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Spry1 and *Spry2* Are Essential for Development of the Temporomandibular Joint

ABSTRACT

The temporomandibular joint (TMJ) is a specialized synovial joint essential for the function of the mammalian jaw. The main components of the TMJ are the mandibular condyle, the glenoid fossa of the temporal bone, and a fibrocartilagenous disc interposed between them. The genetic program for the development of the TMJ remains poorly understood. Here we show the crucial role of sprouty (*Spry*) genes in TMJ development. Sprouty genes encode intracellular inhibitors of receptor tyrosine kinase (RTK) signaling pathways, including those triggered by fibroblast growth factors (Fgfs). Using *in situ* hybridization, we show that *Spry1* and *Spry2* are highly expressed in muscles attached to the TMJ, including the lateral pterygoid and temporalis muscles. The combined inactivation of *Spry1* and *Spry2* results in overgrowth of these muscles, which disrupts normal development of the glenoid fossa. Remarkably, condyle and disc formation are not affected in these mutants, demonstrating that the glenoid fossa is not required for development of these structures. Our findings demonstrate the importance of regulated RTK signaling during TMJ development and suggest multiple skeletal origins for the fossa. Notably, our work provides the evidence that the TMJ condyle and disc develop independently of the mandibular fossa.

KEY WORDS: TMJ, condyle, disc, glenoid fossa, temporalis, pterygoid, bone.

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INTRODUCTION

The temporomandibular joint (TMJ) is a mammalian synovial joint essential for jaw function. The TMJ consists of multiple tissues, including the glenoid fossa of the temporal bone, the condylar head of the mandible, a fibrocartilaginous disc located between these two bones, and associated muscles and tendons (Avery, 2001). Although structural features of the TMJ are well-documented, little information is available regarding the genetic, cellular, and molecular mechanisms involved in TMJ morphogenesis.

TMJ development starts with the appearance of two distinct mesenchymal condensations, the temporal and condylar blastemas, at embryonic day (E) 13.5. The condylar blastema grows toward the temporal blastema, and at E15.5, the glenoid fossa, condyle, disc, and muscles are clearly visible. At E16.5, all TMJ components are well-formed, with the fossa and condyle in complementary shapes with the disc between them (Sperber, 1992). The condyle is endochondral in origin and an important growth site in the mandible. Proliferating and hypertrophic chondrocytes become arranged in columns forming a growth-plate-like zone found at the end of the expanding condylar cartilage (Sarnat, 1966; Silbermann and Frommer, 1972). The fossa forms by a combination of intramembranous and endochondral ossification (Silbermann and Frommer, 1972; Purcell *et al*., 2009; Wang *et al*., 2011), although this process is poorly understood. An important gene in TMJ development is *Ihh* (indian hedgehog), which is crucial for disc formation, cellular organization of the condyle, and maintenance of the jaw joint (Shibukawa *et al*., 2007; Purcell *et al*., 2009; Ochiai *et al*., 2010).

In our previous microarray studies, several components of the Fgf signaling pathway were discovered to be highly expressed in the TMJ at E16.5 (Purcell *et al*., 2009, GEO Series accession number GSE17473; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc_GSE17473). These included several Fgfs, their receptors (Fgfrs), and sprouty genes, which encode antagonists of RTK signaling including Fgf signaling. Specifically, *Spry1* and *Spry2* were expressed at high levels in the TMJ (Purcell *et al*., 2009). Components of the Fgf signaling pathway are highly conserved throughout evolution and are known to play crucial roles in development (reviewed in Dorey and Amaya, 2010; Hatch, 2010; Itoh and Ornitz, 2011). Here, we provide the first evidence that sprouty genes are essential in TMJ development and that the growth of the condyle and disc is independent of the fossa.

MATERIALS & METHODS

Mouse Lines and Embryo Collection

All animal procedures were performed according to guidelines approved by Harvard Medical Area and UCSF animal care committees. Embryos deficient in *Spry1* and *Spry2* were produced as reported previously (Petersen *et al*., 2011). Jaws from double-heterozygous embryos were indistinguishable from wild-type CD-1 embryos; such jaws were used as controls. The presence of a vaginal plug indicated embryonic day (E) 0.5. Osteoblast- and chondrocyte-specific inactivation of *Spry1* (Basson *et al*., 2005) and *Spry2* (Shim *et al*., 2005), alone and in combination, was achieved in the 2.3 kb *Col1α1*-Cre (Liu *et al*., 2004) and *Col2α1*-Cre (Ovchinnikov *et al*., 2000) mouse lines, respectively.

