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Mandible and Tongue Development

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Abstract

The tongue and mandible have common origins. They arise simultaneously from the mandibular arch and are coordinated in their development and growth, which is evident from several clinical conditions such as Pierre Robin sequence. Here, we review in detail the molecular networks controlling both mandible and tongue development. We also discuss their mechanical relationship and evolution as well as the potential for stem cell-based therapies for disorders affecting these organs.

1. EARLY DEVELOPMENT OF THE MANDIBULAR ARCH

1.1 Induction, Delamination, and Migration of Neural Crest Cells

The vertebrate neural crest is a pluripotent cell population that gives rise to a wide array of cell types (Le Douarin, Creuzet, Couly, & Dupin, 2004). Although this differentiation ability has been assumed for decades, only recently has *in vivo* evidence based on mouse genetic tools confirmed the multipotent nature of neural crest cells (NCCs) (Baggiolini et al., 2015). The neural crest is induced at the dorsal region of the neural folds between the surface ectoderm and the neural plate via molecular interactions involving BMP, FGF, and WNT proteins (Trainor & Krumlauf, 2000). Simultaneously with their induction, NCCs undergo epithelial-to-mesenchymal transformation, which leads to their delamination and consequent migration from the neural tube to precise destinations. The neural crest can be subdivided into four distinct axial populations, namely the cranial, cardiac, vagal, and trunk NCC, each of which contributes to a distinctive set of specific cell and tissue types. Cranial neural crest cells (CNCCs) can be further subdivided into forebrain-, midbrain-, and hindbrain-derived populations. This subdivision is achieved through the action of neuroepithelial organizing centers and gradients of FGF, retinoic acid, and WNT signals that specify the character of cells located in these three cephalic vesicles (Gavalas, Trainor, Ariza-McNaughton, & Krumlauf, 2001).

Following this regionalization, two different domains are defined by the expression of Hox genes. Hox genes are expressed along the cranial–caudal axis that defines the posterior hindbrain neuroepithelium (r4 to r8) and its NCC (Trainor & Krumlauf, 2000, 2001). In contrast, NCC from the forebrain, midbrain, and anterior hindbrain (r1 to r2) do not express any Hox gene (Couly, Grapin-Botton, Coltey, Ruhin, & Le Douarin, 2002). This division

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produces the specific derivatives of the NCC: a rostral, Hox-negative domain that originates the entire facial skeleton, and a caudal, Hox-positive domain. This pattern of expression highlights the influence of Hox genes in craniofacial evolution (see below; Couly et al., 2002). Interestingly, the skeletogenic capacities of the Hox-negative and Hox-positive NCC domains are different: both are able to generate cartilage whereas only the anterior region yields intramembranous bones (Creuzet, Couly, & Le Douarin, 2005).

Segmental streaming and migration of CNCC are controlled locally by a combination of intrinsic factors and paraxial exclusion zones in the ectoderm and mesoderm, which restrict the migration of CNCC through the territory adjacent to the odd-numbered rhombomeres (r3 and r5) (Kulesa, Bailey, Kasemeier-Kulesa, & McLennan, 2010; Trainor, Sobieszczuk, Wilkinson, & Krumlauf, 2002). This constraint on the streams avoids fusion of the cranial ganglia and mixing of CNCC with distinct anterior-posterior (AP) genetic identities (Trainor & Krumlauf, 2000). The analysis of *Wnt1-Cre;R26R* transgenic embryos has provided valuable information regarding the contribution of postmigratory CNCC to the head in mammals (Chai & Maxson, 2006; Chai et al., 2000). Upon arrival of CNCC at the ventral region of the embryo, they have contact with both ectoderm and endoderm, and their proliferative activity produces distinct swellings known as branchial arches (BAs) as well as the frontonasal prominence. The first BA and the frontonasal prominence give rise to most of the structures in the mammalian face.

1.2 Interarch Patterning

Patterning of the BAs that are Hox-negative is mainly controlled by the specific expression of distal-less (Dlx) genes. A Dlx code provides CNCC with patterning information and intra-arch polarity along the dorsal–ventral (DV)/proximal–distal axis. In each BA, *Dlx1/2*, *Dlx5/6*, and *Dlx3/4* transcripts overlap distally but exhibit offset proximal expression boundaries. In the first BA, *Dlx1* and *Dlx2* are expressed in both the maxillary and mandibular processes. *Dlx5* and *Dlx6* are expressed only in the mandibular process; their expression extends close to the position of the future hinge region between the maxilla and mandible (Depew, Simpson, Morasso, & Rubenstein, 2005). *Dlx3* and *Dlx4* expression domains are further restricted to the distal-most end of the mandibular process (Depew, Lufkin, & Rubenstein, 2002; Depew et al., 1999; Jeong et al., 2008). In posterior BAs, the nested DV expression domains of *Dlx* genes intersect with the AP Hox code in NCC (Santagati & Rijli, 2003). Because *Dlx5/6* control *Dlx3/4* expression (Depew et al., 2002; Jeong et al., 2008), the subdivision of the first BA into maxilla and mandible is mainly achieved with two Dlx combinations: *Dlx1/2* for the maxillary and *Dlx1/2/5/6* for the mandibular process. Thus, a Dlx combinatorial code in CNCC establishes intra-arch polarity (Depew et al., 2005; Minoux & Rijli, 2010). Loss of *Dlx5* and *Dlx6* results in a homeotic transformation of mandible to maxilla, supporting a model of patterning within the BA that relies on a nested pattern of *Dlx* gene expression (Depew et al., 2002). This observation suggests that at one point during development, the upper and lower jaws are very similar in terms of gene expression and that *Dlx5/6* genes constitute the main difference. In addition, jaw development is sensitive to the dosage of *Dlx* genes as haploinsufficiency of single or multiple *Dlx* genes has a gradient effect on mandibular development (Depew et al., 2005). This observation clearly suggests that the expression of *Dlx* genes must be tightly regulated.

