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Review

Congenital tracheal defects: embryonic development and animal models

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Abstract: Tracheal anomalies are potentially catastrophic congenital defects. As a newborn begins to breathe, the trachea needs to maintain an appropriate balance of elasticity and rigidity. If the tracheal cartilages are disorganized or structurally weak, the airways can collapse, obstructing breathing. Cartilage rings that are too small or too rigid can also obstruct breathing. These anomalies are frequently associated with craniofacial syndromes, and, despite the importance, are poorly understood. In this review, we summarize the spectrum of pathological phenotypes of the trachea and correlate them with the molecular events uncovered in mouse models.

Keywords: trachea; mouse models; human congenital anomaly; cartilage

1. Introduction

1.1. The problem: Why is it important to understand tracheal pathology?

The high morbidity associated with upper airway defects is now an increasingly recognised clinical problem. Congenital tracheal defects can be fatal due to intrinsic airway cartilage pathology, especially at birth when the neonate must take a first breath. These tracheal defects most commonly present as part of a syndrome, such as the tracheal stenosis associated with Apert or Crouzon syndromes [1]. Other examples include VACTERL association (vertebral defects, anal atresia, cardiac anomalies, tracheoesophageal fistula with oesophageal atresia, radial or renal dysplasia, and limb defects) in which the incidence of trachea-oesophageal fistula is well documented [2]. Syndromic patients often have other medical problems requiring urgent attention, and the trachea may not be prioritised in difficult cases. However, due to the poor quality of life and lack of

non-invasive, non-surgical treatment, tracheal development is now an important area of clinical and genetic research.

Most congenital anomalies of the upper respiratory tract are associated with malformed cartilage. Thus, it is essential to understand the molecular embryology of the upper respiratory tract, particularly the development of the cartilage components [3]. Increasing knowledge of respiratory organogenesis will elucidate where and when development is disrupted, providing clues towards pathophysiology and eventually directing clinicians towards the development of effective intervention. With recent advances in regenerative medicine, tracheal transplants, though currently controversial, may provide treatment for some patients [4]. Even though these tracheal transplants are groundbreaking, new and can claim to be 'curative', the fundamentals of embryological tracheal morphogenesis and aetiology of disease processes affecting the trachea remain obscure. A better understanding of the underlying developmental events will certainly improve future treatment protocols.

Here, we review the known aspects of tracheal development, describe the spectrum of pathology affecting the trachea and then relate the analysis of animal models to the disease phenotypes encountered in the clinic.

1.2. Anatomy, function and development

The large airways of the respiratory tract are made up of the larynx, trachea and two main bronchi (Figure 1a, with key cartilaginous elements depicted in blue). The trachea extends from the cricoid cartilage (Cr) of the larynx above, and ends at the carina (Ca), which is a cartilage segment demarcating the bifurcation of the trachea into the two bronchi. In humans, it is made up of up to 22 semi-circular cartilage rings, connected to each other by the annular ligaments (Al), and attached dorsally to the trachealis muscle (TM). The trachea is essentially the connection for the passage of air between the lungs and the outer environment. Its structure is adapted to maintain the active force of inspiration and passive expiration. Aside from breathing mechanics, the inner epithelium of the trachea is comprised of adapted ciliated columnar cells which allow the clearing of secretions and conditioning of inhaled air.

The anatomical structure of the mammalian larynx, trachea, and lungs has been unravelled by studying serial sections from various stages of development, as in mice [5]. Murine knockout studies have uncovered many of the genetic interactions which lead to congenital disease. Mouse models have also been developed where mutation of particular genes mimics human anomalies, ultimately allowing deeper understanding of human disease processes. Studies have shown in mice that around embryonic day (E) 7.5, the larynx and trachea begin development as an out-pouching from the ventral side of the foregut, namely the laryngotracheal groove (Figure 2A). Lung buds form at the caudal end and the trachea grows by extending downward in length. At E9.5, the ventral tracheal endoderm starts separating from the more dorsal endoderm which will form the oesophagus. As development proceeds, by E11.5 the two distinct tracheal and oesophageal tubes eventually emerge. The trachea then becomes surrounded by mesenchymal cells derived from the splanchnic mesoderm, which are committed to the chondrocyte lineage [6]. The committed mesenchymal cells proliferate, aggregate, and condense into pre-cartilaginous nodules, which subsequently differentiate into chondrocytes forming the semi-circular cartilage rings of the trachea [7].

