Foxp2 regulates anatomical features that may be relevant for vocal behaviors and bipedal locomotion

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Fundamental human traits, such as language and bipedalism, are associated with a range of anatomical adaptations in craniofacial shaping and skeletal remodeling. However, it is unclear how such morphological features arose during hominin evolution. FOXP2 is a brain-expressed transcription factor implicated in a rare disorder involving speech apraxia and language impairments. Analysis of its evolutionary history suggests that this gene may have contributed to the emergence of proficient spoken language. In the present study, through analyses of skeleton-specific knockout mice, we identified roles of Foxp2 in skull shaping and bone remodeling. Selective ablation of Foxp2 in cartilage disrupted pup vocalizations in a similar way to that of global Foxp2 mutants, which may be due to pleiotropic effects on craniofacial morphogenesis. Our findings also indicate that Foxp2 helps to regulate strength and length of hind limbs and maintenance of joint cartilage and intervertebral discs, which are all anatomical features that are susceptible to adaptations for bipedal locomotion. In light of the known roles of Foxp2 in brain circuits that are important for motor skills and spoken language, we suggest that this gene may have been well placed to contribute to coevolution of neural and anatomical adaptations related to speech and bipedal locomotion.

Significance

Speech and bipedalism are key aspects of behavior that emerged during human evolution. Foxp2, a gene implicated in a human speech and language disorder, has been suggested to contribute to language evolution. Here, through knockout studies of mouse Foxp2, we show that this gene is not only important for neural circuits involved in vocal behaviors, it also helps regulate relevant anatomical substrates. We additionally demonstrate that Foxp2 influences skeletal features that may be relevant for bipedal locomotion. Our findings raise the possibility that Foxp2 might be important for anatomical features contributing to derived human traits, including speech and bipedalism.


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functions of Foxp1/2 in regulating endochondral ossification during embryonic bone development (30).

Building on our demonstration of a Foxp2 role in embryonic bone development, and in light of prior hypothesized involvement of this gene in human evolution, we here used skeleton-specific loss-of-function analyses in mice to investigate how it might help regulate anatomy. Unexpectedly, skeletal Foxp2 loss led to disruption of pup vocalizations, similar to phenotypes previously reported for global mutant or knockout lines (17, 31). Endochondral ossification of the Bo, the basiophenoid bone (Bs) and the nasal bone, were attenuated in Foxp2 Δ/Δ mice at E15.5, as evidenced by diminished Alizarin red staining (arrows in Fig. 1C). In particular, broader funnel-shaped presphenoids were observed in Foxp2 Δ/Δ mice at P13 (arrows in Fig. 1 D and F). Minor alterations in presphenoid morphology were also detected at P7 in Foxp2 Δ/Δ mutant mice, which carry a point mutation matching that found in affected members of the KE family (Fig. 1 E and G and SI Appendix, Fig. S1A). Altered presphenoid morphology was much more evident in homozygous Foxp2 ΔR552H/ΔR552H mice at P0 (arrows in Fig. 1H and SI Appendix, Fig. S1B). At the histological level, Foxp2 Δ/Δ mice showed delayed chondrocyte hypertrophy and ossification within sphenocostal synchondroses, as revealed by Safranin O staining and immunohistochemistry (IHC) and immunofluorescence (IF) examination using Col X, Osterix (Osx), and Foxp2 antibodies (SI Appendix, Figs. S1C and S2A). Collectively, these data indicate that Foxp2 is important for sphenoid development and cranial shaping.

Foxp1/2 redundantly regulate endochondral ossification during embryonic development (30). Therefore, we also examined the impact of Foxp1 on craniofacial development by generating cartilage-specific knockout mice. As observed in Foxp2 Δ/Δ mice, homozygous Foxp1 Δ/Δ mice had shorter nasal bones (SI Appendix, Fig. S2 B and C) and minor morphological deformities in their presphenoid bone (arrows in SI Appendix, Fig. S2 D and E), with similarities to features observed in cases of heterozygous human FOXP1 disruption (10, 11). Then, we compared craniofacial shaping within the single (Foxp1 Δ/Δ and Foxp2 Δ/Δ) and the double (Foxp1/2 Δ/Δ) cKO mice at E18.5. Shortening of nasal bones and vaulted skulls were evident in the Foxp1/2 Δ/Δ double mutant compared with controls (SI Appendix, Fig. S2F, Upper). Defective sphenoid formation was more pronounced in Foxp1/2 Δ/Δ double mutants than either single mutant or Foxp1/2 Δ/Δ controls (yellow arrows in SI Appendix, Fig. S2F, Lower). The additive effect of double Foxp1/2 deficiency on skull shaping was also observed in heterozygous knockout mice (Foxp1 Δ/+Foxp2 Δ/Δ, Foxp1 Δ/+Foxp2 Δ/Δ, Foxp1 Δ/ΔFoxp2 Δ/Δ, and Foxp1 Δ/ΔFoxp2 Δ/Δ) at P10 (SI Appendix, Fig. S2G).

