FGF10 controls the patterning of the tracheal cartilage rings via Shh

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SUMMARY
During embryonic development, appropriate dorsoventral patterning of the trachea leads to the formation of periodic cartilage rings from the ventral mesenchyme and continuous smooth muscle from the dorsal mesenchyme. In this work, we have investigated the role of two crucial morphogens, fibroblast growth factor 10 and sonic hedgehog, in the formation of periodically alternating cartilaginous and non-cartilaginous domains in the ventral mesenchyme. Using a combination of gain- and loss-of-function approaches for FGF10 and SHH, we demonstrate that precise spatio-temporal patterns and appropriate levels of expression of these two signaling molecules in the ventral area are crucial between embryonic day 11.5 and 13.5 for the proper patterning of the cartilage rings. We conclude that the expression level of FGF10 in the mesenchyme has to be within a critical range to allow for periodic expression of Shh in the ventral epithelium, and consequently for the correct patterning of the cartilage rings. We propose that disturbed balances of Fgf10 and Shh may explain a subset of human tracheomalacia without tracheo-esophageal fistula or tracheal atresia.

KEY WORDS: Fgf10, Shh, Trachea, Cartilage formation, Mouse

INTRODUCTION
The mouse trachea starts to develop at E9.5 from the laryngotracheal groove, a ventral outgrowth of the foregut endoderm into the surrounding mesoderm. This structure elongates to form a tube extending from the larynx to the two main bronchi of the lung. Although the endoderm differentiates into a ciliated pseudo-stratified epithelium, the mesenchyme on the ventral side of the trachea matures progressively into C-shaped cartilage rings that maintain patency of the lumen, while the dorsal mesenchyme differentiates into smooth muscle. Proper periodic development of the tracheal cartilage ring is crucial for normal breathing, as the rings provide support to the trachea lumen by limiting its expansion during inhalation and preventing its collapse upon exhalation.

Tracheal malformations in human involving cartilage defects can be grouped into two classes (for a review, see Kay and Goldsmith, 2006). Malformations in the first class are characterized by an almost continuous tracheal cartilaginous sleeve involving multiple cartilage rings. In extreme cases, one single ring throughout the entire length of the trachea can be observed. This defect can be associated with tracheal stenosis, which is a narrowing of the lumen of the trachea (Ho and Koltai, 2008). The second class of defects is tracheomalacia, where the cartilage rings are too floppy, resulting in weakness of the tracheal walls leading to an eventual collapse of these walls (Austin and Ali, 2003). Depending on the severity of these tracheal cartilage defects, lethal alteration of respiratory function can be observed. Unfortunately, outcomes of reparative surgery for these malformations are often unsatisfactory. There is therefore a need to expand our knowledge on the molecular bases of tracheal cartilage formation to design novel approaches to ameliorate cartilage formation.

A few regulators of cartilage formation are known (Goldring et al., 2006) and most of these molecules seem to converge at the level of the transcription factor SOX9. For example, β-catenin in the mesenchyme interacts with SOX9 to regulate the differentiation of the chondroprogenitor cells (Akiyama et al., 2004). Bone morphogenetic protein 4 (BMP4) induces differentiation of the mesenchymal cells into chondroprogenitors via SOX9 (Hatakeyama et al., 2004). Moreover, impaired BMP signaling induces esophageal atresia and tracheo-esophageal fistula with extensive defects in tracheal cartilage ring formation (Que et al., 2006). Sonic hedgehog (SHH) also induces cartilage differentiation via activation of SOX9 (Park et al., 2010), and partial inactivation of Shh results in tracheobronchial cartilage ring abnormalities (Miller et al., 2004). Finally, fibroblast growth factor (FGF) family members such as Fgf4 and Fgf8 have been described to play an important role during cartilage formation (Goldring et al., 2006). Recently, we demonstrated that FGF10 causes the formation of a uniform cartilaginous sleeve in the trachea, when it activates ectopically expressed FGFR2B in the mesenchyme, reminiscent of the anomaly observed in humans with Apert syndrome (Tiozzo et al., 2009).

In this study, we further determined the normal role of mesenchymal FGF10, signaling via FGFR2B in the epithelium, in tracheal cartilage formation in the mouse embryo. We demonstrate that proper levels of mesenchymal Fgf10 expression between E11.5 and E13.5 are crucial for the induction of a periodic pattern of Shh expression in the ventral epithelium, and for the correct patterning of the cartilage rings downstream of Shh signaling. Deregulation of the expression of one of these two morphogens during this critical time window could therefore be a novel mechanism for abnormal cartilage ring formation in humans.
MATERIALS AND METHODS

