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An *in situ* hybridization study of *Runx2*, *Osterix*, and *Sox9* in the anlagen of mouse mandibular condylar cartilage in the early stages of embryogenesis

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Abstract

Mandibular condylar cartilage is the best-studied mammalian secondary cartilage, differing from primary cartilage in that it originates from alkaline phosphatase-positive progenitor cells. We previously demonstrated that three transcription factors related to bone and cartilage formation, namely *Runx2*, *Osterix* and *Sox9*, are simultaneously expressed in the anlage of mandibular condylar cartilage (condylar anlage) at embryonic day (E)14. In this study, expression of these transcription factors was investigated in the anlagen of mandibular bone (mandibular anlagen) from E11.0 to 14.0. *Runx2* mRNA was first expressed in the mandibular anlage at E11.5. *Osterix* mRNA was first expressed at E12.0, and showed a different expression pattern from that of *Runx2* from E12.5 to E14.0, confirming that *Osterix* acts downstream of *Runx2*. *Sox9* mRNA was expressed in Meckel's cartilage and its anlagen throughout the experimental period, but not clearly in the mandibular anlagen until E13.0. At E13.5, the condylar anlage was morphologically identified at the posterior end of the mandibular anlage, and enhanced *Sox9* mRNA expression was detected here. At this stage, *Runx2* and *Osterix* mRNA were simultaneously detected in the condylar anlage. These results indicate that the *Sox9* mRNA-expressing condylar anlage is derived from *Runx2*/*Osterix* mRNA-expressing mandibular anlage, and that upregulation of *Sox9* in this region acts as a trigger for subsequent condylar cartilage formation.

Key words: Meckel's cartilage; secondary cartilage; *Sox5*; transcription factor.

Introduction

Secondary cartilage has several definitions, and detailed information relevant to this topic has been well reviewed (Beresford, 1981; Vinnka, 1982). One acceptable definition of secondary cartilage is that it arises from the periostea of membrane bone after (secondary to) bone formation.

Avian secondary cartilage, including the quadratojugal, has been extensively studied and characterized (Hall, 2005), and the mandibular condylar cartilage is the best-studied mammalian secondary cartilage. Secondary cartilage differs from primary cartilage in various ways, one of which is that condylar cartilage is derived from alkaline phosphatase (ALP)-positive progenitor cells of the periosteum continuous to the preliminarily formed ossifying mandible (Shibata et al. 1996, 1997; Miyake et al. 1997; Fukada et al. 1999; Fukuoka et al. 2007). These observations indicate the close relationship of condylar cartilage to the developing mandibular bone, although some differences exist among species (Hall, 2005). Progenitor cells of this cartilage (namely skeletoblasts, see Silbermann et al. 1987) rapidly differentiate into hypertrophic chondrocytes (Shibata et al. 1997; Fukada et al. 1999; Fukuoka et al. 2007), after which the temporomandibular joint, consisting of condylar cartilage, an articular disc, and upper and lower joint cavities, can be morphologically identified until E18.5 (Shibukawa et al. 2007). In addition, the periostea/perichondria of the dentary have different characteristics to the perichondria of the limb bud (primary) cartilage. The limb bud cartilage forms mesenchymal cell condensations before the perichondria are present, and the periostea replaces the perichondria adjacent to hypertrophic chondrocytes. Meanwhile, the periostea of the dentary are neural crest in origin, and do not form via chondrogenesis, but have bipotentiality that can differentiate into both bone and secondary cartilage (Fang & Hall, 1996, 1997).

In the last few years, several transcription factors essentially involved in bone and/or cartilage formation have been identified. *Sox9* (SRY-box containing gene 9) is

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an essential factor for chondrocyte differentiation; it is expressed in the chondrogenic region (Wright et al. 1995; Ng et al. 1997; Zhao et al. 1997; Bi et al. 1999), directly regulates cartilage-specific genes (Bell et al. 1997; Lefebvre et al. 1997; Xie et al. 1999; Sekiya et al. 2000), and induces ectopic cartilage formation when mis-expressed (Bell et al. 1997; Healy et al. 1999). In addition, *Sox9* interacts with *Sox5* (*L-Sox5*) and *Sox6* (Lefebvre et al. 1998; Smits et al. 2001). Using the Cre/loxP recombination system, Akiyama et al. (2002) demonstrated that *Sox9* plays essential roles in the successive steps from undifferentiated mesenchymal cells to proliferating chondrocytes, and is required for *Sox5* and *Sox6* expression. Using *Sox9*-Cre;R26R mice, Akiyama et al. (2005) further demonstrated that osteochondroprogenitor cells are derived from *Sox9*-expressing precursors during mouse embryogenesis.

Runx2 (runt-related transcription factor 2) is an essential transcription factor for bone formation and *Runx2* gene knockout mice completely lack bone tissue (Komori et al. 1997; Otto et al. 1997; Inada et al. 1999). *Runx2* also regulates hypertrophic chondrocyte differentiation (Inada et al. 1999; Kim et al. 1999; Enomoto et al. 2000; Takeda et al. 2001; Ueta et al. 2001). Yoshida et al. (2004) demonstrated that *Runx2* and *Runx3* are essential for chondrocyte maturation, and that *Runx2* regulates limb growth through the induction of Indian hedgehog (Ihh).

Further, Nakashima et al. (2002) reported that the gene knockout of a novel zinc-finger-containing transcription factor called *Osterix* causes a complete lack of bone formation, demonstrating that *Osterix* is another essential factor for osteoblast differentiation. Previous studies of these transcription factors have mainly focused on primary cartilage or long bone formation, but rarely on secondary cartilage formation.

Buxton et al. (2003) first reported that in avian secondary cartilage (quadratojugal), *Runx2*-expressing preosteoblasts exit from the cell cycle and rapidly differentiate into hypertrophic chondrocytes; a process that correlates with the upregulation of *Sox9*. We reported that mandibular condylar cartilage is completely absent in *Runx2*-deficient mice (Shibata et al. 2004), and bone morphogenetic protein 2 (BMP2) can rescue secondary cartilage formation in these mice, although BMP2 cannot rescue chondrocyte hypertrophy (Fukuoka et al. 2007).