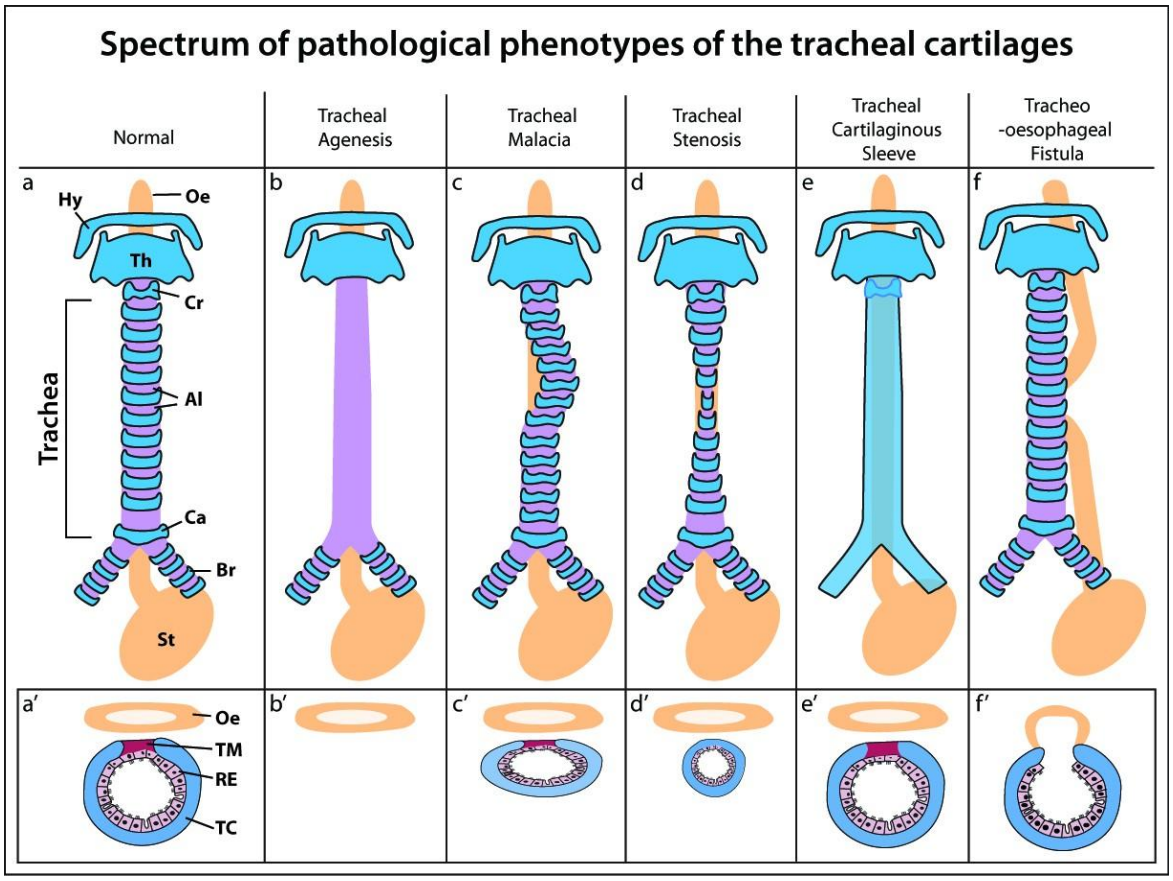

Figure 1. Spectrum of pathological phenotypes of the tracheal cartilages. 1a) Schematic representation of normal anatomy, frontal view. Cartilages are depicted in blue, epithelium in pink and oesophagus/stomach in orange. Oe: oesophagus; Hy: hyoid; Th: thyroid cartilage; Cr: cricoid cartilage; Al: annular ligaments; Ca: carina; Br: Bronchus; St: stomach.

1a') Schematic cross-section through tracheal cartilages. Shown are Oe: oesophageal tube; TM: trachealis muscle; RE: respiratory epithelium; TC: tracheal cartilage.

1b–b') Depiction of tracheal agenesis showing the absence of the tracheal cartilages below the larynx.

1c–c') Tracheal malacia results from a weakness in the cartilage rings making the trachea prone to collapse. This can be seen as a flattening of the tracheal rings in cross-section (1c').

1d–d') Tracheal stenosis is a narrowing of the tracheal lumen. Rather than the normal c-shaped cartilage rings with intervening tissue allowing expansion, in tracheal stenosis the cartilage rings are o-shaped, and therefore unable to grow.

1e–e') In tracheal cartilaginous sleeve, cartilage rings are fused, leading to a loss of the intervening fibrous annular ligaments.

1f–f') Tracheo-esophageal fistula occurs when the foregut tube fails to separate into trachea and oesophagus.