Although FGF8-soaked beads in the first arch epithelium are able to induce *Dlx2* and *Dlx5* expression in the mandibular mesenchyme, loss of *Fgf8* in the ectoderm results in unaltered *Dlx2* and *Dlx5* expression in the first BA (Trumpp, Depew, Rubenstein, Bishop, & Martin, 1999). Similarly, BMP-soaked beads are able to induce *Dlx* gene expression, but the endogenous BMP and *Dlx* expression patterns do not suggest a direct regulatory relationship. Overall, *Dlx* genes are clearly important for intrabranial arch patterning (for more information on *Dlx* genes, see Section 4). Once specified, maxillary and mandibular prominences are established through local migration and regionalized proliferation of CNCC. The maxillary prominence gives rise to part of the upper lip, the maxillary bone, and the secondary palate, whereas the mandibular prominence forms the mandible and part of the tongue.

1.3 Intra-Arch Patterning: Proximal–Distal and Oral–Aboral Axis

CNCC-derived mesenchyme in the mandibular prominence is patterned along the proximal–distal and oral–aboral (rostral–caudal) axis in a complex manner, with the activity of the epithelium being essential (Chai & Maxson, 2006). The mandibular ectoderm can be generally divided into the proximal domain, which expresses *Fgf8*, and the distal domain, which expresses *Bmp4*. FGF and BMP act antagonistically to restrict *Barx1* and *Dlx2* expression to the proximal domain of the first BA mesenchyme and *Msx1*, *Msx2*, and *Alx4* expression to the distal domain (Chai & Maxson, 2006). The biological significance of such a regional molecular specification has been demonstrated by inhibiting BMP signaling, resulting in ectopic *Barx-1* expression in the distal mesenchyme, and producing a morphological change of tooth shape from incisor to molar configuration (Tucker, Matthews, & Sharpe, 1998). The function of *Barx1* is not restricted to the dentition but is also involved in the development of other structures of the digestive system, which are essential for mammalian evolution (Miletich, Buchner, & Sharpe, 2005). Overall, these studies show that FGF and BMP growth factor gradients are critical determinants for specifying the proximal–distal pattern.

Furthermore, FGF signals contribute to the establishment of the oral–aboral (rostral–caudal) axis. Postmigratory CNCC distribution significantly overlaps with the expression of two specific Lim-homeobox domain genes, *Lhx6* and *Lhx7*, within the oral mesenchyme. In the aboral region, the homeobox gene *Gooseoid* (*Gsc*) is expressed in an *Lhx6/7*-negative area. Thus, the mandibular arch mesenchyme can be roughly divided into the *Lhx*-positive rostral (oral) domain and the *Gsc*-positive caudal (aboral) domain (Tucker, Yamada, Grigoriou, Pachnis, & Sharpe, 1999). Apparently, FGF8 is responsible for the regulation of *Lhx* and *Gsc* expression and thereby controls the establishment of the oral–aboral axis in the mandible and the maxilla.

2. MANDIBULAR BONE DEVELOPMENT

2.1 Meckel's Cartilage

After the mandibular arch is formed and patterned, condensation of the CNCC-derived mesenchyme occurs at the level of the presumptive first molar (Fig. 1). This condensation is the first step in the formation of the Meckel's cartilage (MC). Mesenchymal cells then

differentiate into chondrocytes and organize into a characteristic bilateral rod-shaped cartilage prior to any sign of ossification in the mandible. The MC is surrounded by a perichondrium composed of fibrous mesenchymal cells, which separates it from neighboring nonchondrogenic osteogenic cells (Fig. 1). The MC first lengthens ventromedially and dorsolaterally on both sides of the mandibular arch and fuses at the most distal tip. The distal ends of the MC develop into the mandibular symphysis, whereas the proximal ends curve to develop the primordium of the malleus and incus bones of the middle ear (Fig. 1E; Richany, Bast, & Anson, 1956). The intermediate portion of the MC degrades in mammals. In the most posterior part of this intermediate region, the chondrocytes transform into fibroblasts to form the sphenomandibular ligament after resorption of the cartilaginous matrix.

Histologically, the MC is a typical hyaline cartilage. Chondrocytes are embedded in extracellular matrix enriched in type 2 collagen and proliferate in the cartilage lacuna. In addition, the MC undergoes additional growth resulting from activity of the chondrocytes located in the perichondrium (Amano et al., 2010). The molecular mechanism underlying the formation and degeneration of the MC is still unknown. *Sox9*, a well-known chondrogenic master gene, is expressed in all chondroblasts of the MC. Some recent evidence suggests that *Sox9* expression in the mandible is necessary for MC initiation and development. In the absence of *Sox9* in the CNCC-derived mesenchyme, the MC is completely absent (Mori-Akiyama, Akiyama, Rowitch, & de Crombrughe, 2003). Although the mandible is smaller than in control mice, mandibular gross morphology and bone formation are not severely affected in *Sox9*-mutant mice. This study suggests that the MC may primarily control the size of the mandible but not its initial development including patterning (Mori-Akiyama et al., 2003).

Another factor potentially involved in the formation of the MC is connective tissue growth factor (CTGF). CTGF is expressed along the entire length of the MC from E12.5, when the cartilaginous condensation is first detectable, to E15.5. At this stage, differentiation into mature chondrocytes is accompanied by a significant reduction in CTGF expression although strong expression is still detectable in the peripheral chondrocytes and the perichondrium (Parada, Li, Iwata, Suzuki, & Chai, 2013; Shimo et al., 2004). Ablation of CTGF leads to severe alteration of the MC morphology, including folding of the proximal ends. This phenotype is associated with micrognathia (Ivkovic et al., 2003). The cause of the small mandible in *Ctgf*^{-/-} mutant mice is not known but researchers speculate that it is due to the disruption of the mechanical properties of the MC, similar to the defects in other cartilages in these mice (Ivkovic et al., 2003). The function of CTGF in MC development has also been addressed using *Wnt1-Cre;Tgfb2^{fl/fl}* mice, which exhibit micrognathia and a defect in chondroblast proliferation in the MC. In these mice, *Ctgf* expression is downregulated in the MC and, interestingly, exogenous CTGF rescues the proliferation defect *in vitro* (Oka et al., 2007). Deletion of *Alk5* (Type 1 receptor of TGFβ) causes a similar phenotype including a smaller mandible and the defective MC morphology. *Ctgf*, *Tgfb2*, and *Alk5* mutant mice display a common defect in which the MC is severely disrupted in the proximal region, whereas the distal region is comparable to controls. Ossification is also normal in the distal region.