These results indicate that Foxp2 and Foxp1 regulate craniofacial development cooperatively.

**Results**

**Cartilage-Specific Deletion of Foxp2 Impairs Cranial Bone Development.** Cranial base morphogenesis is a major determinant of skull shaping (32). Basicranial skeletons, such as the sphenoid and basioccipital bones (Bos), are primarily formed through endochondral ossification. To test roles of Foxp2 in cranial base development, we firstly examined its expression in the synchondrosis joint—the unique growth plate sustaining endochondral ossification in sphenoid bones. We detected expression of Foxp2 protein, as well as its parologue Foxp1, in mesenchymal progenitor cells in resting zone and/or perichondrium (white arrows in Fig. 1A). We then generated chondrocyte-specific Foxp2 conditional knockout (cKO) mice by crossing a homozygous floxed line with Col2-Cre, which targeted cartilage in appendicular skeletons and partial craniofacial mesenchyme. We observed that craniofacial elements were consistently shortened in homozygous Foxp2 Δ/Δ cKO mice compared with controls at postnatal day 10 (P10) and P30 stages (double-headed arrows in Fig. 1B).

**Ablation of Foxp2 in Cartilage Disrupts Pup USVs.** Morphogenesis and elasticity of the larynx and vocal tract are rudimentary for animal sound production (33). In our observations, complete loss of Foxp2 in Foxp2 Δ/Δ cartilage tissue resulted in a minor perturbation of the morphogenesis of laryngeal thyroid and trachea cricoid cartilage, revealed by reduced Alcian blue staining in cricoid and trachea cartilage at P13 (Fig. 2A) and ectopic ventral expansion of the esophagus below the glottis (arrows in Fig. 2B). Subtle decreases in size of laryngeal cartilage were also observed in Foxp2 ΔR552H/ΔR552H or Foxp2 ΔR552H/ΔR552H mutant mice, at P0 and P7, respectively, as indicated by brackets in Fig. 2C and SI Appendix, Fig. S3. Meanwhile, development of trachea cartilage was relatively attenuated in homozygous Foxp2 ΔR552H/ΔR552H mutant mice, as evidenced by Alcian blue staining (black arrows in Fig. 2A and D and SI Appendix, Fig. S3).

We next examined the consequences of homozygous cartilage-specific Foxp2 loss for mouse pup vocalizations. Foxp2 Δ/Δ cKO mice at P10 were subjected to sound recording and spectrogram analyses. According to our bioacoustic analysis, Foxp2 Δ/Δ pup calls were significantly perturbed compared with that of controls (Fig. 2E). Of note, approximately one-third of the Foxp2 Δ/Δ pups presented no detectable calls. For the other two-thirds of Foxp2 Δ/Δ mice, the call rate (Z = 5.392, P < 0.01) and the proportion of complex syllables, t(34) = −3.237, P < 0.01, were both significantly reduced in Foxp2 Δ/Δ pups compared with...
expression was detected in Osterix\(^+\) skeletal progenitor cells in developing interparietal bones, as indicated by IHC examination of sections of skull from Osx-\textit{GFP}\text{-Cre} embryos at E15.5 (arrow in SI Appendix, Fig. S5A). To investigate the contributions of Foxp2 to skull vault development, we generated cKO mice with Foxp2 deletion strictly in mesenchymal progenitor cells by crossing Foxp2\textsuperscript{R552H/R552H} animals to a Prx1-Cre line. The Foxp2\textsubscript{Prx1}\textsubscript{−/−}\textsubscript{Δ/Δ} cKO mice were grossly indistinguishable from their wild-type littermates (SI Appendix, Fig. S5B), with significant deletion of Foxp2 in mesenchymal stem cells (MSCs) from bone marrow (SI Appendix, Fig. S5 C and D). Loss of Foxp2 from mesenchymal progenitors perturbed osteogenesis of interparietal bones (Fig. 3A), as evidenced by diminished Osterix\(^+\) osteoblasts at the suture (SI Appendix, Fig. S5E), and decreased expression of osteogenic genes (Osx, Runx2, Colla1, and Alpl) in mesenchymal progenitor cells (SI Appendix, Fig. S5F). Effects on lambdoid suture fusion were also observed in Foxp2\textsuperscript{R552H/R552H} or Foxp2\textsuperscript{R552H/+} perinatal mutant mice (Fig. 3 B and C and SI Appendix, Fig. S6). Attenuation in lambdoid suture closure was much more penetrant in Foxp2\textsubscript{Prx1}\textsubscript{−/−}\textsubscript{Δ/Δ} double knockout mice (Fig. 3D). Our findings suggest that Foxp2 helps to regulate posterior skull integrity, including interparietal bone development and lambdoid suture closure, by promoting osteogenic differentiation of MSCs.

