Published in final edited form as:

Gene Expr Patterns. 2016 March ; 20(2): 111–119. doi:10.1016/j.gep.2016.03.001.

Expression of Forkhead box transcription factor genes *Foxp1* and *Foxp2* during jaw development

Jeffrey M. Cesario¹, Asma A. Almaidhan², and Juhee Jeong^{1,*}

¹Department of Basic Science and Craniofacial Biology, New York University College of Dentistry, 345 E. 24th street, New York, NY 10010

²Department of Orthodontics, New York University College of Dentistry, 345 E. 24th street, New York, NY 10010

Abstract

Development of the face is regulated by a large number of genes that are expressed in temporally and spatially specific patterns. While significant progress has been made on characterizing the genes that operate in the oral region of the face, those regulating development of the aboral (lateral) region remain largely unknown. Recently, we discovered that transcription factors LIM homeobox (LHX) 6 and LHX8, which are key regulators of oral development, repressed the expression of the genes encoding forkhead box transcription factors, *Foxp1* and *Foxp2*, in the oral region. To gain insights into the potential role of the *Foxp* genes in region-specific development of the face, we examined their expression patterns in the first pharyngeal arch (primordium for the jaw) of mouse embryos at a high spatial and temporal resolution.

Foxp1 and *Foxp2* were preferentially expressed in the aboral and posterior parts of the first pharyngeal arch, including the developing temporomandibular joint. Through double immunofluorescence and double fluorescent RNA in situ hybridization, we found that *Foxp1* was expressed in the progenitor cells for the muscle, bone, and connective tissue. *Foxp2* was expressed in subsets of bone and connective tissue progenitors but not in the myoblasts. Neither gene was expressed in the dental mesenchyme nor in the oral half of the palatal shelf undergoing extensive growth and morphogenesis. Together, we demonstrated for the first time that *Foxp1* and *Foxp2* are expressed during craniofacial development. Our data suggest that the *Foxp* genes may regulate development of the aboral and posterior regions of the jaw.

Keywords

Foxp1; *Foxp2*; craniofacial development; pharyngeal arch; temporomandibular joint; mouse

*Author for Correspondence. jj78@nyu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

The forkhead box (FOX) proteins are an ancient family of transcription factors, characterized by the conserved DNA binding domain called a forkhead domain (also known as a winged helix domain). They play important roles in a wide variety of biological processes, such as development, carcinogenesis, metabolism, and immunity (Hannenhalli and Kaestner 2009, Jackson, Carpenter et al. 2010, Lam et al. 2013). There are 50 members of FOX family in humans and 44 members in mice. The FOX proteins are further divided into subgroups, FOXA through FOXS, with 1-6 members in each subgroup (Jackson et al. 2010).

The FOXP subgroup is encoded by four genes (*Foxp1-Foxp4*), and they contain a leucine zipper motif and a zinc finger domain in addition to the forkhead domain (Takahashi et al. 2009). Among the four FOXP proteins, FOXP1 and FOXP2 show a particularly high homology to each other throughout their amino acid sequences (Takahashi et al. 2009). *Foxp1* and *Foxp2* are expressed in diverse organs during embryonic development, often in partially overlapping but distinct patterns (Shu et al. 2001, Wang et al. 2004, Shu et al. 2007, Takahashi et al. 2009, Leishman et al. 2013, Zhao et al. 2015). Analyses of mouse mutants have shown that *Foxp1* plays critical roles in development of the spinal motor neurons, lymphocytes, and the cardiomyocytes (Wang et al. 2004, Hu et al. 2006, Dasen et al. 2008, Zhang et al. 2010). In addition, *Foxp1* and *Foxp2* co-regulate the development of the foregut and the skeleton (Shu et al. 2007, Zhao et al. 2015).

In humans, mutations affecting *FOXP1* and *FOXP2* have been found in patients with language and speech defects, often accompanied by additional intellectual disability and/or autism spectrum disorder (Lai et al. 2001, Feuk et al. 2006, Zeesman et al. 2006, Takahashi et al. 2009, Horn et al. 2010, Le Fevre et al. 2013, Lozano et al. 2015). Many of the patients also exhibited generalized functional deficiencies of the face and neck muscles (difficulties in chewing, swallowing, coughing, laughing), and characteristic facial features (triangular face, prominent forehead, short and broad nose, low set ears, downward slanting eyes, high-arched palate, wide-spaced teeth) (Feuk et al. 2006, Zeesman et al. 2006, Horn et al. 2010, Le Fevre et al. 2013, Lozano et al. 2015). The language and mental defects clearly implicate problems in brain development, and the musculoskeletal phenotypes of the face may be secondary to the disruption in the motor control from the brain for the muscles. Alternatively, the latter phenotypes can also be explained by abnormal development of the face itself. To date, potential roles of *Foxp1* and *Foxp2* in orofacial development have not been examined.