Histological Analyses and Tissue Measurements

E14.5-E18.5 embryo heads were fixed in 4% paraformaldehyde and embedded in paraffin or OCT compound (Tissue-Tek, Torrance, CA, USA). Ten-micrometer sections were stained with hematoxylin and eosin according to standard procedures. Area measurements were performed with Adobe Photoshop software.

Cell Proliferation and Apoptosis

Pregnant mice at E14.5 and E17.5 were injected with 1 mg BrdU (Invitrogen, Carlsbad, CA, USA) for 2 hrs at E14.5 and E17.5. Ten-micrometer cryosections were stained with anti-BrdU antibody (Abcam, Cambridge, MA, USA) for analysis of proliferation and Caspase-3 (Cell Signaling, Danvers, MA, USA) for measurement of apoptosis. Immunohistochemistry was performed as described in Petersen *et al*. (2011).

Gene Expression

In situ hybridization was performed on 10-μm cryo or paraffin sections with digoxigenin-labeled probes as described (Purcell *et al*., 2009). RNA probe information is available upon request.

MicroCT Scans

MicroCT scans were taken with the Siemens MicroCAT System (Malvern, PA, USA) and data analyzed by Dolphin Imaging V.11.5 software (Chatsworth, CA, USA).

RESULTS

Expression of *Spry*, *Fgf*, and *Fgfr* Genes in Embryonic TMJ

Components of the Fgf signaling pathway, in particular *Spry1* and *Spry2*, were observed to be enriched by microarray in the TMJ at E16.5 (Purcell *et al*., 2009); therefore, we analyzed their expression by *in situ* hybridization. *Spry1, Spry2,* and *Spry4* were expressed in the lateral pterygoid and temporalis muscles that surround the TMJ (Figs. 1A, 1B, 1D), whereas *Spry3* was not detected (Fig. 1C). Expression of Fgfrs was examined to determine co-localization with sprouty genes. *Fgfr1* was expressed in the periosteum and *Fgfr2* in the perichondrium of the fossa and the condyle; *Fgfr3* in the immature chondrocytes of the condyle (Figs. 1E-1G), consistent with previous observations (Purcell *et al*., 2009); *Fgfr4* was expressed in the lateral pterygoid and temporalis muscles (Fig. 1H), consistent with its role in myogenesis (Lagha *et al*., 2008). Numerous candidate Fgf genes were also analyzed by *in situ* hybridization, including *Fgf3*, *Fgf4*, *Fgf6*, *Fgf7*, *Fgf8*, and *Fgf18*; *Fgf6* was the only gene in this group to show strong expression during the examined stages of TMJ development (Fig. 1I). Notably, *Spry1*, *Spry2*, *Spry4*, *Fgfr4*, and *Fgf6* were co-expressed in the lateral pterygoid and temporalis muscles surrounding the TMJ, suggesting the importance of Fgf signaling in these tissues (Figs. 1A, 1B, 1D, 1H, 1I).

Spry1$^{-/-}$;*Spry2*$^{-/-}$ Mice Do Not Form a Glenoid Fossa

To define the role of sprouty genes in TMJ development, we examined mouse lines carrying null alleles of *Spry1* and/or *Spry2*. Mice null for either *Spry1* or *Spry2* did not show any TMJ abnormalities (data not shown). However, there was an absence of the glenoid fossa in *Spry1*$^{-/-}$;*Spry2*$^{-/-}$mice, herein also referred to as mutant mice (Fig. 2). The temporalis muscle, which is normally located superior and lateral to the fossa, was enlarged in mutant mice, expanding into the space that would normally be occupied by the fossa (Figs. 2G, 2H).

