Bidirectional Wnt signaling between endoderm and mesoderm confers tracheal identity in mouse and human cells

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The periodic cartilage and smooth muscle structures in mammalian trachea are derived from tracheal mesoderm, and tracheal malformations result in serious respiratory defects in neonates. Here we show that canonical Wnt signaling in mesoderm is critical to confer trachea mesenchymal identity in human and mouse. At the initiation of tracheal development, endoderm begins to express *Nkx2.1*, and then mesoderm expresses the *Tbx4* gene. Loss of β-catenin in fetal mouse mesoderm causes loss of Tbx4+ tracheal mesoderm and tracheal cartilage agenesis. The mesenchymal Tbx4 expression relies on endodermal Wnt activation and Wnt ligand secretion but is independent of known Nkx2.1-mediated respiratory development, suggesting that bidirectional Wnt signaling between endoderm and mesoderm promotes trachea development. Activating Wnt, Bmp signaling in mouse embryonic stem cell (ESC)-derived lateral plate mesoderm (LPM) generates tracheal mesoderm containing chondrocytes and smooth muscle cells. For human ESC-derived LPM, SHH activation is required along with WNT to generate proper tracheal mesoderm. Together, these findings may contribute to developing applications for human tracheal tissue repair.
The mammalian respiratory system is crucial for postnatal survival, and defects in the development of the respiratory system cause life-threatening diseases in breathing at birth. The trachea is a large tubular air path that delivers external air to the lung. Abnormal development of the tracheal mesenchyme, including cartilage and smooth muscle (SM), is associated with congenital defects in cartilage and SM such as tracheo-esophageal fistula (TEF) and tracheal agenesis (TA). Thus, understanding tracheal development is crucial to better understand TEF/TA and establish a protocol to reconstruct trachea from pluripotent stem cells for human tissue repair.

Tracheal/lung organogenesis is coordinated by endodermal-mesodermal interactions during embryogenesis. The primordial tracheal/lung endoderm appears at the ventral side of the anterior foregut at embryonic day 9–9.5 (E9.0–9.5) in mouse (Fig. 1a). Previous studies have demonstrated that development of tracheal/lung endoderm is initiated by gradually expression of mesodermal Wnt2b and Bmp4 along the dorsal-ventral axis. This mesodermal-to-endodermal Wnt and Bmp signaling drives expression of Nkx2.1, the key transcription factor of tracheal/lung endoderm. At the same time, the endodermal cells from the esophageal lineage. The Nkx2.1+ mesodermal cells secrete growth factors (e.g., Wnt and Bmp) to induce respiratory endoderm identity. The mechanism underlying the initial induction of tracheal mesoderm is still unclear.

Here, we propose that this communication is bidirectional between endoderm and mesoderm. In our model, once tracheal endoderm is specified around E9.5, endodermal cells express Wnt ligands to induce Tbx4 expression in tracheal mesoderm after E10.5. To substantiate the model, we address the following key issues: (1) tracheal endoderm secretes Wnt ligands; (2) tracheal mesoderm responds to endodermal Wnt ligands to specify the tracheal mesoderm originates in the ventral fold of lateral plate mesoderm (LPM) surrounding the anterior foregut endoderm. After induction of endodermal Nkx2.1 expression at E9.5, tracheal/lung mesoderm is defined by Tbx4 at E10.5, which are markers for tracheal/lung mesoderm and required for proper mesenchymal development (Fig. 1a). In contrast to Tbx5 which is also expressed in LPM and cardiac mesoderm, Tbx4 expression is restricted to respiratory tissue. At E9.5, Tbx4 is only detected in lung bud mesoderm but not tracheal mesoderm (Supplementary Fig. 1). Tbx4 expression is then detected in tracheal mesoderm from E10.5. Tbx4 and Tbx5 cooperate to steer normal trachea development. Both genes are required for mesodermal development of the trachea, particularly for cartilage and smooth muscle differentiation as well as morphogenesis. The crucial functions of these genes are validated by Tbx4, 5 double mutants exhibiting the phenotypes of tracheal stenosis. We previously reported that synchronized polarization of mesodermal cells and temporal initiation of cartilage development regulate tracheal tube morphogenesis by coordinating the length and diameter of the mouse trachea, respectively. However, the mechanism underlying the initial induction of tracheal mesoderm is still unclear.

Fig. 1 Activation of Wnt signaling in endoderm, but not Nkx2.1 expression, is activated to promote mesodermal development of the mouse trachea.

a Schematic model of tracheoesophageal segregation. b Transverse sections of Nkx2.1+/− mouse embryos and littermate controls. Sections were stained for Sox2 (green), Tbx4 (magenta), and DAPI (blue). Arrowheads indicate Tbx4+/− tracheal mesoderm. Asterisks indicate nonspecific background signal of blood cells in dorsal aorta. n = 3/3 embryos per genotype. c Transverse sections of ShhCre, Ctnnb1flox/flox mouse embryos and littermate controls. Sections were stained by Sox2 (green), Tbx4 (magenta), and DAPI (blue). Arrowheads indicate Tbx4+/− tracheal mesoderm. n = 3/3 embryos per genotype. d Transverse sections of ShhCre, Ctnnb1flox/flox mouse embryos, and littermate controls. Sections were stained by Nkx2.1 (magenta) and DAPI (blue). n = 3/3 embryos per genotype. n neural tube, a aorta, Es Esophagus, Tr Trachea, Tr-E Trachea-Esophageal tube. Scale bar, 40 μm.
mesodermal identity through Tbx4 expression; (3) Tbx4 is a direct Wnt target gene.