In vertebrates, the face develops from the embryonic primordia called frontonasal prominence and the first pharyngeal arches (PA1) (Minoux and Rijli 2010, Frisdal and Trainor 2014, Cesario et al. 2015a). PA1 gives rise to the jaw including the palate and the teeth, and it is further divided into the mandibular arch, which is the prospective lower jaw, and the maxillary arch, which is the prospective upper jaw. The mesenchyme of the developing face is of dual origin: the mesoderm-derived cells occupy the core of PA1, and they become skeletal muscles (Noden 1983, Noden and Trainor 2005). On the other hand, the cells that have migrated from the neural crest (called ectomesenchyme cells) give rise to

all the bone and cartilage of the face, as well as connective tissues such as tendons and dermis (Noden 1978, Le Douarin and Kalcheim 1999).

Recently, we discovered that *Lhx6* and *Lhx8* repressed *Foxp1* and *Foxp2* in PA1, where the *Lhx* (LIM homeobox) genes are crucial to development of the palate and the molars on the oral side (Cesario et al. 2015b). To gain insights into the biological significance of the repression of *Foxp* by LHX, we examined the expression patterns of *Foxp1* and *Foxp2* during normal development of PA1 in mouse embryos. Our results suggest that the *Foxp* genes may be involved in development of the muscle, bone, and connective tissue in the face.

2. Results

At embryonic day (E) 10.5, *Lhx6* and *Lhx8* are expressed in the areas of PA1 facing the oral cavity, with the broad expression anteriorly (Fig. 1A; Grigoriou et al. 1998). On the other hand, the expression of *Foxp1* and *Foxp2* was excluded from the oral and anterior regions of PA1 (Fig. 1B,C).

Examination on E11.5 sections revealed that *Foxp1* was expressed in both the epithelium and the mesenchyme of PA1 (Fig. 1F,J,N). In the maxillary mesenchyme, *Foxp1* expression was restricted to the dorsal-lateral (aboral) domain in the anterior and middle sections (Fig. 1F,J), complementary to the expression of *Lhx6* and *Lhx8* in the oral domain (Fig. 1D,E,H,I). This relationship was less pronounced in the posterior section (Fig. 1L-N). *Foxp1* expression in the mandibular arch was mostly concentrated in and around the tongue (Fig. 1J,N). *Foxp2* was expressed in the mesenchyme, but not the epithelium, of PA1 at E11.5, partially overlapping with *Foxp1* (Fig. 1G,K,O). However, the expression domain of *Foxp2* was significantly smaller than that of *Foxp1*.

At E12.5, *Foxp1* was strongly expressed in the mesenchymal condensations in the lateral PA1 (dotted box in Fig. 2F). This area contained the primordia for the cheek bone (zygomatic arch), muscles of mastication (masseter), and the tendons between the bone and muscle, each marked by *Runx2* (runt-related transcription factor 2, a marker of pre-osteoblasts; Karsenty 2008, Long 2012), *MyoD1* (myogenic differentiation-1, a marker of myoblasts; Berkes and Tapscott 2005), and *Scx* (Scleraxis, a marker of tenocytes; Schweitzer et al. 2001) (Fig. 2H,I,J). *Foxp1* was also abundantly expressed in the posterior PA1, where the maxillary arch and the mandibular arch joined (Fig. 2K). *Foxp2* expression was strongest in the mesenchyme just under the eye (Fig. 2G), but low expression extended ventrally into the area where *Foxp1* was expressed (arrow in Fig. 2G). In the posterior section, the expression of *Foxp2* appeared nearly complementary to that of *Foxp1* (Fig. 2L).

To confirm the expression of *Foxp1* in the progenitors for the bone, muscle, and tendon, we used double immunofluorescence and double fluorescent RNA in situ hybridization to co-label FOXP1 protein or mRNA and the markers of each progenitor population. FOXP1 was detected in RUNX2⁺ cells in the dorsal part of the skeletogenic condensation for the zygomatic arch (Fig. 3A-C; arrow in C). In addition, FOXP1 was expressed in most of the MyoD1⁺ cells of the masseter although the intensity of FOXP1 showed a dorsal-to-ventral

decline (Fig. 3D-E). *Scx* was expressed in the strip of cells between the *RUNX2*⁺ domain and *MyoD1*⁺ domain (Fig. 3B,E,H). *Scx* expression also partly overlapped with *Runx2* at this stage (data not shown), which is reminiscent of the presence of *Scx* and *Sox9* double-positive cells during early development of the tendon-bone attachment unit in the limbs (Blitz et al. 2013, Zelzer et al. 2014). *Foxp1* mRNA was detected in a subset of *Scx*⁺ cells (Fig. 3G-I).