To determine whether sprouty genes are required for glenoid fossa development or its maintenance, we examined the developing TMJ between E14.5 and E18.5 in control and mutant embryos (Figs. 2A-2H). At E14.5, the TMJ had not yet formed, but the condyle and fossa were clearly visible as mesenchymal condensations in controls (Fig. 2A). However, in mutant embryos, the fossa condensation was not detected, and the temporalis muscle appeared enlarged (Fig. 2B). At E15.5 in control embryos, the fossa began to ossify and assume its complementary shape with respect to the adjacent head of the condyle. The temporalis muscle was situated lateral to the fossa, and the disc had become more condensed (Fig. 2C). In mutants, the temporalis muscle was dramatically enlarged, such that it filled the space normally occupied by the fossa (Fig. 2D). Remarkably, the condyle and disc appeared normal (Figs. 2C, 2D). At E16.5 and E18.5 in mutants, the condyle and disc continued to develop normally, but the fossa was absent, with its usual location occupied by the enlarged temporalis muscle (Figs. 2E-2H). Notably, a small lateral distal tip of the fossa, a part of the zygomatic arch, was present in mutant mice (Figs. 2B, 2D, 2F, 2H). As expected, the lateral pterygoid muscle was also enlarged in mutant embryos compared with control littermates (Figs. 2A-2H).

To further confirm the absence of the glenoid fossa in *Spry1*$^{-/-}$;*Spry2*$^{-/-}$mice, we examined the TMJ at E18.5 using microCT analysis. In controls, the fossa was clearly distinguished by its characteristic deep concave shape, which complements the configuration of the condylar head for articulation (Fig. 2I). In contrast, mutants did not exhibit the depression of the temporal bone that forms the concave fossa, but instead possessed a flat temporal bone (Fig. 2J). In addition, even though the composition and shape of the mutant condyle appeared normal in the histological studies, the CT scan showed that the size of the condyle was markedly reduced (Figs. 2I, 2J). Together,