**Results**

**Endodermal Wnt activity but not Nkx2.1 initiates Tbx4 expression in mouse tracheal mesoderm.** To study the initiation of the mesodermal development of the trachea, we validated the involvement of Nkx2.1 in mesodermal Tbx4 expression because endodermal-mesodermal interactions orchestrate organogenesis throughout development in general. Nkx2.1 is an endodermal transcription factor necessary for tracheal and lung development and its genetic ablation results in TEF9. We examined Nkx2.1<sup>+/−</sup> mouse embryos and confirmed the TEF phenotype with a single trachea–esophageal (Tr–E) tube (Fig. 1b). Interestingly, Nkx2.1<sup>−/−</sup> embryos retained Tbx4 expression in the ventrolateral mesoderm of a single Tr–E tube, although the segregation was defective (Fig. 1b), indicating that mesodermal induction of the trachea is independent of endodermal Nkx2.1. We compared the phenotype of Nkx2.1<sup>−/−</sup> with that of Shh<sup>Cre</sup>, Ctnmb1<sup>flx/flx</sup> embryos, which also show anterior foregut endoderm segregation defect and loss of Nkx2.1 expression (Fig. 1c, d)4,5. In contrast to Nkx2.1<sup>−/−</sup> embryos, Shh<sup>Cre</sup>, Ctnmb1<sup>flx/flx</sup> embryos did not express Tbx4. To eliminate the possibility that the lack of LPM caused no Tbx4 expression phenotype, we assessed the expression of Foxf1, a pan-LPM marker at this developmental stage (Supplementary Fig. 2a). Foxf1 was still expressed in the mesoderm. This observation suggests that the activation of endodermal Wnt signaling, but not Nkx2.1 expression, is required for following mesodermal Tbx4 expression. Thus, the initial induction of tracheal mesoderm is independent of known Nkx2.1-mediated respiratory endoderm development, but dependent on the activation of Wnt signaling at the ventral anterior foregut endoderm.

**Endodermal-to-mesodermal Wnt signaling induces Tbx4 expression in tracheal mesoderm.** To further study the spatio-temporal regulation of canonical Wnt signaling during trachea–esophageal segregation at E9.5 to E11.5, we used a reporter line LEP<sup>EFGP</sup> and examined the distribution of EGFP in the canonical Wnt signaling response (Fig. 2a, b)15. At E9.5, EGFP was detected in the ventral half of the anterior foregut endoderm where trachea endodermal cells appear and express Nkx2.1 (Fig. 2a, b, arrowheads) and then decreased temporally at E10.5. After E10.5, the EGFP reporter was activated in the surrounding mesoderm and its intensity increased at E11.5 (Fig. 2a, b, arrowheads), which was similar to the patterning of Axin2-LacZ, another reporter line for the response of canonical Wnt signaling.16 We further conducted RNAseq in situ hybridization (ISH) against Axin2, an endogenous Wnt target gene, to confirm activation of Wnt signaling in mesoderm. Axin2 was highly expressed in surrounding mesoderm at E10.5 compared to endoderm, similar to the pattern observed in the reporter line (Fig. 2c). Because these Wnt-responsive mesodermal cells expressed Tbx4 (Fig. 2b), we hypothesized that Wnt signaling in the early mesoderm is involved in the initiation of the tracheal mesoderm.

To validate the role of mesodermal Wnt signaling, we genetically ablated Ctnmb1, also known as β-catenin, which is a core component of canonical Wnt signaling, from embryonic mesoderm. We employed the Dermol<sup>Cre</sup> line, which targets embryonic mesoderm, including tracheal/lung mesoderm, and generated Dermol<sup>Cre</sup>, Ctnmb1<sup>flx/flx</sup> mice.17−20 In the mutant embryos, Tbx4 expression was absent but Foxf1 was retained in the mesoderm at E10.5 (Fig. 2d and Supplementary Fig. 2b), indicating that mesodermal canonical Wnt signaling is necessary for Tbx4 expression. In contrast, endodermal Nkx2.1 expression and tracheoesophageal segregation were not affected, implying that mesodermal Wnt signaling and Tbx4 is dispensable for endodermal development. Supporting the observation of lung buds in Der<sup>Cre</sup>, Ctnmb1<sup>flx/flx</sup> embryos,9,20,21 the mutant lung buds still expressed Tbx4 in mesoderm (Supplementary Fig. 3a). Disruption of Wnt signaling in the mesoderm eliminated Tbx4 expression in the tracheal but still detectable in lung mesoderm, suggesting that Wnt-mediated mesodermal Tbx4 induction is a unique system in trachea development but not lung development. We further found that the Dermol<sup>Cre</sup>, Ctnmb1<sup>flx/flx</sup> mutant exhibits tracheal cartilage agenesis. In the mutants, a periodic cartilage ring structure labeled with Sox9 failed to develop at E16.5, and circumferential SM bundles labeled with smooth muscle actin (SMA) were also malformed (Fig. 2e, f). Therefore, mesodermal Wnt signaling is crucial for trachea mesenchymal development, particularly for tracheal cartilage development.