Mice

Fgf10+/− mice (Sekine et al., 1999) were maintained on a C57BL/6 background. Topgal mice (DasGupta and Fuchs, 1999) were maintained on a mixed background. These strains were intercrossed to obtain [Fgf10+/−;Topgal] embryos. The Fgf10lacZ transgenic mice (Kelly et al., 2001) (hereafter called Fgf10αLacZ) were maintained on a mixed agouti background. Dermo-1Cre/+ mice (Yu et al., 2003), on a C57BL6 background, were crossed with rtTA+/+Fluc/+ (Beltel et al., 2005) and tet(O)Fgf10 mice (Clark et al., 2001), on a mixed background, to generate [Dermo-1Cre/+;rtTA+/+Fluc/+;tet(O)Fgf10] (hereafter tet(O)Fgf10(Dermo)) embryos. Tff1-Cre (also known as Nkx2.1α+/−/−) mice (Tiozzo et al., 2009), on a 129P3/J background, were crossed with rtTA+/+Fluc/+ and tet(O)Shh+/+ mice (Miller et al., 2004), on a 129P3/J background, to generate [Tff1-Cre;rtTA+/+Fluc/+;tet(O)Shh+] (hereafter tet(O)Shh+/−−) embryos. Tff1-Cre mice were crossed with Shh+/−− mice (The Jackson Laboratory) to generate [F2] [Tff1-Cre; Shh+/−−] embryos. Tff1-Cre mice were crossed with Rosa26 reporter line (B6.129S4-Gt(Rosa)26SorSor1, The Jackson Laboratory) to follow the expression pattern of the Tff-1 promoter.

Induction of the rtTA/tet(O) system

Timed pregnant mice were fed with doxycycline chow (Rodent diet with 0.0625% doxycycline, Harlan Teklad TD01306) at specific gestational stages.

Whole-mount in situ hybridization

Tracheas were isolated from the embryos and fixed for 2 hours in 4% paraformaldehyde (PFA) in PBS at 4°C. The samples were washed twice in PBS for 5 minutes, transferred in 70% ethanol overnight and stored in 100% ethanol until needed. Whole-mount in situ hybridization was performed as described (Winnier et al., 1995) with riboprobes transcribed from murine cDNA templates encoding a cDNA fragment of 584 bp for Fgf10 (Belluscì et al., 1997), 622 bp for Fgf10b cDNA and 642 bp for Shh (both kind gifts from Dr Andrew McMahon, Harvard University, Boston, MA), 500 bp for Sox9 (a kind gift from Brigid Hogan, Duke University, Durham, NC, USA) and 1.5 kb for Bmp4 (Winnier et al., 1995).

Measurement of pixel intensity of the whole-mount in situ hybridization signal

In photographs of tracheas stained by whole-mount in situ hybridization with specific probe for Shh, lines were drawn in the ventral and dorsal epithelial domain of Shh expression, parallel to the outer surface of the trachea. Along these lines, pixel intensity of the in situ hybridization signal was measured and graphs were generated using the Leica AF6000 software.

lacZ staining

Samples were briefly fixed in 4% PFA. lacZ expression on Fgf10lacZ and Topgal tracheal was monitored by β-galactosidase activity using 1 mg X-gal/ml dimethylformamide in 5 mM K3Fe(CN)6/5 mM K4Fe(CN)6/2 mM MgCl2 in D-PBS (pH 7.4).

Proliferation analysis

Two pregnant females were injected at the 13th day of pregnancy with 0.2 ml of bromodeoxyuridine (BrdU, Amersham Biosciences, UK) 20 minutes before sacrifice. The first female carried one mutant (M1). The second female carried two mutants (M2 and M3). Mutant M1 was compared with its own wild-type littermate control, and mutants M2 and M3 were compared with their own wild-type littermate controls.

The tracheas were dissected from the embryos, preserved in 4% PFA solution, paraffin embedded and sectioned at 5 µm. The sections were then incubated with monoclonal anti-bromodeoxyuridine (Clone BU-1) RPN 202 as recommended by the manufacturer (Amersham Biosciences), followed by Cy3-labeled anti-mouse secondary antibodies (Jackson ImmunoResearch). Slides were mounted with Vectashield containing DAPI. The sections were photographed using an epi-fluorescence photomicroscope (Leica, DM4000). Proliferation rate was determined as the number of BrdU-positive cells per total number cells (average of 400 cells counted per section) in epithelium and mesenchyme. For analysis of statistical significance, we have considered the three mutant tracheas and their corresponding controls as three pairs and performed a two-tail paired t-test on the data.

Immunostaining

The tracheas were fixed in 4% PFA, paraffin embedded and sectioned at 5 µm. Antigen was retrieved by boiling the slides using a microwave for 20 minutes in 10 mM sodium citrate (pH 6.0). The slides were incubated overnight at 4°C with the following primary antibodies: type II collagen (1/40, Chemicon International), β-tubulin (1/100, BioGenex) and rabbit anti-pro-SP-C (kindly provided by Dr J. Whitsett). Cy3- or FITC-conjugated secondary antibodies were used. Slides were mounted with Vectashield containing DAPI.