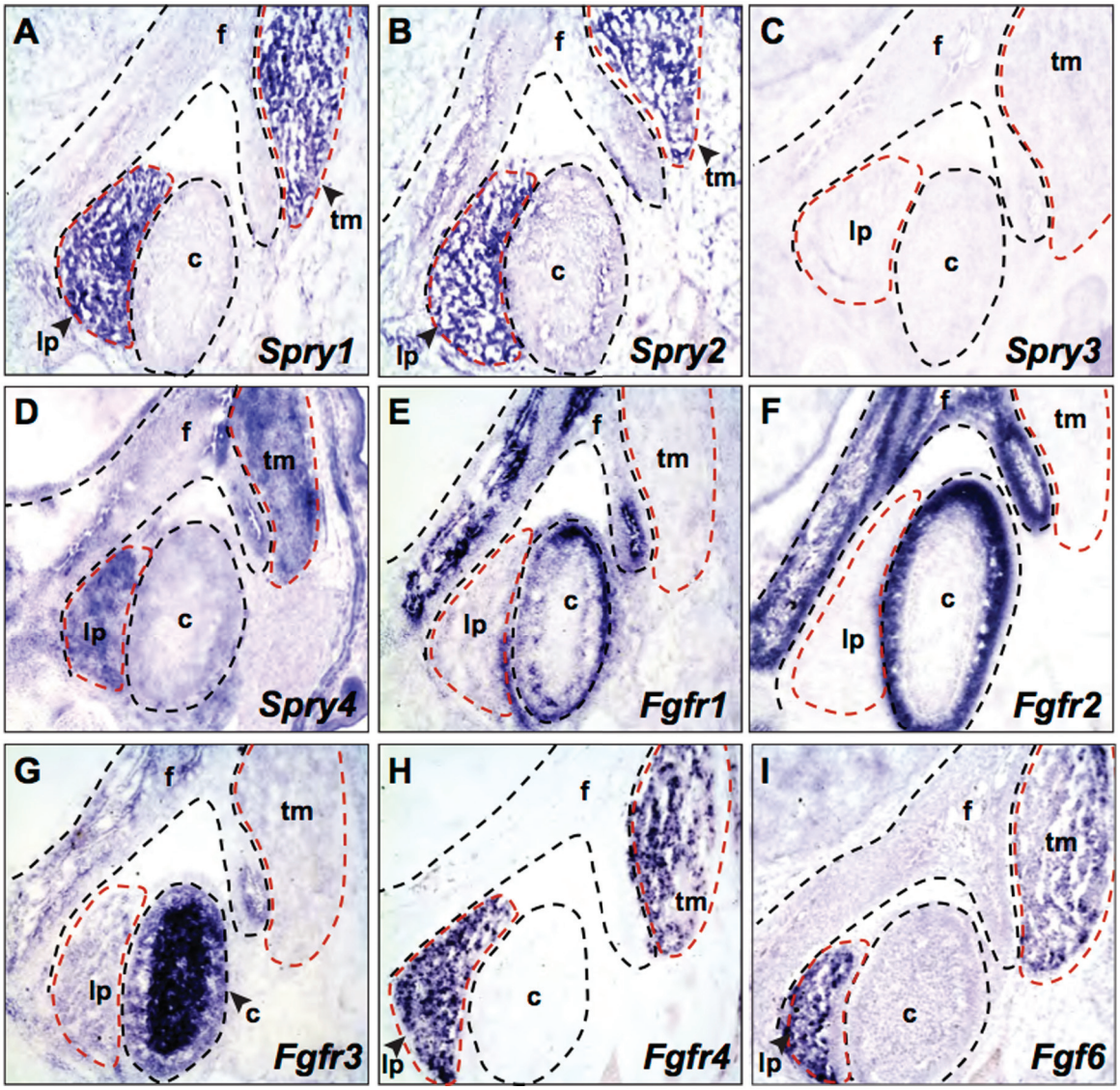

Figure 1. Expression of members of the Fgf signaling pathway in the mouse TMJ region. Fgf signaling components were highly enriched in the mouse TMJ at E16.5 (Purcell *et al.*, 2009). (A-I) Representative *in situ* hybridization in the mouse TMJ at E16.5. **(A-D)** *Spry1*, *Spry2*, and *Spry4* are expressed in the lateral pterygoid and temporalis muscles, with *Spry1* and *Spry2* being highly expressed; *Spry3* is not expressed. **(E-H)** *Fgfr1* is expressed in the osteoblasts and periosteum of the condyle and fossa; *Fgfr2* is expressed in perichondrium of the developing skeletal structures of the glenoid fossa and the condyle; *Fgfr3* is present in the immature chondrocytes of the condyle; and *Fgfr4* is expressed in the lateral pterygoid and temporalis muscles surrounding the TMJ. **(I)** *Fgf6* is expressed in the lateral pterygoid and temporalis muscles. 10x magnification. c, condyle; f, glenoid fossa; lp, lateral pterygoid muscle; tm, temporalis muscle. Condyle and fossa have been outlined in black and muscles in red.

our results substantiate the lack of glenoid fossa development when *Spry1* and *Spry2* are absent.

The increase in size of the temporalis and lateral pterygoid muscles was quantified in control and mutant embryos (Fig. 2K). In mutants, the temporalis and lateral pterygoid muscles were 48% and 69% larger relative to control embryos. No significant size difference in Meckel's cartilage was observed, indicating that the effects of *Spry1* and *Spry2* deletion were specific to muscle. To better understand the mechanism responsible for muscle enlargement, we analyzed cell proliferation and apoptosis in the temporalis and lateral pterygoid muscles of control and mutant littermates at E14.5 and E17.5. High cell proliferation activity was observed at E14.5 throughout the head, and no apparent difference between controls and mutants was detected (data not shown). At E17.5, we detected a 33.1% and 46.2% increase in proliferating muscle progenitors in mutant pterygoid and temporalis muscles, respectively (Fig. 2L). No significant apoptosis at E14.5 or E17.5 was observed in the TMJ and no differences between controls and mutants (data not shown). These results suggest that the increase in muscle size in the mutants is likely due to an increase in proliferation of muscle progenitors.

