Mesenchyme-dependent BMP signaling directs the timing of mandibular osteogenesis

Amy E. Merrill, B. Frank Eames, Scott J. Weston, Thayer Heath and Richard A. Schneider*

To identify molecular and cellular mechanisms that determine when bone forms, and to elucidate the role played by osteogenic mesenchyme, we employed an avian chimeric system that draws upon the divergent embryonic maturation rates of quail and duck. Pre-migratory neural crest mesenchyme destined to form bone in the mandible was transplanted from quail to duck. In resulting chimeras, quail donor mesenchyme established significantly faster molecular and histological programs for osteogenesis within the relatively slower-progressing duck host environment. To understand this phenotype, we assayed for changes in the timing of epithelial-mesenchymal interactions required for bone formation and found that such interactions were accelerated in chimeras. In situ hybridization analyses uncovered donor-dependent changes in the spatiotemporal expression of genes, including the osteo-inductive growth factor Bmp4. Mesenchymal expression of Bmp4 correlated with an ability of quail donor cells to form bone precociously without duck host epithelium, and also relied upon epithelial interactions until mesenchyme could form bone independently. Treating control mandibles with exogenous BMP4 recapitulated the capacity of chimeras to express molecular mediators of osteogenesis prematurely and led to the early differentiation of bone. Inhibiting BMP signaling delayed bone formation in a stage-dependent manner that was accelerated in chimeras. Thus, mandibular mesenchyme dictates when bone forms by temporally regulating its interactions with epithelium and its own expression of Bmp4. Our findings offer a developmental mechanism to explain how neural crest-derived mesenchyme and BMP signaling underlie the evolution of species-specific skeletal morphology.

KEY WORDS: Epithelial-mesenchymal interactions, BMP signaling, Mandibular primordia, Neural crest, Intramembranous ossification, Quail-duck chimeras, Evolutionary developmental biology

INTRODUCTION

Precisely timed interactions between mesenchyme and epithelium are fundamental to intramembranous ossification. That these interactions are osteo-inductive and stage dependent has been demonstrated primarily through tissue recombination and epithelial removal experiments involving chick and mouse embryos (Tyler and Hall, 1977; Hall, 1978; Bradamante and Hall, 1980; Tyler and McCobb, 1980; Hall, 1982; Van Exan and Hall, 1984; Wedden, 1987; Hall and Coffin-Collins, 1990; Dunlop and Hall, 1995; Francis-West et al., 1998; Vaglia and Hall, 1999). What remain to be clarified are the molecular and cellular mechanisms that control the timing of these interactions and the ensuing formation of bone.

Numerous studies have focused on the mandibular primordia, which give rise to the lower jaw, as a model to define the nature of epithelial-mesenchymal interactions. Mandibular osteogenic mesenchyme is derived entirely from cranial neural crest cells that migrate out of the midbrain and rostral hindbrain, whereas mandibular epithelium arises from non-neural ectoderm along the midbrain and rostral hindbrain, as well as from pharyngeal endoderm (Noden, 1978; Couly and Le Douarin, 1990; Couly et al., 1993; Köntges and Lumsden, 1996). Mandibular bone formation commences with the condensation of mesenchyme (Hall and Miyake, 1992; Hall and Miyake, 1995; Schneider and Helms, 1998; Hall and Miyake, 2000; Eames et al., 2003; Helms and Schneider, 2003; Eames and Helms, 2004; Eames et al., 2004), which in chick embryos occurs around Hamburger-Hamilton stage (HH) 27 (Hamburger and Hamilton, 1951). These condensations differentiate into osteoblasts by HH31, and form bone through the intramembranous deposition and calcification of bone matrix by HH34 (Tyler and Hall, 1977). When mesenchyme is isolated from chick mandibles before HH25 and cultured without epithelium, bone does not form (Tyler and Hall, 1977; Dunlop and Hall, 1995).

Shortly thereafter, bone can form without epithelium, which indicates that a prior epithelial-mesenchymal interaction is crucial for osteogenesis. In characterizing this interaction, some studies have proposed that epithelia act permissively, as flank epithelium, which normally overlies non-osteogenic mesenchyme, can function in place of mandibular epithelium and sustain bone formation (Hall, 1978; Hall, 1981). Similarly, our recent studies on bird beak and feather development suggest that mesenchyme signals to the epithelium instructively, by establishing the timing of interactions and regulating gene expression (Schneider and Helms, 2003; Eames and Schneider, 2005; Schneider, 2005).