FGF signaling is also involved in MC development. Injection of a dominant negative form of FGFR3 in the developing mandible of chick embryos at early stages leads to truncation of the rostral end, shortening of the MC, and the absence of five of the mandibular bones (Havens et al., 2008). Conversely, overexpression of Fgf10 causes deformation of the MC and a significant increase in size. Fgf10 overexpression also induces upregulation of cartilage-specific genes such as *Col2a1* and *Sox9* *in vitro* (Terao et al., 2011). The function of the WNT pathway in the development of the MC is not clear. It has been suggested from studies on limb chondrogenesis that WNT is an inhibitor of this process. Accordingly, chick MC and mandibles treated with WNT5a are severely malformed *in vivo* and *in vitro*. Both the cartilage and mandibular bones are affected (Hosseini-Farahabadi et al., 2013). However, in *Fuz*^{-/-} mice, the MC is expanded due to increased cell proliferation linked to the upregulation of Wnt canonical target genes (Zhang et al., 2011). Overall, these findings suggest that Sox9 might control initiation of MC development, whereas CTGF, FGF, and TGFβ signaling might be involved in regulating later events such as the establishment of cell fate.

Degeneration of the MC appears to involve autophagia. At around E16.5, chondrocytes of the central portion of the MC become hypertrophic and express Beclin1 and LC3. Moreover, caspase 3 is expressed in the lateral population of terminal hypertrophic chondrocytes along the degeneration area of the MC, which indicates that autophagy occurs in hypertrophic chondrocytes during the degradation of the MC and happens prior to chondrocyte cell death (Yang, Zhang, Liu, Zhou, & Li, 2012). During the degeneration process, hypertrophic chondrocytes also become p53-positive (Trichilis & Wroblewski, 1997). Recent studies have suggested that BMP signaling participates in the control of the degeneration process. In the absence of Noggin, the MC is significantly thickened due to elevated cell proliferation, enhanced phosphorylated Smad1/5/8 expression, and lack of degeneration (Wang, Zheng, Chen, & Chen, 2013). The size of the mandibular bone in *Noggin*^{-/-} mice appears to be increased. The MC in these mice does not degrade; instead, it differentiates into bone, mimicking the process of mandibular bone development in other species (Wang et al., 2013). Although the MC was previously proposed to serve as a template for bone deposition and to control endochondral and intramembranous bone formation during mammalian mandibular development, the phenotypes of the mutant mice described above do not directly support this hypothesis.

2.2 Mandibular Osteogenesis

Formation of the mandibular bone occurs in different ways along the proximal–distal axis. As mentioned before, the distal region undergoes endochondral-like ossification to form the symphysis. The middle (and largest) part undergoes intramembranous ossification. The proximal region of the mandible is classified as secondary cartilage and is formed by endochondral ossification. Intramembranous ossification is characterized by an initial condensation of mesenchymal cells followed by differentiation of those cells into osteoblasts. Next, osteoblasts secrete a collagen–proteoglycan (osteoid) matrix that is able to bind calcium salts (Amano et al., 2010). Osteoblast differentiation occurs through a multistep molecular pathway regulated by different transcription factors and signaling proteins. *Dlx5*, which is expressed from the early stages, induces the expression of *Runx2*.

Runx2 (also known as *cbfa1*) is one of the first genes expressed by mesenchymal cells committed to the osteogenic lineage and is required for differentiation of mesenchymal cells into preosteoblasts (Baek, Kim, de Crombrughe, & Kim, 2013). Ablation of *Runx2* causes generalized malformations of the skeleton. In the mandible, *Runx2*-null mice display ectopic cartilaginous processes in the MC and lack the condylar cartilage and the mandibular bone (Shibata et al., 2004). In addition to *Dlx5*, *Hand2* also controls *Runx2* expression but in an inhibitory manner, resulting in the negative regulation of osteoblast differentiation. In the mandibular primordium, downregulation of *Hand2* precedes *Runx2*-driven osteoblast differentiation (Funato et al., 2009). *Osterix* (*Osx* or *SP7*), a *Runx2* downstream gene, is required for the differentiation of preosteoblasts into mature osteoblasts and is specifically expressed in all osteoblasts (Zhang, 2010). Mutation of *Osx* in CNCC derivatives leads to a tiny and rudimentary mandible although the MC is unaffected (Baek et al., 2013). This finding suggests again that the development of the mandibular bone and MC are not interdependent.

Endochondral ossification occurs when bone is formed after the establishment of a cartilaginous template (Wagner & Karsenty, 2001). At the onset of this process, mesenchymal cells aggregate into condensations that define the future skeletal elements. FGF and BMP are essential for the formation of chondrogenic condensations. Following mesenchymal condensation, cells in the core of the condensations differentiate into chondrocytes that secrete collagen types II, IX, and XI and aggrecan. Cells at the periphery of the condensation form the perichondrium. As mentioned above, *Sox9* is the earliest identified factor required for chondrogenesis. Although it is dispensable for condensation, it is necessary for the subsequent steps toward chondrocyte differentiation (Barna & Niswander, 2007). BMP signaling also stimulates chondrocyte differentiation (Yoon et al., 2005), whereas retinoic acid, WNT, and NOTCH pathways inhibit late stages of chondrogenesis. Chondrocytes within the cartilage progressively undergo maturation from proliferating to prehypertrophic to hypertrophic chondrocytes (Wagner & Karsenty, 2001). Prehypertrophic chondrocytes are characterized by the expression of Indian hedgehog (*Ihh*) (Vortkamp et al., 1996) and hypertrophic chondrocytes by the expression of type X collagen. Coupled with chondrocyte hypertrophy, osteoblast differentiation first occurs within the perichondrium and continues within the marrow cavity. The differentiation of osteoblasts from their mesenchymal progenitors is regulated by signaling pathways such as *IHH*, NOTCH, WNT, and BMP and also controlled by the activity of transcription factors including *Dlx5*, *Runx2*, and *Osx*, among others (Long & Ornitz, 2013).

