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Anatomy and embryology of tracheo-esophageal fistula

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ABSTRACT

Anomalies in tracheo-esophageal development result in a spectrum of congenital malformations ranging from, most commonly, esophageal atresia with or without trachea-esophageal fistula (EA+/-TEF) to esophageal web, duplication, stricture, tracheomalacia and tracheal agenesis. Despite the relative frequency of EA, however, the underlying etiology remains unknown and is likely due to a combination of genetic, epigenetic and environmental factors. In recent years, animal models have dramatically increased our understanding of the molecular and morphological processes involved in normal esophageal development during the key stages of anterior-posterior regionalization, dorsal-ventral patterning and morphogenic separation. Moreover, the use of animal models in conjunction with increasingly advanced techniques such as genomic sequencing, sophisticated live imaging studies and organoid models have more recently cast light on potential mechanisms involved in EA pathogenesis. This article aims to unravel some of the mysteries behind the anatomy and embryology of EA whilst providing insights into future directions for research.

Introduction

Anatomically, the esophagus and trachea are two distinct tubes intimately related along their course from the neck to the abdomen, arising after morphogenic separation of the common foregut endoderm at 25-35 days in humans. Despite sharing a common embryological origin, their functions vary distinctly, reflected by their respective architecture. The esophagus consists of a stratified squamous epithelium interspersed with submucosal glands and surrounded by a bi-directional circular and longitudinal muscle layer. The epithelium provides lubrication and acts as a barrier against injury during the passage of food and the muscle co-ordinates peristalsis to propel the food bolus to the stomach. Conversely, the trachea has a pseudostratified, columnar epithelium composed of ciliated, secretory and basal cells. This is surrounded by C-shaped ventral cartilaginous rings joined dorsally by smooth muscle, the trachealis, providing both structural integrity and elasticity required for efficient gas exchange. Given their common developmental origin, it is no surprise that abnormalities in tracheo-esophageal (T-E) organogenesis give rise to abnormalities affecting both the esophagus and trachea. The most common of these is esophageal atresia (EA), a congenital malformation where there is a partial or complete interruption of esophageal continuity in approximately 1 in 4000 live births.[1] Rarer defects of faulty T-E separation also exist under the broader banner of tracheal-esophageal defects (TEDs) including tracheal agenesis and laryngo-esophageal clefts (LETC) which likely have similar underlying etiologies (Fig. 1).

Despite the relative frequency of EA, the etiology is not well understood. Approximately 10% of EA cases have been shown to have a genetic basis across a spectrum of over 70 defined genetic syndromes. These include single gene mutations, structural chromosomal anomalies and copy number variations.[2] Additionally, over 50% of patients with EA have at least one other congenital abnormality in organ systems with differing developmental pathways.[1] This makes it likely that the etiology of EA is multifactorial; the result of mutations in genes with pleiotropic effects on developmental pathways in conjunction with epigenetic and environmental exposures.

Animal models, and more recently single cell sequencing techniques, have been instrumental in developing insights into the molecular and cellular processes involved in normal TE morphogenesis and subsequently EA pathogenesis. Key signaling pathways and transcription factors have been shown to co-ordinate the sep-

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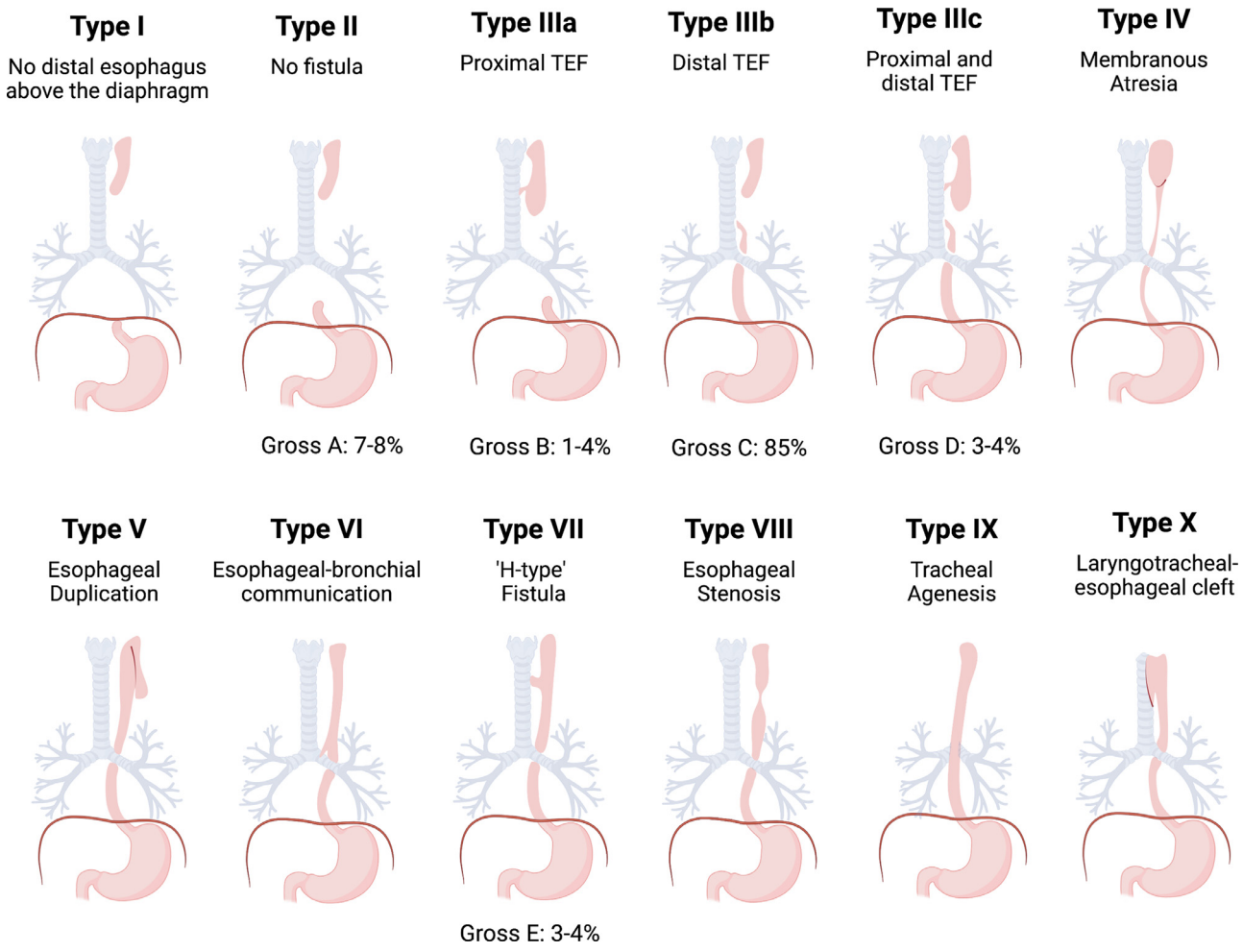

Fig. 1. Variants of Tracheo-Esophageal Defects – Adapted from Kluth, 1976.

aration of the foregut endoderm into the trachea and esophagus in the 4-5th week of development. The four sequential stages of this process (anterior-posterior patterning, dorsal-ventral patterning, T-E separation and maturation and elongation) are mediated through changes in the spatial-temporal expression of six intertwined molecular pathways acting on both the endoderm and mesoderm; Bone Morphogenic Protein (BMP), Wingless-related integration site (Wnt), Sonic Hedeghog (Shh), Retinoic Acid (RA), Fibroblast Growth Factor (FGF) and Notch. Disruption in these patterning processes in animal models commonly result in EA/TEF phenotypes. However, aberrations in the same genes in humans often do not correlate with human EA/TEF. Similarly, the significance of novel mutations found in human genomic studies of EA patients remain largely unknown. This article aims to describe our current understanding of normal trachea-esophageal development and how our understanding of this can inform and direct new lines of enquiry into the basis of EA formation in patients using the most recent evidence from animal models and emerging technologies.