Ablation of Foxp2 Impairs Leg Grace and Cartilage Maintenance. For appendicular long bones, postnatal elongation occurs and depends on the growth plates, which progressively narrow down and ultimately disappear with age. Compared with control littersmates, Foxp2\textsubscript{Prx1}\textsubscript{−/−}\textsubscript{Δ/Δ} femur bones were shortened in both males and females at 2 mo of age (Fig. 4A and B). In cultures of MSCs prepared from wild-type bone marrow, Foxp2 showed overlapping expression with Nestin (SI Appendix, Fig. S7A). Chondrogenic differentiation of MSCs from Foxp2\textsubscript{Prx1}\textsubscript{−/−}\textsubscript{Δ/Δ} mutants was impaired compared with controls, as evaluated by Alcian blue staining and qPCR of chondrogenic markers (SI Appendix, Fig. S7 B and C). Consistent with this observation, growth plates in Foxp2\textsubscript{Prx1}\textsubscript{−/−}\textsubscript{Δ/Δ} femurs were narrower at 6 mo and manifested obvious signs of cessation/disruption at 12 mo (Fig. 4C). Thus, it appears that loss of Foxp2 from mesenchymal progenitors leads to precocious arrest in the growth plate, partially accounting for the shortening of lower limbs. Given that our previous study showed that Foxp2 sustains chondrocyte proliferation and protects from apoptosis in embryonic growth plates (30), the effect of Foxp2 on chondrogenesis may underlie the defective maintenance of the postnatal growth plate. As a consequence of bipedal locomotion, the articular cartilage in humans endures much more pressure than in other primates. Histological analyses of Safranin O-stained sections revealed

wild-type controls (Fig. 2E). Pup calls were also significantly shorter in duration, \(t(42) = -3.691, P < 0.01\), broader in bandwidth, \(t(42) = 2.093, P < 0.05\), and higher in entropy, \(t(42) = 5.099, P < 0.01\), than those of controls (Fig. 2F). No significant differences were observed in the USVs of male and female cKO mice. Similar vocalization defects were observed in Foxp1\textsubscript{Col2}\textsubscript{−/−}\textsubscript{Δ/Δ} mice at P10 (SI Appendix, Fig. S4 A and B). Together these observations suggest that Foxp2 is involved in regulating multiple aspects of vocal tract configuration, including morphological features of the trachea and larynx that are important for vocal production.

**Foxp2 Loss Perturbs Skull Integrity.** The interparietal bone is the boundary component between the parietal and occipital bones, which is considered to be a “hot spot” that is susceptible to cranial remodeling (34). Parietal and interparietal bones are formed in a process of intramembranous ossification. Foxp2

Fig. 2. Ablation of Foxp2 in cartilage impairs USVs in pup calls. (A) Alcian blue staining of larynx cartilages from Foxp2\textsuperscript{Col2}\textsubscript{−/−}\textsubscript{Δ/Δ} (Col2-cKO) mice. CC, cricoid cartilage; TC, thyroid cartilage; Tr, trachea cartilage. (B) Safranin O staining for the transverse sections of larynx at P10. (Scale bar, 500 μm.) E, esophagus; G, glottis. (C and D) Alcian blue staining of larynx cartilages from Foxp2\textsuperscript{R552H/R552H} (R552H/+), C) at P0 and Foxp2\textsuperscript{R552H/+} (R552H/+), D) mutant mice at P7. (E) Representative spectrograms of pup isolation calls in Foxp2\textsuperscript{Col2}\textsubscript{−/−}\textsubscript{Δ/Δ} (Col2-cKO) mice at P10. The y axis indicates the frequency change of the USVs in the kilohertz range, whereas the x axis indicates time in seconds. Color depths in the sonograms represent relative intensity strength in decibels. C, complex syllable; S, simple syllable. (F) The sonic characteristics of pup calls, including syllable rate, proportion of complex syllables, syllable duration, peak frequency, wiener entropy, and bandwidth in Foxp2\textsuperscript{Δ/Δ} (Contr) mice. *P < 0.05; **P < 0.01; ***P < 0.001. Foxp2\textsuperscript{Δ/Δ} mice, n = 27; Foxp2\textsuperscript{−/−}\textsubscript{Δ/Δ} knockouts, n = 26.