Furthermore, we described that mRNAs for *Runx2*, *Osterix* and *Sox9* are expressed in the condylar anlage, consisting of preosteoblasts/skeletoblasts, at E14.0, and that reduced expression of *Osterix* in combination with *Sox9-Sox5* expression is important for the onset of condylar cartilage formation at E15.0 (Shibata et al. 2006). As described above, *Sox9* is strongly expressed in the chondrogenic region, and condylar cartilage is derived from progenitor cells in the periosteum continuous to the preliminarily formed ossifying mandible. These findings led us to hypothesize that a strong *Sox9*-positive area may appear in the anlagen of mandibular bone at earlier stages of embryogenesis. The main purpose of this study was to confirm this hypothesis by standard *in situ* hybridization analysis.

Nakashima et al. (2002) argued that *Osterix* acts downstream of *Runx2* in the process of osteoblast differentiation, and Akiyama et al. (2005) reported that *Osterix* mRNA is temporally expressed later than *Runx2* during limb bud development. However, no *in situ* hybridization studies have been done relevant to the expression pattern of both transcription factors in mandibular bone formation. Furthermore, using the *Wnt-1* Cre/loxP recombination system, Mori-Akiyama et al. (2003) reported that *Sox9* is essential for cartilage derived from neural crest cells, including Meckel's cartilage. However, these findings have not been confirmed in the formation of Meckel's cartilage by *in situ* hybridization. Thus, another purpose of this study was to confirm the expression pattern of these transcription factors in the formation of Meckel's cartilage and mandibular bone using standard *in situ* hybridization.

Materials and methods

All animals were housed in facilities approved by the Health Sciences University of Hokkaido. Our animal-use protocol conformed to the 'Institutional Administrator's Manual for Laboratory Animal Care and Use' (NIH publication No. 88–2959) and was reviewed and approved by the Screening Committee for Animal Research of the Health Sciences University of Hokkaido.

Tissue preparation

A total of 10 pregnant ICR mice, of E11.0–14.0 (08:00 hours on the day of the vaginal plug was designated as E0), were used for this study. At each time point, the pregnant mice were killed by cervical dislocation under ether anesthesia, after which each fetal mouse was killed by cervical dislocation. The heads were then removed and immersed in 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4) for 1 day at 4 °C. The specimens were embedded in paraffin using standard procedures. Sections (5 μm) were cut in the horizontal or coronal (perpendicular to the sagittal plane and parallel to the long axis of condylar process) plane and stained with 0.1% Toluidine blue (0.1 M phosphate buffer, pH 7.4) for histological observation. ALP activity was detected using the Azo-dye method as previously described (Shibata et al. 1997; Fukuoka et al. 2007).

In situ hybridization

Digoxigenin-labeled RNA probes for *aggrecan* and *bone sialoprotein (BSP)* from our previous studies were used (Fukada et al. 1999; Shibata et al. 2002, 2003). ^{35}S-UTP-labeled RNA probes for *Runx2*, *Osterix* and *Sox9*, kindly donated by Dr. Kazuhisa Nakashima (Molecular Pharmacology, Department of Regulation of Internal Environment and Reproduction, Graduate School, Tokyo Medical and Dental University) and a probe for *Sox5* synthesized by reverse transcriptase-polymerase chain reaction were used as in our previous *in situ* hybridization study (Shibata et al. 2006). *In situ* hybridization using the digoxigenin-labeled probes and the Nucleic Acid Detection Kit (Roche Diagnostics, Mannheim, Germany) was performed as previously described (Fukada et al. 1999; Shibata et al. 2002,

2003). When using ^{35}S-UTP labeled probes, sections were dipped in emulsion (NTB, Kodak, Rochester, NY, USA) after hybridization and RNAase treatment, then exposed for 1 week at 4 °C for autoradiography. Sections were observed after counterstaining with nuclear fast red or hematoxylin. Sense probes were used as negative controls.

Results

At E11.0, the mandibular nerve was formed, and a mesenchymal cell condensation of future Meckel's cartilage (termed Meckel's anlage) was faintly recognizable in the mandibular region (Fig. 1a). ALP activity was detected in the anterior part of the mandible and in the brain (Fig. 1b). *Runx2* and *Osterix* mRNA were not expressed (Fig. 1c,d), but *Sox9* and *Sox5* mRNA were expressed in Meckel's anlage (Fig. 1e,f). However, *aggrecan* and *BSP* mRNA were not expressed (Fig. 1g,h).

At E11.5, Meckel's anlage was clearly recognizable and future mandibular bone (termed the mandibular anlage) was also identified (Fig. 2a). ALP activity was detected in the mandibular anlage (Fig. 2b). *Runx2* mRNA was slightly expressed in the mandibular anlage (Fig. 2c) but *Osterix* was not (Fig. 2d). *Sox9* mRNA was clearly expressed in Meckel's anlage but not in the mandibular anlage (Fig. 2e). *Sox5* mRNA was also expressed in Meckel's anlage (Fig. 2f). *Aggrecan* and *BSP* mRNA were not expressed in either Meckel's anlage or in the mandibular anlage (Fig. 2g,h).

At E12.0, Meckel's cartilage was clearly formed and the mandibular anlage had expanded antero-posteriorly (Fig. 3a). ALP activity was detected in the expanded mandibular anlage (Fig. 3b). *Runx2* and *Osterix* mRNA were identically detected in the mandibular anlage (Fig. 3c,d). *Sox9*, *Sox5* and *aggrecan* mRNA were expressed in Meckel's cartilage but not in the mandibular anlage (Fig. 3e,f,g). *BSP* mRNA was also not expressed in the mandibular anlage at this stage (Fig. 3h).

At E12.5, mandibular bone containing mature osteoblasts was clearly formed within the mandibular anlage lateral to Meckel's cartilage (Fig. 4a) and ALP activity was