Anatomy, classification and associated anomalies of EA/TEF

EA is characterized by interrupted continuity of the esophagus with or without an aberrant communication with the trachea. Prevalence varies in reported case series however a recent European registry estimated it to be 2.43/10,000 births with a slight male predominance.[1] The original classification system was first described by Vogt in 1929, redeveloped by Ladd in 1945 and revised by Gross in 1953 which remains the predominant system in use today (Fig. 1). By far the most common variant is Type C (82-85%), where a distal fistula connects the esophagus to the trachea. The absence of a fistula, Type A, also known as 'long-gap OA,' is found in 7-8%. Finally, those with proximal, double, or H-type fistulas (corresponding to Gross type B, D and E) decrease in incidence respectively.[3] Whilst these are the most recognized subtypes of EA, the full spectrum of congenital esophageal malformations is far more extensive. In 1976, Kluth produced an extremely comprehensive 'Atlas of Esophageal Atresia' referencing a total of 96 malformations categorized into 10 major groups by referencing cases cited as far back as 1670.[4] The classically described groups are further divided into all phenotypes of that variant ever described in the literature; Type C EA, for example, had twenty different morphological descriptions. In addition, more unusual variants of esophageal malformations are described including membranous atresia, partial or complete esophageal duplication, esophageal-bronchial communications, stenoses, tracheal atresia and laryngeo-esophageo 'fissures' (clefts). Whilst its complexity precludes practicality as a routinely used classification system, this atlas indicates the huge heterogeneity in the spectrum of esophageal and tracheal malformations, indicating that a single genetic cause is unlikely to be the etiology.

Clinical presentation varies depending on phenotype of EA. Those without a distal fistula are more likely to be detected antenatally due to the presence of a triad of classic ultrasound (USS)

findings including polyhydramnios, a persistent small or absent stomach and a distended proximal esophageal pouch. If there is an ongoing connection between the stomach and the trachea, however, the stomach fills with amniotic fluid and antenatal detection rates are lower; one series of 76 EA/TEF cases found polyhydramnios in 60% of those with a fistula, but an esophageal pouch and absent stomach in only 22% and 27% respectively. No USS findings indicative of EA were found in 36% of all patients.[5] More recently, MRI has started to play a major role in confirmation of prenatal diagnosis once suspected at ultrasound.[6] Where not detected antenatally, presentation is usually in the first days of life with excess saliva, coughing and gagging particularly during feeds. The diagnosis is confirmed by failure of passage of a nasogastric tube.

Associated congenital defects are reported in 55% of patients with EA. The VACTERL association is the most commonly recognized constellation of anomalies; defined as the presence of three or more malformations in vertebral, anorectal, cardiac, tracheoesophageal, renal and limb systems. This was found in 9.6% of EA/TEF patients in the European registry of 1,222 patients.[7] Singular associated anomalies were found in 31.6% of patients, with cardiac being the most common in 29.4%, urinary tract anomalies in 16.4% and other gastrointestinal abnormalities including duodenal atresia and pyloric stenosis in 15.5%.[1]

In addition to VACTERL associations, there are many tracheal anomalies which occur in association with or independent of EA due to the common developmental origin of the two organs. The most common of these is tracheomalacia where abnormal cartilage development results in a soft trachea leading to airway collapse with changes in pressure.[8] The incidence of primary tracheomalacia is 1 in 2100 children,[9] however this is reported to be as high as 11-33% in children with EA.[10,11] Interestingly, a study of 40 patients with TEF in 1979 showed only 10% had histologically normal tracheal cartilage with a deficiency in 75%.[12] Laryngeal tracheoesophageal clefts (LTEC), whereby a posterior sagittal communication exists between the larynx and the pharynx due to failure of separation of the foregut tube, also appear to be more prevalent in EA patients. Severity is related to the degree of downwards extension of the cleft; with the communication in type I at the supraglottic level whereas type IV extends into the thoracic trachea and esophagus. Estimated overall incidence is reported at 1 in 10,000-20,000 live births,[13] however this is as high as 19.6% in one large series of EA patients, again indicating a common underlying aetiology.[14,15] Much rarer but clinically highly significant associated pathologies include complete tracheal ring deformities leading to tracheostenosis, tracheal cartilagenous sleeve, and congenital high airway obstruction syndrome (CHAOS) and tracheal atresia/agenesis, which are incompatible with life unless a surgical airway is immediately established at birth. Genome sequencing of patients with many of these conditions have shown aberrations in molecular pathways associated with hedgehog, WNT and FGF signaling, all genes known to be key mediators in tracheoesophageal separation.[16–18] This not only explains the increase in incidence of congenital tracheal malformations in the EA/TEF cohort but also highlights the importance of clinical suspicion and investigation of these anatomical abnormalities in EA/TEF patients with ongoing respiratory symptoms after surgical repair.

Finally, vascular anomalies have also been reported in up to 18% of EA patients in one cohort. These range from right sided aortic arch (RAA) to aberrant right and left subclavian arteries (ARSA/ALSA). The incidence of vascular malformations appears to be significantly higher in patients with long-gap EA or those with associated cardiac abnormalities, again rsuggesting that a common developmental abnormality is at play.[19,20] The anatomical combination of a RAA with ALSA forms a complete vascular ring due to the presence of the ductus on the left and results in dysphagia and respiratory distress in over 50% of patients. ARSA forms an incomplete vascular ring and as such is frequently asymptomatic and often undiagnosed as it is not visualized in the operative field. However, extrinsic posterior compression can lead to symptoms, particularly where the esophagus and trachea have reduced underlying rigidity, leading to dyspnea, recurrent cough, aspiration, and exacerbation of dysphagia.[20] Fistula formation between aberrant vessels and the esophagus after stent or NG tube placement has been reported so whilst incidence is rare, recognition of these anatomical variants is important.

Normal embryogenesis of the trachea and oesophagus

Since the early twentieth century, clinicians have speculated about the processes behind tracheo-esophageal development, recognizing early on that an understanding of this may hold the key to the pathogenesis behind EA.[21] As such, we now have a clear understanding of the four sequential processes involved. Firstly, **anterior-posterior patterning** of the primitive gut tube regionalizes it into foregut, midgut and hindgut. **Dorsal-ventral patterning** then leads to lineage specification of the anterior foregut endoderm into tracheal and esophageal fates resulting in **separation of the foregut** into distinct esophageal and tracheal tubes. Finally, the respective organs undergo **elongation and maturationo** with an increase in diameter. These four stages are mediated by the complex interplay of several signaling cascades between the foregut endoderm and its surrounding mesoderm in key regulatory pathways. These lead to the cellular and morphogenic processes required for appropriate tracheo-esophageal separation. Interruption to these signaling pathways has been shown in animal models to result in variants of tracheo-esophageal defects, offering some insights into the underlying etiology of TEF.