To determine whether Tbx4 is a direct or indirect target of canonical Wnt signaling in respiratory mesoderm, we analyzed the presence of Tcf/Lef-binding sequences in the Tbx4 lung mesenchyme element (Tbx4-LME)22,23. We identified five putative Tcf/Lef-binding sites in Tbx4-LME using Jaspar 2020 database (Fig. 2g)24. Intersecting these binding sites with public databases, such as UCSC browser and ENCODE, showed that these putative Tcf/Lef-binding sites are highly conserved among different vertebrates except for zebrafish and are localized in a region that contains epigenetic marks of distal regulatory elements H3K27Ac, E14.5 lung; H3K4me1, E15.5 lung; p300, postnatal day (PND) 0 lung and chromatin accessibility at E14.5 lung in which the transcription of Tbx4 gene is active.10,25–27

Next, we sought to identify a source of Wnt ligands that initiate mesodermal Tbx4 expression. Due to the essential role of Wnt2 at early tracheal/lung development4, we used ISH for Wnt2. Wnt2 is transiently expressed in the ventrolateral mesoderm of the anterior foregut at E9.5, which was obviously reduced by E10.5 when Tbx4 was expressed (Figs. 2b and 3a). Wnt2 is most likely not involved in Tbx4 expression after E10.5. This observation prompted us to hypothesize that an endodermal-to-mesodermal interaction but not mesodermal autonomous induction is required for Tbx4 expression. To test this hypothesis, we generated Shh<sup>Cre</sup>, Wls<sup>flx/flx</sup> mice, in which endodermal Wnt ligand secretion is inhibited by targeting Wntless (Wls) gene essential for exocytosis of Wnt ligands.28 This endoderm-specific deletion of Wls results in loss of Tbx4 expression in the mesoderm, but retained Nkx2.1 expression in the endoderm and Wnt2, and Foxf1 in the mesoderm (Fig. 3b, c and Supplementary Fig. 2e)28, making these mice a phenocopy to Dermol<sup>Cre</sup>, Ctnmb1<sup>flx/flx</sup> mice (Fig. 2d). Shh<sup>Cre</sup>, Wls<sup>flx/flx</sup> embryos also formed lung buds and expressed Tbx4 in the distal lung mesoderm (Supplementary Fig. 3b), supporting our idea that Wnt signaling in mesoderm mainly contributes to initiation of mesodermal development of the trachea, but not of the lung. These findings indicate that the endodermal Wnt ligands are sufficient for trachea mesodermal development. From these observations, we conclude that endodermal Wnt2 activates endodermal canonical Wnt signaling to promote endodermal Wnt ligand expression independent of Nkx2.1. These Wnt ligands then induce endodermal-to-mesodermal canonical Wnt signaling to initiate tracheal mesoderm specific identity (Fig. 3d). These results also suggest that specification in the tracheal endodermal lineage is not necessary for the initial induction of the tracheal mesoderm.

In the developing mouse trachea, several Wnt ligands are expressed in the endoderm between E11.5 to E13.5, such as Wnt3a, 4, 5a, 6, 7b, 11, and 16.21,28 Current single-cell RNA-seq data have shown the presence of several Wnt ligands including Wnt4, 5a, 5b, 6, and 7b in the respiratory endoderm of mouse...
embryos at E9.5. We performed ISH against these Wnt ligands at E10.5 to determine the particular ligand inducing Tbx4 expression in trachea development (Fig. 3e, Supplementary Fig. 5). Wnt4 was expressed in esophageal mesoderm and barely detected in tracheal endoderm. Wnt5a, 5b, and 6 were detected in both the endoderm and mesoderm of the trachea. More importantly, Wnt7b was abundantly expressed in tracheal endoderm, suggesting that Wnt7b might be responsible for the ensuing induction of mesodermal Tbx4 expression.

In vitro recapitulation of trachea mesodermal development using mouse and human embryonic stem cells. By recapitulating developmental processes in vitro, trachea/lung endodermal
Fig. 2 Wnt signaling is activated to promote mesodermal development of the mouse trachea. **a** Transverse sections of LEF1EGFP reporter mouse embryos at E9.5 to E11.5. Sections were stained for EGFP (green), Nkx2.1 (magenta), and DAPI (blue). Arrowheads indicate GFP+ cells. **b** Transverse sections of LEF1EGFP reporter mouse embryos at E9.5 to E11.5. Sections were stained for EGFP (green), Tbx4 (magenta), and DAPI (blue). Arrowheads indicate GFP+ cells. **c** Transverse section of mouse embryo at E10.5. Section were stained for Axin2 (green), Nkx2.1 (magenta) and DAPI (blue) by RNAseq experiment. Arrowheads indicate Axin2+ cells. **d** Transverse sections of Dermo1Cre, Ctnnb1fl/fl mouse embryos and littermate controls at E10.5. Upper panels show sections stained for Sox2 (green), Tbx4 (magenta), and DAPI (blue). Lower panels show sections stained for Nkx2.1 (magenta) and DAPI (blue). Arrowhead indicates Tbx4+ cells. Arrows indicate Nkx2.1+ cells. 