Similar to FOXP1, FOXP2 was co-expressed with RUNX2 in the dorsal part of the zygomatic arch primordium (Fig. 3J-L; arrow in L). In addition, FOXP2, but not FOXP1, was expressed in some of the pre-osteoblasts in the developing palatine bone (dotted box in Fig. 2G; Fig. 3M-O). In contrast, FOXP2 was not expressed in the muscle cells (Fig. 3P-R). Limited overlap was detected between *Foxp2* and *Scx* mRNA (Fig. 3S-U).

At E13.5, *Foxp1* continued to be expressed in the mesenchymal mass of the developing zygomatic bone, masseter, and the tendons (Fig. 4G,I-L). In the posterior section, *Foxp1* was expressed in the muscles and the surrounding connective tissues, labeled by *MyoD1* and *Scx* (Fig. 4M,Q,R). RNA in situ hybridization on the sagittal sections of the head confirmed the overall pattern of *Foxp1* expression identified from the coronal sections, namely, abundant in the lateral mesenchyme of PA1 (Fig. 4S) but minimal in the medial mesenchyme (Fig. 4X,c,h). *Foxp2* continued to be expressed in the mesenchyme under the eye at E13.5, but with sharp boundaries instead of the ventral tail seen at E12.5 (Fig. 4H). The intensity was also significantly reduced. *Foxp2* expression appeared to be mostly in the undifferentiated mesenchyme that did not express *Runx2*, *Sp7* (also known as *Osterix*, an osteogenic transcription factor acting downstream of RUNX2; Nakashima et al. 2002, Long 2012), *Scx*, nor *MyoD1* (Fig. 4).

Double immunofluorescence showed that the overlap between FOXP1 and RUNX2 increased substantially at E13.5 compared with E12.5 (Fig. 5A-C). FOXP1 was also co-expressed with SP7 (Fig. 5D-F). Almost all the *RUNX2*⁺ cells and *OSX*⁺ cells appeared to express FOXP1 although the level of FOXP1 varied widely within the population of cells. Similarly, *Foxp1* mRNA was detected more abundantly in *Scx*⁺ cells at this stage than E12.5 (Fig. 5G-I).

At E13.5, FOXP2 was expressed only in a small subset of the pre-osteoblasts for the zygomatic bone (Fig. 5J-O). Also, in the developing palatine, the levels of FOXP2 protein were sharply reduced in the cells co-expressing RUNX2 and SP7, which correspond to more differentiated cells than *RUNX2*⁺ *SP7*⁻ cells (Fig. 5P-U).

Foxp1 was expressed in the head muscles and the tendons surrounding them at E14.5 (Fig. 6A,F,K,P), but its expression in the zygomatic arch was down-regulated compared with the earlier stages (Fig. 6A,F). *Foxp2* expression was further reduced, and it was maintained only in small areas just under the eye and at the root of the tongue in the anterior sections (Fig. 6B,G). In the posterior section, the developing temporo-mandibular joint (TMJ) was recognizable for the first time at this stage. It appeared as the osteogenic condensations for the glenoid fossa of the temporal (squamosal) bone and the condyle of the dentary bone (Fig. 6R), with the intervening mesenchyme that will form the fibrocartilagenous articular

disc (arrowhead in Fig. 6S). Both *Foxp1* and *Foxp2* were expressed in this mesenchyme between the glenoid fossa and the condyle (arrowheads in Fig. 6P,Q).

At E15.5, only faint expression of *Foxp1* and *Foxp2* was detected in the jaw mesenchyme (Fig. 7). *Foxp1* expression was noticeable in the muscles and the tendons (arrows in Fig. 7B,H). In addition, as the articular disc of TMJ takes shape (arrowhead in Fig. 7G), *Foxp1* was specifically expressed in the disc (arrowhead in Fig. 7H). *Foxp2* was also expressed in the disc area (arrowhead in Fig. 7I). However, *Foxp2* was expressed in the mesenchyme around the condyle in all directions instead of being localized to the interface with the glenoid fossa (Fig. 7I). The expression of *Foxp1* and *Foxp2* were no longer detectable in the facial mesenchyme at E16.5 (data now shown).

To summarize, we found that *Foxp1* was expressed in the developing zygomatic arch bone, the muscles and tendons of the jaw, and the articular disc of TMJ. *Foxp1* expression in the bone was dynamic, being most robust at E13.5. *Foxp2* showed limited expression in the zygomatic arch and palatine bone at earlier stages, but it was not expressed in the muscles. Both *Foxp1* and *Foxp2* were down-regulated later in gestation.