Development of the trachea is a complex process, which mirrors the spectrum of tracheal pathology encountered in the clinic. Several steps in the morphogenesis of respiratory organs have been identified and studied using transgenic mouse models. Although this has increased our

understanding of tracheal organogenesis, there are still many aspects of upper airway development which are not as well understood. In order to unveil the aetiology of tracheal disease, it has become important to define the molecular mechanisms which direct foregut morphogenesis [8]. Much of the research into respiratory development has focused on the signalling factors which control branching morphogenesis of the lung, and only minimal data has been derived in relation to tracheal ontogenesis. The trachea, being a foregut derived organ, similarly develops through a process of reciprocal inductive interactions between the endoderm and surrounding mesoderm. Defective signalling is thought to be the potential cause of some of the disease presentations seen in clinics. The signalling networks guiding the processes of tracheal development and the spectrum of tracheal disorders are discussed below.

1.3. The spectrum of congenital tracheal anomalies encountered in the clinic

Congenital tracheal defects in humans can range from complete agenesis of the trachea to excessive production of cartilage, resulting in stenosis. This review aims to gather and classify tracheal disease according to pathophysiology. A number of studies have been discussed which aim to consolidate what we know so far about tracheal development. These studies have been carried out mainly using the mouse as a model organism.

Tracheal Atresia/Agenesis (TA): Tracheal agenesis (Figure 1b) is the rarest and most fatal of congenital anomalies affecting the trachea. The presentation ranges from complete absence of the trachea in tracheal agenesis or a partly atretic tube. The Floyds' classification system categorizes the disease according to anatomical presentation of the tracheal-bronchial tree. When there is tracheal atresia in a newborn, the affected neonate fails to take a breath, which is diagnosed as congenital high airway obstruction syndrome (CHAOS). Because of the structural defects of the airways, intubation and resuscitation is unsuccessful in most, if not all cases. Only around 150 human cases have been reported to date, with just one survivor [9]. During delivery of a foetus with CHAOS syndrome, the head alone can be initially delivered via a maternal Caesarean section, which then allows intubation prior to placental blood flow being cut. This is the ex-utero intrapartum therapy (EXIT procedure). However, prognosis still remains poor, as ventilation problems continue due to the abnormal structure of the trachea [10]. No causative genes have been identified in humans as of yet [11]. However, genetic research on mutant mouse models is now indentifying a number of transcription factors which may be important in the pathophysiology of tracheal agenesis. These will be discussed below.

Tracheomalacia: Tracheaomalacia is the most common congenital tracheal defect, with a prevalence of 1:1500 children [12]. Malacia refers to dynamic collapsibility of the trachea, due to softness of the cartilage (Figure 1c). The affected portion of the trachea is prone to collapse, under conditions where extraluminal pressure exceeds the intraluminal pressure, i.e. during inspiration [13]. Clinical features include dyspnoea, stridor and cyanosis. Narrowing and collapse of the airway results in obstruction, which may be relieved by passing a bronchoscope down the trachea to open it again. Prognosis ranges from a very high mortality rate to manageable, with most surviving patients requiring prolonged respiratory support via a tracheostomy [14]. Congenital tracheal malacia is often associated with Down's syndrome, Trisomy 9, 11p13 deletion (Wilms/aniridia), 22q11 deletion, 18–22 translocation, chondrodysplasias, Williams–Campbell syndrome, Di George syndrome, CHARGE association, Hunter's syndrome and Hurler's syndrome [13]. This anomaly has not been

attributed to a single causative gene, although some genetic knockout mouse models have explored the aetiology of the defect.

Tracheal Stenosis: This is a fixed narrowing of the tracheal lumen due to intrinsic cartilaginous defects (Figure 1d). It cannot be re-opened by a bronchoscope as opposed to the dynamic collapsible 'stenosis' of tracheomalacia. The epidemiology of tracheal stenosis is unknown, however one study has suggested an incidence of 1 in 64,500 births [15]. Tracheal stenosis is associated with cardiovascular anomalies such as atrial-septal defects, in around 50% of patients. Frequently, cartilage rings are constricted and 'complete' at the dorsal end, with an O-shape, rather than the normal C-shape (Figure 1d). A rare cause of tracheal stenosis is Tracheobronchopathia Osteochondroplastica, a disease in which benign cartilaginous/bony nodules grow in the submucosa, and block the airway. Tracheo-oesophageal fistula and tracheal stenosis may also co-exist, so both might not be two entirely separate entities in the critically ill patient [14].

Tracheal Cartilaginous Sleeve: This is a rare congenital malformation of unknown epidemiology, which occurs exclusively in Apert, Goldenhar, Pfeiffers syndrome and Crouzon disease—all of which are examples of craniosynostosis syndromes [16-18]. The C-shaped tracheal rings are frequently fused together, with a loss of the intervening fibrous tissue (Figure 1e). This results in a tubular trachea, which may also be stenotic. The pathophysiology of this defect is unclear, although some studies have proposed that there may be a common defect in the development of all structures derived from hyaline cartilage. For example, in Apert syndrome there is defective limb skeletogenesis as well as cranial suture fusion. The morphology of the tracheal cartilaginous sleeve anomaly could mirror the overproduction of cartilage in the cranial sutures in these patients. Prognosis is very poor, as mortality rate in patients with a tracheal cartilaginous sleeve plus craniosynostosis is up to 90% [18]).