Histological staining

Cross-sections (5 µm) were stained with Hematoxylin and Eosin according to standard procedures or with Alcian Blue as described previously (see http://www.theworld.com/_protocols/special_stains/alcian_blue.htm).

RESULTS

Fgf10 and Fgfr2b are expressed early during trachea development in the mouse

Expression of Fgf10 was investigated between E11.5 and E18.5 using whole-mount in situ hybridization of wild type, and lacZ staining of Fgf10lacZ tracheae. We were not able to detect Fgf10 expression at E11.5 (data not shown). In E12.5-E14.5 embryos, we could not identify any lacZ staining in Fgf10lacZ reporter mice (data not shown), and needed to develop the in situ hybridization signal for 12 hours at room temperature, suggesting that the expression level of Fgf10 is very low at these stages (Fig. 1A-C). Although Fgf10 expression is initially expressed uniformly in the ventral mesenchyme at E12.5 and E13.5 (Fig. 1A,B, respectively), it becomes progressively regionalized in a periodic pattern, as seen at E14.5 (Fig. 1C) and E16.5 (Fig. 1D and the corresponding sagittal section shown in 1E). From E12.5 to E16.5, Fgf10 is not expressed in the dorsal mesenchyme of the trachea (Fig. 1A-E). Analysis of whole-mount Fgf10lacZ tracheae (Fig. 1F,G) and frontal and transversal vibratome sections thereof (Fig. 1H,I) at E18.5 confirmed the restriction of Fgf10 expression to the ventral side and in between the cartilage rings at that late stage as well.

As FGFR2B is the main receptor for FGFR10 (De Moerlooze et al., 2000; Ohuchi, 2000), its expression pattern was also investigated between E12.5 and E14.5 using whole-mount in situ hybridization. At all stages, Fgfr2b is uniformly expressed in the epithelium of the trachea (Fig. 11-K), strongly suggesting that mesenchymal FGF10 can evoke signaling in the tracheal epithelium via activation of this receptor.

Fgf10 inactivation results in a shortened trachea with severe defects in patterning of the cartilage rings

In order to follow the formation of the cartilage rings during tracheal development, we determined the expression of type II collagen, an early marker of cartilage differentiation (Sandell et al., 1994). At E11.5, type II collagen is expressed only in the lamina propria at the ventral side of the wild-type tracheae (Fig. 2A). The staining expands into the ventral mesenchyme by E12.5 (Fig. 2B). At E13.5, the onset of periodicity in type II collagen expression becomes visible (Fig. 2C), and is fully determined by E14.5 (Fig. 2D). Thus, the patterning of the tracheal cartilage is progressively established, starting at E13.5 and defined by E14.5 in wild-type embryos. In the Fgf10+/− trachea, the expression of type II collagen appears normal at E11.5 and E12.5 (Fig. 2E,F). The first defect is observed at E13.5 when collagen expression fails to become
segmented (Fig. 2G). By E14.5, the segmentation is present (Fig. 2H), but although the centers of the proximal cartilage condensations in Fgf10 knockout tracheas are roughly similarly distributed as in the wild type, the condensations are smaller and less uniformly shaped compared with the wild type. Moreover, in the distal part of the trachea type II collagen expression is not segmented at all (Fig. 2H). This early defect of cartilage patterning results in severely disorganized cartilage rings in Fgf10-/- tracheas at E18.5 (Fig. 2J). Whereas the tracheal cartilage forms uniform C-shaped rings in the wild type (Fig. 2I), most of the rings do not span an entire C-shape in the Fgf10-/- tracheas (Fig. 2J). Transversal vibratome sections of E18.5 tracheas show that the width of the Fgf10-/- trachea is roughly the same as in the wild type, yet it is collapsed, reminiscent of the human tracheomalacia phenotype (insets in Fig. 2I,J). This early developmental defect results in a collapse of the airway reminiscent of a tracheomalacia phenotype. (K) The same phenotype was observed in E18.5 Fgfr2b-/- trachea.

RESEARCH ARTICLE

FGF10 and cartilage formation

Fig. 2. Time course following the formation of the cartilage rings in Fgf10-/- and wild-type tracheas. (A-H) E11.5 to E14.5 tracheas, from (A-D) wild-type and (E-H) Fgf10-null embryos, were sectioned in sagittal plane and immunostained for type II collagen. In wild-type tracheas, the patterning of the cartilage rings is established by the condensation of the mesenchyme (white arrows in D) between E12.5 and E14.5. In the Fgf10-/- tracheas, the mesenchyme fails to condensate properly at E13.5. Mis-shapen condensations appear 1 day later (white arrows in H). (I,J) This early developmental defect results in totally disorganized cartilage rings, as seen by Alcian Blue staining of (I) Fgf10 knockout tracheas at birth compared with (I) wild type. Transverse vibratome sections of the tracheas in I and J (insets) show that the malformations of the cartilage rings observed in the mutant result in a collapse of the airway reminiscent of a tracheomalacia phenotype. (K) The same phenotype was observed in E18.5 Fgfr2b-/- trachea.