Prior to regionalization, gastrulation leads to the development of the three primary germ layers (endoderm, mesoderm, ectoderm) from the blastocyst during week three of human embryo development. The lateral plate mesoderm emerges from the primitive streak and endoderm cells migrate over its outer surface to form a bi-layered, flat endodermal-mesodermal structure. This then folds, converting the flat structure into the primitive endodermal gut tube surrounded by mesoderm. The lateral plate mesoderm splits into two layers; the outer somatic layer which gives rise to limbs and body wall and the inner splanchnic mesoderm (SM) which surrounds the endodermal gut tube. This endoderm gives rise to the epithelial lining and parenchyma of the respiratory and digestive systems including the thyroid, thymus, lung, intestine and biliary system whilst the splanchnic mesoderm gives rise to the mesenchymal tissues such as smooth muscle, fibroblasts and mesentery surrounding visceral organs.[22] Several studies have identified the dose-dependent expression of Nodal, a growth factor, and its downstream signaling pathway as key in initiation of gastrulation; high levels of nodal signaling promote an endodermal commitment with lower levels promoting a mesodermal fate with repression of endodermal expression by inducing the expression of FGF.[23]

Regionalization with AP patterning (E7-8.5)

Reciprocal signaling between the primitive gut endoderm and its surrounding mesoderm begins to regionalize the endodermal gut tube along the anterior-posterior axis into different foregut, midgut and hindgut domains in a process named A-P patterning. Initially, high Nodal levels prime the endoderm to an anterior fate, leading to expression of transcription factors (TFs) essential for subsequent foregut development such as Sox2. Simultaneous secretion of Wnt, BMP and FGF4 induce a posterior foregut identity in the prospective hindgut and suppress expression of foregut TFs. Retinoic Acid (RA) production defines the subsequent foregut-midgut boundary by inhibiting the expression of anterior

genes. Recently, single cell RNA sequencing of the mouse embryonic foregut has identified that the splanchnic mesoderm is the primary source of BMP, FGF, RA and Wnt ligands and as such is responsible for the autocrine and paracrine signaling to the corresponding endoderm which results in A-P patterning.[22] Interestingly, the importance of the mesoderm on organogenesis has been demonstrated as early as in the 1960s, when it was shown that transplanted SM from differing A-P regions resulted in patterning of the endoderm to the organ lineage from the original location of the SM.[24]

Over time, distinct cell populations develop lineage-specific expression of transcription factors which refine these broad foregut, midgut and hindgut domains into more precise regions. The foregut endoderm, for example, ultimately gives rise to the primitive pharynx, thymus, thyroid, respiratory, upper gastrointestinal and hepatobiliary systems. Recently established single cell transcriptomes compared across the entire foregut of mouse embryos have shown that endodermal and mesodermal cells begin to express a continuum of distinct regional transcriptional signatures which overlap with adjacent cells on the AP axis compared to spatially distant cell groups, marking the beginnings of organ specification.[22] Using this technique, groups have identified novel TF involved in these early patterning pathways. Osr1, for example, has recently been identified as a regional TF exclusively expressed by respiratory, esophageal and gastric epithelium and mesenchyme during later stages of AP patterning. As development continues, different cell populations become increasingly more distinct based on refined expression of transcriptional signatures. These distinct endodermal cell populations form organ buds which integrate with their surrounding mesoderm to form primitive organs at precise locations along the A-P axis of the foregut.

The underlying signaling which co-ordinates upper gastrointestinal and respiratory specification in the foregut appears to hinge upon RA. Retinoic acid synthesis enzymes enriched in the lateral plate mesoderm trigger a regionally restricted expression of sonic hedgehog (Shh) from the endoderm. This in turn co-ordinates expression of BMP, FGF and Wnt in the localized mesoderm which specifically promotes a lung fate.[25] Interestingly, cells behave differently in response to the same signaling pathways during different timepoints in development; the day after Wnt, BMP and RA suppress an anterior endoderm fate to promote that of the posterior *hindgut*, they actively direct the *foregut* progenitor cells into different organ lineages.[26]

Dorsal-ventral patterning of the anterior foregut endoderm (E8.5-10.5)

At approximately 22-23 days post ovulation in the human, the naïve anterior foregut endoderm is compartmentalized into two domains along the dorsal-ventral (DV) axis. This process, called dorsal-ventral patterning, is ultimately responsible for separation of the common foregut tube into two distinct esophageal and tracheal tubes. The ventral common foregut endoderm specializes into a respiratory-specific lineage marked by the expression of Nkx2.1, and the dorsal foregut endoderm into an esophageal-specific lineage, marked by the expression of Sox2.[27] Knockout mouse experiments with Sox2 and Nkx2.1 mutants have shown that expression of these two transcription factors mutually repress each other resulting in proper DV patterning.[28,29]

Upstream molecular signaling required for regionalized Nkx2.1 and Sox2 expression in the foregut is predominantly mediated by the regional expression of Shh. Through its downstream modulators in the ventral mesoderm, Gli2 and Gli3, it establishes a gradient of Wnt/BMP expression, high in the ventral endoderm and lower in the dorsal endoderm. This gradient promotes the ventral expression of Nkx2.1 whilst repressing Sox2 expression in the endoderm and therefore a respiratory identity.[25] Simultaneously, low Wnt and BMP signaling in the dorsal foregut is reinforced through the establishment of feedback loops. Noggin, a BMP inhibitor from the dorsal endoderm and notochord, maintains Sox2 expression dorsally which in turn promotes the expression of Wnt antagonists such as Dkk1 and Sfrp1/2, inhibiting Nkx2.1 expression.[30–32] This patterning process completes at E8.5 with the formation of the bronchopulmonary buds at the caudal border of the Nkx2.1+ ventral foregut. RA, Shh, Wnt, BMP and FGF expression continue to expand Nkx2.1+ epithelial progenitor cells and promote invasion of the surrounding splanchnic mesenchyme through downstream FGFR-2 expression.[33,34] Repetitive branching, known as branching morphogenesis, through an FGF-10 mediated mechanism, marks the beginning of lung organogenesis.[35] These findings, identified through knockout models of key genes, have been recently validated by the development of splanchnic mesoderm *in vitro* using induced pluripotent stem cells (iPSCs), where cells such as fibroblasts from adults can be reprogrammed to become pluripotent and then driven to differentiate into specific cell types using molecular pathways identified from developmental animal models. The addition of RA, BMP4 and Wnt to foregut endoderm and mesoderm cells promotes gene expression consistent with respiratory mesenchyme, whereas the addition of RA and a BMP4-antagonist resulted in gene expression profiles consistent with gastric and esophageal identities.[22]

Previously, Nkx2.1 and Sox2 transcription factors were believed to be the earliest and most important markers of differentiation of the foregut endoderm to esophageal and tracheal lineages, thought of as master regulators of tracheal and esophageal fates leading to T-E separation. However, critical lung specific mesenchymal markers such as Tbx4 are still present in the ventral mesoderm of Nkx2.1-null mice indicating that many other as yet undiscovered signaling pathways are at play.[36] The application of single cell RNA sequencing techniques in mice has detected the expression of many previously unidentified respiratory and esophageal lineage-specific genes specifically enriched in the dorsal (e.g Klf5) and ventral foreguts (e.g Tppp3). Their expression both prior to and post foregut separation suggests they play a role in endodermal D-V patterning, although this role remains unknown. Interestingly, many were shown not be influenced by Nkx2.1 expression, suggesting other as yet unidentified TFs likely play a role in subsequent tracheo-esophageal separation.[37] Traditionally, the success of tracheo-esophageal separation, has been attributed to *endodermal* D-V patterning. More recently, however, regional differences have also been identified in the mesoderm of mouse embryos using single cell RNA sequencing techniques. Distinct organ-specific transcriptional profiles are seen in the mesoderm adjacent to and in conjunction with foregut endoderm patterning including Osr1/Hic1 in the esophagus and Sp5/Hoxa5 in the respiratory domains, indicating a patterning process also occurs here.[22] As the mesoderm ultimately differentiates into the smooth muscle and tracheal cartilage surrounding the esophagus and trachea, identification of novel patterning pathways in this layer may start to unravel affected pathways in clinical conditions affecting these such as tracheomalacia.