Tracheo-esophageal fistula (TEF) with or without esophageal atresia (OA): This is a relatively common abnormality of the foregut, affecting 1 in 3500 births [19]. The common foregut tube fails to separate into the trachea and oesophagus, resulting in an abnormal connection between the two (Figure 1f). This may rarely manifest as an isolated TEF, however 87% of have one blind-ended oesophagus, and a more distal oesophagus which abnormally connects with the trachea [8]. As with most tracheal defects, TEF/OA also usually occurs as part of a genetic syndrome in humans, such as the VATER/VACTERL association. Table 1 below highlights some mouse models which also exhibit a TEF/OA type phenotype. These studies have been reviewed in Que J et al 2006, so an in-depth discussion is not provided here [8].

TEF/OA is the most commonly researched defect. Trying to interpret the pathophysiology of this condition has led to two main theories of foregut separation. One theory proposes that foregut division results from fusion of the lateral ridges of the foregut, starting caudally and elongating cranially. The septum thus separates the ventral trachea from the dorsal oesophagus. A more recently postulated theory suggests that the respiratory diverticulum develops from the ventral aspect of the foregut and continues to elongate caudally as the trachea. The mesenchyme which is in between the respiratory and digestive tubes, forms the septum, which is apparently fixed and does not move during caudal growth of the trachea [20].

Table 1. Congenital tracheal anomalies in mouse models.

Mouse gene	Tracheal Defect					
	TEF/OA	TA	TM	TS	Excess/ Distortion	Reference
Foxf1$^{+/-}$ (forkhead transcription factor)	x					Mahlapuu et al., (2001)
Gli2 and *Gli3* (Hedgehog pathway)	x				x	Motoyama et al., (1998)
Nkx2.1 (Homeodomain transcription factor)	x	x				Minoo et al., (1999)
Noggin (Secreted BMP antagonist)	x					Que J et al., (2006)
Shh (Secreted hedgehog family ligand)	x				x	Litingtung et al., (1998) Miller LA et al., (2004)
RARα, RARβ2 (Retinoic acid receptors)	x				x	Mendelsohn et al., (1994)
BMP4 (Bone morphogenetic protein4)		x				Domyan ET et al., (2011)
TBX4/*TBX5* (T-box transcription factors)		x				Arora M et al., (2012
CV2 (BMP pathway)		x				Zakin L et al., (2008)
Tmem16a (transmembrane protein)			x			Rock J et al., (2008)
Trps1 (trichorhino-phalangeal sydrome gene)			x			Suemoto H et al., (2007)
Traf4 (TNF receptor associated factor, scaffold protein)				x		Regnier, C.H (2002)
Sox9 (SRY domain transcription factor)					x	Park et al., (2010)
FGF10 (Fibroblast Growth Factor 10)					x	Tiozzo C et al., (2009), Sala et al., (2011)
Mek1/Mek2 (kinases)		x				Boucherat et al., (2014)

TEF usually does not occur as an isolated tracheal defect. The development of TEF commonly occurs alongside oesophageal pathophysiology, therefore pointing to a defect in overall foregut morphogenesis. Although not separate from tracheal development, the aetiology of TEF/OA involves non-cartilage defects of the oesophagus likely due to failure of the complex processes of epithelial-to-mesenchymal signalling during embryogenesis. These factors have to be considered when discussing tracheal abnormalities, and therefore TEF/OA is excluded from in-depth discussion in this review.

2. Stages of tracheal development in mice

Tracheal development in mice can be organised into 3 consecutive stages for the purpose of this review: specification, outgrowth/proliferation and cartilage condensation. These stages can be used

to help explain normal development of the trachea, with an aim to identify time points where development is disrupted.

2.1. Specification

The initial specification of the respiratory primordium is governed by a number of cellular events, which will then allow patterning of the cartilage itself. There are some uncertainties surrounding what we know about the genes and factors responsible for commitment of cartilage precursor cells to the chondrocyte lineage. Many genetic knock-out studies have revealed the function of some genes in the development of the respiratory tree. Several key signalling pathways are Hedgehog (Hh), Fibroblast Growth Factors (FGF) and bone morphogenetic protein (BMP), detailed below. The earliest requirement appears to be the Hh ligand Sonic Hedgehog, as mutation of *Shh* leads to a loss of the transcription factor *Foxf1* and early foregut malformations leading to tracheo-esophageal fistula [21].