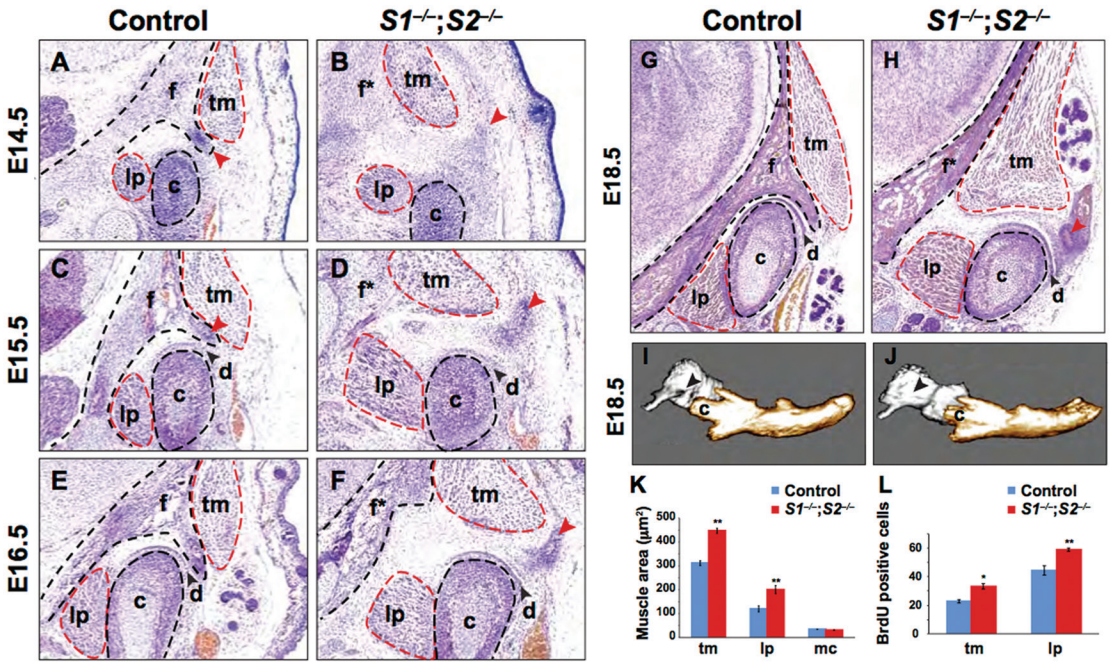

Figure 2. *Spry1*$^{-/-}$;*Spry2*$^{-/-}$ embryos do not form a glenoid fossa. (A-H) Representative H&E staining from 4 different control and *Spry1*$^{-/-}$;*Spry2*$^{-/-}$ (*S1*$^{-/-}$;*S2*$^{-/-}$) littermates at E14.5, E15.5, E16.5, and E18.5. **(A,C,E,G)** In control embryos, the glenoid fossa anlage (f*) and condyle (c) condensations are visible at E14.5, and the disc (d) is visible at E15.5 (black arrowhead). At all stages analyzed, the lateral pterygoid (lp) and temporalis muscles (tm) are detected (outlined in red). **(B,D,F,H)** In mutant embryos, the glenoid fossa is not visible at any stage, and its space is filled by an enlarged temporalis muscle. Instead of a fossa (f), a distal bony element forms at the region equivalent to the distal lateral tip of the fossa (*i.e.*, zygomatic arch) in control TMJ (red arrowheads in A-D). The lateral pterygoid muscle is also enlarged starting at E15.5. The condyle and disc appear normal at all stages analyzed. (A-F) 10x magnification; (G,H) 4X magnification. **(I,J)** 3D reconstructions of microCT scans taken from 3 control and mutant heads at E18.5. To allow for better visibility of the glenoid fossa and the mandibular condyle, we removed the zygomatic bone and zygomatic processes of the temporal bone using the sculpting module in the Dolphin imaging software. MicroCT scans show a clear depression of the temporal bone in the control mice corresponding to the concave shape of the fossa (I; arrowhead). In mutant embryos, instead of the concave shape of the temporal bone, a flat surface is observed, confirming the absence of the glenoid fossa (J; arrowhead). The mandible is shown in yellow. The mandibular condylar process (c) of the mutant mouse is smaller by 50% in length and 25% in width compared with that in its control littermates. Analysis was restricted to embryos due to perinatal lethality of mutant mice. **(K)** The sizes of the lateral pterygoid and temporalis muscles at E18.5 were quantified. A 10-mm microscopic ruler (Klarmann Rulings, Inc.) was used to convert image pixel length to an SI unit of measurement (*i.e.*, 1 mm = 533 pixels). Meckel's cartilage (mc) showed no size difference between control and mutant mice. **(L)** Quantification of cell proliferation indicated by BrdU-positive cells at E17.5. n = 4; Student *t* test: *p < 0.05; **p < 0.01. c, condyle; d, disc; f, glenoid fossa; f*, fossa anlage; lp, lateral pterygoid muscle; mc, Meckel's cartilage; tm, temporalis muscle.