**Fig. 3** Wnt ligands induce Tbx4 expression for tracheal mesoderm development of mouse trachea. **a** In situ hybridization for Wnt2 mRNA during tracheoesophageal segmentation. Arrowheads indicate Wnt2 expression in the ventrolateral mesoderm at E9.5 and E10.5. n = 2/2 embryos. **b** Transverse sections of ShhCre, Wlsfllox/fllox mouse embryos and littermate controls at E10.5. Left panels show sections stained with Sox2 (green), Tbx4 (magenta), and DAPI (blue). Right panels show sections stained for Nkx2.1 (magenta) and DAPI (blue). n = 3/3 embryos per genotype. **c** In situ hybridization for Wnt2 mRNA in ShhCre, Wlsfllox/fllox mouse embryos and littermate controls at E9.5. Arrowheads indicate Wnt2 expression in the ventrolateral mesoderm. n = 2/2 embryos. **d** Refined model of tracheoesophageal segmentation and tracheal mesodermal differentiation. In situ hybridization for Wnt7b mRNA in mouse embryo at E10.5. Arrowhead indicates Wnt7b+ cells. n = 2/2 embryos. Eso Esophagus, Lu Lung, Tr Trachea, Tr-E Tracheoesophageal tube. Scale bar: 40 μm (**a, b**), 50 μm (**c, d**), 300 μm (**e**).
Foxf1 and Gata4, which are known to be expressed in LPM including splanchnic mesoderm, ICC showed that 89% of total cells were Foxf1+ LPM. Furthermore, qRT-PCR also showed obvious upregulation of LPM marker genes, such as Foxf1, Gata4, Hoxb6, Prrx1, and Bmp4. Given that previous mouse genetic studies have identified Bmp4 as a crucial regulator of trachea development, we tested whether canonical Wnt and Bmp4 signaling are sufficient to direct the differentiation of LPM into the tracheal mesoderm (Foxf1+/Tbx4+). mESC-derived LPM cells were cultured with CHIR99021, a GSK3β inhibitor to stabilize β-catenin and activate canonical Wnt signaling, and Bmp4. At day 6, 89% of total cells...
became double positive for Foxf1 and Tbx4. qRT-PCR further demonstrated elevated expression of tracheal marker genes such as Tbx5, Wnt2, Bmp4 in addition to Tbx4 (Fig. 4d, e). To further confirm the respiratory characteristics of these cells, we took advantage of the 5 Tcf/lef-binding sequences in Tbx4-LME, which we described in Fig. 2g. We established a luciferase reporter assay by reporter plasmids that express luciferase under the control of Tbx4-LME (Fig. 4f). The reporter plasmid was transfected into mESC-derived LPM and luciferase activity was assessed during differentiation. After 24 h (at day 6), the luciferase activity significantly increased in the presence of CHIR99021 (Fig. 4g). Importantly, the mutated reporter, in which all Tcf/lef-binding sequences were changed to random sequences (Figs. 2g and 4f), did not respond to CHIR99021. The modest increase of luciferase activity might be due to low transfection efficiency. These results determined that the mESC-derived cells were differentiated into proper tracheal mesoderm at day 6.

Because tracheal mesenchyme includes cartilage and smooth muscle, we wondered whether our protocol induces mESC to differentiate into these tissues. At day 12, Sox9+ aggregated cell masses positive for Alcian blue staining appeared on the dish, indicative of chondrocytes (Fig. 4h–i). Smooth muscle cells (SMA+ cells) concurrently appeared to show fibroblastic morphology and filled the space not filled by the Sox9+ cells (Fig. 4i, j). Other chondrogenic markers (Aggrecan, Collagen2a1, Sox5/6, Ephipheny) and smooth muscle markers (Tagln, Collagen1a1) were also present in the differentiated cells (Fig. 4k, l and Supplementary Fig. 7a). These data suggest that the mESC-derived tracheal mesoderm is able to develop into tracheal mesenchyme, including chondrocytes and smooth muscle cells.

Finally, we tested the role of Wnt signaling in the human tracheal mesoderm using human ESCs (hESCs). Human LPM induction was performed by following an established protocol (Fig. 5a). Subsequently, the cells were directed to tracheal mesoderm by using CHIR99021 and BMP4. For validating hESC-derived LPM, we checked the common LPM markers at day 2 and confirmed that these markers were abundantly expressed in the LPM (Fig. 5b, c and Supplementary Fig. 8k). Immunostaining determined that 95% of the total cells expressed FOXF1 at day 2 (Fig. 5b). Because Tbx4 is also expressed in the limbs and other fetal mouse tissues, we sought additional genetic markers for the tracheal mesoderm. We searched the single-cell transcriptomics dataset of the developing splanchnic mesoderm at E9.5 and identified Nkx6.1 as a marker for mesodermal cells surrounding the trachea, lung, and esophagus.