Fig. 1. Fgf10 and Fgfr2b expression pattern in the developing trachea. (A-E) Whole-mount in situ hybridization shows Fgf10 expression in the ventral mesenchyme of the trachea. At E14.5 onwards, we observe a regionalization of the expression in the non-cartilaginous mesenchyme (black arrows in C). The dorsal side of the trachea does not stain for Fgf10 (white arrows). (E) Sagittal vibratome section of the E16.5 trachea shown in D. Arrows in E indicate Fgf10 expression in between the cartilage rings. (F-H) This expression pattern is confirmed using the Fgf10lacZ at E18.5. (H') Frontal vibratome section of the E18.5 trachea shown in F and G. (H') Transverse vibratome section of the E18.5 trachea shown in F and G. (I-K) Whole-mount in situ hybridization shows uniform Fgfr2b expression in the tracheal epithelium from E12.5 to E14.5. V, ventral side of the trachea; D, dorsal side; R, right side; L, left side; tr, trachea.

The height of which seems slightly reduced in Fgf10-/- embryos

width of the Fgf10-/- trachea is roughly the same as in the wild type, yet it is collapsed, reminiscent of the human tracheomalacia phenotype (insets in Fig. 2I,J). This same phenotype is also observed in the Fgfr2b-/- tracheas (Fig. 2K, inset), supporting our hypothesis that FGF10 triggers signaling in the epithelium via FGFR2B. Of interest, both mutants have tracheas that are about half the length of a normal trachea, and did not display tracheoesophageal fistula or tracheal atresia.

Fgf10-null tracheas display normal differentiation but decreased proliferation of the epithelium and mesenchyme

As it is well established that FGF10 plays an important role in controlling the proliferation and/or the differentiation of the epithelium in various organs (Bellusci et al., 1997; Sala et al., 2006; Spencer-Dene et al., 2006; Veltmaat et al., 2006), we next examined these cell decisions in tracheal development.

Hematoxylin and Eosin staining of sagittal sections of E18.5 tracheas shows columnar epithelium in control embryos (Fig. 3A), the height of which seems slightly reduced in Fgf10-/- embryos.
showed no apoptosis in both epithelium and mesenchyme of the \(Fgf10^{+/+}\) tracheas (data not shown), suggesting that the smaller size of mutant tracheas is solely due to a lack of proliferation.

**Overexpression of \(Fgf10\) from E11.5 to E13.5 induces tracheal cartilage ring malformations**

In order to explore further the role of \(Fgf10\) during the formation of the tracheal cartilage rings, we generated transgenic mice that conditionally overexpressed \(Fgf10\) under the control of the mesenchymal specific promoter Dermo1 (Yu et al., 2003) \((\text{tet}(O)/Fgf10^{\text{Dermo}-1}\) hereafter). Overexpression of \(Fgf10\) was confirmed by feeding doxycycline to females pregnant with E12.5 embryos for 24 hours prior to sacrifice. Compared with control littermates (Fig. 4A), \(Fgf10\) mRNA is expressed at a higher level in E13.5 \(\text{tet}(O)/Fgf10^{\text{Dermo}-1}\) trachea (Fig. 4B). Although such short overexpression did not induce branching of the epithelium (Fig. 4C,D), it did stimulate ectopic SP-C expression on the ventral epithelium (Fig. 4E,F), as previously described by Hyatt et al. (Hyatt et al., 2004), which further confirmed that \(Fgf10\) was overexpressed.

Overexpression was induced for 24 or 48 hours, starting at specific time points during the formation of the cartilage rings (E10.5 to E13.5). The cartilage rings were analyzed at E18.5 by Alcian Blue staining. When induced from E10.5 to E11.5, before the cartilage starts to form, overexpression of \(Fgf10\) did not alter the patterning of the cartilage rings (Fig. 4G,G’). However, when induced from E11.5 to E13.5, overexpression of \(Fgf10\) resulted in totally disorganized cartilage rings. We observed an overall reduction of cartilage formation. The cartilage rings fail to form continuous C-shaped rings in the ventral part but fused in the lateral side of the trachea in a proximal-distal fashion. These proximal-distal cartilaginous fusions extend also to the two main bronchi (Fig. 4H,H’). Overexpression for 24 hours between E11.5 and E12.5 or E12.5 and E13.5 also resulted in altered cartilage rings (data not shown). Interestingly, when \(Fgf10\) is overexpressed after E13.5 for 48 hours, at the time when the cartilage is already patterned, the patterning of the rings is no longer affected (Fig. 4I,I’). Under all conditions, \(Fgf10\) overexpression did not result in cartilage differentiation in the dorsal side of the trachea, in tracheoesophageal fistula (data not shown) or in tracheal atresia. The patterning of the cartilage rings of the littermate controls was not affected by the doxycycline treatment (Fig. 4J,J’). Overall, these results demonstrate that E11.5 to E13.5 is the crucial developmental phase where \(Fgf10\) controls the patterning of the mesenchyme, allowing the formation of the alternating cartilage segments.