Molecules that participate in mandibular osteogenesis include Bone Morphogenetic Proteins (BMPs) and their targets. BMPs are secreted factors capable of inducing de novo bone postnatally (Urist, 1965; Wozney et al., 1987; Wang et al., 1990); they also function as osteo-inductive factors embryonically (Kingsley et al., 1992; Luo et al., 1995; Solloway et al., 1998). Bmp2, Bmp4 and Bmp7, and their receptors (Bmpr1a, Bmpr1b and Alk2) are expressed in mandibular epithelium and/or mesenchyme (Francis-West et al., 1994; Bennett et al., 1995; Wall and Hogan, 1995; Ashique et al., 2002a), and are essential for osteogenesis (Francis-West et al., 1998; Wang et al., 1998; Ashique et al., 2002b). Conditional knockout of Bmp4 in mandibular epithelium eliminates the lower jaw (Liu et al., 2005), and BMP4 signaling influences the differentiation of neural crest-derived mesenchyme into bone (Abzhanov et al., 2007). BMPs regulate osteogenesis through a highly conserved pathway (Heldin et al., 1997;
were rinsed in Ca²⁺/Mg²⁺-free PBS (Sigma), and incubated for 60 minutes following published protocols (Tyler and Hall, 1977). Briefly, mandibles were incubated in BGJb differentiation medium (Fig. 1C-E). Epithelium was removed from mandibular primordia and cultured on Transwell membranes immersed in a control on the unoperated host side. After surgery, embryos were incubated until stages appropriate for analysis and/or organ culture. Organ culture was performed after BC, and chimeric mesenchyme was isolated from surgically extracted control and chimeric mesenchyme was isolated from surgically extracted control and chimeric mesenchyme was isolated from surgically extracted control and chimeric mesenchyme was isolated from surgically extracted control and chimeric mesenchyme was isolated from surgically extracted control and chimeric mesenchyme was isolated from surgically extracted embryos, which have distinct maturation rates (17 versus 28 days to hatching), and performed in ovo transplants of pre-migratory neural crest cells destined to form mandibular mesenchyme (Fig. 1). This permitted progressively asynchronous quail donor mesenchyme and duck host epithelium to interact with one another continuously from the moment they first meet (as opposed to in vitro recombinations of later-staged tissues), and allowed us to observe the resultant mesenchyme-mediated changes to events during osteogenesis. Our experiments reveal that neural crest-derived mesenchyme concomitantly governs the timing of epithelial interactions, mesenchymal BMP signaling and bone formation. This salient feature of mesenchyme to exert temporal control over key osteogenic events, and especially the complicity of the BMP pathway in the process, likely provides a developmental mechanism facilitating species-specific evolution of the facial skeleton.

Materials and Methods

Generation of chimeras

Eggs from Japanese quail (Coturnix coturnix japonica) and white Pekin duck (Anas platyrhynchos) (AA Labs, Westminster, CA) were incubated at 37°C until reaching HH9.5 (Fig. 1A). Embryos were handled following University and NIH guidelines. Tungsten needles and Spemann pipettes were used for operations (Schneider, 1999; Schneider and Helms, 2003). Unilateral grafts of rostral hindbrain and midbrain neural crest were excised from quail donors and transplanted into stage-matched duck hosts, producing chimeric ‘duck’ (Schneider and Helms, 2003; Tucker and Lumsden, 2004; Eames and Schneider, 2005). Equivalent transplants were also made from duck to quail (‘duai’). Donor tissue was inserted into a host that had comparable regions of tissue removed (Fig. 1B). Control orthotopic grafts and sham operations were made within each species. Controls were incubated alongside chimeras to ensure that stages of grafted cells were accurately assessed. In addition, unilateral transplants provided an internal control on the unoperated host side. After surgery, embryos were incubated until stages appropriate for analysis and/or organ culture. Organ culture was used to facilitate subsequent tissue manipulations and bead experiments.

Epithelial removal experiments

Control and chimeric mesenchyme was isolated from surgically extracted mandibular primordia and cultured on Transwell membranes immersed in BGJb differentiation medium (Fig. 1C-E). Epithelium was removed following published protocols (Tyler and Hall, 1977). Briefly, mandibles were rinsed in Ca²⁺/Mg²⁺-free PBS (Sigma), and incubated for 60 minutes at 4°C in 3% (6:1 trypsin:pancreatin solution in PBS. Digestion was stopped in 1:1 BGJb medium:FBS solution at 4°C, and mesenchyme was separated from epithelium using forceps. Epithelial removal was confirmed by inspection of each sample. Because these tissue layers are anatomically distinct, they can be easily distinguished during surgery. Some control and chimeric mandibles were processed similarly, but epithelium was not removed. Mesenchyme was cultured for up to 8 days and analyzed for gene expression and bone histology.

Histology and immunohistochemistry

Tissues were fixed in Serra’s (6:3:1,100% ethanol:37% formaldehyde:glacial acetic acid) overnight at 4°C, dehydrated, paraffin embedded, and cut into 7 μm frontal sections. To detect bone matrix in control and chimeric mandibles, sections were processed with Osteoid stain (Ralis and Ralis, 1975). Sections were viewed using brightfield. To detect quail cells in chimeric mandibles, representative sections were immunostained with the quail nuclei-specific Q¢PN antibody (Developmental Studies Hybridoma Bank) and a horseradish peroxidase-conjugated secondary antibody (Schneider, 1999). Sections were viewed using differential interference contrast.

Gene expression analysis

Mandibles from successive stages were assayed for temporal changes in gene expression. Whole-mount in situ hybridization was performed (Hogan et al., 1994; Kwang et al., 2002). Briefly, mandibles were fixed in 4%
parafomaldehyde for 20 minutes, permeabilized with 25 µg/ml Proteinase K for 5 minutes, and hybridized overnight at 60°C with digoxigenin (DIG)-labeled antisense riboprobes for either chick Bmp4, Bmp7, Bmpr1a, Bmpr1b, Noggin, Runx2, Msx1, Col2a1 or Twist. Mandibles were incubated with a 1:2000 dilution of anti-DIG-Alkaline phosphatase (AP) antibody (Roche) overnight at 4°C. BM Purple AP substrate (Roche) was used for colorimetric detection of hybridized riboprobes. In situ hybridization was performed on paraffin sections (Albrecht et al., 1997). 35S-labeled antisense riboprobes were generated to chick Bmp2, Bmp4, Bmp5, Bmp7, Alk2, Bmpr1a and Bmpr1b. These chick probes specifically and equivalently identified counterparts in quail and duck tissue (data not shown). Sections were counterstained with Hoechst nuclear dye (Sigma). Hybridization signals were detected using darkfield and the nuclear stain with epifluorescence.