In the most distal region of the mandible, *Tgfb β -1*/*Runx2*-expressing mesenchymal cells condense at the prospective symphysis. From embryonic day 16.5 to 18.5, chondrocytes expressing *Sox9* form in this region and organize into a structure that resembles a growth plate with distinct *Ihh*, collagen X, and osteopontin expression patterns. *Ihh* signaling appears to be essential for symphyseal cartilage development. In *Ihh*^{-/-} mice, the development of the symphysis is defective due to enhanced chondrocyte maturation and reduced proliferation of the chondroblast progenitors. This phenotype is rescued upon ablation of *Gli3*, which thus acts as a negative regulator of symphyseal development. *Gli3* function is specifically related to the control of chondroblast proliferation (Sugito et al., 2011). In postnatal life, *Ihh* also plays an important role. Mesenchymal cells expressing the

Ihh receptor Patched1 are present anterior to the Ihh-expressing secondary cartilage, proliferate, differentiate into chondrocytes, and contribute to anterior growth of alveolar bone (Sugito et al., 2011).

In the proximal region, development of the condyle and mandibular angle starts at around E14.5. Both structures are first observed as Sox9-positive, highly condensed mesenchymal cell areas in the posterior osteogenic front of the mandibular bone. These cells in the proximal part of the mandible have the potential to become either osteoblasts or chondrocytes; thus, they are called osteochondroprogenitor cells (Shibata, Suda, Suzuki, Fukuoka, & Yamashita, 2006; Silbermann et al., 1987). Reduced expression of Osterix in combination with Sox9–Sox5 expression appears to be important for the specification of the chondrogenic fate of those cells and consequently the onset of condylar cartilage formation (Shibata et al., 2006). By E15.5, cartilage matrix is clearly detectable in both the condylar and angular processes. TGF β signaling controls the fate of the osteochondrogenic progenitors in the proximal region of the mandible. This pathway is crucial for cell lineage determination during endochondral ossification, as a positive regulator for chondrocytes and an inhibitor of osteoblasts, via regulation of the expression of critical transcriptional factors. In *Wnt1-Cre;Tg fbr2^{fl/fl}* mice, *Sox9* expression is reduced in osteochondroprogenitor cells in the proximal region of the mandible, whereas osteoblast markers such as *Runx2* and *Dlx5* expression are enhanced. Changes in the expression levels of these three genes result in bone formation without a cartilaginous intermediate in these mice (Oka et al., 2007). Ihh is also involved in the development of proximal structures of the mandible. Ihh expression is detectable in the condylar cartilage by E15.5, and expression of its receptors and effector genes, such as *Gli1*, *Gli2*, *Gli3*, and *PTHrP*, suggest that its range of activity extends to apical condylar tissue layers, including the polymorphic chondroprogenitor layer. In *Ihh*^{−/−} mice, cartilage growth, cell proliferation, and PTHrP expression are reduced as well as chondrocyte gene expression. As in the symphyseal region, these severe alterations are partially rescued in double *Ihh*^{−/−}; *Gli3*^{−/−} mice, confirming that Gli3 modulates the action of Ihh (Shibukawa et al., 2007). Details on the molecular regulation of the condyle and the temporomandibular junction can be found elsewhere in this volume (Hinton, Jing, & Feng, 2015).

3. TONGUE DEVELOPMENT

The development of the tongue and mandible is tightly connected. The tongue begins with the formation of a medial triangular elevation on top of the mandibular arch called the median lingual swelling. If the early mandibular arch is abnormal, tongue development is disrupted in most cases (see below). Next, lateral lingual swellings form on each side of the median tongue bud at E10.5. At this stage, the tongue primordium is exclusively composed of CNCC-derived mesenchyme and covered by epithelium (Han et al., 2012). The lateral swellings grow, fuse with each other, and overgrow the median lingual swelling. Myoblasts from the occipital somites start invading the tongue primordium at E11.5. The merged lateral lingual swellings form the anterior two-thirds of the tongue. The fibrous CNCC-derived lingual septum is the fusion site of those lateral swellings. Two outgrowths from the third BA, the copula and the hypopharyngeal eminence, compose the posterior third of the tongue. As development advances, the copula is progressively overgrown by the hypopharyngeal

eminence and disappears (Moore & Persaud, 2008). Finally, the tongue primordium undergoes rapid enlargement and the muscular component occupies most of it (Huang, Zhi, Izpisua-Belmonte, Christ, & Patel, 1999). Tongue connective tissue and vasculature are derived from CNCC, whereas the skeletal muscles originate from myoblasts (Noden & Francis-West, 2006). Reciprocal interactions between CNCC and myogenic cells play an important role in regulating tongue development.

3.1 Development of the Muscular Component of the Tongue

Myoblasts from the occipital somites migrate to the tongue primordium along the hypoglossal cord (Huang et al., 1999). This process is tightly controlled by at least three main molecules: *c-met*, *Gab1*, and *Lbx1* (Amano et al., 2002; Gross et al., 2000; Sachs et al., 2000). *Pax3* and *Pax7* regulate proliferation and differentiation of myogenic progenitors shortly after their arrival in the limb or the tongue primordium (Tajbakhsh & Buckingham, 2000; reviewed in Parada, Han, & Chai, 2012). *Pax3* is essential for muscle development because it controls the gene hierarchy that activates myogenic regulatory factors (MRFs), including myogenic factor 5 (*Myf5*), muscle-specific regulatory factor 4 (*MRF4*), myoblast determination protein (*MyoD*), and myogenin (Tajbakhsh & Cossu, 1997). Together, these MRFs direct the determination and differentiation of myoblasts in the limbs (Berkes & Tapscott, 2005; Nabeshima et al., 1993; Rudnicki et al., 1993). In the developing tongue, MRFs are expressed in different subpopulations of myoblasts throughout development (Han et al., 2012; Hosokawa et al., 2010; Zhong, Zhao, Mayo, & Chai, 2015). Recent studies have shown that ablation of the *Myf5*-expressing progenitors in the tongue does not significantly affect muscle pattern or size (Zhong et al., 2015). This finding suggests that a *Myf5*-independent myogenic lineage can compensate for loss of *Myf5*-expressing myogenic cells, as suggested by other studies of limb muscles (Gensch, Borchardt, Schneider, Riethmacher, & Braun, 2008; Haldar, Karan, Tvrdik, & Capecchi, 2008). In contrast, ablation of the *MyoD*-positive population in the tongue leads to microglossia that is associated with a striking reduction in muscle fiber formation in the tongue (Zhong et al., 2015). Myogenic differentiation is followed by myoblast fusion. Individual myoblasts fuse with one another to generate myotubes. Afterward, additional differentiated myoblasts incorporate into the forming myotubes, leading to the further maturation of the myofibers (Rochlin, Yu, Roy, & Baylies, 2010). Myoblast fusion requires extracellular calcium and changes in cell membrane topography and cytoskeletal organization. Recently, studies have identified several cell adhesion proteins, transmembrane lipids, and intracellular domain-associated signaling or adaptor proteins that accumulate at sites of contact between two myogenic cells (Hindi, Tajrishi, & Kumar, 2013).