\(Fgf10\) controls the amplitude of periodicity of \(Shh\) expression in the ventral epithelium of the developing trachea

We next sought to understand the molecular mechanism by which \(Fgf10\) (via \(FGFR2B\)) regulates the periodicity in tracheal mesenchyme differentiation. Therefore, we examined the expression of \(Sox9, Bmp4\) and \(Shh\), known molecular regulators of tracheal cartilage formation (Hatakeyama et al., 2004; Que et al., 2006; Park et al., 2010; Miller et al., 2004), in wild-type and \(Fgf10^{+/+}\) embryos. We primarily focused our study at E13.5, as this is a crucial stage where cartilage patterning is detectable. We also examined wild-type and mutant tracheas for activated signaling via \(\beta\)-catenin, another known regulator of cartilage formation (Akiyama et al., 2004) and a potential downstream target of \(Fgf10/FGFR2B\) signaling pathway (Berg et al., 2007), using the \(Topgal\) reporter line, a \(\text{lacZ}\)-reporter for \(\beta\)-catenin signaling.

**Fig. 3.** Differentiation and proliferation analysis in the \(Fgf10^{+/+}\) and wild-type tracheas at birth. The tracheal lumen is on the left in each panel. (A,B) Hematoxylin and Eosin staining showing normal pseudo-stratified epithelium in control (A) and \(Fgf10\)-null (B) tracheas. (C,D) Alcian Blue staining of the goblet cells (white arrows) in control (C) and \(Fgf10\)-null (D) tracheas. (E,F) \(\beta\)-tubulin immunostaining of ciliated cells in control (E) and \(Fgf10\)-null (F) tracheas. (G,H) Smooth muscle actin (SMA) staining of the muscle cells in the dorsal side of control (G) and \(Fgf10\)-null (H) tracheas. (I,J) BrdU incorporation in E13.5 wild-type (I) and \(Fgf10^{+/+}\) (J) tracheas. (K) Quantification of BrdU incorporation in mesenchyme and epithelium. Data are mean ± s.e.m. *P<0.05; **P<0.005.

(Fig. 3B). Alcian Blue staining (Fig. 3C,D) and \(\beta\)-tubulin immunostaining (Fig. 3E,F) demonstrate the presence of properly differentiated goblet and ciliated cells, respectively, perhaps with slightly different distributions or shapes in the \(Fgf10^{+/+}\) tracheas. Immunostaining for smooth muscle actin (SMA) showed no defect in the differentiation of the smooth muscle cells in the dorsal mesenchyme of \(Fgf10^{+/+}\) tracheas compared with wild-type tracheas (Fig. 3G,H). The section in Fig. 3G is parasagittal, crossing the dorsoventral transition between cartilage and smooth muscle tissue, which explains the undulating pattern of SMA expression. By contrast, the section in Fig. 3H is a midsagittal section showing the straight pattern of SMA expression. In conclusion, \(Fgf10^{+/+}\) tracheas do not display notable defects in epithelial differentiation.

As \(Fgf10^{+/+}\) and \(Fgfr2b^{+/+}\) tracheas are about half the length of wild-type trachea (Fig. 2I-K), we next analyzed the proliferation of the mesenchyme and the epithelium by BrdU incorporation in three pairs of mutant and littermate control embryos from two different mothers. The mutant tracheas showed a 15% decrease in the proliferation of the epithelium (from 29.7% in controls to 25.1%) and a 35% decrease in the proliferation of the mesenchyme (from 14.3% in controls to 9.2%) at E13.5 (Fig. 3I-K). A paired two-tailed \(t\)-test showed that these decreases were significant in both tissues \((P=0.017\) for the epithelium, and 0.003 for the mesenchyme). At that same stage of development, TUNEL assay
Inactivation of Fgf10 did not affect the expression level of Sox9 and Bmp4 mRNA (Fig. 5A-D) or that of Topgal (Fig. 5E,F). However, we did observe a change in the pattern of Shh expression (Fig. 6).