Two T-box transcription factors, *Tbx4* and *Tbx5* are expressed in the mesenchyme of the developing foregut, and appear to be important in the initial specification of the trachea. *Tbx5* (but not *Tbx4*) is required for the initial specification of the trachea, as its expression is detected as early as E9.0, in the laryngotracheal groove [22]. Lack of *Tbx5* results in no expression of *Nkx2.1*, hence complete absence of tracheal specification. These data place *Tbx5* upstream of *Nkx2.1*, which was **c**lassically seen as the earliest marker of respiratory development in mice MinooGuoshanDrum et al. [23]. Nkx2.1 is a homeodomain transcription factor which is important for the epithelial-mesenchymal interactions required for tracheal development. Its expression in the epithelium demarcates the ventral boundary of the anterior foregut, distinguishing pulmonary from oesophageal tissue. When *Nkx2.1* is knocked out in mice, the resulting phenotype is similar to the tracheosophageal fistula anomaly seen in humans. As there is still some development of tracheal cartilage rings albeit fused and truncated, it would appear that another gene must be responsible for initial specification instead of *Nkx2.1*, which could be *Tbx5*.

The retinoic acid receptor pathway also appears important. Retinoic acid receptors (RARs) are expressed in the foregut endoderm of developing mouse embryos. Knocking out the different types of retinoic acid receptors in mouse models results in a variety of defects. These range from agenesis of the trachea to shortening of the tracheal cartilage leading to developmental abnormalities [24]. As the effects are quite severe in nature, the RARs may be important not only for specification, but also the maintenance of epithelial to mesenchymal signals during development.

Epithelial-mesenchymal interactions during tracheal specification: Nkx2.1 is epithelial whereas *Tbx5* is mesenchymal. It appears that both factors interact with one another to initiate tracheal specification. *Nkx2.1* mutants show TEF whereas *Tbx5* mutants have tracheal agenesis. This is a similar phenotype to *bone morphogenetic protein receptor 1* and *2* (*Bmpr1* and *Bmpr2*) mutants in which lung bud specification occurs, but the ventral foregut fails to acquire tracheal identity. Signalling via the BMP-R1 and -R2 receptor proteins is required to initiate tracheal identity, via repression of the oesophageal marker *Sox2* [25]. Therefore, based on the overall phenotype of the two knock out mice models, *Tbx5* may be upstream of *Nkx2.1* in tracheal specification, and acts in parallel with or in the BMP signalling pathway to direct the ventral foregut into a trachea. Furthermore, *Tbx4* and *Tbx5* interact genetically and affect tracheal/bronchial cartilage development via *Sox9*, but independently of the Fgf10 signalling pathway. *Tbx4* and *Tbx5* heterozygous tracheas

have fewer cartilage rings, and further reduction of these genes in the lung-specific *Tbx4*$^{+/-}$*; Tbx5*$^{-/-}$ mutants, leads to severely disrupted tracheal and bronchial rings, as mesenchymal cells fail to condense. Downstream targets of Sox9 (*Sox2* and *Sox6*) are also down-regulated in these mutants [22].

2.2. Outgrowth/proliferation

Following on from specification, at E11.5–E14.50, the mouse laryngotracheal groove then elongates bidirectionally, to produce the larynx above and trachea below, with lung buds at the distal end. This process of outgrowth is regulated by cell to cell signalling via the recruitment of downstream signalling factors. *Tbx4/Tbx5* interact with each other and are upstream of *Sox9* during chondrogenesis (Figure 2B). This leads into the process of 'outgrowth' where downstream targets of *Tbx4* and *Tbx5/Nkx2.1* are required specifically at the time of mesenchymal cell condensation and proliferation. At E11.5 and E13.5 both *Tbx4* and *Tbx5* are expressed throughout the tracheal mesenchyme in ventral tracheal cells that also express *Sox9*. At E15.5, their expression is noted in the mesenchyme between the cartilage condensations; this is in a pattern complementary to that of *Sox9*, which is restricted to the condensing mesenchyme of the cartilage rings at this stage [22].

Sox9 is a transcription factor which is required for every step of chondrogenesis [7]. It is expressed in pre-cartilaginous mesenchymal cells which are committed to the chondrocyte lineage, even at the specification stage at E9. Its expression is later detected in the ventral tracheal mesenchyme from E11.5, and it becomes segmented at E13.5. This segmented pattern indicates where the tracheal cartilage rings will form. The same study has shown that induction of this ventral/segmented pattern of expression is dependent on the Sonic Hedgehog (SHH) signalling pathway. By knocking out the *Shh* gene in a mouse model, it can be seen that *Sox9* expression is disrupted. This loss could be partially rescued when *Shh* mutant tracheas were explanted and treated with Bmp4, leading to some cartilage production. Thus, this places *Shh* upstream of *Bmp4* and *sox9* in tracheal chondrogenesis (Figure 2B).