Tracheo-esophageal morphogenesis (E 10.5-12.5)

After D-V patterning is complete, the common endodermal foregut splits into two distinct tubes; the primitive esophagus and trachea. Whilst the molecular pathways preceding T-E separation have been clearly defined, the actual morphogenic events involved in the separation itself have been the subject of much conjecture with the proposal of many different models. Until the last decade, the outgrowth, septation and watershed models were proposed as mechanisms by which T-E separation occurred. In the septation

model, epithelial mesenchymal ridges are proposed to fuse across the length of the D-V midline resulting in the formation of a T-E septum along the foregut lumen and separation into two tubes.[38] In the outgrowth model, the trachea is suggested to develop as an evagination from the ventral common foregut as a result of rapid growth of the respiratory primordium and lung budswhich elongates distally whilst the remaining common foregut forms the esophagus.[39,40] Finally, the watershed model proposed that the presence of a mesenchymal wedge at the D-V midline prevents caudal growth of the foregut as a singular tube resulting in growth of two tubes distal to this point. However, all these theories have flaws. In 2010, Ioannides et al showed the common foregut decreases in absolute length during T-E separation, with no evidence of increased proliferation at the origin of the trachea compared to the common foregut. This indicates that the formation of the trachea and esophagus is due to separation of one tube rather than proliferative growth of one from the other, essentially disproving the outgrowth and watershed models.[41] The finding of the foregut shortening was confirmed in scanning electron microscopy of chick embryos, which also showed no evidence of lateral ridges forming a trachea-esophageal septum proposed in the septation model.[42]

In 2015, a new model of 'splitting and extension' was proposed based on findings from live imaging of actively separating cultured mouse foreguts *in vitro* using fluorescent labelling of Sox2 cells. A 'saddle-like' epithelial structure was identified at the distal end of the foregut at the site of lung bud origination which moves in a caudal to cranial direction, splitting the foregut in two until the level of the pharynx. Simultaneously, caudal elongation of newly formed tracheal and esophageal tubes occurs. Interestingly, mesenchymal cells were seen to migrate away from the epithelium, suggesting that rather than forming a wedge as proposed in the watershed model, TE separation may be predominantly driven by epithelial cells.[43]

In 2019, Nasr et al were able to demonstrate events occurring at a cellular level during T-E separation using wholemount confocal imaging of both xenopus and mouse embryos which has led to a much clearer understanding of the processes involved.[44] Four key cellular events appear to occur; **medial constriction** at the boundary of Sox2/Nkx2.1 epithelial cells which fuse forming a **transient septum, remodeling of the septum** and finally **mesenchymal invasion** which separates the trachea and esophagus into two tubes (Fig. 2). The constriction of the foregut lumen at the Sox2/Nkx2.1 boundary is due to both an increased proliferation of midline Foxf1+ mesoderm and a corresponding localized thinning of the epithelial cells. This process appears to be initiated by the mesenchyme, because when this is removed, medial constriction does not occur. As this midline constriction occurs, the two opposing epithelial cell walls touch and adhere to form a short, transient epithelial septum. Endosome recycling and degradation of the basement membrane by matrix metalloproteinases results in new polarity of the epithelial cells from the common foregut to incorporation into the esophageal or tracheal epithelium. Concomitant migration of Foxf1+ mesenchymal cells result in an invasion of the bi-layered epithelial septum, forming the trachea and esophagus. Interestingly, for the first time, it was reported that the epithelial cells which fuse specifically co-express Sox2 and Nkx2.1. This was corroborated by Kim *et al* who showed that a small number of Nkx2.1+ cells incorporate at the ventral aspect of the esophagus after separation and a small number of Sox2+ cells incorporate on the dorsal aspect of the trachea, although expression is transient.[45] The length of this dual-positive boundary appears to decrease cranially as the length of the newly formed trachea and esophagus increase distally, confirming caudal to cranial orientation of this process and that the 'splitting and extension' model most likely represents the underlying morphological process of TE separation. In the last two years, EPHRIN-B2 and the transcription factor Isl1 have been shown to be integral to the development of the Sox2/Nkx2.1 boundary and have started to identify how key regulators of dorsal-ventral patterning actually result in tissue separation.[45,46]

Lengthening and maturation (E12.5-18.5)

After separation, at around 4-5 weeks of human gestation, both the trachea and esophagus undergo elongate and widen with maturation of epithelial and mesenchymal layers. In the trachea, Wnt signaling initially promotes tracheal elongation whilst restricting diameter expansion by determining smooth muscle cell polarity.[34] From E14.5, formation of cartilage rings result in an expansion of the tracheal diameter. In animal models, disruption of Sox9 results in absence of tracheal chondrocytes and failure of tracheal diameter increase, potentially leading to human developmental anomalies such as tracheostenosis. Interestingly, the formation of cartilage has also been shown to influence tracheal epithelial proliferation. As such, disruption to the stiffness or differentiation of the cartilage as seen in human conditions such as tracheomalacia may also influence the integrity of the tracheal epithelium. The esophageal epithelium begins as a single layer of columnar cells. At 11 weeks, a transient subpopulation of ciliated cells develops until 17 weeks where these are lost and the whole epithelium is replaced by a non-keratinized, stratified squamous epithelium with progenitor basal cells and a fully differentiated superficial layer.[47] Residual islands of columnar epithelium grow into the mesenchyme to form submucosal glands.[48] The mesenchyme gives rise to the muscularis mucosae and muscularis externa, in which the proximal third is composed of skeletal muscle and the distal two-thirds of smooth muscle. Mouse and human embryonic studies suggest the entire esophageal mesenchyme begins as smooth muscle with subsequent conversion to skeletal muscle from the cranial to caudal direction regulated by Foxp1 and Foxp2 expression.[49]

A fine balance of BMP, Noggin and P63 signaling are instrumental in epithelial maturation. High BMP expression prevents the transition from columnar to squamous epithelium, however high levels of its antagonist Noggin promotes its formation but prevents subsequent differentiation.[50,51] Sox2 is also required for the ongoing organization of the esophageal epithelium; downregulation results in a disorganized epithelium, reduces commitment to a squamous cell fate and enhances mucin production.[28] Recent studies have suggested that these TFs play crucial roles in the ongoing maintenance of stratified squamous epithelium in adults, long after their role in its development. Many responsible for epithelial maturation and homeostasis have also been implicated in the pathogenesis of EA/TEF, potentially explaining why EA patients are more predisposed to pathologies of the esophageal epithelium such as Barrett's esophagus than the background population.

Models of EA/TEF: lessons from animal models

Whilst significant advances have been made in the understanding of normal tracheo-esophageal development, a fuller understanding of abnormal T-E morphogenesis remains elusive, due to the rarity of EA and a lack of human embryos to study at such an early gestation. Over the past 40 years, animal models have been instrumental in developing a greater insight into the developmental basis for tracheo-esophageal defects in humans. The similarity in mouse and xenopus tracheo-esophageal development is remarkable and has therefore been used as a proxy to study underlying processes in human T-E morphogenesis.[44] Initial attempts to produce surgical models of EA by esophageal ligation or hyperflexion of chick embryos in early development were largely unsuccessful as they did not reflect the full spectrum of EA malformations.