Gain- and loss-of-function experiments

Mandibular primordia were dissected in sterile PBS and placed on Transwell membranes (0.4 µm pore size, Corning) immersed in minimal BGJb medium. Mandibles were treated contralaterally with Affigel beads (Bio-Rad) equilibrated in BMP4, Noggin (R&D Systems), or control Bovine Serum Albumin (BSA). Beads were prepared by soaking in 100 ng/µl recombinant BMP4, Noggin or 0.1% BSA. After 24 hours of treatment, mandibles were either collected for whole-mount in situ hybridization or cultured in BGJb differentiation medium with 10% FBS, 50 µg/ml ascorbic acid, 10 mM β-glycerol phosphate. BMP4 and Noggin dosage was based on protein concentrations known to induce a response in mandibular mesenchyme (Barlow and Francis-West, 1997; Ekanayake and Hall, 1997; Tucker et al., 1998a; Tucker et al., 1998b; Wang et al., 1998). HH23 explants were cultured for 3 to 8 days and assayed for bone.

Bone quantification

To quantify bone in cultured mandibles, histological sections were digitized. Adobe Photoshop was used to count pixels comprising bone matrix, whereas Zeiss AxioVision LE 4.4 was used to count pixels in a given condensation around bone matrix. Bone matrix volume (BV) and condensation volume (CV) were estimated using the equation for a conical frustum: BV or CV=(1/3h)[(A1+A2)+(A1A2)/h]; where h is distance between sections (7 µm), and A1 and A2 are areas (in µm) of bone in sequential sections (Colnot et al., 2003; Lu et al., 2005). P-values were calculated using a paired Student’s t-test with a one-tailed distribution.

RESULTS

Neural crest-derived mesenchyme establishes the timing of osteogenesis

To determine the extent to which mesenchyme governs the timing of bone formation, we transplanted neural crest cells from quail to duck (Fig. 1), producing chimeric ‘quck’. Quick mandibles were extracted at HH23, grown in organ culture, and assayed for donor-induced changes to osteogenesis. We stained bone matrix on sections adjacent to those containing abundant quail donor mesenchyme. We find that quail donor mesenchyme develops on the same timetable for osteogenesis as quail control mesenchyme, but does so within the slower developmental environment of the duck host (Fig. 2A,B). As early as 5 days of culture, quck mesenchyme stained for bone only coincident with QePN-positive cells (Table 1). Mandibles cultured for less than 5 days never formed bone. Quick mandibles grown for up to 6 days had well-developed bone in quail-derived mesenchyme, but not in the contralateral side derived from duck host mesenchyme (Fig. 2A,B).

Table 1. Timing of bone formation in HH23 mandible cultures

<table>
<thead>
<tr>
<th>Days cultured</th>
<th>Duck control</th>
<th>Duck host side (QePN)</th>
<th>Quail donor side (QePN)</th>
<th>Quail control</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0% (0/2)</td>
<td>0% (0/2)</td>
<td>0% (0/2)</td>
<td>0% (0/7)</td>
</tr>
<tr>
<td>4</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
<td>0% (0/6)</td>
</tr>
<tr>
<td>5</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
<td>100% (4/4)</td>
<td>66% (10/15)</td>
</tr>
<tr>
<td>6</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
<td>100% (4/4)</td>
<td>100% (11/11)</td>
</tr>
<tr>
<td>7</td>
<td>77% (7/9)</td>
<td>100% (3/3)</td>
<td>100% (3/3)</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>100% (11/11)</td>
<td>100% (2/2)</td>
<td>100% (2/2)</td>
<td>100% (4/4)</td>
</tr>
</tbody>
</table>

In no case was duck host mesenchyme positive for bone before day 7 of culture (Table 1).
In reciprocal transplants (i.e. duail), duck donor mesenchyme was delayed in its osteogenic differentiation relative to that observed in quail host mesenchyme (Fig. 2C,D). At 6 days of culture, we observed abundant quail host-derived bone in chimeric mandibles but no duck donor-derived bone. Only after 7 and 8 days of culture, which is equivalent to the amount of time normally required for bone to form in control duck, did duck-derived mesenchyme (i.e. Q¢PN-negative) stain positively for bone (Fig. 2E,F).

Neural crest-derived mesenchyme also altered the expression of genes known to affect bone development. Quail donor mesenchyme upregulated Runx2 at a stage when duck host mesenchyme expressed this gene at much lower and more restricted levels (n=3; Fig. 2G). Expression of Msx1 was upregulated on the donor-derived side relative to that observed in host-derived mesenchyme (n=3; Fig. 2H).

Mesenchyme controls the timing of interactions with epithelium

To uncover a mechanism through which mesenchyme could exert its control over osteogenesis, we defined embryonic stages in quail and duck during which tissue interactions are required for mandibular bone formation, and determined the extent to which mesenchyme governs these interactions. We extracted mandibular primordia at successive stages (HH21-HH27) from quail, duck and quck embryos, removed overlying epithelium, cultured the mesenchyme in vitro, and assayed for histological evidence of bone (Fig. 1C,D). Consistent with previous reports for chick (Tyler and Hall, 1977; Van Exan and Hall, 1984), our experiments confirm that the ability of mandibular mesenchyme to form bone when cultured in the absence of epithelium depends upon the time of mesenchymal isolation. Intact control quail mandibles harvested at HH23 and cultured for 5 days formed bone (Fig. 3A, Table 1), whereas HH23 quail mesenchyme cultured without epithelium, was unable to form bone even after 8 days (0%, n=6; Table 2). Although all samples of intact HH25-HH26 quail mandibles formed bone (100%, n=8) following 6 days of culture, none of the corresponding samples of quail mesenchyme cultured without epithelium formed bone even after 8 days (0%, n=18; Fig. 3B,C). The same stage-dependent effects were observed in duck mandibles cultured with and without epithelium (Table 2). All samples contained abundant amounts of cartilage, which serves as an internal control for tissue viability, as mandibular cartilage formation can occur in the absence of epithelium (Tyler and Hall, 1977). By sharp contrast, mesenchyme from HH27 quail embryos formed bone without overlying epithelium after 6 days of culture (100%, n=5; Fig. 3D), as did mesenchyme from HH27 duck embryos cultured without epithelium for 7 days (n=10; Table 2).