Using whole-mount in situ hybridization, we followed the expression pattern of Shh during a wider time-window of tracheal development. In wild types, Shh is uniformly expressed in the entire tracheal epithelium at E11.5 and E12.5 either along the left/right axis or the ventral/dorsal axis (Fig. 6A,B, respectively). At E13.5, although remaining uniformly expressed throughout the dorsal tracheal epithelium, Shh acquires a periodic expression pattern in the ventral epithelium (Fig. 6C). This periodic Shh expression pattern is more pronounced at E14.5 (Fig. 6D). Shh expression in the ventral side seems lower than in the dorsal side. To further illustrate the periodicity of Shh expression, we measured the hybridization signal for Shh mRNA along a precise location on the ventral side (green line) using pixel intensity in digital photographs of tracheas (Fig. 6E-G). In wild-type trachea, the presence of minima and maxima in pixel intensity (illustrated as double-headed arrows in the green line in Fig. 6G) indicates a periodicity of Shh expression in the ventral side of the trachea. Quantitative analysis indicates that the average peak-to-peak amplitude in intensity is about 22 pixels (average minimum intensity=37 pixels; average maximum intensity=59 pixels). By contrast, Shh expression in the dorsal side is uniform. The dorsal expression of Shh is also stronger than ventral confirming the previous observation. This ventral periodic expression at E13.5 correlates with a ventral periodic expression of type II collagen at that same developmental stage (Fig. 6H). Macroscopic analysis of E13.5 Fgf10 maternal tracheas after whole-mount in situ hybridization suggests a reduced amplitude in periodicity of Shh expression in the ventral side. In addition, the difference in Shh expression levels between ventral and dorsal sides appears reduced (Fig. 6I). Measurement of the pixel intensities revealed that dorsal epithelial Shh expression is indeed still higher than ventrally. Furthermore, periodicity in Shh expression was still detected in the ventral epithelium (green line in Fig. 6K), but the average peak-to-peak amplitude is reduced to only 13.5 pixels (average minimum intensity=68.5; average maximum intensity=82). Interestingly, Shh expression in the dorsal epithelium is less uniform along the anterior/posterior axis. The reduced amplitude and regularity of periodicity of ventral Shh expression in Fgf10 maternal trachea is associated with an absence of periodicity of type II collagen expression (Fig. 6L). In tet(0)Fgf10 maternal tracheas that overexpress Fgf10, Shh expression levels progressively increase along the ventrodorsal axis but remain uniform along the anteroposterior axis (Fig. 6M-O). The complete absence of periodicity of Shh expression in the ventral epithelium (Fig. 6M-O) is again associated with a loss of periodicity of type II collagen expression (Fig. 6P).
These data indicate that mesenchymal Fgf10 is not required for epithelial Shh expression per se, and strongly suggest that the expression of Fgf10 must be critically balanced to allow for periodicity of Shh expression. It is interesting to note that Shh expression in the esophageal epithelium is unchanged in the Fgf10−/− mutants (Fig. 6I), and completely lost in the tet(O)Fgf10Dermo-1 mutants (Fig. 6M), suggesting that FGF10 negatively regulates Shh transcription in the esophagus.

**Changes in periodicity of epithelial Shh expression cause defects in mesenchyme condensation, resulting in cartilage ring malformations**

To further explore the role of SHH in tracheal cartilage formation, we made use of the previously reported Ttf-1cre (or Nkx2.1cre) driver line either to delete or overexpress Shh in the tracheal epithelium. To verify the activity of this line, we crossed the Ttf-1cre driver line with the Rosa26R reporter line containing a floxed lacZ gene in the Rosa26-locus (Soriano, 1999). As expected, β-galactosidase was activated in wild-type (E) and Fgf10–/– (F) tracheas using the Topgal reporter line, a lacZ reporter for β-catenin signaling.

At the ventral midline (Fig. 7K, K′), similar to what we observed in Fgf10 overexpressing trachea. It is interesting to note that, despite an absence of supporting rings, the lumen of the trachea did not collapse (inset in Fig. 7K). Overexpression of Shh in tet(O)ShhFgf10−/− tracheas from E12.5 to E13.5 upon exposure of the pregnant females to doxycycline food results at E13.5 in sustained type II collagen deposition with absence of segmentation (Fig. 7I), similar to the Fgf10−/− tracheal phenotype (Fig. 2G, Fig. 6L). At E18.5, transient Shh overexpression during the E12.5/E13.5 time frame induced a strong differentiation of the mesenchyme into cartilage, resulting in mis-shaped rings. The cartilage rings fuse laterally (Fig. 7L, L′). This anomaly resulted in a narrowing of the lumen of the trachea (inset in Fig. 7L) compared with the lumen of a wild-type trachea (inset in Fig. 7I). Similar to the Fgf10-null and over-expressing mutants, Shh deletion and overexpressing mutants do not form cartilage dorsally, and do not display tracheo-esophageal fistula or tracheal atresia. To demonstrate that Fgf10 and Shh interact at the genetic level, we have generated wild type, single heterozygous, as well as double heterozygous (Fgf10+/−; Shh+/−) tracheas at E18.5 and determined the formation of the cartilage by Alcian Blue staining. Double heterozygous tracheas (n=5) demonstrate significant cartilage defects not observed in single heterozygous (n=5) or wild-type tracheas (n=5). These defects include aborted rings and mis-shapen rings (Fig. 8). These defects are reminiscent of the defects observed in Fgf10-null mutants and indicate that there is indeed a genetic interaction between Fgf10 and Shh.