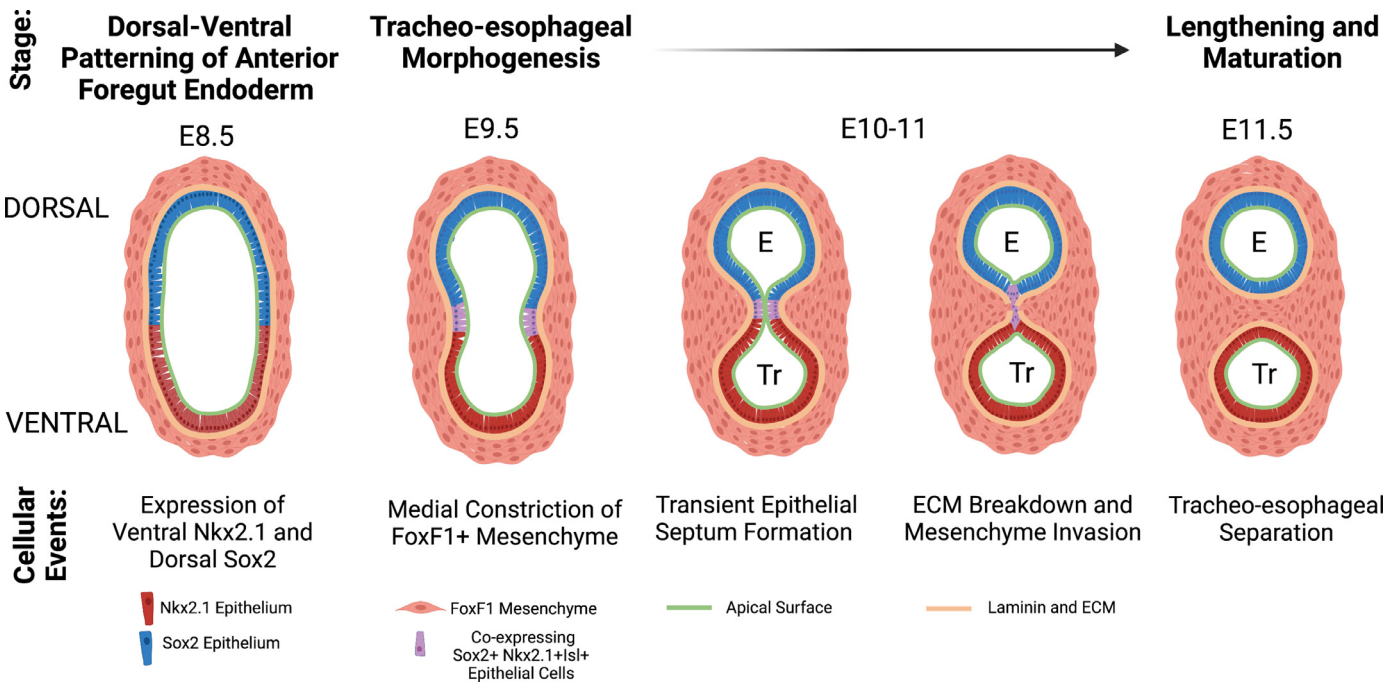

Fig. 2. Cellular morphogenesis of T-E separation: adapted from Nasr et al., 2019.

Their fundamental flaw was an assumption that EA was a purely structural abnormality, neglecting the huge influence of molecular and cellular changes in the etiology.[52,53] Subsequent studies showed vitamin A- and riboflavin-deficient diets in pregnant rats led to isolated TEF and EA respectively, but not consistently, giving some clues as to possible pathways which have subsequently been shown to be involved in esophageal embryogenesis (e.g. retinoic acid).[54]

Exposure of fetal rats to Adriamycin resulted in the first reproducible animal model of EA/TEF leading to a spectrum of T-E malformations including Type C EA in up to 45% of fetuses.[55] Interestingly, a high incidence of VACTERL associations were also found in these models including DA, ARM, renal and limb malformations. This provided the first platform used to identify molecular and cellular processes involved in T-E development. Using this model, Crisera et al highlighted the presence of Nkx2.1, at the time a transcription factor known to be important for *lung* development, in the esophageal fistula of Adriamycin treated fetuses. Although this finding was subsequently contradicted in an Adriamycin mouse model, this was the first suggestion that an imbalance in respiratory and gastrointestinal fates may lead to EA.[56,57] Further work began identifying several potential molecular pathways implicated in the development of Adriamycin-induced malformations including Shh, Gli-2 and FGF signaling.[56,58] This model was also used to identify differences in cellular processes between Adriamycin-TEF and control rodents, identifying a potential role of disturbed apoptosis in EA pathogenesis.[59,60] The precise mechanism by which Adriamycin induces EA/TEF in animal models is unknown and it is important to note that Adriamycin exposure at high doses does not result in EA in humans. Frequently, however, a spectrum of notochord abnormalities have also been noted in Adriamycin TEF models, including ectopic positioning, abnormal morphology or tethering to the foregut.[61] The notochord plays a key role in patterning of the neural tube and coordinating hepatic specification and is known to express Shh.[62] This has led to the suggestion that notochord abnormalities may be integral to the development of EA in Adriamycin animal models, due to ectopic and disrupted Shh signaling leading to a disturbance in D-V patterning.[63]

The use of the Adriamycin animal model has largely been surpassed by the advent of genetic knockout mutant mice models in the 1990s. Disrupting gene loci by partial or complete knockout results in production of non or hypo-functioning proteins, allowing investigation of these on signaling pathways known to be involved in T-E embryogenesis. The first identification of an EA mouse model was serendipitous. During lung development, Nkx2.1 is expressed in all epithelial cells and suppression leads to disruption of branching morphogenesis. The creation of an Nkx2.1 mouse knockout model to investigate this resulted in postnatal death in all mice. Three years later, Minoo et al studied Nkx2.1-/- mutant embryos at an earlier stage and found they not only had markedly dysmorphic, hypoplastic lungs, but also a complete failure of separation of the foregut tube. The foregut was short, wide and led directly to the stomach with the bronchi emerging from the ventral side in what would be classified in humans as tracheal agenesis.[27] Interestingly, the entire foregut was composed of smooth muscle with a complete absence of tracheal rings, a finding later substantiated by other groups who have found that Nkx2.1 appears to control several downstream genes which contribute to cartilage, rather than smooth muscle, formation.[28,37] They went on to find that in wildtype embryos, Nkx2.1 is only expressed on the ventral aspect of the foregut endoderm prior to separation, leading to the discovery of dorsal-ventral patterning in the foregut. The development of Sox2-null mutants to identify their role in D-V patterning was fatal prior to gastrulation. Subsequent generation of mutant mice with hypomorphic Sox2 alleles, however, resulted in a type-C EA in 60%. The remaining embryos with 'successful' T-E separation exhibited variable esophageal diameters which diminished altogether in some, similar to that seen in the human type A variant.[28] Further interrogation of the relationship showed the phenotype is dose-dependent on the expression of Sox2. When Sox2 expression fell below a threshold, ectopic expression of dorsal Nkx2.1 occurred with 'ventralization' of the foregut leading to incomplete separation and a 'trachealization' of the distal TEF expressing NKx2.1 positive cells and a tracheal phenotype. Interestingly, the Sox2 pathway is one of the only genetic pathways shown to be associated with a human phenotype of EA; heterozygocity in

humans is associated with Anopthalmia-Esophageal-Genital (AEG) syndrome. Nasr et al recently showed the failure of T-E separation in Nkx2.1 and Sox2 mutant mice is due to a failure of medial constriction of the mesoderm during the T-E separation phase, arresting all subsequent separation and resulting in the common foregut tube phenotype seen.

As seen above, the relationship between gene knockouts and their effects is not linear. The severity of the phenotype is dependent on multiple factors; how crucial the transcription factor is to the T-E separation process, whether the protein loss is complete or partial, or how far downstream the molecule is in the signaling cascade. Interestingly, the majority of animal models with a loss of signaling in RA, HH, Wnt2 or BMP pathways are too fundamental to T-E separation resulting in the often-lethal combination of a single undivided foregut and lung hypoplasia.[64,65] Partial, downstream loss of these signaling cascades, however, often results in normal D-V patterning but less severe phenotypes of the full trachea-esophageal defects spectrum including esophageal stenosis, tracheomalacia, and laryngotracheal clefts (Table 1). The Shh-Gli2/3-Foxf1 pathway demonstrates this. Complete loss of Shh or Gli2 and 3 results in a common foregut tube (tracheal agenesis), whereas one copy of Gli3 in a Gli 2-/-;Gli3+/- model is enough to support D-V patterning but not separation; medial constriction is initiated however insufficient to allow the two epithelial walls to touch leading to a failure in separation. Further downstream, loss of FoxF1 expression allows for complete separation but results in esophageal stenosis or mild LTECs.[25,44] Interestingly, Gli2/3 null mutants have a worse phenotype than its upstream Shh null mutant, suggesting other as yet unidentified signaling axes influence downstream effects of Gli, highlighting the complexity of signaling pathways.