Sonic Hedgehog signalling is required in the epithelium to initiate tracheal chondrogenesis: The Sonic Hedgehog (SHH) ligand is secreted by the endodermal epithelium (Figure 2B). The SHH ligand binds to Patched1 (Ptc1), which is expressed in the pre-cartilaginous condensations of the trachea. The *Shh* null mutant is not viable and dies postnatally, due to severe developmental defects. These include a single tracheo-oesophageal remnant, devoid of cartilage [26]. The mutants have incomplete, misaligned and constricted rings. In the null mouse there is agenesis, and expressing SHH in the SHH rescue mouse does not rescue the defects. Deletion between E8 and 12.5 causes tracheal cartilage abnormalities, but no effects are seen after E12.5. Furthermore, adding SHH to explants does not result in rescuing the defects. Therefore, a major role for SHH in tracheal development may be that it is required locally for the induction of cartilage condensations, via epithelial to mesenchymal signalling [26]. Consistent with this, *Shh* appears necessary to initiate and maintain the ventral-specific expression of *Sox9* in the tracheal mesenchyme. Furthermore, the Gli proteins, the downstream effectors of Hedgehog signalling, are also expressed in the tracheal mesenchyme. *Gli2/3* null mutants lack the tracheal primordium at E9.5, and the *Gli2*$^{-/-}$*; Gli3*$^{-/+}$ mutants have TEF [27]. Mutations in *GLI3* are also associated with three human dominant genetic disorders, Greig cephalopolysyndactyly syndrome, Pallister-Hall syndrome (PHS) and postaxial polydactyly type A Syndrome. Foregut malformations including lung lobulation defects, tracheal

stenosis and tracheo-oesophageal fistula have been observed in some PHS patients [28]. Interestingly, in the absence of SHH function, BMP4 and Noggin can partially induce chondrogenesis, suggesting that chondrogenic induction is sensitive to relative levels of BMP activity. Taken together, these results suggest that both *Bmp4* and *noggin* genes are downstream of *Shh*, and they can promote cartilage formation in trachea through regulation of chondrogenic genes such as *Sox9* (Figure 2B).

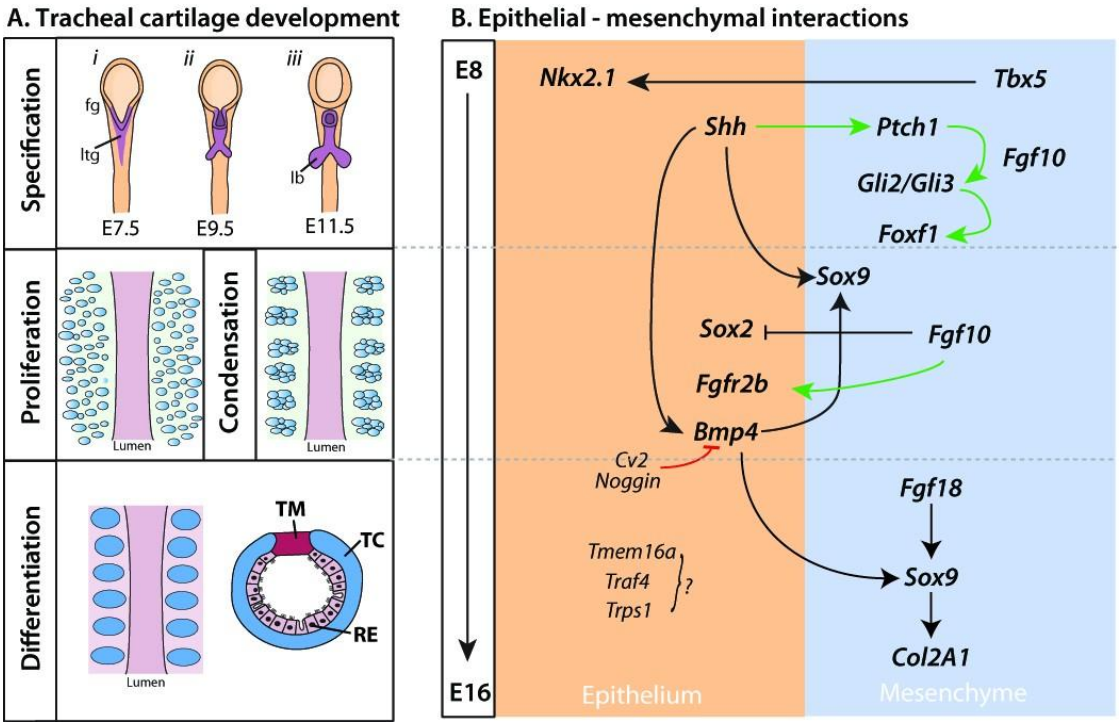

Figure 2A. **Schematized stages of tracheal development from initial specification to proliferation/condensation and subsequent differentiation.** Fg: foregut; ltg: laryngotracheal groove; lb: lung bud; TM: trachealis muscle; RE: respiratory epithelium; TC: tracheal cartilage. **Figure 2B. Key signals mediating the epithelial-mesenchymal interactions necessary for tracheal morphogenesis.** Genes identified are correlated with the stages depicted in 2A, with the top third showing genes involved in specification of the tracheal primordia, the middle third showing cues important for proliferation and condensation of the cartilage progenitors, and the bottom third outlining factors important in differentiation of the cartilage rings. Direct protein-protein interactions are marked by green arrows while indirect interactions are marked with black lines.

