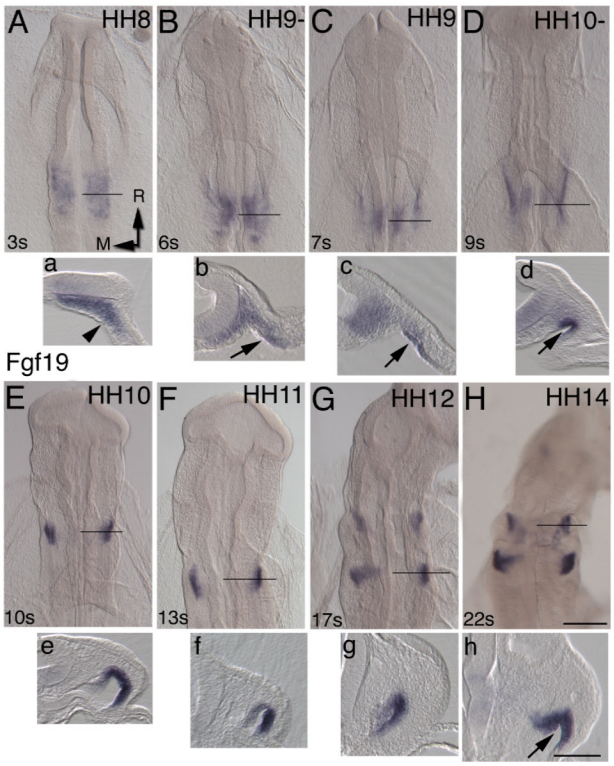

Figure 5. *Fgf19* expression in the ear region

(A-H) HH8-14 embryos with rostral towards the top of the page and (a-h) corresponding transverse 50 μm gelatin sections. The black line on the whole mount indicates the level of the section. (A) Initial *Fgf19* expression is in the mesoderm and is weakly expressed in the ventral neural folds, but not the pharyngeal endoderm (arrowhead). (B, C) At HH9, expression is detectable in the future pouch endoderm. (D-F) Expression is particularly noticeable in the fold of the pharyngeal endoderm (arrowed). (G, g) As NC cells migrate into the 2nd arch the cells are exposed to *Fgf19* signaling in the endoderm. A second region of *Fgf19* expression is detected at level of the 4th arch from HH12. (H) At the conclusion of NC migration *Fgf19* expression is directly subjacent to the putative columella mesenchyme population. Scale bars: (A-D) 100 μm, (a-d) 100 μm.

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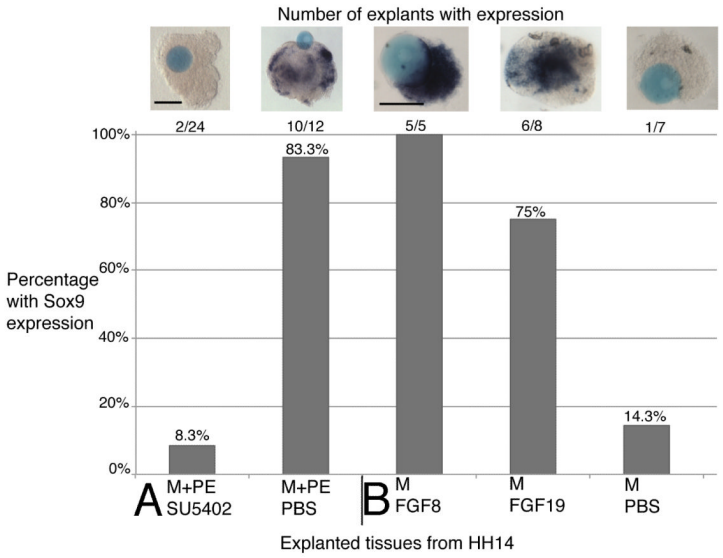

Figure 6. The role of FGF signaling in inducing *Sox9* in mesenchyme

The graph shows the number and percentage of explants that tested positive for *Sox9*. The tissues included in each graph are listed on the horizontal axis. (A) Mesenchyme and pharyngeal endoderm cultured for 24 hours with SU5402 soaked beads failed to express *Sox9*, whereas *Sox9* expression was unaffected by addition of PBS control beads. (B) In the converse experiment adding FGF8 or FGF19 soaked beads to mesenchyme alone, induced *Sox9* expression. PBS beads in control explants did not have *Sox9* expression. Scale bars: (A) 100 μm, (B) 100 μm.

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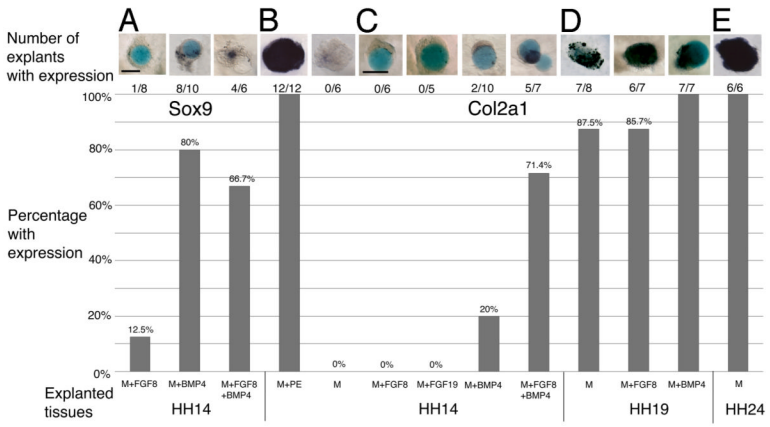