To evaluate the effects that these tissue interactions have on molecular mediators of skeletal differentiation, we carried out whole-mount in situ hybridization experiments on quail mandibles collected at HH25 and cultured for 24 hours. For controls, we assayed for Col2a1 transcripts in mesenchyme cultured with (n=3) and without (n=3) epithelium, and observed no difference in expression (Fig. 3E,F). Because Col2a1 is expressed in regions that will form cartilage, these experiments provide an internal control for tissue viability and affirm that mesenchyme can synthesize mRNA without epithelium. Contralateral sides of control quail mandibles also expressed Runx2 when cultured with epithelium (n=3; Fig. 3G). However, contralateral mesenchyme cultured without epithelium demonstrated a significant downregulation of Runx2 within 24 hours of epithelial removal (n=3; Fig. 3H).

Fig. 3. Mesenchyme sets the timing of tissue interactions required for osteogenesis. (A) HH23 quail mandibles cultured for 6 days are positive for Osteoid (blue). (B,C) By contrast, quail mesenchyme harvested without epithelium at HH25 and HH26, and cultured for up to 8 days forms cartilage but not bone. (D) By HH27, mesenchyme cultured without epithelium makes bone (arrow). (E,F) Mandibles harvested from quail at HH25 and cultured with and without epithelium for 24 hours express Col2a1 in Meckel's cartilage as shown in lateral view. (G) HH25 mesenchyme cultured with epithelium for 24 hours expresses Runx2 (arrow). (H) When mandibles are cultured without epithelium, Runx2 expression is significantly downregulated (asterisk). (I,J) Quck mandibles harvested at HH25 and cultured for 6 days without epithelium form bone (arrow) due to faster developing quail donor mesenchyme (arrow).

To investigate the extent to which mesenchyme controls the timing of signaling interactions necessary for bone formation, mandibles from quck embryos were cultured with and without epithelium. Again, control mandibles at HH25 did not form bone when cultured without epithelium and could only do so after HH27 (Fig. 3B,D). Yet chimeric mandibles at HH25 formed abundant bone when cultured without epithelium (Fig. 3I; 100%, n=5). Osteoid stain within these quck mandibles was coincident only with quail-derived osteoblasts (Fig. 3J). Thus, in comparison to controls, chimeric mandibles showed a two-stage shift in the time at which
epithelia were no longer required for osteogenesis. This appeared to be the earliest that quail cells could make bone in chimeras without epithelium, as HH23 \((n=6)\) and HH24 \((n=3)\) quck mandibles did not form bone (data not shown).

**BMP genes are expressed at stages when tissue interactions are required for osteogenesis**

To identify a molecular mechanism through which mesenchyme achieves temporal regulatory control over osteogenesis, we examined spatiotemporal expression patterns of BMPs and their receptors during the period when tissue interactions are critical for mandibular osteogenesis, which is after HH23 but before HH27 (Tyler and Hall, 1977; Hall, 1978). *Bmp2* expression, although detected at low levels in epithelium at HH23, was observed throughout the mesenchyme and epithelium at HH25 (Fig. 4A,B). *Bmp4* transcripts were first restricted to the epithelium at HH23 and were subsequently localized to the mesenchyme only at HH25 (Fig. 4C,D). *Bmp5* gene activity was not detected until HH25, when transcripts were localized to the mesenchyme (Fig. 4E,F). *Bmp7* expression was observed in both epithelium and mesenchyme at HH23 and HH25 (Fig. 4G,H). *Alk2*, a receptor for BMP ligands, was expressed throughout mesenchyme and epithelium at HH23 and HH25 (Fig. 4I,J). *Bmpr1a*, although detected at low levels at HH23, was upregulated throughout the mandible by HH25 (Fig. 4K,L). *Bmpr1b* transcripts were concentrated in lateral mesenchyme and epithelium at HH23, and subsequently became localized to medial mesenchyme by HH25 (Fig. 4M,N). Equivalent stage- and tissue-specific expression patterns were observed for duck (data not shown).

**Neural crest-derived mesenchyme regulates the expression of BMP pathway members**

To discern the ability of mesenchyme to regulate BMP signaling, we performed whole-mount in situ hybridization. Chimeric mandibles harvested at HH23 and cultured for 24 hours showed donor-induced changes to BMP signaling. Although *Bmp4* transcripts were restricted to epithelium in duck control mandibles (Fig. 5A) and on the duck host side of quck mandibles (Fig. 5B), we observed a substantial upregulation of *Bmp4* transcripts on the quail-derived side \((n=2)\). We also observed that *Bmpr1a* \((n=2)\) and *Bmpr1b* \((n=2)\) had altered patterns of expression that were consistent with the upregulation observed for *Bmp4* in chimeric mandibles (Fig. 5C,D). Despite the role of *Alk2* (*ActR1*) in mandibular development (Dudas et al., 2004), no alterations in *Alk2* expression were detected \((n=2);\) data not shown). We did not observe changes in *Bmp2* \((n=4)\), *Bmp5* \((n=2)\) or *Bmp7* \((n=2)\) in chimeras (Fig. 5E, data not shown). Additionally, the BMP inhibitor *Noggin* was expanded less in quail-derived mesenchyme \((n=2)\), which would allow for a net increase in BMP4 signaling activity (Fig. 5F). We also found that Runx2 and Msx1, which function downstream of BMP signaling, were upregulated, similar to that of *Bmp4* (Fig. 2G,H).