BMP signalling and the outgrowth of the trachea: Signalling through BMP4 is crucial to the subsequent outgrowth of the trachea. This is demonstrated by a *Bmp4* knock-out mouse model, in which the outgrowth stage was affected (Li et al 2008). At E10.5 the BMP conditional knock-out mice had a single endodermal tube, as opposed to the two tubes (representing the oesophagus and trachea). This tube has oesophageal characteristics, as it stained for the oesophageal marker *Pax9* and not the respiratory specific marker *Nkx2.1* suggesting a tracheal agenesis type of defect in the *BMP4* knock out embryos. It is worth noting that tracheal induction is actually not affected in these *BMP4* knock out mice as there is comparable *Nkx2.1* expression in the mutant and wild-type controls at the

embryonic stage of initial tracheal specification, E9.25. The results of this study show that there is reduced proliferation of epithelial and mesenchymal cells in the foreguts of *BMP4* knock-out mice. Interestingly, *BMPR1a* and *BMPR1b* are both important in maintaining the fate of the ventral foregut, via *Nkx2.1* (Domyan et al 2011). These studies indicate that while *BMP4* is essential for the normal outgrowth of the trachea, BMP signalling via these receptors is required earlier, possibly through other ligands. Another mouse mutant which lacks a trachea is the *CV2 (crossveinless-2)* mutant. CV2 is a feedback inhibitor of the BMP pathway, discovered to genetically interact with BMP4 in formation of crossveins in *Drosophila* wings [29]. Although this gene's function is less known in murine tracheal development, it may be that BMP4 is upstream of CV2 in tracheal development, as CV2 has a role in the maintenance of BMP4 gradients during mouse vertebral development (Figure 2B, middle). Together, this confirms the importance of BMP signalling, via various downstream targets, in the outgrowth stage of normal tracheal development.

2.3. Cartilage differentiation

Initiation of the cartilage condensation: Sox9 and Col2a1: Sox9 is expressed throughout the developing trachea at the stages when cartilage formation occurs, beginning at E9. Induction of the cartilage specific collagen gene *Col2A1* is *Sox9*-dependent and its initial expression is detected a day later than *Sox9*. These chondrocytes then proliferate and migrate along the lateral and anterior trachea, in a periodic pattern, eventually forming the mature tracheal cartilage rings [3]. The expression of *Col2A1* is uniform at E12.5, and becomes periodic at E13.5 to E14.5 in the ventral mesenchyme of the trachea [30]. *FGF10* expression is detected in the tracheal mesenchymal at E12.5 becoming segmented by E14.5. When *Fgf10* is knocked out in mice, the segmented pattern of expression is disrupted at E13.5, resulting in shortened and misformed cartilage rings, due to failure of the mesenchymal cells to condense [30]. This appears due to reduced proliferation of cells, rather than differentiation.

At E13.5, *Shh* expression is reduced in the *Fgf10* mutant trachea. This reduction in periodic expression of ventral *Shh* in the $Fgf10^{-/-}$ trachea is associated with an absence of periodicity of type II collagen expression [30]. This places both FGF10 and Shh upstream of *Col2a1* during chondrogenesis. These studies also show that *Shh* is required for a second step, during the maintenance of cartilage differentiation. As for FGF10, the level of *Shh* expression needs to be within a critical range for proper patterning of tracheal cartilage indicating that neither SHH nor FGF10 alone is sufficient as an instructive signal for cartilage formation, in fact, both are dependent on each other, and act via epithelial to mesenchymal signalling on order to produce tracheal cartilage. No changes in *Bmp4* and *Sox9* have been noted in FGF10 mutants.

3. What have mouse models taught us?

Tracheomalacia mouse models: The *Tmem16a* mouse mutant has a malacic airway, in which the cartilage ring incompletely forms, with ventral gaps between the segments [31]. The multiple cartilaginous rudiments formed along the trachea are the result of abnormal condensations during chondrogenesis. Although a defect of this type has not been identified in humans, this study may still help increase knowledge of the epithelial to mesenchymal interactions involved in the pathophysiology of tracheomalacia. For example, *Tmem16a* is expressed in the tracheal epithelium,

as opposed to most of the other chondrogenic genes discussed above, which are expressed in the mesenchyme.