It is noteworthy that the expression of *Foxp1* and *Foxp2* was absent from the odontogenic mesenchyme of all the teeth (Fig. 2F,G; Fig. 4A,B,G,H,c,d; Fig. 7B,C), and also from the mesenchyme of the ventral (oral) domain of the palatal shelf during the major growth and morphogenesis of the palate (Fig. 2A,B,F,G,K,L; Fig. 4A,B,G,H,M,N). In addition, we failed to detect any *Foxp1* or *Foxp2* expression in the developing dentary bone of the mandibular arch.

3. Discussion

In this report, we describe the expression of *Foxp1* and *Foxp2* during craniofacial development for the first time. Our data suggest that *Foxp1* may regulate development of the muscular, skeletal, and connective tissue in the aboral part of the face, including the TMJ. While we know of many genes that are expressed in the oral mesenchyme and play essential roles in development of the palate and/or teeth (Bei 2009, Bush and Jiang 2012), little is known about the factors that regulate aboral development. Therefore, *Foxp1* is highly unique among the genes implicated in craniofacial development. Another unusual feature of *Foxp1* is that it is expressed in both neural crest-derived cells (precursors of bone and tendon) and mesoderm-derived cells (myoblasts) within PA1 mesenchyme, whereas most genes are expressed in one of the two lineages only (Cesario et al. 2015a). Unlike *Foxp1*, *Foxp2* expression was specific to the neural crest-derived tissue, some in the osteogenic condensations and the connective tissue, but mostly in the undifferentiated mesenchyme.

Foxp1 and *Foxp2* have been implicated in bone development in other parts of the body. During endochondral ossification of the long bones, FOXP1 and FOXP2 suppressed osteoblast differentiation by inhibiting the activity of RUNX2 through physical interactions (Zhao et al. 2015). Surprisingly, however, deletion of *Foxp1* and *Foxp2* in the calvaria led to deficient ossification, indicating that the FOXP proteins are positive regulators of bone formation in this context (Zhao et al. 2015). The bones of the face are similar to the calvarial

bone in that both develop intra-membraneously. Therefore, we speculate that *Foxp1* and *Foxp2* may be required for the normal development of the zygomatic arch and the palatine bone from PA1. However, the fact that two genes were down-regulated in the osteogenic condensations after E13.5 suggests that their role would be limited to the early specification of the cell fate.

In addition to the bone, *Foxp1* has also been implicated in tendon and muscle development. Whole transcriptome profiling of limb tendons identified *Foxp1* as one of the genes activated during early tendon development (Liu et al. 2015). In the heart, *Foxp1* was required cell-autonomously to promote differentiation and repress proliferation of the cardiomyocytes (Zhang et al. 2010). Cardiomyocytes and the skeletal muscles of the pharyngeal arches (branchiomeric muscles) develop from a common pool of mesodermal progenitors termed “cardiopharyngeal field”, and are regulated by many of the same genes (Tzahor 2009, Diogo et al. 2015). Therefore, it is plausible that *Foxp1* plays a similar role in the muscles of the heart and the jaw.

In conclusion, we have demonstrated that *Foxp1* and *Foxp2* are expressed in the developing face. Given that human patients with mutations in *FOXP1* or *FOXP2* frequently exhibit dysfunction and dysmorphology of the orofacial region, future investigation is necessary to elucidate the function of the *Foxp* genes in craniofacial development.

4. Experimental Procedures

4.1. Preparation of tissue

All the tissue sections used in this study are frozen sections. Wild type mouse embryos of mixed strain background were used. All the experiments using mice were performed following protocols approved by New York University institutional committee on the use of laboratory animals, and in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Tissue sections were prepared as previously described (Jeong et al. 2012). Some sections were stained with cresyl violet to visualize tissue morphology as previously described (Jeong et al. 2012).

4.2. RNA In situ hybridization

For in situ hybridization for a single gene, antisense probes were labeled with digoxigenin (DIG RNA labeling mix, Roche) and the hybridization was performed following a standard protocol (Schaeren-Wiemers and Gerfin-Moser 1993). After incubating with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche), BM purple (Roche) was used to visualize the signal.

For double fluorescent in situ hybridization, Tyramide Signal Amplification (TSA) system (PerkinElmer) was used. One antisense probe was labeled with digoxigenin, and the other probe was labeled with fluorescein (Fluorescein RNA labeling mix, Roche). Two probes were mixed and added to the sections. After unbound probes were washed away, the slides were first incubated with anti-digoxigenin antibody conjugated with peroxidase (Roche). Biotin-tyramide → streptavidin-peroxidase → Cy3-tyramide were added sequentially, which allowed the detection of the digoxigenin-labeled probe through Cy3 fluorescence. The

peroxidase reaction was then quenched by incubating the slides in 3% hydrogen peroxide. Next, the slides were treated with anti-fluorescein-peroxidase antibody (Roche) → fluorescein-tyramide → anti-fluorescein-peroxidase antibody → fluorescein-tyramide sequentially, to amplify the signal and visualize the fluorescein-labeled probe. The nuclei were stained with DAPI.