Fig. 3. Disruption of posterior skull integrity in Foxp2 knockout mice. (A) Dorsal view of skulls of Foxp2\textsuperscript{Prx1}\textsuperscript{−/−}\textsuperscript{Δ/Δ} mice (Prx1-cKO) at E18.5. Ip, interparietal bone. (B and C) Dorsal view of skulls of Foxp2\textsuperscript{R552H/+}\textsuperscript{Δ/Δ} mice (R552H/+). (D) Dorsal view of skulls of Foxp2\textsuperscript{R552H/+}\textsuperscript{Δ/Δ} (Prx1-cKO), and Foxp2\textsuperscript{12}\textsubscript{Δ/Δ} (Prx1-cKO) (P1/2) mice at 1 mo of age. Dashed lines outline the lambdoid suture.

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that Foxp2Δ/Δ cKO animals manifested osteoarthritic (OA)-like pathology in their knee joints from the age of 6 mo, as well as reduction of superficial zones and proteoglycan content in distal femurs (Fig. 4D). Signs of OA in mutant knee joints were exacerbated by destabilization of the medial meniscus (DMM) at 2 mo of age (Fig. 4E). Interestingly, precocious signs of intervertebral disc (IVD) degeneration could be detected in the lumbar IVD of Foxp2Δ/Δ mutant mice at 2 mo of age, as evidenced by decreased Safranin O staining in annulus fibrosus (Fig. 4F).

Strong and less massive legs have been suggested to represent evolutionary adaptations to improve walking economy (35). A key indicator for bone strength is stiffness, a parameter reflecting evolutionary adaptations to improve walking economy (35). A key indicator for bone strength is stiffness, a parameter reflecting evolutionary adaptations to improve walking economy (35). A key indicator for bone strength is stiffness, a parameter reflecting evolutionary adaptations to improve walking economy (35). A key indicator for bone strength is stiffness, a parameter reflecting evolutionary adaptations to improve walking economy (35).
**Discussion**

To date, the majority of investigations into the genetic bases of vocal communication and language functions have focused on neural pathways (37). Here we used conditional knockouts in mice to extend the examination of Foxp2 function to skull shaping and long bone development. As shown in the model of Fig. S3, our work suggests that Foxp2 exerts pleiotropic influences on skeletal development by helping to regulate: (i) skull shaping, including cranial base formation and interparietal bone development; (ii) vocal tract geometry, including the sphenoid bone and laryngeal cartilage, anatomical substrates that are important for speech; and (iii) development of gracile and strong hind limbs, and maintenance of cartilage integrity in knee joint and IVD. In sum, Foxp2 influences multiple skeletal features conferring susceptibility to anatomical variances in vocal production and, we speculate, maybe also bipedal locomotion.

In line with the speech and language disorders observed in people with heterozygous FOXP2 mutations (i.e., with haploinsufficiency of the gene), prior studies of humans and animals have given substantial evidence that the gene is important for development and function of relevant brain circuits (38, 39). For example, neural investigations of mice with mutated Foxp2 have identified significant effects on neurite outgrowth and synaptic plasticity of the corticostriatal and corticocerebellar circuits where it is typically expressed (40–42). The core behavioral phenotype associated with heterozygous disruptions of human FOXP2 is still a matter of debate (39). The most obvious diagnostic feature is CAS, involving problems with the neural control of sequences of orofacial movements (6), and expressive skills are more profound than problems with receptive language and/or grammar. Recent work also points to cognitive deficits in phonological working memory in FOX2 mutation carriers in the KE family (43). Craniofacial and/or skeletal abnormalities have seldom been documented for human heterozygous FOX2 mutation cases. Interestingly, studies of people with FOX2 variants have anecdotaly reported difficulties in infant feeding and coughing in a few cases (12, 13, 44, 45), which could feasibly relate to larynx cartilage changes. In the present study, cartilage-specific ablation of Foxp2 in mouse pups disrupted the production of innate USVs, despite normal neural expression in key brain structures (SI Appendix, Fig. S10). The primary findings stem from homozygous skeleton-specific deletions of Foxp2. Thus, besides its important actions in the central nervous system and in vocal production learning (46), Foxp2 also helps to establish anatomical substrates important for vocal communication. On the other hand, our investigations also revealed that Foxp2R552H homozygous mutant mice showed alterations in presphenoid and larynx cartilage, although heterozygous mutants displayed only minor changes (SI Appendix, Figs. S1 and S3). The potential existence of subtle anatomical anomalies should be taken into consideration when dissecting the etiology of speech and language disorders.