**DISCUSSION**

As periodicity in the tracheal cartilage is essential for an optimal breathing function, we sought to understand how periodic patterning of the tracheal cartilage is established. Using mouse embryos, we show here that the ventral tracheal mesenchyme expresses type II collagen, an early marker of cartilage formation, as a continuum along the proximal-distal axis starting at E11.5. Expression becomes periodic around E13.5. We found that perturbation of Fgf10 expression levels results in incomplete and irregularly shaped cartilaginous C-shaped rings. We therefore investigated its role in tracheal cartilage formation in more detail.

We did not detect any Fgf10 mRNA expression in the trachea at E11.5, when cartilaginous differentiation of the ventral tracheal mesenchyme has already begun. Moreover, while Fgf10 expression is detectable in the ventral tracheal mesenchyme from E12.5 onwards, it is continuous along the proximal-distal axis at that time and only becomes periodic at around E14.5, at least one day later than periodic pre-cartilaginous condensations are observed in the ventral tracheal mesenchyme. At that time, Fgf10 is expressed in between those condensations. In Fgf10−/− mutants, cartilage forms as incomplete rings. Together, these data demonstrate that Fgf10 expression is neither required for the induction of cartilaginous differentiation of the tracheal mesenchyme, nor for the establishment or maintenance of periodicity within the tracheal mesenchyme. However, Fgf10 is required for the proper patterning of tracheal mesenchymal differentiation, i.e. for complete and properly shaped cartilage rings. Using mutant mice conditionally overexpressing Fgf10 in the mesenchyme, we established that the critical time-window for this function of Fgf10 is between E11.5 and E13.5, which nicely coincides with the time-frame during which periodic patterning of the tracheal mesenchyme is observed.

Interestingly, aside from the shorter tracheal length (and lack of lung development) in Fgf10−/− mutants, the tracheal cartilage phenotypes of constitutive Fgf10-null mutants and mutants
overexpressing Fgf10 during a 48-hour period between E11.5 and E13.5 share a common feature: the cartilage rings are induced but they are truncated. The rings do not extend far enough dorsally, resulting in a flaccid tracheal support, characteristic of tracheomalacia in humans. In both gain- and loss-of-function of Fgf10, we found a change in localized expression of Shh, a key regulator of cartilage formation (Miller et al., 2004). We propose that the FGF10-controlled segmentation of Shh expression is crucial for the proper patterning of the cartilage and that changes in the expression level of Shh along the proximal-distal axis will directly impact the integrity of the rings being formed. The proposed interaction between these two signaling pathways is supported at the genetic level as double heterozygous Fgf10+/–;Shh+/– tracheas display both aborted rings and misshapen rings, a defect not observed in wild-type and single heterozygous (Fig. 8). Collectively, these data strongly suggest that the Fgf10 expression level needs to be within a crucial range to allow for proper patterning of the tracheal mesenchyme. Importantly, ablation or temporal overexpression of mesenchymal Fgf10 does not lead to tracheo-esophageal fistula, which sometimes accompanies tracheomalacia in humans.

As FGFR2B is the main receptor for FGF10 expressed on epithelial cells (De Moerlooze et al., 2000) and, moreover, the tracheal phenotype of Fgfr2b+/– mutants is strikingly similar to that
of Fgf10−/− mutants, we conclude that in a normal situation, FGF10 exerts its role in mesenchymal patterning via activation of the tracheal epithelium. This is very different from the role we established for FGF10 in a mouse model of Apert syndrome (Tiozzo et al., 2009), in which FGF10 activates ectopically expressed FGFR2B receptors in the mesenchyme, resulting in cartilaginous overgrowth. Although those previous data indicate that FGF signaling in the mesenchyme can induce its differentiation into cartilage, our current data show that FGF10 is normally not an autocrine activator of FGF signaling in the mesenchyme, but an activator of FGFR2B signaling in the tracheal epithelium. Interestingly, this activation seems to have no or little importance for the differentiation of the epithelium itself, as we observed the presence of both ciliated and goblet cells in trachea of Fgf10−/− mutants. Thus, the main role of FGF10 during this early developmental stage may be to coordinate mesenchymal patterning, using the epithelium as an intermediate.