Other than hypomorphic Sox2 mutants, the only other genetic models which have reliably produced a TEF phenotype like that seen in humans have been in Noggin and Isl knockouts. Loss of Noggin results in increased Sox2 expression and a type C TEF in 70-82% of homozygotes. Simultaneous knockout of BMP7 in these models rescued the TEF anomaly, highlighting the potential role of Noggin-mediated BMP antagonism in EA/TEF pathogenesis and more broadly, how disruption of the fine balance of agonists and antagonists in D-V patterning can lead to EA. Interestingly, as in the Adriamycin model, the Noggin-/- model resulted in several notochord abnormalities including delayed detachment, again implicating a potential role for notochord-mediated signaling in EA pathogenesis.[66] Finally, in 2019, Kim et al made the novel discovery of the role the transcription factor Isl1 plays in T-E separation. Isl1 null embryos die at E10.5 due to severe cardiac abnormalities, however the development of mutants with selective loss Isl1 in the ventral foregut endoderm at E9.5 resulted in a Type C TEF phenotype in 50%.[45] Interestingly, it also resulted in fusion of lung lobes but normal epithelial and alveolar differentiation, a phenotype occasionally seen in EA patients with horseshoe lung.[45,67] Significantly, Kim et al identified Isl1 is co-expressed in the specialized midline dual-positive Nkx2.1/Sox2+ epithelial cells seen at the D-V boundary previously reported by Nasr et al in 2019. When Isl1 was selectively lost in these midline epithelial cells, TEF was seen in 100% of embryos, suggesting Isl1 is critical for T-E separation and may also be key to the pathogenesis of EA/TEF. Of note, chromosomal deletions in 5q11.2, the region encompassing Isl1, have previously been described in patients with abnormal T-E separation.

In addition to attempting to understand the etiology of EA, the genetic and molecular pathways identified in animal models also have a clinical relevance. A recent systematic review reported that the prevalence of respiratory and gastrointestinal symptoms in adolescent and adult patients is astonishingly high; recurrent respiratory tract infections, cough, wheeze and asthma were seen in 24, 14, 34 and 22%, respectively.[68] The basis for respiratory tract disease in EA survivors is multifactorial. Although respiratory symptoms in EA patients may be accounted for by tracheomalacia, gastro-esophageal reflux, prematurity or surgical complications such as stricture, up to 75% of adult survivors have obstructive and restrictive changes to their respiratory function not related to these conditions.[67] Additionally, structural malformations including horseshoe lung and pulmonary agenesis/hypoplasia reported in EA animal models have been reported in EA patients.[69] Several of the animal models of EA have simultaneous anomalies of lung lobulation, branching morphogenesis and epithelial differentiation, reflecting how aberrations in transcription factors and signaling pathways involved in T-E separation also influence subsequent lung development. It is likely, therefore, that the specific genetic changes leading to the development of EA likely also contribute in part to the underlying and ongoing lung pathologies seen clinically in EA patients.

Similarly, the esophageal epithelium in patients with EA appears to have differences to that in non-EA patients. The prevalence of Barrett's esophagus is four times higher in EA patients than in the background adult population, with a 108-fold increase reported in esophageal squamous cell carcinoma.[70,68] Genes known to be important in the morphogenesis and maturation of the developing esophageal epithelium such as Sox2 and p63 have also been shown to play an ongoing role in adult epithelial homeostasis.[51,71] A reduction in Sox2 protein levels and increased BMP signaling have been found to be associated with the development of Barrett's esophagus in non-EA patients.[72] Similarly, gene amplifications of Sox2 have been found in 30% of squamous cell carcinomas.[73] Finally, eosinophilic esophagitis, reported to be found in up to 11% in the EA population, has recently been linked to abnormalities in the FoxF1 gene, associated with esophageal stenosis and LTEC in mouse models, and BMP, known to cause tracheal agenesis.[71,74–76] Taken together, these results indicate that aberrations in the pathways shown to result in tracheal-esophageal defects in animal models which may also be disrupted in human EA, probably result in impaired homeostasis of the epithelium into adulthood and may explain the predisposition of EA patients to chronic esophageal and respiratory dysfunction.

Morphological theories of EA/TEF formation

How these molecular and transcriptional changes described above actually result in the morphological changes seen in EA/TEF remains to be seen. Previous suggestions such as a localized failure of the formation of the tracheo-esophageal septation and failure of tracheal outgrowth were intrinsically linked to old models of tracheo-esophageal morphogenesis which now appear to be disproved. The suggestion of a vascular event resulting in an atretic upper pouch with a compensatory TEF to the stomach appears to have little grounding.[38,39,77] In 2016, in conjunction with the suggestion of the splitting and extension model, Que suggested that a constriction in the esophageal endoderm may result in a 'roadblock' of the ascending saddle during the T-E separation process with the development of a second wave of caudal to rostral movement above the first resulting in the TEF rather than a completely unseparated foregut tube. They suggest two proposed mechanisms for this phenomenon. Firstly, abnormal D-V patterning could shift the Nkx2.1/Sox2 boundary and so block the movement of the saddle. Secondly, they propose the abnormal detachment of the notochord from the early endoderm, as seen in models of EA such as in Noggin null or Adriamycin treated mice, could result in inappropriate removal of endodermal cells leaving too few cells to establish the transient esophageal septum, halting the progress of the saddle rostrally.[2,43] Ultimately, there is currently limited evidence for either of these morphological theories however this may become

Table 1
Mouse knockout models of tracheo-esophageal defects. Signalling pathways and specific genes implicated in EA/TEF aetiology are shown with mechanism, associated defects and human equivalents listed where known. Those in bold indicate the finding of a clinically similar TEF phenotype in mouse models to that seen most commonly in humans (type C).