**Table 2. Bone formation in mesenchyme cultured without epithelium**

<table>
<thead>
<tr>
<th>Surgery stage</th>
<th>Duck control</th>
<th>Duck host side (Q¢PN–)</th>
<th>Quail donor side (Q¢PN+)</th>
<th>Quail control</th>
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</thead>
<tbody>
<tr>
<td>HH23</td>
<td>0% (0/8)</td>
<td>0% (0/6)</td>
<td>0% (0/6)</td>
<td>0% (0/6)</td>
</tr>
<tr>
<td>HH24</td>
<td>–</td>
<td>0% (0/3)</td>
<td>0% (0/3)</td>
<td>–</td>
</tr>
<tr>
<td>HH25</td>
<td>0% (0/5)</td>
<td>0% (0/5)</td>
<td>100% (5/5)</td>
<td>0% (0/5)</td>
</tr>
<tr>
<td>HH26</td>
<td>0% (0/7)</td>
<td>–</td>
<td>–</td>
<td>0% (0/12)</td>
</tr>
<tr>
<td>HH27</td>
<td>100% (10/10)</td>
<td>–</td>
<td>–</td>
<td>100% (5/5)</td>
</tr>
</tbody>
</table>

Fig. 4. Spatiotemporal expression patterns of BMP pathway members in quail. (A,B) *Bmp2* (white), detected at low levels in epithelium at HH23, is expressed across the mandible at HH25. (C) *Bmp4* transcripts are restricted to epithelium at HH23 (arrow). (D) By HH25, *Bmp4* transcripts are expressed only in mesenchyme (arrow). (E,F) *Bmp5* is not expressed at HH23 but is detected at HH25 in mesenchyme. (G,H) *Bmp7* expression is observed in both epithelium and mesenchyme at HH23 and HH25. (I,J) *Alk2* is uniformly expressed throughout the mandible at HH23 and HH25. (K,L) *Bmpr1a*, although not detected at HH23, is expressed across the mandible by HH25. (M,N) *Bmpr1b* transcripts are concentrated in lateral mesenchyme and epithelium at HH23 and become localized to medial mesenchyme by HH25.
Epithelium is initially required for mesenchymal Bmp4 expression

Given that epithelial-mesenchymal interactions are necessary for intramembranous ossification, we investigated the extent to which such interactions regulate Bmp4. We challenged mesenchyme to maintain Bmp4 expression, which normally appears by HH25 (Fig. 5D), in the absence of epithelium. Control mandibles harvested at HH25, treated with digestive enzymes, and cultured for 24 hours, expressed Bmp4 broadly across the distal mesenchyme (n=3; Fig. 5G). In the distal mesenchyme of control mandibles cultured at HH26, Bmp4 transcripts were localized to three distinct regions (data not shown) in an expression pattern equivalent to that reported for mouse mandibles at E11.5 (Tucker et al., 1998a). In control mandibles cultured at HH27, Bmp4 expression in the distal mesenchyme was further restricted peripherally (n=3; Fig. 5H). However, upon removal of epithelium from HH25 (n=3) and HH26 (n=3) mandibles, distal mesenchyme lost the ability to express Bmp4 (Fig. 5I, data not shown). Bmp4 expression was still detected proximolaterally, indicating that neither enzymatic digestion nor epithelial removal destroyed mesenchymal synthesis of mRNA. Furthermore, HH27 mesenchyme expressed Bmp4 distally, even without overlying epithelium (n=6; Fig. 5J).

Exogenous BMP4 accelerates bone formation

The correlation between the timing of epithelial-mesenchymal interactions and Bmp4 expression strongly reinforced the notion that Bmp4 mediates the osteogenic effects of interactions between mandibular epithelium and mesenchyme. As a further test, we used a biochemical approach to examine the capacity of BMP signaling to control the expression of endogenous molecular regulators of osteogenesis and to influence the timing of bone formation. Specifically, mandibles from quail embryos at HH21 and HH23 were surgically extracted and treated with BMP4. Mandibles were cultured for 24 hours and then analyzed for changes in gene expression (Fig. 6A-D). Whole-mount in situ hybridization revealed that exogenous BMP4 directly expresses the transcription factors Mxl1, Twist1 and Runx2. Mxl1 is normally expressed in the mandible medially after HH23 (Mina et al., 1995). Upon treatment with BMP4, Mxl1 expression was expanded laterally to surround the beak (n=4; Fig. 6B). Twist1, a known inhibitor of osteoblast differentiation (Bialek et al., 2004), is normally expressed medially (Soo et al., 2002). BMP4 treatment resulted in the downregulation of Twist1 (n=4; Fig. 6C), whereas Runx2 was slightly upregulated (n=4; Fig. 6D).

To ascertain the potential of BMP signaling to regulate the timing of bone formation, we delivered BMP4 to quail and duck mandibles at HH23. We selected this approach because the effects of protein-soaked beads allowed us to achieve a transient period of protein augmentation and to mimic the premature upregulation of Bmp4 that occurs in donor-derived mesenchyme of quck mandibles (Fig. 5B). Mandibles were cultured for 3 to 6 days (Fig. 6E) and bone was assayed histologically. Representative sections (Fig. 6F) were digitized, and bone (Fig. 6G) and condensation (Fig. 6H) volume were estimated (Colnot et al., 2003; Lu et al., 2005). Stage HH23 quail mandibles cultured for up to four days (n=12), and duck mandibles cultured for up to 6 days (n=17), did not present any histological evidence of bone on either the BMP4- or the BSA-treated side (Table 3, data not shown). Following extended cultures for 5 or more days for quail (n=24), and 7 or more days for duck (n=15, data not shown), mandibular explants showed a statistically significant increase in bone and condensation volume on the side treated with BMP4 compared with the control side treated with BSA (Table 3). For example, those quail mandibles treated with BMP4 and cultured for 5 days exhibited an average 3.3-fold (P=0.0007) increase in bone volume and a 2.4-fold (P<0.0004) increase in volume of the condensation around the bone, relative to the control side. Interestingly, the control side for five of these 11 mandibles formed neither matrix nor condensations at all, demonstrating that BMP4 accelerates the timing of bone formation.