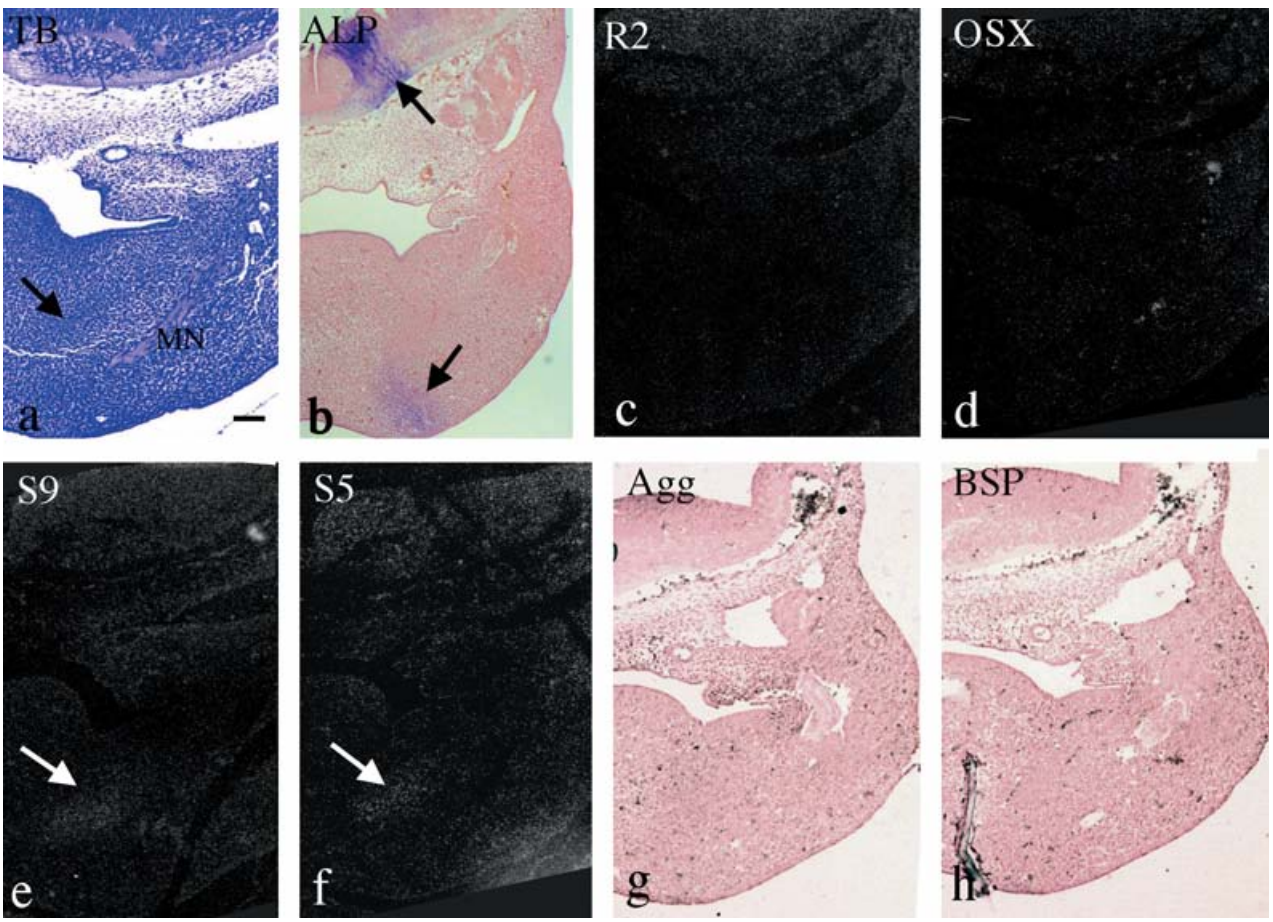

Fig. 1 Mandible in the horizontal plane at E11.0. (a,c–f) Serial sections and (b,g,h) other sections cut in a similar plane. Toluidine blue staining (a), ALP staining (b) and *in situ* hybridization using ^{35}S-UTP labeled probes of dark fields (c–f) and digoxigenin-labeled probes (g,h). (a) Meckel's anlage is faintly recognizable in the mandibular region (arrow). MN, mandibular nerve (b) ALP activity is detected in the anterior part of mandible and in the brain (arrows). (c–h) *Runx2* (c), *Osterix* (d), *aggrecan* (g) and *BSP* (h) mRNA are not expressed, but *Sox9* (e) and *Sox5* (f) mRNA are expressed in the mandibular anlage (arrows in e and f). Width of view = 100 μm.