To investigate whether *Spry1* and *Spry2* act cell-autonomously in muscle or whether they affect bone or cartilage, we generated mice harboring bone- or cartilage-specific inactivation of *Spry1* and/or *Spry2*. The resultant mice did not show any phenotype in the TMJ (data not shown). Although we did not delete sprouty genes specifically in the muscle, the comparison of tissue-specific *vs*. global *Spry1*$^{-/-}$;*Spry2*$^{-/-}$ mice suggests a cell-autonomous role for *Spry1* and *Spry2* in regulating the sizes of cranial muscles.

Molecular Analysis of Developing TMJ in *Spry1*$^{-/-}$;*Spry2*$^{-/-}$ Embryos

To confirm the cellular and molecular integrity of the condyle and disc formed in the absence of glenoid fossa in mutant mice, we analyzed the expression of key genes involved in cartilage and bone formation (Fig. 3). *Sox9* and *Acan* (aggrecan), markers of proliferating and mature chondrocytes, respectively, showed similar expression patterns in control and mutant embryos (Figs. 3A-3D). The expression of *ColX* (collagen type X), a marker for hypertrophic chondrocytes, was maintained in mutants, although it appeared to be reduced relative to controls (Figs. 3E, 3F). This difference is likely due to the smaller size of the condyle in mutant embryos. The expression of *ColI* (collagen type I), a marker for osteoblasts, remained unchanged in the condyle of control and mutant littermates (Figs. 3G, 3H). However, the strong expression of *ColI* in control fossa was not detected in mutants, consistent with the lack of a fossa in these embryos. To test the integrity of the disc and the attachment sites of the muscles to the bones in the absence of *Spry1* and *Spry2*, we studied the expression pattern of *Scx* (scleraxis), a marker for tendons and ligaments, described to be regulated by Fgf signaling (Brent and Tabin, 2004). We observed that *Scx* was strongly expressed in the disc and the attachment points of muscle to bone in mutant and control mice (Figs. 3I, 3J). Thus, molecular analysis of the TMJ in control and mutant embryos confirmed normal development of the condyle and disc, which remarkably were not affected by the absence of the fossa.

DISCUSSION

The TMJ consists of multiple interacting tissues that are prone to injury- and disease-related degeneration. According to the National Institutes of Health (NIH), an estimated 3% to 5% of Americans suffer from a TMJ disorder. The lack of understanding of the development and function of the TMJ at the molecular level has hampered progress toward the diagnosis and treatment of TMJ disorders.

In the present work, we evaluated the role of sprouty genes during TMJ development. *Fgf* and *Spry* genes have previously been shown to have important roles during the development of various organs, including ear, tooth, lens, mandible, palate, and muscle (Shim *et al*., 2005; Boros *et al*., 2006; Klein *et al*., 2006; Goodnough *et al*., 2007; Mina *et al*., 2007; Welsh *et al*., 2007; Yang *et al*., 2010; Matsumura *et al*., 2011). We observed strong expression of *Spry1* and *Spry2* in the lateral pterygoid and temporalis muscles. We discovered that the combined inactivation of *Spry1* and *Spry2* resulted in overgrowth of these muscles, leading to the disruption of normal glenoid fossa development. Surprisingly, *Spry1*$^{-/-}$;*Spry2*$^{-/-}$embryos formed a complete condyle and joint disc, providing the first evidence that the condyle and disc form independently of the fossa.

The failure of glenoid fossa formation in *Spry1*$^{-/-}$;*Spry2*$^{-/-}$embryos may be due to one of three possibilities. First, the absence of the fossa could result from increased Fgf signaling in the absence of *Spry1* and *Spry2* in the muscle, which may inhibit the ossification of the forming bridge that ultimately gives rise to the temporal bone. Consequently, an overgrowth of the muscle could fill the space that otherwise would have been occupied by the fossa. Second, the lack of fossa may be due to a physical impediment to bone formation, perhaps due to excessive growth of the temporalis muscle that occupies the space between the two individual cartilages, preventing their fusion for fossa formation. This hypothesis is supported by the fact that cranial bones form by the fusion of many individual ossification centers (McBratney-Owen *et al*., 2008). We observed the formation of an isolated lateral bony fragment as early as E14.5. At E15.5 in control animals, it becomes part of the fossa. By contrast, in *Spry1*$^{-/-}$;*Spry2*$^{-/-}$embryos, the small bony fragment remains isolated in a position equivalent to the lateral distal tip of the fossa. Because this fragment expresses *ColI* and *ColX* , it is likely that bone has formed *via* endochondral ossification. In fact, only this portion of the fossa is missing in a *Sox9* conditional knockout (Wang *et al*., 2011), suggesting that more than one mesenchymal condensation may give rise to the fossa, and that the fossa possesses multiple skeletal origins. Third, hyperactivated Fgf signaling may alter the fate of mesenchymal cells. The specific fate of an individual neural crest cell is determined by the signals they receive from the surrounding tissues (for review, see Trainor, 2010). The glenoid fossa and cranial muscles derive partly from cranial neural crest cells (Gu *et al*., 2008; Tzahor, 2009), and hyperactivation of *Fgfr4* might favor muscle rather than bone differentiation, thereby generating temporalis muscle instead of fossa.