The plasmid templates for *Lhx6*, *Lhx8*, *Runx2*, *Sp7*, and *MyoD1* probes were obtained from other researchers (Davis et al. 1987, Ducy et al. 1997, Grigoriou et al. 1998, Nakashima et al. 2002). For *Scx*, a full length cDNA clone (NCBI accession # BC062161) was purchased from GE Dharmacon, and it was linearized and used for in vitro transcription. For *Foxp1* and *Foxp2* probes, full-length cDNA plasmids were obtained from another laboratory (Shu et al. 2001), and parts of them were amplified by PCR and used as templates. The PCR primers for *Foxp1* were 5'-AGCAACCAGCTCTTCAGGTTCC-3' and 5'-AATTAACCCTCACTAAAGGGACGTTGTATTTGTCTGAGTACCG-3' (underlined is T3 polymerase promoter); the amplified region is nucleotide 500-1563 of NCBI accession # BC064764. The PCR primers for *Foxp2* were 5'-CTGTGATGTTGCAGCAGCAGCA-3' and 5'-AATTAACCCTCACTAAAGGGGAAGGGAGGTCTAACATCTGCG-3'; the amplified region is nucleotide 699-1774 of NCBI accession # BC058960. While *Foxp1* and *Foxp2* have large numbers of isoforms (10 and 5, respectively, according to University of California Santa Cruz Genome Browser), the probes were designed against the regions common to almost all the isoforms. Therefore, our in situ hybridization likely detected all of *Foxp1* and *Foxp2* expression in the region irrespective of the isoforms.

4.3. Immunofluorescence

Immunofluorescence was performed as described (Jeong and McMahon 2005). The primary antibodies were: goat polyclonal anti-FOXP1 (R&D Systems, catalog # AF4534, 1:300), goat polyclonal anti-FOXP2 (Abcam, catalog # ab1307, 1:1000), rabbit polyclonal anti-MyoD (C-20) (Santa Cruz Biotech, catalog # sc-304, 1:100), rabbit polyclonal anti-RUNX2 (Santa Cruz Biotech, catalog # sc-10758X, 1:500), and rabbit polyclonal anti-Sp7 (Abcam, catalog # ab22552, 1:150). All the secondary antibodies were Alexa Fluor antibodies from Life Technologies. DAPI was used to stain the nuclei.

Acknowledgments

This work was funded by National Institute of Dental and Craniofacial Research, National Institutes of Health (R00 DE019486 to J.J.).

References

- Bei M. Molecular genetics of tooth development. *Curr Opin Genet Dev.* 2009; 19(5):504–510.10.1016/j.gde.2009.09.002 [PubMed: 19875280]
- Berkes CA, Tapscott SJ. MyoD and the transcriptional control of myogenesis. *Semin Cell Dev Biol.* 2005; 16(4-5):585–595.10.1016/j.semdb.2005.07.006 [PubMed: 16099183]
- Blitz E, Sharir A, Akiyama H, Zelzer E. Tendon-bone attachment unit is formed modularly by a distinct pool of Scx- and Sox9-positive progenitors. *Development.* 2013; 140(13):2680–2690.10.1242/dev.093906 [PubMed: 23720048]
- Bush JO, Jiang R. Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development. *Development.* 2012; 139(2):231–243.10.1242/dev.067082 [PubMed: 22186724]