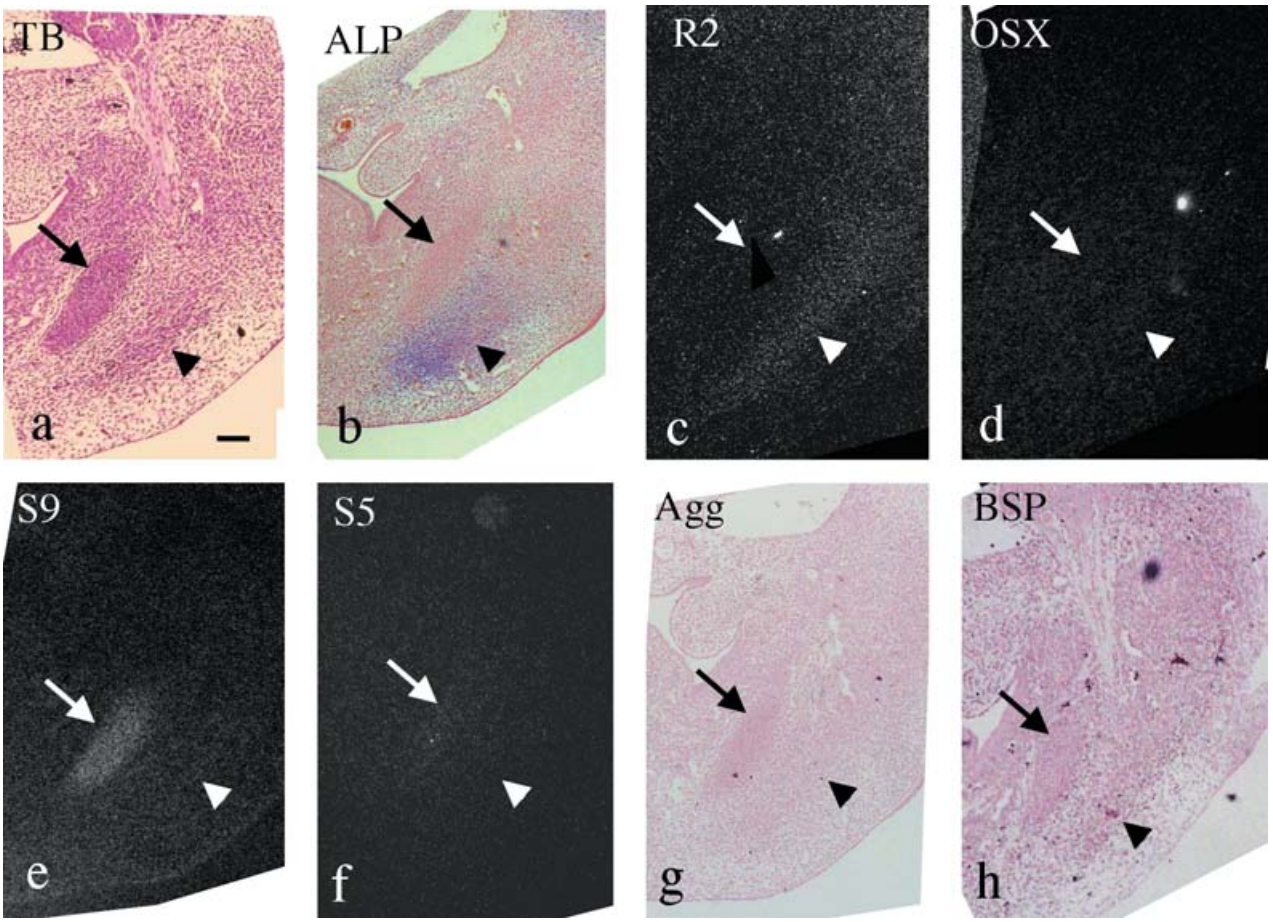

Fig. 2 Mandible in the horizontal plane at E11.5. (a,c–f) Serial sections and (b,g,h) other sections cut in a similar plane. Toluidine blue staining (a), ALP staining (b) and *in situ* hybridization using ^{35}S-UTP labeled probes of dark fields (c–f) and digoxigenin-labeled probes (g,h). (a) Meckel's anlage (arrow) and the mandibular anlage (arrowhead) are recognizable as mesenchymal cell condensations. (b) ALP activity is detected in the mandibular anlage (arrowhead), but not in Meckel's anlage (arrow). (c–h) *Runx2* mRNA is expressed in the mandibular anlage (arrowhead in c), but *Osterix* and other mRNAs are not (arrowheads in d–h). *Sox9* and *Sox5* mRNA are expressed in Meckel's anlage (arrows in e and f) but other mRNAs are not (arrows in c,d,g,h). Width of view = 100 μm.

detected in the mandibular anlage (Fig. 4b). *Runx2* and *Osterix* mRNA were expressed in the mandibular anlage, and *Osterix* tended to be expressed strongly in the center (Fig. 4c,d). *Sox9*, *Sox5* and *aggrecan* mRNA were detected in Meckel's cartilage (Fig. 4e,f,g) and *BSP* mRNA was first detected in the mandibular bone area (Fig. 4h).

At E13.0, the difference of expression patterns of *Runx2* and *Osterix* mRNA became distinctive, i.e. strong expression of *Osterix* mRNA in the center of the mandibular anlage was clearly recognized (Fig. 4i,j). However, the other mRNAs showed similar expression patterns to those seen at E12.5 (data not shown).

At E13.5, the mandibular anlage and mandibular bone within it had further expanded antero-posteriorly, and the outline of the future condylar head and mandibular fossa consisting of mesenchymal cell condensation of future temporal bone were morphologically recognizable at the posterior end of mandibular anlage. We therefore regarded this area as the condylar anlage (Fig. 5a). ALP activity was detected in the mandibular anlage and in the condylar anlage (Fig. 5b). *Runx2* and *Osterix* mRNA showed similar expression patterns to those at E13.0 in the mandibular anlage, but enhanced expression of *Osterix* was not seen in the condylar anlage. Mesenchymal cell condensation of future temporal bone did not show clear mRNA expression of both molecules at this stage (Fig. 5c,d).

Sox9 mRNA was expressed in Meckel's cartilage and first detected in the condylar anlage but not in the anterior position of the mandibular anlage (Fig. 5e). *Sox5* and *aggrecan* mRNA was expressed in Meckel's cartilage but not in either the condylar anlage or the mandibular anlage (Fig. 5f,g). *BSP* mRNA was detected in the center of the mandibular anlage corresponding to mandibular bone area (Fig. 5h).

At E14.0, mandibular bone had expanded and the condylar anlage was recognizable as a mesenchymal cell condensation at the posterior end of mandibular bone, as previously described (Shibata et al. 2006). The formation