We performed immunostaining and found that Nkx6.1 was expressed in tracheal and esophageal mesenchyme throughout development (Supplementary Fig. 6). Of note, Nkx6.1 was expressed in esophageal and dorsal tracheal mesenchyme but not ventral trachea, which enabled us to define three subtypes of tracheal-esophageal mesenchyme based on the combination of Tbx4 and Nkx6.1 expression (i.e., Tbx4+/Nkx6.1+; dorsal tracheal mesenchyme, Tbx4+/Nkx6.1−; ventral tracheal mesenchyme, Tbx4−/Nkx6.1+; esophageal mesenchyme) (Supplementary Fig. 6). Having characterized the subtypes of tracheoesophageal mesoderm in vivo, the expression of Tbx4 and Nkx6.1 in the hESC-derived tracheal mesoderm was examined by ICC and qRT-PCR. Although Tbx4 was induced in a Wnt activator dose-dependent manner, Nkx6.1 expression was not significantly elevated (Supplementary Fig. 8a, b), suggesting that human trachea mesodermal development requires an additional factor to become more in vitro-like. Because the ventral LPM is exposed to SHH in addition to Wnt and Bmp4 during tracheoesophageal segregation, we assessed whether the SHH activator (PMA; purmorphamine) can improve differentiation from hESC-derived LPM cells into the tracheal mesoderm. As expected, both Tbx4 and Nkx6.1 expression was upregulated by the SHH activator (Supplementary Fig. 8c, d). After day 5, the differentiating cells also expressed respiratory markers such as TBX4, TBX5, WNT2, BMP4, and NKX6.1 (Fig. 5d–h, Supplementary Fig. 8k). In this culture condition, CHIR99021 enhanced the expression of TBX4 and NKX6.1 genes in a dose-dependent manner (Supplementary Fig. 8e, f). We also performed qPCR analysis for FOXF1 expression as a pan-LPM marker. FOXF1 expression was decreased by CHIR99021 but still retained after induction (Supplementary Fig. 8g). This result also reflects the feature of in vivo tracheal mesoderm because FOXF1 expression is decreased in the ventral mesoderm of the mouse trachea (Supplementary Fig. 2a–c). These data indicate that HH/BMP was sufficient to induce FOXF1 expression but HH/BMP/WNT was necessary for human respiratory mesoderm induction. We further estimated the efficiency of the induction by immunostaining for TBX4 and FOXF1, and then confirmed that 83% of total cells were TBX4+/FOXF1− double positive cells at day 5 (Fig. 5d). At day 10, NKX6.1 expression was clearly elevated, and 30.3% of the total cells became TBX4+/NKX6.1− double positive (Fig. 5e–g) while 18.0% were TBX4+/NKX6.1− (Fig. 5g). These data suggest that the half of the cells induced with our protocol are trachea mesodermal cells. Further extended culture induced Sox9+ aggregates, which were positive for Alcian blue staining.

Fig. 4 Generation of tracheal mesodermal cells and chondrocytes from mouse ESCs in vitro. a Experimental design to generate tracheal mesoderm from mESCs. b Differentiating cells from mESCs at day 5. Cells were stained for Foxf1 (magenta) and Gata4 (green), respectively. c was calculated from randomly chosen 3 fields. Images are representative of two independent experiments. c qRT-PCR for LPM markers of mESC-derived LPM (n = 3 independent wells). Experiments were repeated at least twice. d Differentiating cells from mESCs at day 6. Cells were stained for Tbx4 (green) and Foxf1 (magenta). % was calculated from randomly chosen three fields. Images are representative of at least two independent experiments. e qRT-PCR for respiratory mesoderm marker expression of mESC-derived trachea mesodermal cells. (n = 3 independent wells). Experiments were repeated at least twice. f Diagram showing the constructs utilized in luciferase experiments containing Tbx4−/− LME Tbx4-LME wild-type containing five Tcf/lef-binding sites and Mutant. mESC-derived LPMs were transfected with wild-type or mutant Tbx4-LME during 4hrs following by respiratory induction in presence or absence of CHIR99021. g Luciferase assay examining the activation of Tbx4-LME wt and mutant in response to 3 μM CHIR99021. P-values were provided by two-sided Tukey’s multiple comparison. ***p < 0.0001 (n = 3 from independent wells from a single experiment). h Differentiating cells from mESCs at day 12. Cells were stained for Acta2 (magenta) and Sox9 (green). The asterisk indicates Sox9+/SMA+ chondrocyte aggregates. Images are representative of two independent experiments. i qRT-PCR for Sox9 and Acta2 expression of hESC-derived trachea mesodermal cells (n = 3 independent wells). Experiments were repeated twice. j Differentiating cells from mESCs at day 12. Chondrocytes were stained with Alcian blue. The asterisk indicates one of the chondrocyte aggregates. Images are representative of two independent experiments. k Differentiating cells from mESCs at day 12. Cells were stained for Col1a1 (magenta) and Aggrecan (green). Image is representative of two independent experiments. l Differentiating cells from mESCs at day 12. Cells were stained for Tagln (magenta) and Col2a1 (green). Image is representative of two independent experiments. Each column shows the mean with S.D. Scale bar; 50 μm. Source data for b, d, e, g, i are provided in Source data file.
a Wnt activity-dependent manner (Fig. 5h–l). Likewise in mESC-derived cells, ACTA2⁺ smooth muscle-like fibroblastic cells occupied Sox9⁻ region (Fig. 5i). These cells also expressed chondrogenic markers and smooth muscle cell markers (Fig. 5k, l and Supplementary Fig. 7b).