- Cesario, JM.; Malt, A Landin; Jeong, J. Developmental Genetics of the Pharyngeal Arch System, e-book. In: Saint-Jeannet, JP.; Kessler, D., editors. Colloquium Series on Developmental Biology. Vol. 6. Morgan & Claypool Life Sciences Publishers; 2015a.
- Cesario JM, Malt A Landin, Deacon LJ, Sandberg M, Vogt D, Tang Z, Zhao Y, Brown S, Rubenstein JL, Jeong J. Lhx6 and Lhx8 promote palate development through negative regulation of a cell cycle inhibitor gene, p57Kip2. *Hum Mol Genet.* 2015b; 24(17):5024–5039.10.1093/hmg/ddv223 [PubMed: 26071365]
- Dasen JS, De Camilli A, Wang B, Tucker PW, Jessell TM. Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. *Cell.* 2008; 134(2):304–316.10.1016/j.cell.2008.06.019 [PubMed: 18662545]
- Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell.* 1987; 51(6):987–1000. [PubMed: 3690668]
- Diogo R, Kelly RG, Christiaen L, Levine M, Ziermann JM, Molnar JL, Noden DM, Tzahor E. A new heart for a new head in vertebrate cardiopharyngeal evolution. *Nature.* 2015; 520(7548):466–473.10.1038/nature14435 [PubMed: 25903628]
- Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell.* 1997; 89(5):747–754. [PubMed: 9182762]
- Feuk L, Kalervo A, Lipsanen-Nyman M, Skaug J, Nakabayashi K, Finucane B, Hartung D, Innes M, Kerem B, Nowaczyk MJ, Rivlin J, Roberts W, Senman L, Summers A, Szatmari P, Wong V, Vincent JB, Zeesman S, Osborne LR, Cardy JO, Kere J, Scherer SW, Hannula-Jouppi K. Absence of a paternally inherited FOXP2 gene in developmental verbal dyspraxia. *Am J Hum Genet.* 2006; 79(5):965–972.10.1086/508902 [PubMed: 17033973]
- Frisdal A, Trainor PA. Development and evolution of the pharyngeal apparatus. *Wiley Interdiscip Rev Dev Biol.* 2014; 3(6):403–418.10.1002/wdev.147 [PubMed: 25176500]
- Grigoriou M, Tucker AS, Sharpe PT, Pachnis V. Expression and regulation of Lhx6 and Lhx7, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development. *Development.* 1998; 125(11):2063–2074. [PubMed: 9570771]
- Hannenhalli S, Kaestner KH. The evolution of Fox genes and their role in development and disease. *Nat Rev Genet.* 2009; 10(4):233–240.10.1038/nrg2523 [PubMed: 19274050]
- Horn D, Kapeller J, Rivera-Brugues N, Moog U, Lorenz-Depiereux B, Eck S, Hempel M, Wagenstaller J, Gawthrop A, Monaco AP, Bonin M, Riess O, Wohlleber E, Illig T, Bezzina CR, Franke A, Spranger S, Villavicencio-Lorini P, Seifert W, Rosenfeld J, Klopocki E, Rappold GA, Strom TM. Identification of FOXP1 deletions in three unrelated patients with mental retardation and significant speech and language deficits. *Hum Mutat.* 2010; 31(11):E1851–1860.10.1002/humu.21362 [PubMed: 20848658]
- Hu H, Wang B, Borde M, Nardone J, Maika S, Allred L, Tucker PW, Rao A. Foxp1 is an essential transcriptional regulator of B cell development. *Nat Immunol.* 2006; 7(8):819–826.10.1038/ni1358 [PubMed: 16819554]
- Jackson BC, Carpenter C, Nebert DW, Vasiliou V. Update of human and mouse forkhead box (FOX) gene families. *Hum Genomics.* 2010; 4(5):345–352. [PubMed: 20650821]
- Jeong J, Cesario J, Zhao Y, Burns L, Westphal H, Rubenstein JL. Cleft palate defect of *Dlx1/2*^{-/-} mutant mice is caused by lack of vertical outgrowth in the posterior palate. *Dev Dyn.* 2012; 241(11):1757–1769.10.1002/dvdy.23867 [PubMed: 22972697]
- Jeong J, McMahon AP. Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1. *Development.* 2005; 132(1):143–154.10.1242/dev.01566 [PubMed: 15576403]
- Karsenty G. Transcriptional control of skeletogenesis. *Annu Rev Genomics Hum Genet.* 2008; 9:183–196.10.1146/annurev.genom.9.081307.164437 [PubMed: 18767962]
- Lai CS, Fisher SE, Hurst JA, Vargha-Khadem F, Monaco AP. A forkhead-domain gene is mutated in a severe speech and language disorder. *Nature.* 2001; 413(6855):519–523.10.1038/35097076 [PubMed: 11586359]
- Lam EW, Brosens JJ, Gomes AR, Koo CY. Forkhead box proteins: tuning forks for transcriptional harmony. *Nat Rev Cancer.* 2013; 13(7):482–495.10.1038/nrc3539 [PubMed: 23792361]
- Le Douarin, N.; Kalcheim, C. The Neural Crest. Cambridge University Press; 1999.