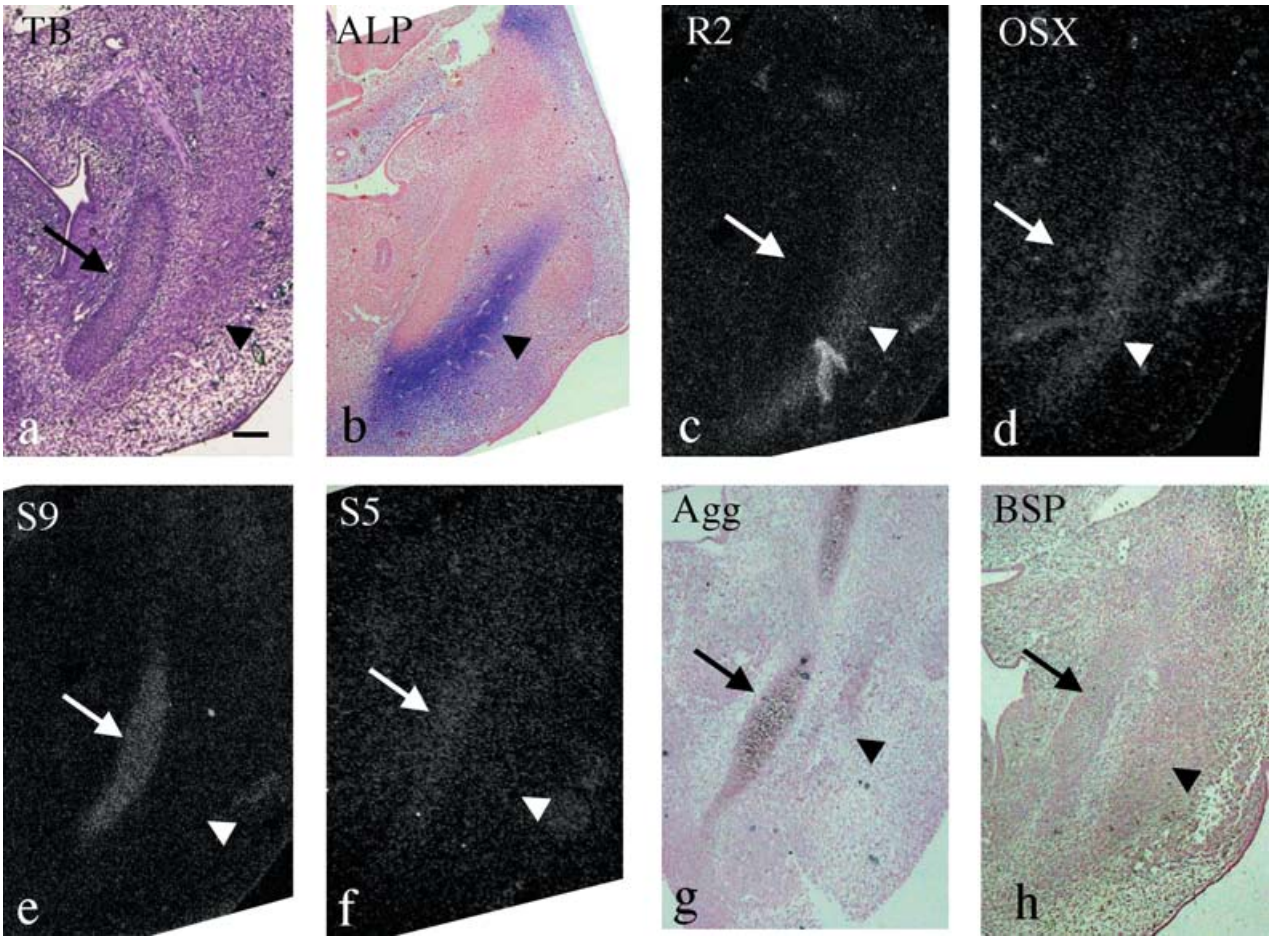

Fig. 3 Mandible in the horizontal plane at E12.0. (a,c–f) Serial sections and (b,g,h) other sections cut in a similar plane. Toluidine blue staining (a), ALP staining (b) and *in situ* hybridization using ^{35}S-UTP labeled probes of dark fields (c– f) and digoxigenin-labeled probes (g,h). (a) Meckel's cartilage has clearly formed (arrow) and the mandibular anlage is recognizable as a mesenchymal cell condensation lateral to Meckel's cartilage (arrowhead). (b) ALP activity is detected in the mandibular anlage (arrowhead). (c–h) *Runx2* and *Osterix* mRNA are expressed in the mandibular anlage (arrowhead in c and d) but other mRNAs are not (arrowheads in e–h). *Sox9*, *Sox5* and *aggrecan* mRNA are expressed in Meckel's cartilage (arrows in e–g) but *Runx2*, *Osterix* and *BSP* mRNA are not (arrows in c,d,h) Width of view = 100 μm.

of temporal bone constituting mandibular fossa was also recognizable. Condylar cartilage had not formed by this stage (Fig. 6a). *Runx2* mRNA was expressed in the condylar anlage and in the periphery of mandibular bone, but weakly in the center (Fig. 6b). In contrast, *Osterix* mRNA was expressed strongly in the center, but still moderately in the condylar anlage (Fig. 6c). Additionally, *Runx2* and *Osterix* mRNA were clearly expressed in the temporal bone at this stage (see Fig. 6b,c).

Sox9 mRNA was expressed in the condylar anlage (Fig. 6d), but *Sox5* mRNA was not (Fig. 6e). Negative controls using sense probes did not show any positive reactions at any stage examined (data not shown).

Discussion

In the present study, ALP activity was diffusely detected in the mesenchymal cells at the anterior part of the mandible at E11.0, then detected in the mandibular anlage at E11.5, and then continuously detected in the mandibular anlagen including mandibular bone until E13.5. This distribution pattern is consistent with previous histochemical studies of ALP activity (Miyake et al. 1997). In addition, the ALP activity-positive areas were identical to the *Runx2*-positive areas from E11.5 to E13.5, indicating that ALP activity is utilized as a histological marker of mandibular bone formation *in vivo*.

Previous gene knockout studies have indicated that *Runx2* and *Osterix* are essential for bone formation (Komori et al. 1997; Otto et al. 1997; Nakashima et al. 2002). Nakashima et al. (2002) reported that *Osterix* acts downstream of *Runx2* in the lineage of osteoblast differentiation, i.e. *Runx2* is mainly involved in the differentiation of mesenchymal cells into preosteoblasts, whereas *Osterix* acts on the differentiation of preosteoblasts into mature osteoblasts. In the present study, *Runx2* mRNA was detected