Noggin delays bone formation in quack

To complement our BMP4 gain-of-function experiments, we employed a biochemical loss-of-function approach. We attempted to delay skeletal formation in control embryos and to ‘rescue’ the
premature skeletal differentiation in chimeras by delivering recombinant Noggin to mandibles. Noggin was the best candidate inhibitor given its endogenous role in the antagonism of BMP4 during axial skeletal development (Wijgerde et al., 2005) and mandibular patterning (Stottmann et al., 2001). Furthermore, exogenous Noggin treatments have been shown to inhibit BMP4 activity in mouse mandibular explants (Tucker et al., 1998b).

If mesenchyme controls the timing of skeletal differentiation by regulating the expression of \(Bmp4\), then the inhibition of BMP signaling should also affect the ability of mesenchyme to form bone. Consistent with published data (Tucker et al., 1998b), HH23 quail mandibles treated unilaterally with Noggin and cultured for 24 hours showed a reduced expression of \(Msx1\) (Fig. 6I). Additionally, we found that Noggin not only inhibited BMP signaling, but also delayed osteogenesis. Stage HH23 quail mandibles were placed in organ culture for 0, 24 or 48 hours, and were then treated unilaterally with Noggin. Mandibles were subsequently grown for 6 days and assayed histologically for bone. Those mandibles exposed to Noggin after 24 hours of culture did not form any bone on the treated side \((n=6)\), despite the formation of abundant bone on the untreated contralateral side (Table 4). More than one-third (38%) of the mandibles treated with Noggin following 48 hours of culture formed bone \((n=5)\). Thus, a block in BMP signaling before 48 hours of culture delays bone formation. These data establish a developmental window during which BMP signaling exercises temporal control over osteogenesis.

### Table 3. Exogenous BMP4 alters the timing of osteogenesis in HH23 quail mandibles

<table>
<thead>
<tr>
<th>Days cultured</th>
<th>(n)</th>
<th>Average untreated volume ((\text{mm}^3))</th>
<th>Average treated volume ((\text{mm}^3))</th>
<th>Fold difference</th>
<th>(P)-value</th>
<th>Average untreated volume ((\text{mm}^3))</th>
<th>Average treated volume ((\text{mm}^3))</th>
<th>Fold difference</th>
<th>(P)-value</th>
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<td>0</td>
<td>–</td>
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<tr>
<td>5</td>
<td>11*</td>
<td>(0.34 \times 10^{-6})</td>
<td>(1.10 \times 10^{-6})</td>
<td>3.3</td>
<td>0.0007</td>
<td>(0.49 \times 10^{-3})</td>
<td>(1.17 \times 10^{-3})</td>
<td>2.4</td>
<td>0.0004</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>(1.73 \times 10^{-6})</td>
<td>(3.72 \times 10^{-6})</td>
<td>2.2</td>
<td>0.0001</td>
<td>(1.30 \times 10^{-3})</td>
<td>(2.83 \times 10^{-3})</td>
<td>2.2</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

*Five out of 11 quail mandibles cultured for 5 days had matrix and condensations only on the BMP4-treated side.
0, 12, 24 or 48 hours, and were treated with Noggin. By day 5 of culture, quack mandibles would have formed early bone on the donor side but not on the host side (Table 1). Noggin treatment at the time of culture delayed premature bone formation in 100% of the chimeric mandibles (n=5; Table 4). However, by 12 hours of culture, Noggin became significantly less effective at delaying the premature differentiation of donor-derived mesenchyme, as only 13% of chimeric mandibles lacked bone (n=8; Table 4, data not shown). By 24 hours of culture, Noggin was unable to delay bone growth in any chimeric mandible (n=7; Fig. 6K,L). This ineffectiveness could not be overcome by administering even a 10-fold increase in Noggin concentration. Thus, in quck mandibles Noggin was merely able to delay bone formation before 24 hours of culture.

**DISCUSSION**

**Mesenchyme temporally controls osteogenesis and requisite interactions with the epithelium**

Our results demonstrate that mesenchyme establishes the timing of bone formation and does so by autonomously executing molecular programs for osteogenesis. Following transplantation, quail donor mesenchyme maintains its faster timetable for osteogenesis within the slower environment of the duck host, which is consistent with that observed in previous experiments (Noden, 1983; Tucker and Lumsden, 2004). Additionally, quail donor mesenchyme expresses increased levels of Msx1 and Runx2 at a stage when duck host mesenchyme expresses these genes only at low levels. In reciprocal transplants of duck neural crest cells into quail (i.e. duall), we find that duck donor mesenchyme is delayed in its bone formation when compared with that of the quail host. Thus, faster-developing quail host epithelium is not sufficient to accelerate the osteogenic differentiation of slower-developing duck-derived mesenchyme in duall mandibles.