Major signaling pathways such as WNT, TGF β , and FGF are involved in the regulation of myogenesis at different key developmental stages. Canonical Wnt signaling plays important roles in dermomyotome and myotome formation (Linker, Lesbros, Stark, & Marcelle, 2003; Otto, Schmidt, & Patel, 2006). In the somites, Wnt1 ligands preferentially activate *Myf5*, whereas Wnt7a ligands activate *MyoD* in myogenic progenitors (Tajbakhsh & Cossu, 1997). Wnt signaling is activated in the same region of the hypoglossal cord as the *Myf5*-derived population. In the tongue primordium, canonical Wnt signaling is activated in the myogenic region right after migration of the myoblasts, and it shifts to the CNCC-derived mesenchyme

at later stages. The Wnt signaling pathway first controls myoblast migration and later regulates differentiation of the myogenic progenitors in the tongue, based on the analysis of *Myf5-Cre;β-catenin^{fl/fl}* mice, which exhibit microglossia (Zhong et al., 2015).

Recent studies have shown that TGFβ pathway members are also involved in the regulation of proliferation, differentiation, and myoblast fusion during tongue myogenesis.

Interestingly, both inhibition and promotion of myogenesis by TGFβ have been reported (McPherron, Lawler, & Lee, 1997; Wang, Noulet, Edom-Vovard, Le Grand, & Duprez, 2010). Ablation of *Smad4*, a mediator of TGFβ signaling, in myogenic progenitors disrupts myogenic terminal differentiation and myoblast fusion in the tongue primordium but does not interfere with early myogenic determination. The disruption in these processes leads to reduced myotube length, decreased average number of myonuclei per myotube, and numerous centrally located nuclei (Han et al., 2012).

FGF pathway members act as downstream mediators of TGFβ signaling that control myogenic differentiation and fusion. *Fgf6* and its receptor, *Fgfr4*, are expressed from early stages in the myogenic component of the mouse developing tongue, although their expression patterns do not overlap entirely (Han et al., 2012). Both members of the FGF family act downstream of Smad4-mediated TGFβ signaling during tongue myogenic differentiation and myoblast fusion. Accordingly, in *Myf5-Cre;Smad4^{fl/fl}* mice, exogenous FGF6 partially rescues the compromised tongue myoblast differentiation and fusion (Han et al., 2012). *Fgf6*^{-/-} mice have a severe regeneration defect with fibrosis and myotube degeneration, consistent with a function during tongue myogenic differentiation (Armand, Laziz, & Chanoine, 2006). Although *Fgfr4*^{-/-} mice show no phenotype at birth, muscle regeneration in *Fgfr4*-null mice becomes highly abnormal at the time point when *Fgfr4* is normally expressed (Zhao et al., 2006). Previous studies suggest that TGFβ2 may function upstream of Smad4 during myogenesis. TGFβ2 promotes fusion of myoblasts *in vitro* and is specifically expressed in the region of myoblast-to-myotube fusion (Olson, Sternberg, Hu, Spizz, & Wilcox, 1986). This expression pattern suggests that myoblasts may induce TGFβ2 to stimulate the fusion of adjacent myoblasts. Taken together, these findings indicate that a genetic hierarchy involving TGFβ and FGF plays a critical role in the regulation of specific myogenic factors during tongue muscle development.

3.2 CNCC-Derived Mesenchyme in the Developing Tongue

CNCCs initiate and potentially direct tongue development (Han et al., 2012). From the few studies published addressing the function of the CNCC-derived mesenchyme in tongue development, it is tempting to speculate that CNCCs in the tongue act as a scaffold for the organization of migrating myoblasts into the myogenic core and also may operate as a niche that releases molecular instructions to direct survival, proliferation, and differentiation of myogenic progenitors as well as patterning of the muscles. Early mandibular development is characterized by the differential expression of specific *Dlx* genes. *Dlx5/6* expression also regulates the development of the anterior (and largest) region of the tongue. Craniofacial myogenesis depends on normal *Dlx5/6* expression by CNCC, because ablation of both genes leads to loss of masticatory muscles and disrupted tongue development (Heude et al., 2010). Because *Dlx5/6* are only expressed by the CNCC-derived mesenchyme in the craniofacial

region, these genes might be playing an early instructive function to control muscle formation. In *Dlx5/6*^{-/-} mice, the intrinsic muscles of the tongue and sublingual muscles are severely affected, although they express both determination and differentiation markers (Heude et al., 2010). *Dlx5/6* work together with the transcription factor *Hand2*, which is also expressed early in the mandibular arch, to establish the dorsoventral/proximodistal pattern of the mandibular arch and, consequently, that of the tongue. *Hand2* contributes to the establishment of the proximal–distal patterning through a negative-feedback loop in which it represses *Dlx5/6* expression in the distal arch mesenchyme following *Dlx5*- and *Dlx6*-mediated induction of *Hand2* expression in the same region. Failure to inhibit distal *Dlx5/6* expression leads to the absence of lateral lingual swelling expansion, resulting in aglossia. Therefore, *Hand2* seems to determine a distal mandibular arch domain that is favorable for lower jaw development, including the induction of tongue morphogenesis (Barron et al., 2011).