Figure 7. FGF and BMP signaling in initiation of the chondrogenic program

The graph shows the number and percentage of explants that tested positive for *Sox9* and *Col2a1*. The tissues included in each graph are listed on the horizontal axis. (A) Cultured HH14 mesenchyme expresses *Sox9* when exposed to BMP4 alone, and in combination with FGF8 soaked beads, but not FGF8 beads alone at E6. (B) Mesenchyme in the presence of pharyngeal endoderm, but not alone, strongly expresses *Col2a1* at E8. (C) Neither FGF8 or FGF19 protein soaked beads is sufficient for *Col2a1* expression. BMP4 is a weak inducer of *Col2a1*. Only when BMP4 and FGF8 are combined does mesenchyme tissue express *Col2a1*at E8. (D) By HH19, mesenchyme explanted in isolation expresses *Col2a1* and is unaffected by the presence of FGF8 or BMP4. (E) *Col2a1* is expressed throughout the mesenchyme in tissue isolated at HH24. Scale bars: (A, B) 100 μm, (C-E) 100 μm.

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Table 1

Sox9 expression at E6 in explanted tissues.

Exp #	Explants	Explant stage	Embryonic Day	# with expression	% with expression	Marker	Stage harvested
1	Mesenchyme + PE	HH14	E2	36/36	100%	*Sox9*	E6
	Mesenchyme alone	HH14	E2	4/26	15.4%	*Sox9*	E6
2	Mesenchyme + PE	HH19	E3	3/4	75%	*Sox9*	E6
	Mesenchyme alone	HH19	E3	3/3	100%	*Sox9*	E3
3	Mesenchyme + PE	HH24	E4	8/8	100%	*Sox9*	E6
	Mesenchyme alone	HH24	E4	8/8	100%	*Sox9*	E6
4	Arch 2 slice - PE	HH14	E2	10/10	100%	*Sox9*	E6
	Arch 2 slice + PE	HH14	E2	10/10	100%	*Sox9*	E6
5	Mesenchyme + otic vesicle	HH14	E2	13/13	100%	PNA	E6
6	Mesenchyme + otic vesicle	HH14	E2	16/16	100%	*Sox9*	E6
7	Mesenchyme + surface ectoderm	HH14	E2	5/20	25%	*Sox9*	E6

Abbreviations: PE, pharyngeal endoderm.

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Table 2

Induction of *Sox9* in explanted tissues.

Exp #	Explants	Explant stage	Embryonic day	# with expression	% with expression	Marker	Stage harvested
8	Mesenchyme + PE + SU5402 bead	HH14	E2	2/24	8.3%	*Sox9*	+ 24 hrs
	Mesenchyme + PE + PBS bead	HH14	E2	10/12	83.3%	*Sox9*	+ 24 hrs
9	Mesenchyme + FGF8 bead	HH14	E2	5/5	100%	*Sox9*	+ 24 hrs
	Mesenchyme + FGF19 bead	HH14	E2	6/8	75%	*Sox9*	+ 24 hrs
	Mesenchyme + PBS bead	HH14	E2	1/7	14.3%	*Sox9*	+ 24 hrs

Abbreviations: PBS, phosphate buffered saline; PE, pharyngeal endoderm.

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Table 3

Initiation of the chondrogenic program in explants.

Exp #	Explants	Explant stage	Embryonic day	# with expression	% with expression	Marker	Stage harvested
10	Mesenchyme + FGF8 bead	HH14	E2	1/8	12.5%	*Sox9*	E6
	Mesenchyme + BMP4 bead	HH14	E2	8/10	80%	*Sox9*	E6
	Mesenchyme + FGF8 bead +BMP4 bead	HH14	E2	4/6	66.7%	*Sox9*	E6
11	Mesenchyme + PE	HH14	E2	12/12	100%	*Col2a1*	E8
	Mesenchyme alone	HH14	E2	0/6	0%	*Col2a1*	E8
12	Mesenchyme + FGF8 bead	HH14	E2	0/6	0%	*Col2a1*	E8
	Mesenchyme + FGF19 bead	HH14	E2	0/5	0%	*Col2a1*	E8
13	Mesenchyme + BMP4 bead	HH14	E2	2/10	20%	*Col2a1*	E8
	Mesenchyme + FGF8 bead +BMP4 bead	HH14	E2	5/7	71.4%	*Col2a1*	E8
14	Mesenchyme alone	HH19	E3	7/8	87.5%	*Col2a1*	E8
	Mesenchyme + FGF8 bead	HH19	E3	6/7	85.7%	*Col2a1*	E8
	Mesenchyme + BMP4 bead	HH19	E3	7/7	100%	*Col2a1*	E8
15	Mesenchyme alone	HH24	E4	6/6	100%	*Col2a1*	E8

Abbreviations: BMP, Bone Morphogenetic Protein; FGF, Fibroblast Growth Factor; PBS, phosphate buffered saline; PE, pharyngeal endoderm.