In this culture system, the removal of BMP4 from the growth factor cocktail did not affect differentiation, implying that exogenous BMP4 activation is dispensable (Supplementary Fig. 8h–j). Because of the obvious upregulation of the endogenous BMP4 gene in the hESC-derived LPM by day 2, endogenous BMP4 may be enough to induce tracheal mesoderm and chondrocytes (Supplementary Fig. 8k). Taken together, these data suggest that Wnt signaling plays a unique role in driving differentiation into tracheal mesoderm and...
Fig. 5 Generation of trachea mesodermal cells and chondrocytes from human ESCs in vitro. a Experimental design to generate tracheal mesoderm from hESCs. b Differentiating cells from hESCs at day 2. Cells were stained for FOXI1 (magenta) and GATA4 (green), respectively. % was calculated from randomly chosen 3 fields. Images are representative of two experiments. c qRT-PCR for LPM marker expression of hESC-derived LPM (n = 3 independent wells). Experiments were repeated at least twice. d Differentiating cells from hESCs at day 5. Cells were stained for TBX4 (green) and FOXF1 (magenta). % was calculated from randomly chosen three fields. Images are representative of three wells in a single experiment. e qRT-PCR for respiratory mesoderm marker expression of hESC-derived trachea mesodermal cells (n = 3 independent wells). Experiments were repeated at least twice. f Differentiating cells from hESCs at day 10. Cells were stained for TBX4 (green) and NKX6.1 (magenta). Images are representative of three wells in a single experiment. White arrows; TBX4+/NKX6.1+ mesodermal cells. White arrowheads; TBX4+/NKX6.1− mesodermal cells. Grey arrows; TBX4−/NKX6.1+ mesodermal cells. g The rate of differentiated cells at day 10. % was calculated from randomly chosen three fields. Images are representative of three wells in a single experiment. h Differentiating cells from hESCs with or without CHIR99021 at day 10. Cells were stained for ACTA2 (magenta) and SOX9 (green). Images are representative of at least two experiments. i qRT-PCR for SOX9 and ACTA2 expression of hESC-derived trachea mesodermal cells with different doses of CHIR99021 (n = 3 independent well). Experiments were repeated twice. j Differentiating cells from hESCs at day 10. Chondrocytes were stained with Alcian blue. The asterisk indicates a chondrocyte aggregate. Images are representative of two experiments. k Differentiating cells from hESCs at day 10. Cells were stained for COL1A1 (magenta) and AGGRECAN (green). Images are representative of two experiments. l Differentiating cells from hESCs at day 10. Cells were stained for TAGLN (magenta) and COL2A1 (green). Images are representative of two experiments. Each column shows the mean with S.D. (n = 3). Scale bar; 50 μm (d, f, j, k, l), 100 μm (b). Source data for b, c, d, e, g, i are provided in Source data file.

Discussion

This study demonstrates that endodermal-to-mesodermal canonical Wnt signaling is the cue that initiates trachea mesodermal development in developing mouse embryos, which is independent of the previously known Nkx2.1-mediated respiratory tissue development. Based on our knowledge of developmental biology, we successfully generated tracheal mesoderm and chondrocytes from mouse and human ESCs. In our protocol, we stimulated ESC-derived LPM with Wnt, Bmp and SHH signaling to mimic spatial information of the ventral anterior foregut. For induction of respiratory endoderm, Wnt, Bmp, and Fgf signaling are required to direct cells in anterior foregut to differentiate into the respiratory lineage30–32. Thus, Wnt, and Bmp signaling are conserved factors that provide spatial information, while Fgf and SHH are required in endoderm and mesoderm induction, respectively, reflecting the unique signaling pathways in each tissue. Mesoderm induction may need fewer exogenous growth factors because the mesodermal cells themselves are sources of spatial information, such as BMP4 in our protocol.

In our culture system, Bmp4 and Wnt activator were sufficient to induce tracheal mesoderm from mouse ES-derived LPM, but HH signalling was also required for the induction of human tracheal mesoderm. To investigate the involvement of HH signalling in mesodermal development, we examined Shh-null mouse embryos (Supplementary Fig. 9). Reflecting the observation in in vitro differentiation, Tbxi4 was still expressed in mutant, suggesting the dispensable role of HH signalling in mouse tracheal mesoderm. By contrast, in human, HH signalling is involved in TEF, but the characteristics of mesoderm is not yet defined yet48. In future study, it would be important to decipher the role of HH signalling on human trachea mesodermal development.