In summary, we showed that *Fgfr4* expression is restricted to the cranial muscles and mimics the expression of *Spry1*, *Spry2*, and *Spry4*, suggesting that sprouty genes modulate

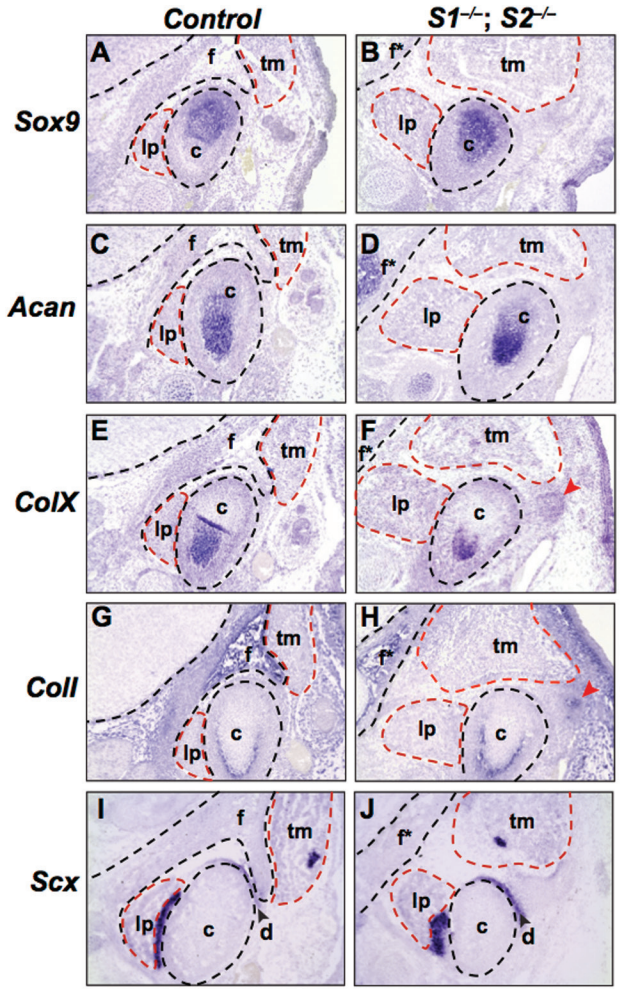

Figure 3. Condyle and disc develop normally in the absence of fossa. (A-J) *In situ* hybridization analysis on representative 10-μm serial coronal cryosections from 4 different control and *Spry1*$^{-/-}$;*Spry2*$^{-/-}$ (*S1*$^{-/-}$;*S2*$^{-/-}$) littermates at E16.5. (**A-D**) *Sox9* is localized to the proliferating chondrocytes, and *Acan* is localized to the cartilage of the condyle growth plate in control and mutant mice. (**E,F**) *ColX* is expressed in the zone of the hypertrophic chondrocytes of the condylar growth plate. (**G,H**) *ColI* expression is localized to the osteoblasts of the glenoid fossa and condyle in control and mutant mice. (**I, J**) *Scx* is expressed in tendons and tendon progenitors of the disc (black arrowheads) and muscle-bone contact sites. The lateral distal bony tip is indicated in mutant mice (F,H; red arrowheads). 10x magnification. c, condyle; f, glenoid fossa; f*, fossa anlage; lp, lateral pterygoid muscle; tm, temporalis muscle; *Acan*, aggrecan; *ColX*, collagen X; *ColI*, collagen I; *Scx*, scleraxis.