Hh signaling in the CNCC is also crucial for early tongue development. *Ptch1*, a *Shh* receptor, is expressed in the mesenchyme of the first BA and in the tongue CNCC-derived mesenchyme, suggesting that this tissue is responsive to Shh signals coming from the tongue epithelium. Interestingly, *Wnt1-Cre;Smo*^{n/c} embryos, in which Shh signaling is disrupted in the CNCC component, exhibit a vestigial tongue. The tongue defect in these mice is detectable early and is associated with the absence of *Myf5*-expressing myogenic progenitors. The CNCC component is also reduced. Thus, Hh signaling in the CNCC-derived mesenchyme might be involved in transmitting information from the epithelium to the myogenic progenitors to coordinate tongue formation (Jeong, Mao, Tenzen, Kottmann, & McMahon, 2004). The relevance of Shh signaling in tongue development is confirmed by the conditional inactivation of *β-catenin* in the lingual epithelium, which causes downregulation of *Shh* expression in the tongue epithelium and reduction of *Ptch1* and *Gli1* expression in the underlying mesenchyme. In *β-catenin*-mutant mice, the two lateral swellings of the primitive tongue are smaller and remain separated. In addition, the merger between the tuberculum impar and two lingual swellings is defective. At late stages, the tongue is much smaller, deformed, and completely lacks taste papillae. The number of CNCC-derived mesenchymal cells is severely reduced, which appears to be the cause of the microglossia (Lin et al., 2011). These findings are also consistent with the phenotype of *Fuz*^{-/-} mice that exhibit severe craniofacial deformities including agenesis of the tongue. Malformations in these mice are associated with downregulation of Hedgehog signaling (Zhang et al., 2011).

TGFβ family members expressed in the CNCC also regulate skeletal muscle development via tissue–tissue interactions. Ablation of TGFβ pathway members in CNCC lead to severe tongue defects. For instance, deletion of *Tgfb2* in CNCC-derived mesenchyme results in defects in tongue myogenesis and a smaller overall tongue size, which are due to a significant decrease in proliferation of myogenic cells (Hosokawa et al., 2010). The reduction in myogenic cell proliferation is associated with downregulation of *Fgf10* expression, which is only detectable in the CNCC component. Interestingly, exogenous FGF10 reverses the reduction of tongue myogenic cell numbers in *Tgfb2*-mutant mice, which suggests noncell autonomous activity (Hosokawa et al., 2010). More recently, another study suggested that Fgf10 overexpression in the CNCC-derived tongue mesenchyme in

Wnt1-Cre;Tak1^{fl/fl} mice also causes tongue malformations (Song et al., 2013). These results are consistent with previous studies showing that FGF signaling is required for skeletal muscle formation and suggest that its function is dose dependent.

Tak1 is an element of one of the noncanonical pathways mediating TGF β signals. In the developing tongue of *Wnt1-Cre;Tgfb β 2^{fl/fl}* mice, non-canonical TGF β signaling via a T β RI/T β RIII complex results in increased phosphorylation of p38 (Iwata, Suzuki, Pelikan, Ho, & Chai, 2013), which is accompanied by overactivation of ABL1 and PKC. The activation of these pathways in *Tgbr2*-mutant mice is associated with upregulation of the expression of *folistatin* and downregulation of *Fgf4*. Additionally, elastic and collagen fibers are poorly organized and immature and *tenascin C* expression is compromised in *Wnt1-Cre;Tgfb β 2^{fl/fl}* tongues (Iwata et al., 2013). Moreover, reduction of altered noncanonical TGF β signaling rescues microglossia in *Wnt1-Cre;Tgfb β 2^{fl/fl}* mice by restoring proliferation of the myogenic progenitors. However, muscle disorganization persists, suggesting that the noncanonical TGF β pathway affects myoblast proliferation, whereas canonical TGF β signaling might be required for muscle organization (Iwata et al., 2013).

3.3 Tendon Development and Connection to Bone

The initial relationship between CNCC and myogenic progenitors is maintained throughout myogenesis and helps establish the attachment of tongue and head muscles to the skeleton during craniofacial development. Both muscle and tendon cells are involved in the formation of this attachment and regulate each other at different stages of development throughout the body. For example, the loss of stripe, which is involved in early steps of tendon differentiation, results in the disruption of the entire somatic muscle pattern (Frommer, Vorbrüggen, Pasca, Jackle, & Volk, 1996). Similarly, ablation of Periostin, an adhesion molecule, in the myoseptum causes a differentiation defect in myoblasts (Kudo, Amizuka, Araki, Inohaya, & Kudo, 2004). On the other hand, although muscles are not necessary for the initiation of tendon formation in the branchiomic and extraocular regions, they are required for further tendon development (Grenier, Teillet, Grifone, Kelly, & Duprez, 2009).

During tendon development, collagen fibrillogenesis and assembly occur to form a structurally and functionally mature tissue. *In vivo* evidence suggests that the condensation, determination, and differentiation of tendon progenitors are dependent on the activity of *Scleraxis* (*Scx*) (Murchison et al., 2007). All of the forming tendons associated with the tongue are of CNCC origin and express *Scx* (Hosokawa et al., 2010 and our unpublished data). One of the relevant functions of *Scx* during tendon formation is the control of collagen expression (Léjard et al., 2007; Terraz, Brideau, Ronco, & Rossert, 2002). *Type 1 collagen* is expressed in the tongue from early stages of development. It is detectable in the CNCC-derived mesenchyme adjacent to the tongue epithelium (Hosokawa et al., 2010) and in the tendons of the extrinsic muscles, which connect the tongue to the mandible (our unpublished results), where *Scx* is also expressed.

TGF β and FGF also act at later stages in the induction of tendon formation and maintenance of tendon progenitor cells (Pryce et al., 2009). TGF β may mediate the recruitment of new tendon cells by promoting the differentiation of muscles and cartilage to establish the connections between tendon primordia and their respective musculoskeletal counterparts

(Pryce et al., 2009). TGF β signaling is a potent inducer of *Scx* throughout the organism. Disruption of TGF β signaling in *Tgfb2*^{-/-}; *Tgfb3*^{-/-} embryos or through inactivation of *Tgfb2* results in the loss of most tendons and ligaments in the limbs, trunk, tail, and head (Pryce et al., 2009). In *Wnt1-Cre;Tgfb2*^{fl/fl} mice, *Scx* expression is diminished in the tendons of the tongue muscles (Hosokawa et al., 2010). Bead implantation experiments indicate that TGF β signaling, particularly TGF β 2, induces *Scx* expression in CNCC during tongue development. Thus, in the tongue primordium, TGF β signaling is required for the cell-autonomous induction of *Scx*, *type I collagen* expression, and the regulation of the fate of CNCC (Hosokawa et al., 2010). Other TGF β ligands, such as growth/differentiation factor 8 (GDF-8, encoded by *MSTN*), could also be involved, as *Mstn*^{-/-} mice have hypocellular tendons, in addition to hypertrophic muscles (Mendias, Bakhurin, & Faulkner, 2008). However, the function of GDF-8 in tendon formation in the craniofacial region is still unclear.