- Le Fevre AK, Taylor S, Malek NH, Horn D, Carr CW, Abdul-Rahman OA, O'Donnell S, Burgess T, Shaw M, Gecz J, Bain N, Fagan K, Hunter MF. FOXP1 mutations cause intellectual disability and a recognizable phenotype. *Am J Med Genet A*. 2013; 161A(12):3166–3175.10.1002/ajmg.a.36174 [PubMed: 24214399]
- Leishman E, Howard JM, Garcia GE, Miao Q, Ku AT, Dekker JD, Tucker H, Nguyen H. Foxp1 maintains hair follicle stem cell quiescence through regulation of Fgf18. *Development*. 2013; 140(18):3809–3818.10.1242/dev.097477 [PubMed: 23946441]
- Liu H, Xu J, Liu CF, Lan Y, Wylie C, Jiang R. Whole transcriptome expression profiling of mouse limb tendon development by using RNA-seq. *J Orthop Res*. 2015; 33(6):840–848.10.1002/jor.22886 [PubMed: 25729011]
- Long F. Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol*. 2012; 13(1):27–38.10.1038/nrm3254 [PubMed: 22189423]
- Lozano R, Vino A, Lozano C, Fisher SE, Deriziotis P. A de novo FOXP1 variant in a patient with autism, intellectual disability and severe speech and language impairment. *Eur J Hum Genet*. 201510.1038/ejhg.2015.66
- Minoux M, Rijli FM. Molecular mechanisms of cranial neural crest cell migration and patterning in craniofacial development. *Development*. 2010; 137(16):2605–2621.10.1242/dev.040048 [PubMed: 20663816]
- Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrughe B. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell*. 2002; 108(1):17–29. [PubMed: 11792318]
- Noden DM. The control of avian cephalic neural crest cytodifferentiation. I. Skeletal and connective tissues. *Dev Biol*. 1978; 67(2):296–312. [PubMed: 738529]
- Noden DM. The embryonic origins of avian cephalic and cervical muscles and associated connective tissues. *Am J Anat*. 1983; 168(3):257–276.10.1002/aja.1001680302 [PubMed: 6650439]
- Noden DM, Trainor PA. Relations and interactions between cranial mesoderm and neural crest populations. *J Anat*. 2005; 207(5):575–601.10.1111/j.1469-7580.2005.00473.x [PubMed: 16313393]
- Schaeren-Wiemers N, Gerfin-Moser A. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry*. 1993; 100(6):431–440. [PubMed: 7512949]
- Schweitzer R, Chyung JH, Murtaugh LC, Brent AE, Rosen V, Olson EN, Lassar A, Tabin CJ. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development*. 2001; 128(19):3855–3866. [PubMed: 11585810]
- Shu W, Lu MM, Zhang Y, Tucker PW, Zhou D, Morrissey EE. Foxp2 and Foxp1 cooperatively regulate lung and esophagus development. *Development*. 2007; 134(10):1991–2000.10.1242/dev.02846 [PubMed: 17428829]
- Shu W, Yang H, Zhang L, Lu MM, Morrissey EE. Characterization of a new subfamily of winged-helix/forkhead (Fox) genes that are expressed in the lung and act as transcriptional repressors. *J Biol Chem*. 2001; 276(29):27488–27497.10.1074/jbc.M100636200 [PubMed: 11358962]
- Takahashi H, Takahashi K, Liu FC. FOXP genes, neural development, speech and language disorders. *Adv Exp Med Biol*. 2009; 665:117–129. [PubMed: 20429420]
- Tzahor E. Heart and craniofacial muscle development: a new developmental theme of distinct myogenic fields. *Dev Biol*. 2009; 327(2):273–279.10.1016/j.ydbio.2008.12.035 [PubMed: 19162003]
- Wang B, Weidenfeld J, Lu MM, Maika S, Kuziel WA, Morrissey EE, Tucker PW. Foxp1 regulates cardiac outflow tract, endocardial cushion morphogenesis and myocyte proliferation and maturation. *Development*. 2004; 131(18):4477–4487.10.1242/dev.01287 [PubMed: 15342473]
- Zeesman S, Nowaczyk MJ, Teshima I, Roberts W, Cardy JO, Brian J, Senman L, Feuk L, Osborne LR, Scherer SW. Speech and language impairment and oromotor dyspraxia due to deletion of 7q31 that involves FOXP2. *Am J Med Genet A*. 2006; 140(5):509–514.10.1002/ajmg.a.31110 [PubMed: 16470794]

- Zelzer E, Blitz E, Killian ML, Thomopoulos S. Tendon-to-bone attachment: from development to maturity. *Birth Defects Res C Embryo Today*. 2014; 102(1):101–112.10.1002/bdrc.21056 [PubMed: 24677726]
- Zhang Y, Li S, Yuan L, Tian Y, Weidenfeld J, Yang J, Liu F, Chokas AL, Morrissey EE. Foxp1 coordinates cardiomyocyte proliferation through both cell-autonomous and nonautonomous mechanisms. *Genes Dev*. 2010; 24(16):1746–1757.10.1101/gad.1929210 [PubMed: 20713518]
- Zhao H, Zhou W, Yao Z, Wan Y, Cao J, Zhang L, Zhao J, Li H, Zhou R, Li B, Wei G, Zhang Z, French CA, Dekker JD, Yang Y, Fisher SE, Tucker HO, Guo X. Foxp1/2/4 regulate endochondral ossification as a suppresser complex. *Dev Biol*. 2015; 398(2):242–254.10.1016/j.ydbio.2014.12.007 [PubMed: 25527076]