signaling downstream of FGFR4 in the TMJ. Moreover, FGF6 is a key ligand of FGFR4 and has been reported to play a crucial role in myogenesis (reviewed in Armand *et al*., 2006). In the absence of *Spry1* and *Spry2*, Fgf signaling *via* FGFR4 may be hyperactivated in the muscle, increasing myoblast proliferation, evidenced by increased cell proliferation in the mutants. Therefore, we suggest that condensations of the temporal bone that give rise to the glenoid fossa are able to form in the absence of *Spry1* and *Spry2,* but the overgrown temporalis

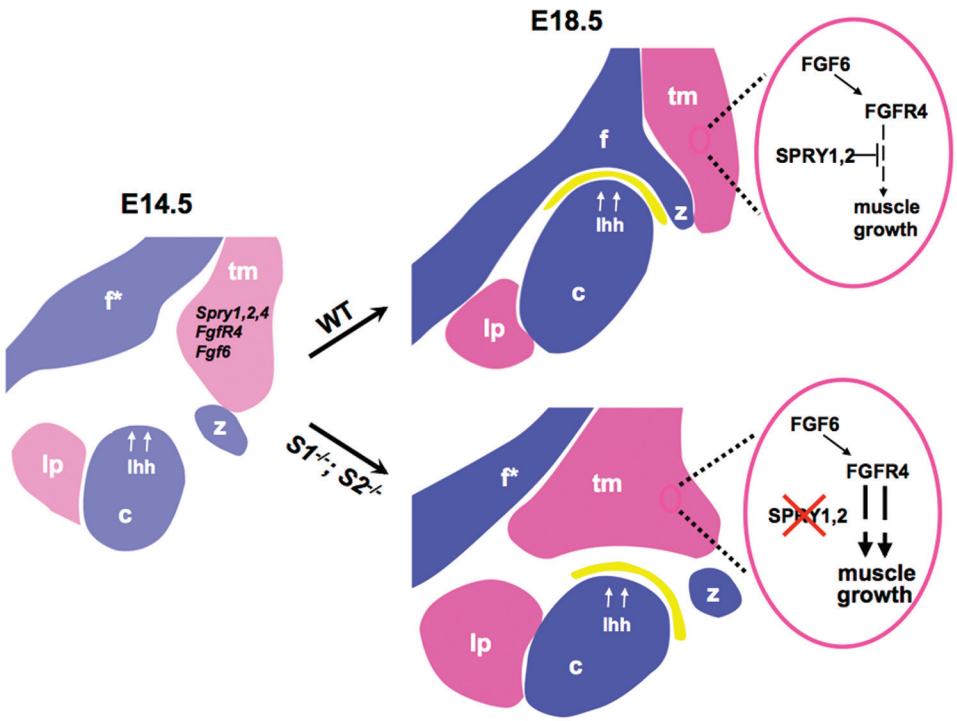

Figure 4. Model showing the requirement of *sprouty* genes in TMJ formation. *Spry1*, *Spry2*, *Spry4*, *Fgfr4*, and *Fgf6* are expressed in the lateral pterygoid (lp) and temporalis (tm) muscle around the TMJ during embryonic development. Conditional inactivation of *Spry1* and *Spry2* in cartilage and bone indicate that these genes act cell-autonomously and exclusively in the muscles. We propose that SPRY1 and SPRY2 control muscle size through the regulation of signaling downstream of FGFR4. In *Spry1*$^{-/-}$;*Spry2*$^{-/-}$ embryos, signaling downstream of FGFR4 is hyperactivated because of the lack of inhibition by sprouty proteins, resulting in uncontrolled muscle outgrowth. Since FGF6 is the key ligand for FGFR4 and is expressed by the muscle, it is likely that muscle growth is promoted by FGF6. The proposed mechanism in the temporalis muscle is also suggested to occur in the lateral pterygoid muscle. c, condyle; f, glenoid fossa; f*, fossa anlage; and z, zygomatic bone. Muscles are denoted in pink; condyle and fossa in blue; and disc in yellow. Light pink and light blue at E14.5 represent mesenchymal condensations for muscle and bone, respectively.

muscle impedes the fusion of these two elements to form the fossa (Fig. 4).

Future studies will need to investigate the relationship between sprouty genes and other signals involved in the growth and differentiation of muscle cells, as well as how these affect the formation of the fossa. Further molecular understanding of TMJ organogenesis is essential to improve diagnoses and develop novel therapeutic approaches for TMJ disorders.

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