FGFs have both early and late functions in tendon development. During somite development, FGF signals secreted from the myotome induce the formation of a *Scx*-expressing tendon progenitor population in the sclerotome, at the juncture between the future muscle and cartilage lineages. The activation of *Pea3* and *Erm* in response to FGF signaling is both necessary and sufficient for *Scx* expression in the somite, which helps restrict the domain of somitic tendon progenitors (Brent & Tabin, 2004). Later, *Pea3* and *Sprouty1* and 2 are expressed in muscles and tendons, and their expression is enhanced at the myotendinous junctions in limbs. Analysis of *Pea3* and *Sprouty* gene expression in muscleless limbs of *Pax3*-mutant mice indicates strong expression in muscles and expression in tendons that depends on muscles (Eloy-Trinquet, Wang, Edom-Vovard, & Duprez, 2009), demonstrating again the importance of interactions between connective tissues and muscle during development and adult stages alike.

4. EVOLUTION OF THE MANDIBLE AND TONGUE

The mandible is a recent evolutionary novelty characteristic of gnathostomes. The anatomical configuration of the BAs is different in agnathans and gnathostomes. This different configuration is related to changes in the CNCC-derived mesenchyme that is distributed in an embryonic domain known as the trigeminal region, the rostral part of the embryonic head corresponding to the peripheral innervation of the trigeminal nerve (Kuratani, 2012). CNCC populating the first BA do not express any Hox genes. The absence of *Hox* genes from the first BA is thought to be a permissive and indispensable condition favoring maxilla and mandible development. Interestingly, in agnathans like lamprey and hagfish, the homologous structures do express Hox genes. The most anteriorly expressed *Hox* gene in gnathostomes is *Hoxa2*, which is only expressed in the more posterior NCC that migrate into the second BA. Accordingly, ectopic expression of *Hoxa2* in the first BA in several animal models impedes jaw development and ablation of *Hoxa2* in mice leads to duplications of some first BA skeletal components in the second BA, but not of the jaw (Creuzet, Couly, Vincent, & Le Douarin, 2002). In agnathans, the *HoxL6* gene is expressed in the first BA, and this is also the case for the homologous gene in amphioxys, which do not exhibit jaws or BAs (Cohn, 2002). It is still not clear how this Hox-negative zone in gnathostomes is controlled from a molecular point of view. Researchers have suggested that

it might be due to the action of the midbrain–hindbrain isthmus, a signaling center that expresses and releases FGF8, which in turns prevents the expression of *Hoxa2* in its rostral domain (Trainor et al., 2002).

The development and evolution of the mandible are highly dependent on the influence of other tissues on the CNCC-derived mesenchyme. For instance, the foregut endoderm provides patterning information to the mesenchyme in the first BA. Interestingly, this tissue can only pattern *Hox*-negative neural crest-derived mesenchyme but not *Hox*-positive cells (Creuzet et al., 2002; Couly et al., 2002). In lamprey, *HoxL6*-positive NCC migrating into the first BA are unresponsive to the endoderm and consequently incapable of developing jaws (Cohn, 2002). Another remarkable feature of lamprey is that they show no *Dlx* code, meaning that there is no restricted expression of any *Dlx* genes along the proximal–distal axis of the BAs, unlike in gnathostomes (Cohn, 2002; Kuratani, Nobusada, Horigome, & Shigetani, 2001). The *Bapx1* gene is also absent in lamprey, which suggests that it might contribute to the acquisition of jaws and the establishment of maxilla in gnathostomes that express it focally in the first BA (Cerny et al., 2010). At later stages, the absence of *Hox* expression might allow the cartilage to develop and then be sculpted by the differential expression of *Dlx* genes. Even at these late developmental stages, *Hoxa2* represses endochondral ossification in the second BA (Kanzler, Kuschert, Liu, & Mallo, 1998), whereas *Dlx5* activates chondrocyte differentiation throughout the body (Ferrari & Kosher, 2002). A fine balance between the opposing activities of these genes could then be the basis for differentiation of the jaw and other skeletal elements of the vertebrate face.

The feeding mechanism is probably the most important element determining the success of adaptation of vertebrates to their environment. In feeding, both the jaws and tongue play a crucial function. The tongue has a characteristic form in tetrapods. Fish have a slight elevation of the mucosa on the floor of the mouth but this structure does not contain any voluntary muscles, unlike the tongues of land vertebrates (Iwasaki, 2002), one exception being the *Xenopus laevis* (Toyoshima & Shimamura, 1982). Most adult amphibians and all known reptiles, birds, and mammals have a tongue. Therefore, it has been suggested that the tongue appeared with the establishment of tetrapods and is related to the terrestrial lifestyle. In agnathans, particularly in lamprey, there is a tongue-like structure with the shape of a piston, but this organ is not homologous to the tongues of gnathostomes. The lamprey tongue and the tongues of tetrapods originated independently during evolution. The musculature of the gnathostome tongue originates in the hypobranchial system. In agnathans and gnathostome fishes, the hypobranchial apparatus forms musculature that is related to the gills and reforms during embryonic development to produce the lingual musculature. With the reduction of the gills in tetrapods, the mobile tongue appeared. Thus, the tongue musculature is derived from the hypobranchial musculature, which is anchored to the hyoid apparatus. Most likely, one of the main roles of the tongue is to facilitate eating on land, in collaboration with other anatomical structures within and near the oral cavity. It has been proposed that, during adaptation from a wet to a dry habitat in the evolution of vertebrates, stratification and keratinization were the most important changes in the lingual epithelium (Iwasaki, 2002).