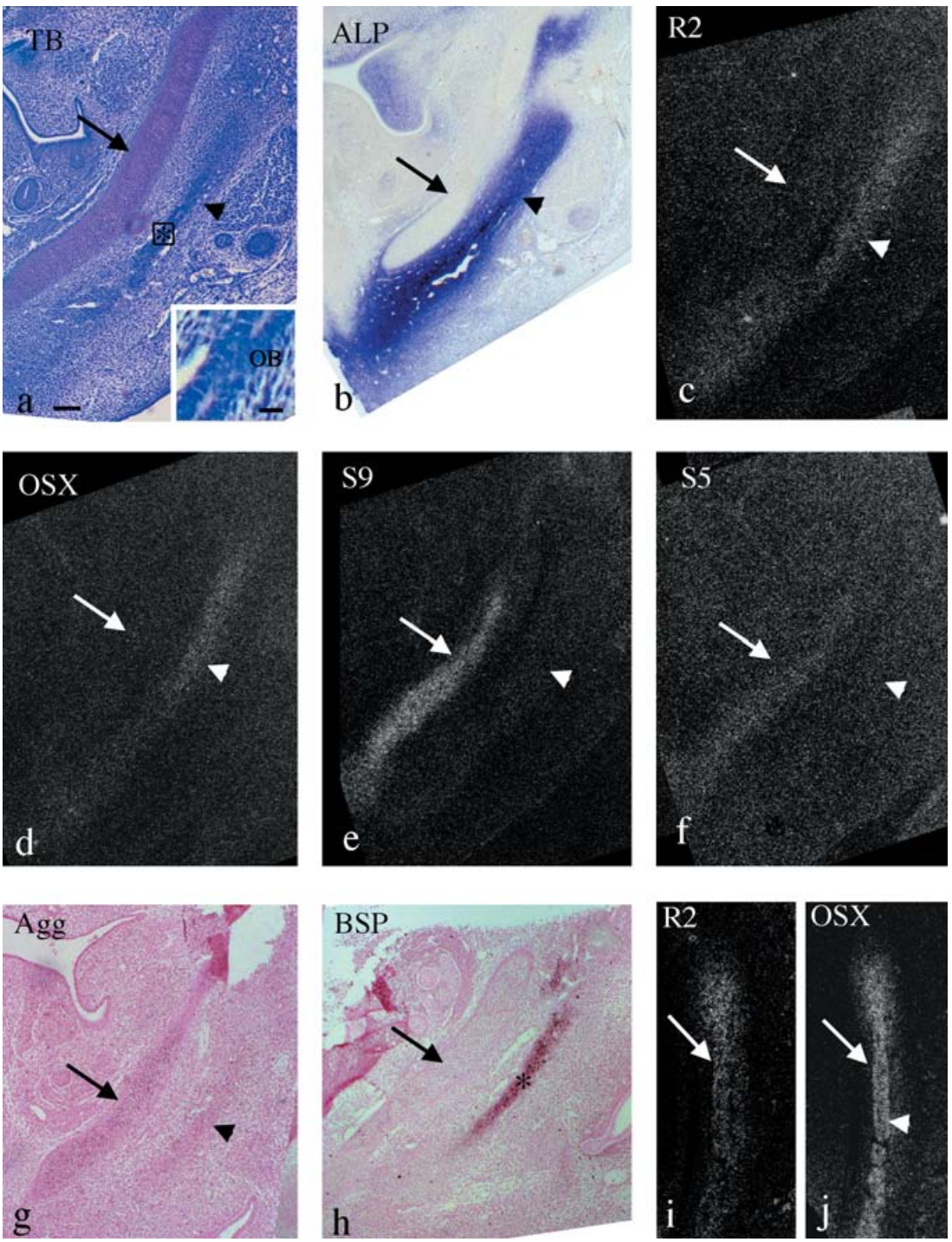

Fig. 4 Mandible in the horizontal plane at E12.5 (a–h) and E13.0 (i,j). (a,c–f) Serial sections and (b,g,h) other sections cut in a similar plane. Toluidine blue staining (a), ALP staining (b) and *in situ* hybridization using ^{35}S-UTP labeled probes of dark fields (c–f,i,j) and digoxigenin-labeled probes (g,h). (a) Meckel's cartilage (arrow) and the mandibular anlage (arrowhead) has expanded, and mandibular bone (*) has formed within it. Inset: enlargement of rectangular area. Mature osteoblasts (OB) are seen in the mandibular bone. (b) ALP activity is detected in the mandibular anlage (arrowhead) but not in Meckel's cartilage (arrow). (c–h) *Runx2* and *Osterix* mRNA are expressed in the mandibular anlage, and *Osterix* tends to be expressed strongly in the center (arrowheads in c and d), but not in Meckel's cartilage (arrows in c and d). *Sox9*, *Sox5* and *aggrecan* mRNA are expressed in Meckel's cartilage (arrows in e–g), but not in the mandibular anlage (arrowheads in e–g). *BSP* mRNA is expressed in the mandibular bone (* in h) within the mandibular anlage, but not in Meckel's cartilage (arrow in h). (i,j) *Runx2* and *Osterix* mRNA are expressed in the mandibular anlage (arrows in i and j), and strong *Osterix* expression is clearly seen in the center (arrowhead in j). Width of view = 100 μm and 20 μm in inset of a.