Pathway	Gene	Animal	Expression Location	Mechanism	T-E Phenotype	Additional findings	Incidence	Lethality	Human Counterpart	Reference
Transcription Factors	Nkx2.1-/-	Mouse	Epithelium	Failure of patterning: Single undivided Sox2+ foregut	Single Sox2+ foregut tube	Small, hypotrophic lungs	100%	100% post natal	Choreoathetosis, hypothyroidism and respiratory distress, no EA	Minoo 1999
	Sox cond (30%)	Mouse	Epithelium	Failure of patterning	**Type C TEF in heterozygotes**	Trachealisation of fistula, dysmorphic anterior stomach with ectopic mucin production	60%	100% post natal	AEG syndrome	Que 2009
	ISL1 cond	Mouse and xenopus	Ventral epithelium	Loss of Nkx2.1 expression in midline epithelial cells at separation boundary	**Type C Tef**	Cardiac and neural abnormalities, lung lobe fusion	50%-100%	100% post natal	5q11.2 deletion in EA/TEF patients overlying Isl1 region (de Jong 2010)	Kim 2019
HH Signalling	Shh-/-	Mouse	Ventral epithelium	Correct patterning, reduced Nkx2.1 and Foxf1, failure of medial constriction	Juxtaposed stenotic trachea and oesophagus with continuous common lumen, absent smooth muscle	Hypoplastic single-lobe lungs	100%	Not described	Holoprosencephaly	Litingtung 1998, Pepicelli 1998, Nasr 2019
	Gli2-/-	Mouse	Mesenchyme	Reduced Gli1 expression	Hypoplastic oesophagus and trachea, abnormal cartilidge, no smooth muscle	Lung hypoplasia, skeletal and neural defects	100%	100% post natal		Motoyama 1998
	Gli2-/-, Gli 3-/-	Mouse, xenopus	Mesenchyme	Loss of Wnt and BMP signalling, no Nkx2.1 expression, reduced Fox f1 mesenchyme	Single hypoplastic Sox 2+ foregut	Lung bud and foregut agenesis, hypoplastic pancreas, liver, thymus	Unreported	Embryonic lethality		Motoyama 1998, Rankin 2016
	Gli2 -/-, Gli3-/+	Mouse, xenopus	Mesenchyme	Appropriate patterning, medial constriction but failure of separation: epithelium fails to touch	Single foregut tube (complete LTEC)	Single hypoplastic lung lobe	100%	Not desrcibed	Pallister Hall Syndrome	Motoyama 1998, Rankin 2016, Nasr 2019
	Foxf1+/-	Mouse	Mesenchyme	Impaires medial constriction of foregut at Sox2/Nkx2.1 boundary	Oesophageal stenosis, anterior LTEC, tracheomalacia, occasional EA	Fusion of lung lobes and hypoplasia, rib anomalies	Unreported	FoxF1-/- embryonic lethality, -/+ 90% perinatal mortality		Mahlaupuu 2001, Nasr 2019

(*continued on next page*)

Table 1 (*continued*)

RA Signalling	Ra2dlh-/-(exogenous RA to rescue)	Mouse	Epithelium, mesenchyme	Failure of HH activation and Wnt signalling	Common foregut	Lung agenesis, blind ending foregut, rudimentary stomach, cardiac defects	100%	100% embryonic unless exogenous RA delivery. Postnatal fatality 100%		Wang 2006
	Tbx4-/+ Tbx/5-/-	Mouse	Mesenchyme	Reduction in Wnt and FGF10	Tracheal stenosis	Defective cartilage, abnormal smooth muscle. Cardiac and allantois defects in homozygotes	Unreported	100% postnatal		Arora 2012
BMP Singalling	BMP1a-/-;b-/-	Mouse	Epithelium	Failure of patterning: Absence of NKX2.1 due to loss of SMAD 1/5/8 (inhibitors of SOx2)	Tracheal agenesis: Single smooth muscle lined foregut tube (Sox2+)	Ectopic lung buds off common foregut	Unreported	100% post natal		Domyan 2011
	BMP4+/-	Mouse	Mesenchyme, epithelium	Failure of patterning: Absence of Nkx2.1	Tracheal agenesis: Single Sox2+ foregut tube with no cartilidge	Lung hypoplasia	100%	100% post natal		Li 2008
	Noggin -/-	Mouse	Dorsal epithelium, notocord	Increased BMP signalling, reduced Sox2 expression	**Type C EA/TEF, ectopic cartilidge in fistula. 18% esophageal stenosis**	Delayed notochord detachment	70%-82%	Not described	Brachydactylyl; EA in <1% Deletion of chromosomal region spanning NOG locus found in patients with EA/TEF (Marsh 2000)	Que 2007, Li 2007
Wnt-signalling	Wnt2-/-;Wnt2b-/-	Mouse	Ventral foregut mesenchyme	Loss of Nkx2.1 FGF10, BMP4 expression	Tracheal agenesis, loss of cartilade and expansion of smooth muscle	Lung agenesis	100%	100% post natal		Goss 2009, Hou 2019, Kishimoto 2019
	Barx 1	Mouse	Midline mesenchyme	Normal patterning, increased Wnt in dorsal foregut	Single foregut tube: Complete LTEC, ectopic Nkx2.1 expression		Unreported	Not reported		Woo 2011
Ephrin B2	EfnB2-/- cond	Mouse	Dorsal foregut endoderm, mesoderm	Disruption of Nkx2.1-Ephrin boundary required for separation	Common foregut tube, disorganised cartilidge	Cardiac, urethra, anorectum	47%	Not reported	Similar to VACTERL	Dravis and Henkemeyer 2011, Lewis 2022
	EfnB2+/- cond	Mouse	Dorsal foregut epithelium, mesoderm	Disruption of Nkx2.1-Ephrin boundary required for separation	LTEC II-III, distally separated, rostral single tube		Unreported	Not reported		Dravis and Henkemeyer 2011, Lewis 2022

Table 2
Genetic Syndromes known to be associated with EA/TEF. Those with correlation between clinical findings and mice models highlighted in bold. TE frequency the incidence of TE in small case series of the condition discussed.

Syndrome	Clinical Features	Gene	TE Frequency	Mouse model
Monogenic Syndromes				
AEG Syndrome	Anopthalmia, EA+/-TEF, urogenital abnormalities	**SOX2**	100% (Williamson 2006)	60% TEF in hypomorphic allele
Feingold Syndrome 1	Digital anomalies, Microcephaly, facial dysmorphism, EA, DA	N-MYC	47% (Cognet 2011)	No TEF, failed lung branching, embryonically lethal
Mandibulofacial Dysostosis with Microcephaly	DD, craniofacial anomalies	EFTUD2	40% (Need 2012)	Embryonically lethal
Di George Syndrome	Parathyroid/thyroid hypoplasia, CHD, EA	**TBX1**	33% (Lee 2008)	Respiratory failure and LTEC
Coffin-Siris Syndrome	DD, dysmorphism, feeding difficulty, hypotonia	SMARCD1	20% (Nixon 2019)	Nil
Treacher Collins Syndrome	Craniofacial Abnormalities, Coloboma, EA, hearing loss	TCOF1	5% (Sutphen 1995)	Respiratory failure
Alveolar Capillary Dysplasia	Heterotexia, VACTERL	**FOXF1**	<1% (Stankiewicz 2009)	LETC, oesophageal stenosis
Pallister Hall Syndrome	Polydactyly, VACTERL	**Gli3**	Rare (Kause 2018)	Tracheal Agenesis
Chromosomal Duplicaitons				
Trisomy 18	CHD, renal anomalies, DD, exomphalos, EA	Multiple	25% (Broesens 2014)	N/A
Trisomy 21	DD, CHD, thyroid, gastrointestinal, eye and hearing abnormalities	Multiple	1% (Felix 2007)	N/A
Trisomy 13	DD, Microcephaly, Eye Defects, Polydactyly, Cleft Palate, Genital, kidney and CHD	Multiple	Rare (Felix 2007)	N/A
Triple X	DD, Limb and GI Abnormalities	Multiple	Rare (Broesens 2014)	N/A
Unknown/Multiple mutations				
VACTERL Syndrome	Vertebral, anorectal, CHD, tracheo-esophageal, renal and limb anomalies	Unknown ? WBP11	50-80% (Solomon 2011)	Adriamycin mouse model, VACTERL with EA in 45% (Martin et al 2020)
Fanconi Anaemia	VACTERL, cancer	FANCA/B/C/D1/G	EA/TEF 1-14% (Brosens 2014)	Embryonically lethal
CHARGE Syndrome	Coloboma, CHD, choanal atresia, DD, genital and ear anomalies	CHD7, SEMA3E	10-15% (de Jong 2010)	CHD7: Embryonically lethal, EA not reported. SEMA3E: Normal

Acronyms:
DD - developmental delay, DA duodenal atresia, CHD congenital heart defects.
AEG - Anopthalmia, Esophageal Genitourinary Syndrome.

more clear in the next few years with improvements ever more sophisticated wholemount imaging techniques.