5. MECHANICAL RELATIONSHIP BETWEEN THE MANDIBLE AND TONGUE

The tongue and mandible have a common origin. They arise simultaneously from the mandibular arch and their development is coordinated (Fig. 1). Mutations affecting early mandibular development have deleterious effects on tongue formation. In wild-type mice, the mandibular primordium grows down and lengthens from E12.5 to newborn stage (Ramaesh & Bard, 2003). This growth pattern seems to provide the tongue with physical space to move downward, and this movement closely coincides with another important event in craniofacial morphogenesis, the reorientation of the palatal shelves from a vertical to a horizontal position (Ferguson, 1977). In humans, morphometric analyses have shown that the growth of the tongue and mandible is intrinsically linked in size and shape between 20 gestational weeks and 24 months postnatally (Hutchinson, Kieser, & Kramer, 2014). The mechanical relationship between the mandible and tongue is also supported by certain clinical conditions in which alteration of one affects the other. One of the best examples is Pierre Robin Sequence (PRS; Fig. 2). PRS was originally described as a form of respiratory obstruction accompanied by malposition of the tongue and caused by dysmorphic atresia of the mandible. The current definition of PRS includes respiratory obstruction, glossoptosis, mandibular hypoplasia, and cleft palate (Fig. 2; Tan, Kilpatrick, & Farlie, 2011). Several previous studies have reported cleft palate due to physical obstruction by the tongue in mice. However, only mutation of *Prdm16* causes failed palate elevation associated with a highly positioned tongue and smaller mandibular bone, mimicking the clefting of human PRS (Bjork, Turbe-Doan, Prysak, Herron, & Beier, 2010). Yet, no direct evidence has been presented showing that the tongue malposition and cleft palate are due to the mandibular malformation. Further investigation is required to clarify the influence of mandibular growth on the size and position of the tongue.

6. STEM CELLS IN MANDIBLE AND TONGUE REGENERATION

Mandibular distraction osteogenesis (MDO) is the conventional treatment for infants with compromised airways due to micrognathia with glossoptosis and other congenital anomalies resulting in unilateral or bilateral mandibular hypoplasia. Typically in MDO, a corticotomy is used to fracture the bone into two segments, and the ends of the bone are gradually moved apart during the distraction phase, allowing new bone to form in the gap. Studies in multiple animal models have demonstrated diverse methods to increase bone-healing processes with the addition of stem cells, growth factors, experimental medications, and laser and ultrasound treatment. Mesenchymal stem cells (MSCs) from the bone marrow also enhance bone growth at distraction sites. Recently, it has been shown that stromal cell-derived factor-1 facilitates migration of endogenous MSCs *in vitro* and *in vivo*, which makes it a potential target that can be manipulated to upregulate an ongoing endogenous process. Moreover, the use of BMP and FGF along with transplantation of MSCs dramatically improves outcomes in osteogenic distraction compared to the cell transplant alone (Earley & Butts, 2014).

Stem cell-mediated tissue regeneration also has the potential to restore tongue tissue lost to cancer. Tongue squamous cell carcinoma is one of the most prevalent malignant cancers of the head and neck region. Surgical management remains the basis of treatment. After

excision of the lesion, reconstruction is required to maintain function. However, almost half of patients have difficulties in eating and drinking after treatment. Stem cell-mediated tissue regeneration would restore the normal tongue volume, shape, and function, which is generally not possible using surgical procedures. The presence of *Pax7*-positive cells in the adult tongue provides the opportunity to develop a stem cell-based regeneration approach. *Pax7* is crucial for the specification and survival of satellite cells, which are quiescent myogenic cells (Morgan & Partridge, 2003). In the adult muscle, they act as a reserve population, able to proliferate in response to injury, and give rise to regenerated muscle and additional satellite cells (Oustanina, Hause, & Braun, 2004). Interestingly, *Pax7*^{-/-} mice do not show muscle defects during embryogenesis but display severely compromised muscle regeneration capability. Overexpression of *Pax7* results in downregulation of *MyoD* and promotes cell-cycle withdrawal from the proliferating state; therefore, *Pax7* plays a critical role in the maintenance of the satellite cell pool (Olguin & Olwin, 2004). In the tongue of adult mice, the number of *Pax7*-positive cells is low but increases after injury, consistent with their role in forming new muscles (our unpublished data). In order to use *Pax7*-positive satellite cells in regenerative medicine, it is necessary to gain a comprehensive understanding of the regulatory mechanisms that control tongue morphogenesis and myogenesis. New approaches will likely be based on stem cell and tissue engineering technologies, using isolated CNCC-derived stem cells and skeletal muscle satellite *Pax7*-positive cells under the precise control of the proper molecular regulatory mechanisms to generate a functional tongue.

7. CONCLUSION

The mandible and tongue share a common origin in the CNCC populating the first BA and develop simultaneously and coordinately. Their relationship is not only based on their CNCC origins, but is also mechanical in nature. Growth of the tongue appears to be sensitive to that of the mandible. In humans, conditions in which one of these two structures is affected usually disrupt the development of the other. Understanding normal development of the mandible and tongue will facilitate the treatment of both developmental syndromes and cancer affecting this region using regenerative medicine and tissue engineering approaches.

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Figure 1.

Scheme of mandible development. (A) The first BA subdivides into maxillary and mandibular prominences at E10.5. At this stage, both prominences are solely constituted by CNCC-derived mesenchyme, which is covered by epithelium. (B) At E12.5, the MC develops in the mandibular primordium and mesenchymal cells condense close to it. (C) Mesenchymal cells start differentiating into osteoblasts. An osteogenic front composed of undifferentiated cells surrounds the differentiated osteoblasts. (D) Differentiation progresses in the mandibular primordium at E14.5. (E) The MC gives rise to the symphysis in the distal region and to the malleus and incus in the proximal region of the mandible. *Abbreviations:* E, eye; MC, Meckel's cartilage; I, incus; M, malleus; PS, palatal shelf; S, symphysis; T, tongue.

Figure 2.

Mechanical relationship between the mandible and tongue. (A) and (B) show schemes of a sagittal view of the skull of control and a PRS mouse model at newborn stage, respectively. (C) and (D) are schemes of corresponding coronal sections.