In chicken embryo, misexpression of Tbxi4 throughout the respiratory-esophageal region ectopically induces Nkx2.1 expression in distal esophageal endoderm underneath manipulated Tbxi4-expressing mesoderm49, indicating that mesodermal Tbxi4 expression defines tracheal identity in chicken. However, in mouse, we determined that Nkx2.1 expression was not affected in Wnt mutant (e.g., Dermo1Cre, Ctnnb1fl/fl/flox, and ShhCre, Whlflox/flox) although Tbxi4 expression was completely lost (Figs. 2d and 3b), suggesting that mesodermal Tbxi4 expression does not trigger tracheal endoderm specification, which is consistent with retaining Nkx2.1 expression in respiratory explants from Tbxi4-null mouse embryos10. These findings indicate that the mechanism inducing tracheal identity is different between mouse and chicken.

Another question is how trachea and lung are differentially specified, despite the strong commonalities between them. Canonical Wnt signalling induces Tbx4 expression in the tracheal mesoderm but not in the lungs. The trachea and lungs share the maker genes, such as endodermal Nkx2.1 and mesodermal Tbx4, but recent mouse genetics have accumulated the evidence that different combination of geneset regulated the trachea and the lungs individually. For instance, Fgf10-null mouse embryos failed lung development, but still develop the trachea50. In contrast, Bmpr1a, b-null embryos show the tracheal agenesis phenotype while the mutant embryos have the lung buds51. It would be interesting to examine expression patterns of receptors for Fgf10, Bmp, and Wnt in the trachea and lung that may distinguish these organs.

Past studies have proposed that Wnt signalling is required for cartilage formation in the late stage of trachea development19,28. Tracheal chondrocytes originate in Tbxi4-expressing cells, and Tbxi4/5 knockout mice show severe defects in chondrogenesis10,22. The early loss of Tbxi4 expression in Wnt mutant might contribute, at least a part, the abnormal structures of cartilage in the late stage of development.

In this study, we were unable to perform tissue-specific targeting for trachea endodermal or mesodermal cells because of multiple Cre-expression patterns in Shh-Cre and Dermo1-Cre mouse lines. For example, Shh is also expressed in the notochord and ventral neural tube51. Future studies with analyses of respiratory tissue-specific Cre lines would strengthen the evidence demonstrating that mutual interaction between respiratory endoderm and mesoderm is required for the induction of trachea development.

Dermo1Cre, Ctnnb1fl/fl/flox/flox mutants display a tracheal cartilage agenesis phenotype. Due to the multiple functions of Ctnnb1 in transcriptional regulation and cellular adhesion, however, it is possible that Ctnnb1 knockout affects not only Wnt-mediated transcriptional regulation but also mesenchymal cell-cell adhesion19. To exclude this possibility, we examined the distribution of Cadh2 as an adhesive molecule in tracheal mesoderm. Cadh2 expressions in the ventral half of tracheal mesoderm were indistinguishable between control and Dermo1Cre, Ctnnb1fl/fl/flox/flox embryos (Supplementary Fig. 3). Furthermore, our luciferase assay showed that respiratory mesenchyme specific cis-regulatory region of Tbxi4 is stimulated by CHIR99021 through Tcf/Lef-binding elements in the developing tracheal mesoderm in vitro.
These findings suggest that Wnt signaling-mediated transcriptional regulation is important for the induction of tracheal mesoderm.

Recently, Han et al. delineated mesodermal development during organ bud specification using single-cell transcriptomics analyses of mouse embryos from E8.5 to E9.5. Based on the trajectory of cell fates and signal activation, this group also generated organ-specific mesoderm, including respiratory mesoderm, from hESCs, thereby determining that Wnt, BMP4, SHH, and retinoic acid direct differentiation of hESC-derived splanchnic mesoderm to respiratory mesoderm, supporting our current findings. In our protocol, retinoic acid was not included in the media, but alternatively vitamin A, a precursor for retinoic acid, was supplemented. Endogenous metabolite of vitamin A might affect the differentiation into respiratory mesoderm.

These culture methods could be a strong tool to study human organogenesis and the aetiology of TEA and TA, as well as to provide cellular resource for human tracheal tissue repair.

Methods

Mice. All mouse experiments were approved by the Institutional Animal Care and Use Committee of RIKEN Kobe Branch. Mouse were handled in accordance with the ethics guidelines of the institute. Mice were housed in 18–23 °C with 40–60% humidity. A 12-h light/12-h dark cycle was used. Nkx2.5<sup>tm1.swa</sup>, Shh<sup>Cre</sup>, Dermo1<sup>Cre</sup>, Cnmbi<sup>b</sup>1<sup>fl</sup>er<sup>ER</sup>, Wnt5b<sup>fl</sup>er<sup>ER</sup>, Wnt6<sup>fl</sup>er<sup>ER</sup> mice were previously generated<sup>53,54</sup>. In all experiments, at least three embryos from more than two litters were analyzed. All attempts were successful, and sample size was not estimated by statistical methods. No data were excluded in this study. All control and mutant embryos were analyzed. We did not distinguish the sex of the embryos. No blinding was done in this study.

Immunostaining. Mouse embryos were fixed by 4% Paraformaldehyde/PBS (PFA) at 4 °C overnight. Specimens were dehydrated by ethanol gradient and embedded in paraffin. Paraffin sections (6-μm) were deparaffinized and rehydrated for staining. Detailed procedure and antibodies of each staining was listed in Supplementary Table 1.