Besides FGF signaling, β-catenin signaling, BMP4 and SHH are known to be involved in tracheal cartilage formation (Akiyama et al., 2004; Hatakeyama et al., 2004; Park et al., 2010). We found that β-catenin signaling and Bmp4 expression are not changed in Fgf10−/− mutants. Although recombinant FGF10 upregulates Bmp4 expression in the tracheal epithelium in tracheo-pulmonary organotypic cultures in vitro (Hyatt et al., 2004), FGF10 is apparently not required for Bmp4 expression in vivo. We did not observe a change in Sox9 expression either, which was unexpected because SOX9 is implicated in cartilage formation mediated by β-catenin signaling, BMP4 and SHH (Akiyama et al., 2004; Hatakeyama et al., 2004; Park et al., 2010). We cannot exclude the possibility that a change was present, but we may have failed to observe it as it might be obscured due to the partial formation of cartilage rings in the mutants. Alternatively, changes may have occurred at the translational or post-translational level instead of at the RNA level.

Interestingly, although Shh expression in the epithelium normally becomes periodic at around E13.5, concomitant with mesenchymal patterning, periodicity of Shh was less pronounced in Fgf10−/− mutants and was absent in Fgf10 overexpressing mutants. This is of particular interest, as the gene encoding PTC1, a transducer and transcriptional target of SHH, is expressed in the pre-cartilaginous condensations at E13.5 (Miller et al., 2004); SHH appears to control the cartilaginous differentiation of the mesenchyme. As for Fgf10, the level of Shh expression needs to be within a critical range for proper patterning of tracheal cartilage. This result indicates that neither SHH nor FGF10 is an instructive signal for cartilage formation. Interestingly, disruption of dorsoventral
partitioning of Fgf10 and Shh expression does not alter dorsoventral patterning of the trachea in terms of separation of the muscle lineage on the dorsal side and cartilage lineage on the ventral side.

Establishment of complex patterns during embryonic development is a major interest in the field of developmental biology. Two conceptual developmental models have been proposed to explain the formation of repetitive patterns. Of these, the clock and wave-front model involves the cyclic expression of specific genes resulting in a repetitive pattern (Cooke and Zeeman, 1976). In contrast to other periodic patterns, such as somite or hair follicle formation, where the new structures form sequentially, the tracheal cartilaginous segments seem to be induced simultaneously and mature over an extended period of time (between E13.5 and E14.5). In addition, we did not observe any cyclic expression of Fgf10 or Shh. These pieces of evidence seem therefore to argue against a clock-and-wavefront model underlying tracheal cartilage formation. In the alternative reaction-diffusion model proposed by Alan Turing, two morphogens – an activator and the activator-dependent inhibitor – establish periodic patterning during development (Turing, 1952; Gierer and Meinhardt, 1972). We wondered whether Turing’s model, with SHH and FGF10 acting as the activating and inhibiting morphogens, would apply to tracheal cartilage formation. We demonstrated that Shh is expressed prior to Fgf10 in the trachea (Figs 1 and 6) and that Fgf10 expression represses Shh expression (Fig. 6). In addition, Shh expression becomes periodic only after Fgf10 expression becomes detectable, and Fgf10 expression becomes periodic only after Shh expression has become periodic. One could propose SHH as an activator and FGF10 as the activator-dependent inhibitor, the periodicity of which reinforces the periodicity of Shh expression, although it remains unclear how the initial periodicity of Shh expression is achieved. However, removal of the activator or inhibitor in Turing’s model would result in the complete absence of periodicity, i.e., either no cartilage formation at all or formation of a continuous cartilaginous sleeve. We still observed residual periodicity in the tracheal cartilage in our loss- and gain-of-function models for Fgf10 and Shh.

In conclusion, we show for the first time that both Fgf10 and Shh expression levels are crucial between E11.5 and E13.5 for tracheal cartilage patterning in the mouse embryo. The similarity in tracheal phenotypes between Fgf10+/- and Fgfr2b+/- embryos strongly suggest that FGF10 exerts its role in cartilage formation via activation of FGFR2B in the epithelium. Although this signaling is not required for epithelial differentiation itself, it may be a regulator of periodic Shh expression in the epithelium. The residual and irregular formation of cartilage rings in the absence of Fgf10 indicate that this molecule does not act in the induction of periodicity, but rather in the fine-tuning of it. Finally, we show that reduced or elevated expression of Fgf10 and Shh did not induce any tracheo-esophageal fistula or atresia. This is in sharp contrast with tracheo-esophageal fistula co-occurring with tracheomalacia, owing to perturbations of BMP signaling (Que et al., 2006), and underscores the distinct role played by FGF10/SHH versus BMP signaling, in tracheal malformations. Thus, our current results not only provide novel insights in the mechanism of action of Fgf10 and Shh on normal tracheal cartilage formation, but may also provide helpful diagnostic criteria for the development and application of future molecular therapies to prevent or correct congenital tracheal cartilage defects.

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Competing interests statement
The authors declare no competing financial interests.

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