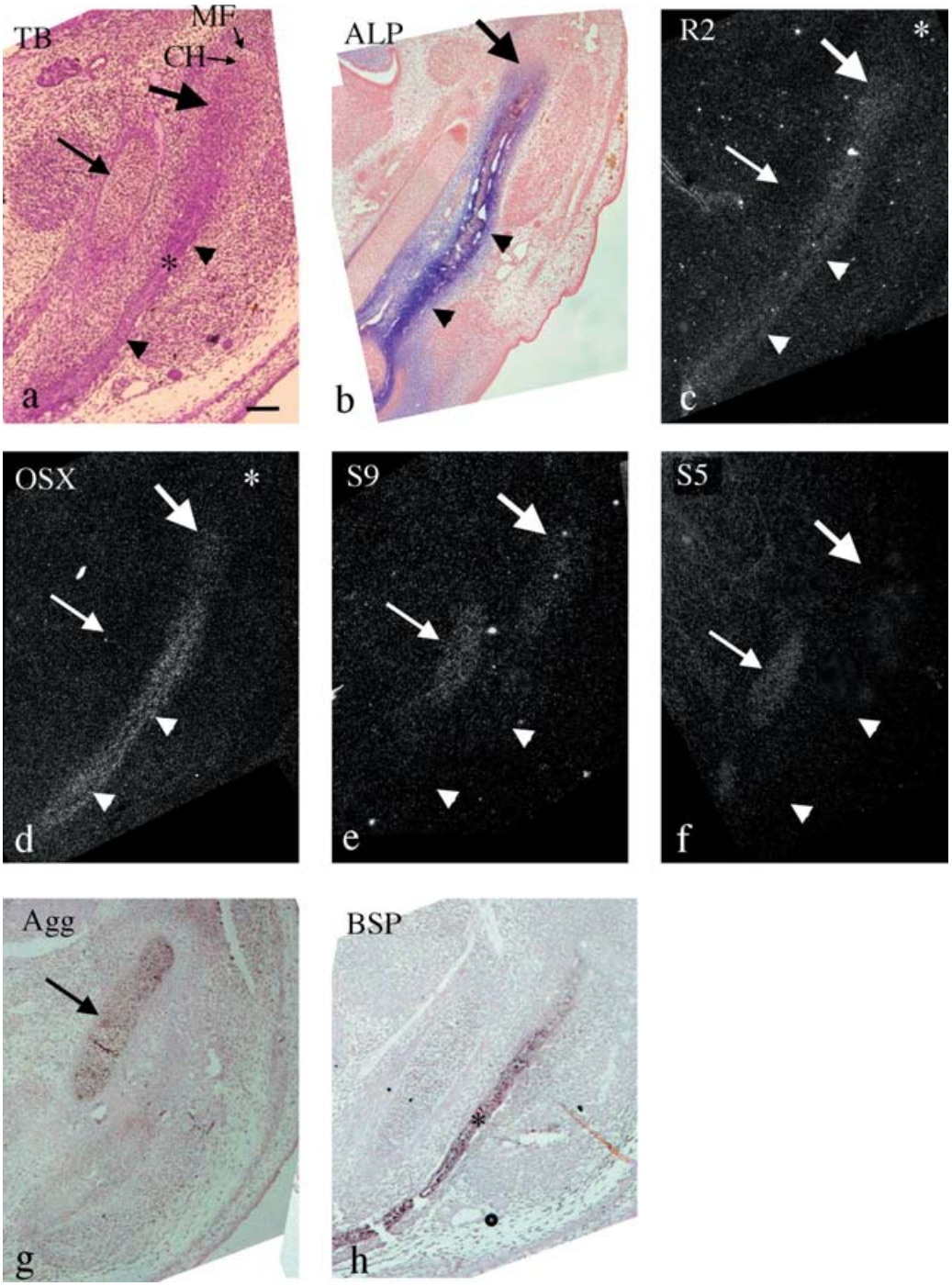

Fig. 5 Mandible in horizontal plane at E13.5. (a,c–f) Serial sections and (b,g,h) other sections cut in a similar plane. Toluidine blue staining (a), ALP staining (b) and *in situ* hybridization using ^{35}S-UTP labeled probes of dark fields (c–f) and digoxigenin-labeled probes (g,h). (a) The mandibular anlage (arrowheads) and mandibular bone (*) within it lateral to Meckel's cartilage (arrow) have expanded. The outline of the future condylar head (CH) and mandibular fossa (MF) consisting of mesenchymal cell condensation of future temporal bone are clearly recognized. Large arrow indicates position of condylar anlage. (b) ALP activity is detected in the mandibular anlage (arrowheads) and in the condylar anlage (large arrow). (c–h) *Runx2* mRNA is evenly expressed in the mandibular anlage (arrowheads in c), and in the condylar anlage (large arrow in c) but not in Meckel's cartilage (arrow in c). *Osterix* mRNA is expressed strongly in the center of the mandibular anlage (arrowheads in d), moderately in the condylar cartilage (large arrow in d), but not in Meckel's cartilage (arrow in d). Note that *Runx2* and *Osterix* mRNA are not clearly expressed in the mesenchymal cell condensation of future temporal bone (* in c and d). *Sox9* mRNA is expressed in Meckel's cartilage (arrow in e) and is expressed in the condylar anlage (large arrow in e), but not in the anterior position of the mandibular anlage (arrowheads in e). *Sox5* mRNA is expressed in Meckel's cartilage (arrow in f), but not in either the mandibular anlage (arrowheads in f) or the condylar anlage (large arrow in f). *Aggrecan* and *BSP* mRNA are expressed in Meckel's cartilage (arrow in g) and mandibular bone (* in h), respectively. Width of view = 100 μm.

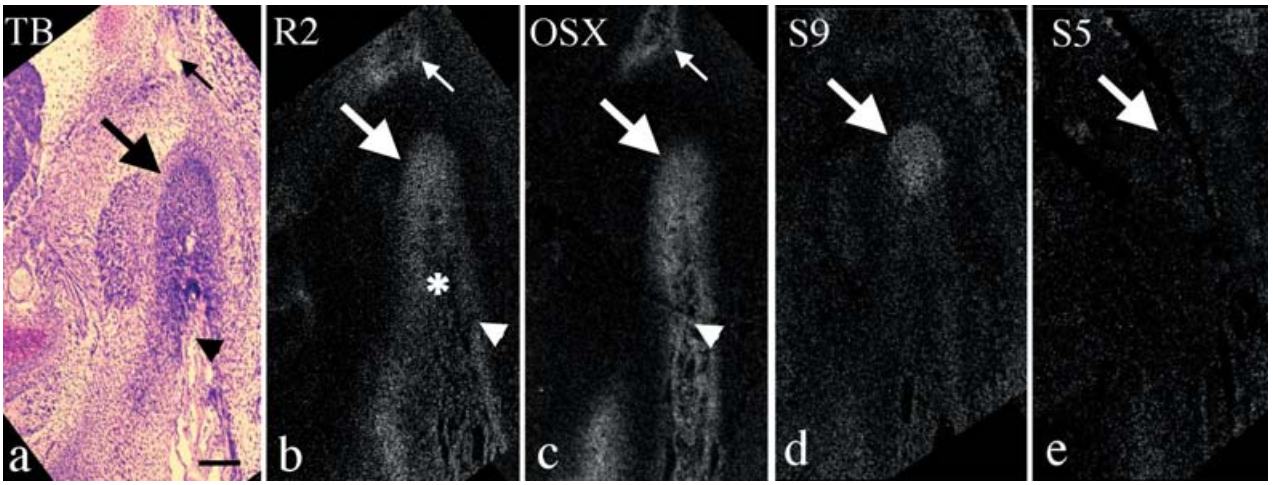

Fig. 6 Mandible in the coronal plane at E14.0. (a–e) Serial sections. Toluidine blue staining (a), and *in situ* hybridization using ^{35}S-UTP labeled probes of dark fields (b–e). The condylar anlage is recognizable as a mesenchymal cell condensation (large arrow in a) at the posterior end of the expanded mandibular bone (arrowhead in a). The formation of temporal bone constituting mandibular fossa is recognizable (small arrow in a). *Runx2* mRNA is expressed in the condylar anlage (large arrow in b) and in the periphery of the mandibular anlage (arrowhead in b), but shows lower expression in the center (* in b). *Osterix* mRNA is expressed strongly in the center (arrowhead in c), but still moderately in the condylar anlage (arrow in c). Note that *Runx2* and *Osterix* mRNA are clearly expressed in the temporal bone (small arrows in b and c). *Sox9* mRNA is expressed in the condylar anlage (arrow in d), but *Sox5* mRNA is not expressed (arrow in e). Width of view = 100 μm.

in the mandibular anlage at E11.5, whereas *Osterix* mRNA was first detected at E12.0. Thus, temporal expression of both molecules at the onset of mandibular bone formation is comparable to that in limb bud development indicated by a gene knockout study (Akiyama et al. 2005). However, the expression patterns of *Runx2* and *Osterix* were different after *BSP*-expressing mature osteoblasts appeared in the center of the mandibular anlage at E12.5. *Runx2* was evenly expressed in the mandibular anlagen, whereas *Osterix* was expressed strongly in the center. This different expression pattern became marked at E14. Therefore, the present findings provide the first support by *in situ* hybridization of mandibular bone formation of the hypothesis of Nakashima et al. (2002) based on the histological viewpoint.