In situ hybridization. Mouse embryos were fixed with 4% PFA/PBS at 4 °C overnight, and then tracheas were dissected. Specimens were incubated in sucrose gradient (10, 20, and 30%) and embedded in OCT compound. Frozen sections (12-μm) were subjected to in situ hybridization. For Wnt2, 4, 5, 7b probe construction, cDNA fragments were amplified by primers listed in Supplementary Table 2. These cDNA fragments were subcloned into pBluescript SK<sup>+</sup> at EcoRI and SaI sites. For Wnt5b and six probes, pSPROT1-Wnt5b (MCH085322) and pSPROT1-Wnt6 (MCH050524) were linearized at SaI sites. The NIA/NH Mouse 15 K and 7.4 K cDNA fragments were cloned into pBluescript SK<sup>+</sup> by REPOK<sup>BC</sup>-<sup>Lo</sup>. Antisense cRNA transcripts were synthesized with DIG labeling mix (Roche Life Science) and T3 or SP6 RNA polymerase (New England Biolabs Inc.). Slides were permeabilized in 0.1% Triton X-100/PBS for 30 min and blocked in acetylation buffer. After prehybridization, slides were hybridized with 500 ng/ml of DIG-labeled cRNA probes overnight at 65 °C. After washing with SSC, slides were incubated with anti-DIG-AP antibodies (1:1000, Roche Life Science, 11093274910). Sections were colored with BM-purple (Roche Life Science, 11442074001). For RNAscope experiments, the RNAscope Multiplex Fluorescent v2 assays (Advanced Cell Diagnostics, 232110) were used. The detailed procedure and probes were listed on Supplementary Table 3.

Cell culture. For mesodermal differentiation from mES cells, C57BL/6J-Chr 12 A/J/Nal AC464/Gsr mES cells (The Jackson Laboratory) and EB3 cells (AESC0193, RIKEN BioResource Center) were used. C57BL/6J-Chr 12 A/J/Nal AC464/Gsr mES cells were kindly provided by Kentaro Iwasawa and Takakuni Takebe (Center for Stem Cell & Organoid Medicine (CuStOM), Perinatal Institute, Division of Gas- troenterology, Hepatology and Nutrition, Cincinnati Children’s Hospital, Cincinnati). EB3 was kindly provided by Dr. Hitoshi Niwa (Department of Pluripotent Stem Cell Biology, Institute of Molecular Embryology and Genetics in Kumamoto University).<sup>55,56</sup> Cells were maintained in 21 ± leukemia inhibitory factor (LIF) media (1000 units ml<sup>−1</sup> LIF, 0.4 μM PD0325901, 3 μM CHIR99021 in N2B27 medium) on orithine-laminin coated dishes.<sup>57</sup> For mesodermal differentiation of mouse ES cells, cells were digested by TrypLE express (Thermo Fisher Scientific, 12604013) and seeded onto Matrigel-coated 12-well plate. EpI-LC were induced by EpLc differentiation medium (1% knockout serum, 20 ng ml<sup>−1</sup> Activin A, 12 ng ml<sup>−1</sup> FGF2, and 10 μM Y27632 in N2B27 Medium)<sup>58</sup> for 2 days. Lateral plate mesoderm was established by using Loh’s protocol with some modification. Cells were digested by TrypLE express to single cells and seeded onto Matrigel-coated 12-well plate at the density of 6 × 10<sup>5</sup> cells per well. The detailed procedure and probes were listed on Supplementary Table 3.

Quantitative RT-PCR. Total mRNA was isolated by using the Nucleosipin kit (TaKaRa, 740955) according to manufacturer’s instruction. cDNA was synthesized with SuperScript<sup>TM</sup> VIVO CDNA synthesis kit (Thermo Fisher Scientific, 11754050). qPCR was performed by PowerUp<sup>TM</sup> SYBR<sup>TM</sup> Green Master Mix on QuantStudio 3 or 6. Primer sequences were listed on Supplementary Tables 5 and 6. Data are expressed as a fold-change and were normalized with undifferentiated cell expression.

Statistical analyses. Statistical analyses were performed with Excel2013 (Microsoft) or PRISM<sup>®</sup> (GraphPad software). For multiple comparison, one-way ANOVA and two-tailed Tukey’s methods were applied. For paired comparison, statistical significance was determined by F-test and Student’s or Welch’s two-tailed t-test.
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Author contributions
K.K. and M.M. designed the project and performed experiments with the aid of A.L.M., A.Y., C.M., and K.T.F. A.M.Z. analysed single-cell transcriptomics for definitive endoderm and splanchic mesoderm. A.L.M. performed enhancer analyses of Tbx4 gene and supported human ES cell experiments. A.Y. supported mouse experiments. K.K., K.T.F., and C.M. performed mouse ES cell experiment. C.A. and M.H. contributed to mouse and human embryonic-stem-cell-based lateral plate mesoderm induction and differentiation experiments. K.K. and M.M. wrote the manuscript with the contribution of all authors.

Competing interests
The authors declare no competing interests.

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