How does mesenchyme establish the timing of mandibular osteogenesis if bone formation relies upon epithelial signaling? To address this question, we defined the precise developmental stage in quail and duck at which mandibular mesenchyme no longer needs epithelial influence for intramembranous ossification, and then used chimeras to determine the extent to which such timing is governed by the mesenchyme. Our epithelial removal experiments show that signaling interactions required to express transcriptional regulators of osteogenesis, such as Runx2, are complete by HH27, which is a stage when osteogenic condensations are defined histologically (Tyler and Hall, 1977). In quck mandibles, these interactions are prematurely finished by HH25 because of the presence of an accelerated population of quail donor mesenchyme. Thus, mesenchyme schedules the epithelial interactions required for intramembranous ossification.

Overall, our results provide evidence that the epithelium signals permissively rather than instructively during intramembranous ossification. The epithelial signal appears to be a proteinaceous component located in the basal lamina (Bradamante and Hall, 1980; Hall and Van Exan, 1982; Hall et al., 1983), which is transmitted via cell-cell contact to mesenchyme (Van Exan and Hall, 1984). Because our quail-duck transplants accelerate the timing of epithelial-mesenchymal interactions, as well as the subsequent formation of bone, we expect that, at earlier stages, donor mesenchyme induces premature signaling from the overlying host epithelium, and/or that epithelial signals stay continuously expressed before their period of necessity, and that mesenchyme regulates its own competence to respond to these signals (Fig. 7). The former scenario is substantiated by prior quail-duck experiments where donor mesenchyme accelerated the expression of genes in host-derived epithelial tissues of quck (Schneider and Helms, 2003; Eames and Schneider, 2005). The latter possibility finds support in previous in vitro heterochronic work in chick that demonstrates a capacity for epithelium to support osteogenesis as early as HH18 (Hall, 1978). Additionally, many genes such as Bmp2, Bmp4 and Bmp7 are expressed in the mandibular epithelium and oropharyngeal cavity as early as HH15 and thereafter (Francis-West et al., 1994; Wall and Hogan, 1995; Shigetani et al., 2000; Ashique et al., 2002b; Mina et al., 2002). But as these epithelial gene expression patterns arise prior to and concurrent with the arrival of neural crest-derived mesenchyme in the mandibular primordia (Tosney, 1982; Noden, 1991), the extent to which their induction is mesenchyme-dependent remains unknown. At this point, the quail-duck chimeric system cannot clearly identify mesenchyme-mediated events prior to HH15 because not enough incubation time passes after surgery at HH9.5 to detect a substantive difference between donor and host populations. Nonetheless, as our experiments make clear, there is little doubt that signaling interactions start right away, and that their outcome is set forth by mesenchyme.

**Mesenchyme regulates mesenchymal BMP signaling**

Our results show that mesenchymal BMP signaling depends upon epithelial-mesenchymal interactions, and furthermore plays a functional role in promoting the subsequent differentiation of bone. Spatiotemporal changes in Bmp4 expression correlate with the timing of osteogenic tissue interactions in the mandible, and they corroborate other BMP gene expression studies (Barlow and Francis-West, 1997; Nonaka et al., 1999). The switch of Bmp4 expression from epithelium to mesenchyme during avian osteogenesis is similar to what has been observed during mouse odontogenesis, and to what has been shown for bone development (Vainio et al., 1993; Aberg et al., 1997; Barlow and Francis-West, 1997; Tucker et al., 1998b; Nonaka et al., 1999; Wang et al., 1999). For example, this expression transition does not occur in Msx1 mutant mice, and, ultimately, results in adontia and lower jaw deficiencies (Satokata and Maas, 1994; Chen et al., 1996).

Our gene expression analyses also yield evidence for regionalized activity of the BMP pathway during mandibular osteogenesis. BMPs promote the differentiation of osteoblasts (Urist, 1965; Wozney et al., 1988; Wang et al., 1990), and bone formation in the avian jaw requires BMP signaling via BMPR1B (Ashique et al., 2002b). The co-expression of Bmp4 and Bmpr1b that we observe in medial mesenchyme suggests an initial restriction of BMP signaling.

**Table 4. Noggin delays bone formation in HH23 mandibular explants**

<table>
<thead>
<tr>
<th>Quail</th>
<th>Time of Tx (hours)</th>
<th>n</th>
<th>Bone matrix treated side</th>
<th>Bone matrix control side*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>n</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>5</td>
<td>8</td>
<td>5</td>
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**Quick**

<table>
<thead>
<tr>
<th>Time of Tx (hours)</th>
<th>n</th>
<th>Q¢PN+ bone matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>0</td>
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<tr>
<td>12</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>24</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>24†</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*Treated with 0.1% BSA.
†10× increase in Noggin concentration.
Tx, treatment.
is also restricted to medial mesenchyme and its abrogation in mice causes medial truncation of the lower jaw, suggesting a relationship between expression and skeletal pattern (Vainio et al., 1993; Satokata and Maas, 1994; Hollnagel et al., 1999; Ishii et al., 2003; Brugger et al., 2004). In quck mandibles, Bmp4, Bmp11b and Mxs1 transcripts are prematurely upregulated in medial mesenchyme, linking their expression to accelerated bone formation.

Our study reveals that mesenchyme regulates its own BMP signaling, that at least initially this signaling depends upon interactions with the overlying epithelium, and that epithelial maintenance eventually stops. Such results are consistent with studies showing that an epithelial source of BMP4 autoregulates mesenchymal expression by activating Mxs1 in the adjacent mesenchyme (Vainio et al., 1993; Tucker et al., 1998b; Wang et al., 1998). In Mxs1 mutant mice, epithelial BMP4 is unable to induce Bmp4 in mandibular mesenchyme (Chen et al., 1996). The removal of epithelial BMP prevents mesenchymal activation of Mxs1 (Tucker et al., 1998b), consequently impeding Bmp4 expression. At HH27, just prior to condensation, we find that mesenchymal expression of Bmp4 becomes fixed. The ability of isolated mesenchyme to sustain Bmp4 independently coincides with its autonomous differentiation into bone. Together, these data suggest that mesenchyme dictates the timing of bone formation by governing mesenchymal BMP4 signaling.