Trps1 knock-out mice are also reported to have cartilage nodule formation rather than the C-shaped rings and appears to regulate chondrocyte proliferation [32]. *Trps1* is a causative gene in trichorhinophalangeal syndrome, which affects the orofacial cartilages. *Trps1* mutation in mice leads to respiratory failure at birth due to the malacic airway. In both examples above, the condensation stage or the proliferation stage is affected in tracheal chondrogenesis, which then results in the tracheal malacia defect in the mouse models.

Mouse model for tracheal cartilaginous sleeve: Ordinarily, FGF10 is expressed segmentally in the tracheal mesenchyme, in the areas between the presumptive cartilage [30]. FGF10 appears to control the periodicity of cartilage rings via SHH [30]. Over-expressing *Fgf10* in a mouse model of Apert Syndrome caused the TCS type of defect via ectopic expression of *Fgfr2b* throughout the mesenchyme [33]. Another mouse model demonstrating tracheal cartilaginous sleeve is Crouzon syndrome, a related craniosynostosis disorder which carries a gain-of-function mutation in FGFR2c [17]. Similarly application of FGF18 to cultured mouse tracheas resulted in an increase in the cartilage marker *Sox9* (Figure 2B bottom) [34]. Thus, both SHH and FGF signalling networks are implicated in the overproduction of cartilage in the tracheal cartilaginous sleeve phenotype.

Mouse model for tracheal stenosis: Traf4, a TNF receptor pathway protein, is an intracellular signalling molecule. *Traf4* has been shown to be required for normal tracheal development, as *raf4* deficient mice have a stenotic trachea with a reduced diameter. The cartilage is disrupted frontally, and segments are laterally fused. As the *traf4* gene is expressed throughout the tracheal epithelium, it may be that the cartilage defects are a result of abnormal signalling from the epithelium to the surrounding mesenchyme [35]. To our knowledge, this is the only single gene model which has the tracheal stenosis phenotype.

4. Conclusions

This review has highlighted how normal tracheal anatomy can be affected at many stages development. The different types of pathology reflect the intricacy in normal development and function of respiratory organs. The spectrum of disease suggests that multiple factors are involved in tracheal ontogenesis; many of these have been identified, and some important time points in the context of murine development have also been identified. Mouse models for each particular syndrome or tracheal abnormality can be developed and used to determine what genes are implicated in the aberrant development of the trachea.

We can establish from numerous studies that tracheal development is reliant on signals from the developing epithelium to the mesenchyme and vice versa. It is also clear that there is a requisite amount and time for the expression of some molecular factors. Some genes are turned on early in the initial specification stage, and they can then recruit other factors for subsequent development of the airways. By knocking out one gene we can try to establish its specific role, however there is not a clear way of knowing the effect on its downstream targets. What we also do not know is the specifics of how development goes wrong to produce the defects we see in the clinic. There are also likely to be other factors involved; for example, recent studies have shown that the smooth muscles are also necessary for cartilage development in the trachea [36].

One further question that arises during the study of congenital tracheal abnormality is whether there are any environmental factors involved. Teratogens, such as the injection of adriamycin in rats, have been shown to cause tracheal defects such as TEF/OA and tracheal agenesis [37]. Could there be some interplay between genetic and environmental factors which cause the syndromes to occur?

It is difficult to say which are the most important genes, as none of the tracheal defects discussed above can be explained by a single gene disorder. Occurring usually as part of sporadic syndromes, it is difficult to categorise tracheal defects into a system based on genetic aetiology. It is evident that loss of some genes and signalling factors in mice may recreate the tracheal pathology that has been encountered in human patients; however, this does not explain what causes the disease in the first instance. Abnormal tracheal samples could be stained for genes/proteins known to be important in cartilage development and may direct us to the causative process of the disease in question. With recent advances in genetic testing and human induced pluripotent stem cells, we now have a great opportunity to learn from affected individuals.

Future Perspectives: Bioengineered windpipes may provide promising evidence of success in some patients [38]. The only successful tracheal transplant carried out on a patient using a tissue-engineered trachea seeded with autologous epithelial cells and chondrocytes of mesenchymal stem-cell-origin is proving successful even at 5 year follow up [4,38]. However, this procedure was carried out on a patient who had acquired tracheal disease, of infective origin. The tracheal defects discussed in this work are mainly congenital anomalies which often occur as part of a syndrome with other accompanying complications. Therefore, it is unclear whether bioengineered organs can play a curative role in congenital airway disease. Nevertheless, it is important to be able to understand the molecular development of the tracheal cartilages, which can then be applied to the reconstruction of tracheal tubes or breathing support devices.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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