Modifications of vocal tract morphology may have played roles in the emergence of human speech (33). Unlike speech, mouse vocalizations are not learned, but acoustic analysis of USVs is a commonly used tool for studying mice carrying mutations associated with communication disorders. Recent work has revealed a novel mechanism of USV production, a planar impinging air jet within the larynx (47). When epiglottis and thyroid cartilage in the larynx is damaged, the production of USVs may be blocked to varying degrees. In the present study, cartilage-specific ablation of Foxp2 occurred around one-third of the knockout pups, which may correlate with dysmorphogenisis of the larynx (Fig. 2A–D). Moreover, a substantial reduction of USV syllable rates was observed in both Foxp1Δcld/Δcld and Foxp2Δcld/Δcld knockout pups (Fig. 2F and SI Appendix, Fig. S4B). However, the peak frequency, which is mostly regulated by laryngeal muscle motor and airflow pressure (48, 49), showed a significant increase in the Foxp1Δcld/Δcld, but not Foxp2Δcld/Δcld knockou tline. These findings also remind us to be cautious about using pup USVs to try to model human speech impairments (50).

**FOX1** and FOX2 show partially overlapping expression patterns in the brain, and heterozygous disruptions of these genes lead to a distinct yet overlapping spectrum of neurodevelopmental disorders (11). The phenotype associated with heterozygous FOX1 mutations is more severe and extensive, including global developmental delay, intellectual disability, autistic features and, notably, a number of documented craniofacial symptoms (9, 31). Interestingly, craniofacial anomalies, including plagiocephaly, cleft palate, telecanthus, and saddle nose (52, 53). In the present study, cartilage-specific knockout of Foxp1 in mice led to impairment of cranial base formation and USVs, just as with knockout of Foxp2. In addition, loss of Foxp1 and Foxp2 displayed additive effects in skull shaping and bone formation (Fig. 3 and SI Appendix, Figs. S2 F and G and S9). Therefore, Foxp1 and Foxp2 cooperatively regulate craniofacial shaping.

Paleoanthropological evidence suggests that bipedalism emerged at an early stage of hominid evolution following the split from chimpanzee lineages (35). Two amino acid changes in FOX2 occurred on the lineage that led to modern humans, after splitting from the chimpanzee but before the divergence of Neandertals, and these changes have been considered as candidates for involvement in the evolution of speech (24, 26). We still know little about the genetic basis of bipedal gait, which is thought to provide advantages in strength and walking economy (35, 54). Given the coordination of osteogenesis and neurogenesis in shaping of the skull and brain (1), it is interesting to speculate on whether Foxp2 may have been relevant for bipedal evolution in early human history. Although we have not tested evolutionary changes in the present study, our findings suggest that Foxp2 may have been well placed to provide resources for adaptations in bone and cartilage that are relevant for human evolution. Firstly, Foxp2 helps regulate craniofacial shaping and skull integrity (Fig. 3), such as sphenoccipital synchondrosis and interparietal bone, which are major evolutionary sources of skull reshaping (55). Secondly, Foxp2 helps to forge gracile but strong
bodies through its dual effects on bone remodeling (Figs. 4A and 5–A,D), improving walking economy and energy expenditure. Finally, Foxp2 sustains growth plate competency for elongation of hind limbs and helps maintain the integrity of knee joint articular cartilage and IVDs. All these features have the potential to protect bones from stress damage during bipedal striding. In summary, this study raises hypotheses about contributions of FOXP2 to human evolution that can be empirically tested through studies of, for example, mice that have been humanized for this locus.

Materials and Methods

All animal experiments were performed according to the guidelines and approved by the ethical committee of Bio-X Institutes of Shanghai Jiao Tong University (SYXK 2011-0112). For skeletal morphological analysis, skeletal preparations for mice of different ages were made by Alcian blue/Alizarin red staining as previously reported. For μCT analysis, femurs were dissected from mice and fixed in 70% ethanol at 4 °C. μCT scanning of bones was performed on SkyScan 1176. A 3D model was reconstructed and structural indices were calculated using CTAn software. The region of interest selected was 5 mm below growth plate of bones.

The details of other materials and methods can be found in SI Appendix.