We have also substantiated the potential of exogenous BMP4 to mediate the expression of transcriptional regulators of osteogenesis and establish the timing of bone formation. BMP4 treatment in control mandibles recapitulates the early bone phenotype of quck chimeras. However, we find that BMP4 alone cannot functionally replace mandibular epithelium and induce bone formation (data not shown). Moreover, others have reported that such treatments, although sufficient to induce early molecular programs for bone (Wang et al., 1998), are not able to induce overt differentiation prior to a stage when mesenchyme can form bone autonomously (Ekanayake and Hall, 1997). This can be explained by the apparent necessity for other epithelial signals, such as Fibroblast Growth Factors, during mandibular development (Trumpp et al., 1999; Tucker et al., 1999; Fergusson et al., 2000; Shigetani et al., 2000; Mina et al., 2002; Wilson and Tucker, 2004; Havens et al., 2006). That the maintenance of mesenchymal BMP4 expression requires epithelium for a period after epithelial BMP4 expression subsists is further evidence for this scenario.

By treating with exogenous Noggin, we tested the biological significance of endogenous BMP signaling during mandibular osteogenesis. Noggin blocks the osteo-inductive potential of Bmps by forming complexes that inhibit the ability of Bmps to interact with their receptors (Zimmerman et al., 1996). For example, overexpression of Noggin in vivo inhibits osteoblast differentiation (Devlin et al., 2003; Wu et al., 2003). We find that Noggin blocks endogenous BMP in mandibular explants and delays mesenchymal differentiation into bone. After 48 hours in culture, quail mandibles are no longer susceptible to the inhibitory effects of Noggin, as they are capable of forming bone following treatment. In quck mandibles, the window of opportunity for Noggin inhibition is reduced to 24 hours, providing additional evidence that mesenchyme exerts temporal control over BMP signaling, which in turn establishes the timing of osteogenesis.

**Temporal changes in epithelial-mesenchymal interactions may influence facial evolution**

The ability of mesenchyme to regulate the timing of tissue interactions underlying bone formation may offer insight into developmental mechanisms facilitating facial evolution. Modifications to the timing of osteogenesis have been proposed as a source of evolutionary variation (Smith and Hall, 1990;
MacDonald and Hall, 2001). Small temporal changes to inductive interactions can alter the balance between growth and differentiation, leading to variation in skeletal size and shape. Also, subtle changes in the timing of osteogenic condensation account for the morphological differences observed in mandibles of inbred mouse strains (Miyake et al., 1997b; Miyake et al., 1997a; Hall and Miyake, 2000). Our study reveals that mesenchyme establishes the timing of key events during intramembranous ossification, including interactions with adjacent epithelium, mesenchymal Bmp4 expression and osteoid matrix deposition.

Such findings are of particular significance, as recent work has implicated both Bmp4 and neural crest-derived mesenchyme in generating species-specific facial morphology. Spatiotemporal changes in the mesenchymal expression of Bmp4 produce variations in avian beak shape (Abzhanov et al., 2004; Wu et al., 2004; Wu et al., 2006; Schneider, 2007). For example, chicken and duck exhibit different levels and domains of Bmp4 expression that correlate with their beak shapes, and overexpression of Bmp4 in the distal mesenchyme increases the width of chicken beaks. A similar role for Bmp4 has also been proposed for bony fish, as variations in jaw shape in zebrasfish and East African cichlids correlate with variable domains of Bmp4 expression, and genetic data link such traits to a chromosomal region that contains Bmp4 (Albertson et al., 2005; Albertson and Kocher, 2006).

Our experiments suggest that mesenchyme employs Bmp4 as a mechanism to implement osteogenic pattern in space and time. BMP signaling probably enhances the recruitment of neural crest-derived mesenchyme and alters cell proliferation through Msx1 (Mina et al., 1995; Kim et al., 1998; Hall and Miyake, 2000; Rice et al., 2000; Goldring et al., 2006). Additionally, our experiments indicate that once neural crest-derived mesenchyme is in place, initial and subsequent events of osteogenesis unfold fairly autonomously. The connection to the neural crest is especially important here because prior experiments using quail and duck reveal that this embryonic population provides species-specific information for facial morphology (Schneider and Helms, 2003; Tucker and Lumsdon, 2004). Quail mesenchyme can generate a quail-like skeleton in duck, and vice versa. We propose that by regulating BMP signaling, and especially the timing of interactions with adjacent epithelium, mesenchyme functions as a mechanism integrating the process of osteogenesis with the species-specific patterning of bone.

We thank K. Butcher, J. Staufinger and J. Hur for technical assistance; R. Yeh, S. Brugger, T. Alliston and R. Maruccio for helpful discussions; and T. Dam at AA Lab Egs. The QtpN antibody was obtained from the DSHB, developed under the auspices of the NICHD and maintained by the University of Iowa. Funded in part by NIH T32/DE07306-09 to A.E.M.; NIH F32 DE016778 to Y., Dobias, S. L., Yi, S. E., Lyons, K., Bell, J. R. et al. (2004). A phylogenetically conserved cis-regulatory module in the Msx2 promoter is sufficient for BMP-dependent transcription in murine and Drosophila embryos. Development 131, 5153-5165.


Francis-West, P. H., Tatla, T. and Brickell, P. M. (1994). Expression patterns of the bone morphogenetic protein genes Bmp-4 and Bmp-2 in the developing chick face suggest a role in outgrowth of the primordia. Dev. Dyn. 201, 168-178.