Many previous standard *in situ* hybridization studies have demonstrated *Sox9* expression just in mesenchymal tissues for the chondrogenic cell population of mesodermal origin (Wright et al. 1995; Ng et al. 1997; Zhao et al. 1997). Using a conditional gene knockout system, Akiyama et al. (2002, 2005) demonstrated that *Sox9* plays essential roles in successive steps from undifferentiated mesenchymal cells to proliferating chondrocytes mesodermal in origin. In the present study, *Sox9* mRNA was first detected in Meckel's anlage at E10.0 and was continuously detected in Meckel's cartilage until E13.5. This result indicates that *Sox9* is also involved in cartilage formation neural crest in origin, and supports the results in a conditional gene knockout study performed by Mori-Akiyama et al. (2003).

In a study related to primary cartilage and subsequent endochondral bone formation, Akiyama et al. (2005) demonstrated that *Runx2*-expressing osteogenic cells are derived from *Sox9*-expressing progenitor cells during limb bud development. Meanwhile, Buxton et al. (2003) reported that chondrocytes of avian secondary cartilage form coincident with *Sox9* upregulation from a precursor population expressing *Runx2*, and they insisted that this is a reversal of the normal sequence. In the present study, *Sox9* mRNA was not clearly expressed in the mandibular anlagen until E13.0, but enhanced expression was seen in the condylar anlage at the posterior end of the mandibular anlage at E13.5. Furthermore, *Runx2* and *Osterix* mRNA were simultaneously expressed in the condylar anlage at this stage, indicating that strong *Sox9*-expressing condylar anlage is derived from the *Runx2*/*Osterix*-expressing mandibular anlage. In addition, our previous study (Shibata et al. 2006) indicated that newly formed condylar cartilage appears within the condylar anlage at E15.0. Thus, upregulation of *Sox9* in the condylar anlage acts as a trigger for subsequent condylar cartilage formation, and this result is comparable with that of Buxton et al. (2003). Therefore, the reversal expression pattern of *Runx2* and *Sox9* is possibly a unique feature of secondary cartilage formation.

Next, as described above, enhanced *Osterix* expression was seen in the center of the mandibular anlage, but not in the condylar anlage at E13.5 and 14.0. Our previous study (Shibata et al. 2006) indicated that downregulation of *Osterix* is apparently involved in the onset of condylar cartilage formation at E15.0. Meanwhile, mesenchymal cells in *Runx2*-deficient mice do not express *Osterix* but do still express *Sox9* (Nakashima et al. 2002; Fukuoka et al. 2007), and condylar cartilage formation is rescued by BMP-2 in the absence of *Runx2* (Fukuoka et al. 2007). In addition, downregulation of *Runx2* is slight at the onset of condylar cartilage formation (Shibata et al. 2006). These

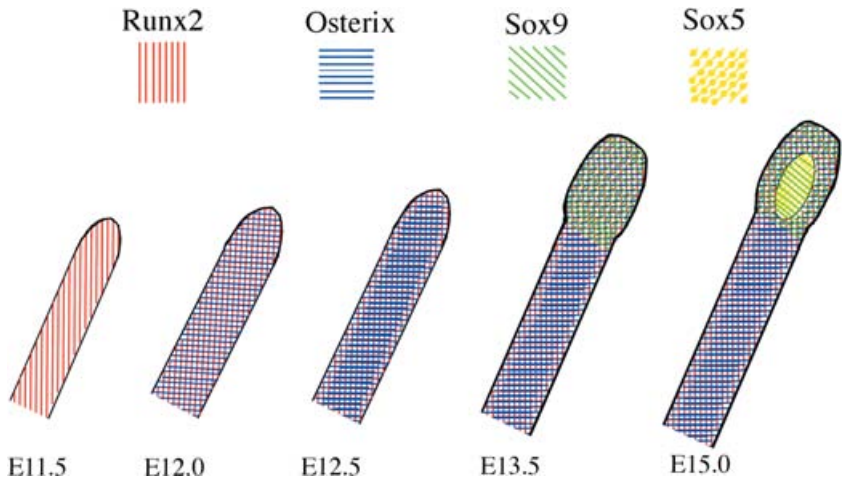

Fig. 7 Schematic representations of the expression pattern of transcription factors during mandibular bone and condylar cartilage formation, based on the present findings and previous reports of Akiyama et al. (2003, 2005), Buxton et al. (2003) and Shibata et al. (2006). *Runx2* mRNA is first expressed in the mandibular anlage at E11.5*, and subsequently, *Osterix* mRNA expression overlaps *Runx2* mRNA expression at E12.0. At E12.5, the mandibular bone containing mature osteoblasts is formed in the center of the mandibular anlage and enhanced *Osterix* mRNA expression is seen in the center. At E13.5, the condylar anlage is morphologically identified and enhanced *Sox9* mRNA expression overlaps the *Runx2*/*Osterix* expression. Enhanced *Osterix* mRNA expression is seen in the center of the mandibular anlage, but not in the condylar anlage. Upregulation of *Sox5* mRNA and downregulation of *Osterix* and *Runx2*** mRNA is seen at the onset of condylar cartilage formation at E15.0. * *Sox9* mRNA may be expressed in the mandibular anlage (Akiyama et al. 2005), but only weakly. ** Downregulation of *Osterix* mRNA is more predominant than that of *Runx2* (Shibata et al. 2006).

results indicate that *Osterix* rather than *Runx2* may have a direct interaction with *Sox9* in condylar cartilage formation. A future study of transfection of *Osterix*/*Runx2* into the condylar anlagen should clarify this issue.

Previous gene knockout studies indicate that interactions between *Sox9* and *Sox5* (*L-Sox5*) or *Sox6* are important for cartilage formation (Lefebvre et al. 1998; Smits et al. 2001; Akiyama et al. 2002). A standard *in situ* hybridization study indicated that *Sox9* and *Sox5* mRNA are expressed in precartilaginous mesenchymal cell condensations during limb bud cartilage formation (Smits et al. 2001). In the present study, *Sox5* mRNA was detected in Meckel's anlage at E11.0 before aggrecan mRNA expression. Thus, interactions between *Sox9* and *Sox5* are also adapted for primary cartilage formation of neural crest in origin. However, the present and previous results (Shibata et al. 2006) have shown that *Sox5* mRNA is first expressed in the newly formed condylar cartilage. Chondrocytes in the mandibular condylar cartilage rapidly differentiate into hypertrophic chondrocytes (Shibata et al. 1997; Fukada et al. 1999; Fukuoka et al. 2007) and this rapid differentiation may account for the discrepancy relevant to *Sox5* expression between condylar (secondary) cartilage and Meckel's (primary) cartilage.

Our model of transcription factors active during mandibular condylar cartilage formation is summarized in Fig. 7.

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