The genetic basis of EA/TEF

In addition to the use of animal models, information about the pathogenesis can also be gleaned from the genetic study of patients with EA/TEF. EA/TEF is predominantly recognized as a sporadic event due to the low familial recurrence rate of <1% in isolated EA cases. Where familial cases do exist, parents are usually unaffected, suggesting a de novo mutation secondary to epigenetic and environmental factors.[78] Several observational studies have suggested risk factors including maternal diabetes, antithyroid drugs, smoking, alcohol and increasing maternal age .[79] Unfortunately analysis of the genetic or molecular aberrations involved in de novo mutations and Copy Number Variations (CNVs) in isolated EA patients is challenging as they rarely impact the same locus or gene.

There is, however, evidence to suggest genetics play a role in EA pathology alongside non-genetic factors. Firstly, the incidence of concordance in isolated EA has been shown to be higher in monozygotic (50%) than in dizygotic twins (26%).[80] Secondly, causal genetic abnormalities can be identified in approximately 11% of EA patients; in a large, long-standing Dutch cohort of 582 patients, 9% were diagnosed with a recognized genetic syndrome. Non-syndromic copy number variations, microdeletions, duplications and chromosomal anomalies were reported in the remaining 2%.[81] In 2021, Edwards et al identified a total of 54 different genes reported to have a causative association with EA/TEF across 35 genetic syndromes.[82] These include the presence of whole chromosome duplications such as trisomy 21, 18 and 13, structural chromosomal anomalies such as translocations and deletions, copy number variations, and monogenetic syndromes with autosomal dominant, recessive or X-linked inheritance patterns e.g. Anophthalmia-Esophageal-Genital (AEG) syndrome. Table 2 demonstrates some of the most recognized EA-associated genetic syndromes with the corresponding gene affected and inheritance pattern, where known.

Advances in our understanding of the human genome have shown that some monogenetic syndromes occur in genes known from animal models to play a role in T-E development including Sox2, MYCN, Gli3 and Foxf1 (all downstream targets of Shh) in AEG, Feingold, Pallister-Hall syndromes and alveolar capillary dysplasia respectively. The penetrance of EA in these disorders however is extremely variable, from 100% in AEG syndrome to very rare in Alveolar capillary dysplasia despite a known mutation in Foxf1. In addition, the rarity of these conditions (<200 cases worldwide of AEG syndrome for example), means there is limited understanding of the direct mechanism of this causation.[83] Therefore, whilst some correlation occurs between candidate genes in animal models and human EA patients, this is limited. Attempts to corroborate several of the molecular targets identified from animal

models in patient cohorts with EA/TEF has also been unproductive. In mouse models, loss of function in Noggin clearly leads to a TEF phenotype. Whilst deletions in the region of chromosome containing the Noggin gene have been sporadically reported in patients with EA, no loss of function mutations were found in the coding region of the Noggin gene after specifically sequencing for it in 50 EA patients.[84,85] Similarly, although loss of function in Shh and its pathway results in severe T-E abnormalities in mice, analysis of VACTERL patient cohorts with EA, albeit small, did not identify pathogenic mutations in this gene.[86]

The genetic basis for VACTERL is unclear. Interestingly, whilst 23% of the large Dutch cohort had a VACTERL diagnosis, 92% of these had no identifiable genetic diagnosis and, unlike in isolated EA, twin concordance rates appear to be equivocal between monozygotic and dizygotic cases.[81,87] Whilst this suggests the role of genetics in VACTERL may be limited, the genes involved in VACTERL like-syndromes (e.g. Pallister Hall syndrome) all involve Shh signaling pathways and some studies have suggested a higher than expected incidence of VACTERL features in first degree relatives (1-5%).[7] The anatomical and morphological range of congenital anomalies associated with EA is interesting, particularly as they occur at different developmental timepoints in different organs. This could either be due to the timing of the occurrence of somatic mutation e.g at gastrulation affecting several organs vs. at dorsal-ventral patterning affecting the foregut specifically, due to mutations in pleiotropic genes affecting multiple pathways or due to other unidentified environmental and epigenetic factors.[88]

Identifying a specific genetic cause for EA is therefore extremely challenging. Many genes are affected by chromosomal duplications or deletions so causal genes remain unidentified. Although we now have a significantly increased capacity to examine genetic changes in EA patients through genome sequencing, identifying background de novo mutations or those with a causal role in EA/TEF is very arduous; CNVs need to be shown to have a specific molecular basis for its effect at a protein level with *in vitro* and *in vivo* evidence of harm.[2] Somatic mutations occur either as a result of parental germline mosaicism and as such are detected in the blood of EA patients, or later in organ development meaning this mutation is only detectable at a tissue level, therefore most likely remains undiagnosed. As survival of patients with EA has improved so significantly, those with previously undetected local de novo mutations can be transmitted to their children and detectable in blood, meaning we can begin to build a clearer picture of which gene mutations play a clinical role and counsel patients appropriately.

Conclusions and future directions

In recent years, huge advances have been made in our understanding of the molecular, cellular and morphogenic processes involved in normal embryogenesis of the trachea and esophagus. Genetic knockout models have dramatically increased our insight into how transcription factors and pathways known to be involved in normal T-E separation may play a role in the pathogenesis of trachea-esophageal deformities. However, whilst some loss of function mutations in candidate genes share the same phenotype as that commonly seen in human EA, they more often result in the clinically less relevant variant of tracheal agenesis and are frequently embryonically lethal. As such, whilst they have provided clues as to the underlying mechanisms and pathways behind the pathogenesis of EA, distinct molecular targets are still to be identified.

More recently, analysis of single cell genomics over different developmental timepoints has helped us validate and expand upon previously implicated signaling pathways. This has led to the development of human esophageal and airway epithelial organoids by exposing iPSCs to components of molecular pathways identified from these developmental animal models including RA, Noggin, BMP and Wnt.[29,89] Organoids provide a novel platform with which to investigate the effects of newly identified molecular targets from genomic studies at a cellular level. Specifically, they could be used to understand differences in cellular behaviors between the respiratory and esophageal epithelium of EA compared to non-EA patients, to help us understand why conditions such as Barrett's esophagus are so prevalent. The main disadvantage of current organoid models is the lack of mesenchymal cells known to play a key role in T-E development. Excitingly, in 2020, Han et al also described the successful derivation of esophageal and tracheal mesenchyme from splanchnic mesoderm by exploiting signaling pathways identified from a mouse single-cell signaling roadmap.[22] The combination of foregut organoids of both epithelial and mesenchymal origin represents a promising new avenue for modelling of T-E development. Using such a system, very recently, esophageal organoids derived from iPSCs from EA/TEF patients and iPSCs and from healthy individuals showed a transient dysregulation of Sox2 and the abnormal expression of Nkx2.1 in patient-derived cells which could be linked to the abnormal foregut compartmentalization.[90] As patients with de novo mutations have children, genome profiling of families with recurrence in addition to genome sequencing of large patient cohorts may identify previously unknown causal genes. The use of CRISPR gene editing in organoids has the potential to explore and validate the functional importance of genetic variants identified in this way. Mutating healthy control cell lines to include the specific defect in question or conversely correcting the genetic variant found in the EA patient cell line to look for causality will allow for prioritization of the investigation of clinically relevant mutations.[90,91] Finally, in the last year, two groups have reported the generation of synthetic mouse embryos from stem cells.'[92,93] Both protocols give rise *in vitro* to structures which mimic mouse development up to E8.5, the precise stage of foregut separation. Whilst these systems are still inefficient, this technology offers an exciting new perspective with which to study the development of the esophagus and trachea, potentially edging us ever closer to understanding the underlying processes involved in tracheo-esophageal development and EA pathogenesis.

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