Highlights

- We describe the expression of *Foxp1* and *Foxp2* during craniofacial development.
- *Foxp1* was expressed in the developing muscle, bone, and tendon of the jaw.
- *Foxp2* was expressed in a subset of bone and tendon progenitors.
- Both *Foxp1* and *Foxp2* were expressed in the temporomandibular joint.
- This is the first report on the expression of *Foxp1* and *Foxp2* in the face.

Figure 1. Complementary patterns of expression between *Lhx* and *Foxp* genes in PA1
 (A-C) Lateral views of the head of E10.5 embryos processed by whole mount RNA in situ hybridization. (D-O) Coronal sections of the head of an E11.5 mouse embryo processed by RNA in situ hybridization. Only the right half of the face is shown. The anterior-posterior axis, medial-lateral axis, and the dorsal-ventral axis are indicated in panels (A), (D), and (E), respectively. Ey, eye; Md, mandibular arch; Mx, maxillary arch; OC, oral cavity; PA2, second pharyngeal arch; To, tongue. Bar, 0.2 mm.

Figure 2. Expression of *Foxp1* and *Foxp2* in PA1 at E12.5

Coronal sections of the head of an E12.5 mouse embryo processed by RNA in situ hybridization. Only the right half of the face is shown. The arrow in G points to the faint extension of *Foxp2* expression. Developing bone, tendon, and muscle are labeled by *Runx2*, *Scx*, and *MyoD1*, respectively. The dotted boxes in F and G indicate the areas shown in Figure 3. De, dentary bone; LM, lower molar; Ma, masseter muscle; MC, Meckel's cartilage; Pa, palatine bone; PS, palatal shelf; TgG, trigeminal ganglion; UM, upper molar; Zy, zygomatic arch.

Figure 3. Co-expression analyses for the *Foxp* genes and the markers of the developing bone, tendon, and muscle at E12.5

(A-L) and (P-U) correspond to the boxed area in Fig. 2F. (M-O) correspond to the boxed area in Fig. 2G. (A-F) and (J-R) are the results of double immunofluorescence. (G-I) and (S-U) are the results of double fluorescent RNA in situ hybridization. Each channel is shown in the first two columns, and then the merged image is shown in the third column. The arrows in (C) and (L) point to the expression of FOXP1 and FOXP2 in the dorsal part of the developing zygomatic arch bone. The arrow in (O) points to the expression of FOXP2 in the palatine bone. The insets in (I) and (U) are the enlargements of the dotted box in each panel.

Figure 4. Expression of *Foxp1* and *Foxp2* in PA1 at E13.5

(A-R) Coronal sections of the head of E13.5 mouse embryos processed by RNA in situ hybridization. Only the right half of the face is shown. The dotted boxes in (G) and (H) indicate the areas shown in Figure 5. (S-L) Sagittal sections of the head of an E13.5 mouse embryo processed by RNA in situ hybridization. LI, lower incisor; Pt, pterygoid muscle; Te, temporalis muscle; UI, upper incisor. Bar, 0.5 mm.

Figure 5. Co-expression analyses for the *Foxp* genes and the markers of the developing bone and tendon at E13.5

(A-O) correspond to the boxed area in Fig. 4G. (P-U) correspond to the boxed area in Fig. 4H. (A-F) and (J-U) are the results of double immunofluorescence. (G-I) are the results of double fluorescent RNA in situ hybridization. Each channel is shown in the first two columns, and then the merged image is shown in the third column. The inset in (I) is the enlargement of the dotted box in this panel.

Figure 6. Expression of *Foxp1* and *Foxp2* in PA1 at E14.5

(A-T) Coronal sections of the head of an E14.5 mouse embryo processed by RNA in situ hybridization. The arrowheads in (P,Q,S) point to the expression in the prospective articular disc of TMJ. Co, condyle; GF, glenoid fossa; LPT, lateral pterygoid muscle; MPt, medial pterygoid muscle.

Figure 7. Expression of *Foxp1* and *Foxp2* in PA1 at E15.5

Coronal sections of the head of an E15.5 mouse embryo stained with cresyl violet (A,D,G) or processed by RNA in situ hybridization (B,C,E,F,H,I). The arrows in B and H point to the expression of *Foxp1* in the tendons. The arrowheads in (H,I) point to the expression in the nascent articular disc of TMJ.