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Coordination of heart and lung co-development by a multipotent cardiopulmonary progenitor

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SUMMARY

Co-development of the cardiovascular and pulmonary systems is a recent evolutionary adaption to terrestrial life that couples cardiac output with the gas exchange function of the lung [1]. In this report, we show that the pulmonary vasculature develops even in the absence of lung development. We have identified a population of multi-potent cardiopulmonary mesoderm progenitors (CPPs) within the posterior pole of the heart that are marked by the expression of Wnt2/Gli1/Isl1. We show that CPPs arise from cardiac progenitors prior to lung development. Lineage tracing and clonal analysis demonstrates that CPPs generate the mesoderm lineages within the cardiac inflow tract and lung including cardiomyocytes, pulmonary vascular and airway smooth muscle, proximal vascular endothelium, and pericyte-like cells. CPPs are regulated by hedgehog expression from the foregut endoderm, which is required for connection of the pulmonary vasculature to the heart. Together, these studies identify a novel population of multipotent cardiopulmonary progenitors that coordinates heart and lung co-development that is required for adaptation to terrestrial existence.

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AUTHOR CONTRIBUTIONS

T.P. and E.E.M. designed the experiments. T.P., Y.T., C.J.B., M.M.L., R.S.K., K.M.S. performed experiments. S.M.E. provided animal lines and expertise. T.P. and E.E.M. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests

LETTER

The coordinated development of the cardiovascular and pulmonary organ systems is illustrated in embryonic development, when the lung endoderm protrudes into the cardiac mesoderm as the two organs develop in parallel to form the cardiopulmonary circulation. However, little is known about the origins and pathways involved in co-development of the cardiopulmonary system. We assessed pulmonary vascular development in a model of lung agenesis through conditional deletion of *β-catenin* (*Ctnnb1*) within the anterior foregut (AFG) endoderm [2,3]. The lung fails to develop from the foregut in Shh^{cre}:$Ctnnb1^{flox/flox}$ mutants, while the development of the heart and other foregut-derived organs remains intact (Supplemental Fig. 1) [2,3]. Using CD31 whole mount immunostaining and confocal microscopy to visualize early cardiopulmonary vascular development we show that in wild-type embryos, the paired pulmonary arteries (PA) descending from the outflow tract (OFT) of the heart and the pulmonary veins (PV) extending from the atria connect with the lung bud to form a vascular plexus at E10.5 (Fig. 1a–e and Supplemental Video 1). In Shh^{cre}:$Ctnnb1^{flox/flox}$ lung agenesis mutants, the PA and PV continues to develop and intersect at a region approximately where the lung bud would normally form (Fig. 1f–i and Supplemental Video 2). Although this vascular plexus persists throughout embryonic development in the absence of lung, it fails to branch or develop further (Supplemental Fig. 2a–d). Interestingly, Shh is expressed in the AFG even in the absence of lung development (Supplemental Fig. 2e–h).

The presence of both the PAs and the PVs in the absence of lung development suggests that cardiac progenitors contribute to the generation of these structures. The cardiac progenitors closest to the anterior foregut where the lung arises are defined by expression of Isl1 and are referred to as the second heart field (SHF) (Supplemental Fig. 3) [4]. Nkx2.5 expression further subdivides the Isl1+ domain into a ventral/medial domain that expresses both Isl1 and Nkx2.5, and a lateral/dorsal domain that expresses only Isl1 (Supplemental Figs. 3 and 7). We performed cell lineage tracing using the $Isl1^{cre}$, $Isl1^{MerCreMer}$, and $Nkx2.5^{cre}$ mouse strains to define the contribution of these lineages to pulmonary mesoderm derivatives (Fig. 1 and Supplemental Fig. 3) [4,5]. Lineage tracing using the $Isl1^{MerCreMer}$:$R26R^{tdTomato}$ mice demonstrates that Isl1+ cells tagged at E8.5 prior to lung development give rise to all layers of the pulmonary vasculature as well as the myocardium of the cardiac inflow tract (Fig. 1k–n and data not shown). This is supported by lineage tracing with the constitutive $Isl1^{cre}$ demonstrating that Isl1+ cells generate ventral lung mesenchyme adjoining the inflow tract of the heart, whereas lineage tracing with $Nkx2.5^{cre}$ shows that Nkx2.5+ progenitors only generate the myocardium of the proximal PV (Fig. 1o and Supplemental Fig. 3).

We generated a $Wnt2^{creERT2}$ mouse to delineate the temporal and spatial relationship between the ventral mesoderm flanking the anterior foregut and overlapping the posterior pole of the heart with the development of pulmonary mesoderm lineages (Supplemental Fig. 4). Wnt2 is expressed in a unique pattern that is confined to the posterior pole of the developing heart adjacent to the AFG prior to lung development at E8.5–E9.5 (Fig. 2a and b, [6,7]). Lineage labeling of Wnt2+ cells at E8.5 reveals that Wnt2+ progenitors generate cells within the cardiac inflow tract but not the outflow tract (Supplemental Fig. 5), and these cells progressively move into the developing lung bud (Fig. 2c and d, Supplemental

Fig. 6). By E17.5, Wnt2+ cells labeled at E8.5 generate multiple mesoderm lineages within the developing heart including cardiomyocytes and endocardium (Supplemental Fig. 6). Importantly, Wnt2+ cardiac progenitors can generate all layers of the pulmonary vasculature, airway smooth muscle, and Pdgfrβ+/NG2+ lung pericyte-like cells similar to the Isl1+ cardiac progenitor population (Fig. 2e–h and Supplemental Fig. 6). Thus, prior to lung specification, Wnt2 marks a multipotent progenitor population that generates the majority of the repertoire of mesodermal lineages in the developing lung and cardiac inflow tract.

Gli1 is expressed in a temporal and spatial pattern similar to that of Wnt2 and Isl1 in the ventral mesoderm surrounding the anterior foregut that is activated by Shh[8]. We performed lineage tracing with the *Gli1*^{creERT2} mouse and our data show that Gli1+ cells marked at E8.5 can contribute to the inflow tract mesoderm of the heart as well as cells surrounding the early lung bud similar to the Wnt2+ progenitors (Fig. 2i and j and Supplemental Fig. 7). Examination of the fate of E8.5 Gli1+ progenitors at E17.5 shows that they can generate all of the mesoderm derivatives within the cardiac inflow tract and developing lung including vascular and airway smooth muscle, proximal vessel endothelium, and PV myocardium in a manner indistinguishable from Wnt2+ and Isl1+ progenitors (Fig. 2k–n and Supplemental Fig. 6). Furthermore, Wnt2+ progenitors co-express Isl1 and Gli1 (Supplemental Fig. 7). These data identify a population of multipotent cardiopulmonary mesoderm progenitors (CPPs) defined by Wnt2+/Gli1+/Isl1+ expression that generates the majority of mesoderm lineages in the lung and cardiac inflow tract (Fig. 2o).

Wnt2+, Gli1+, nor Isl1+ progenitors contributed in a significant manner to the distal alveolar capillary endothelium of the lung, but did contribute to the VWF+ proximal endothelium of the pulmonary vessels (Supplemental Fig. 8). Using the VE-cadherin^{creERT2}*:R26R*^{mTmG} mouse line, we show that VE-cadherin+ endothelial cells at E8.5 give rise to both the proximal and distal alveolar capillary endothelium of the lung [9] (Supplemental Fig. 8). This suggests that the distal alveolar capillary endothelium arises prior to lung development from a VE-cadherin+ population distinct from CPPs. Interestingly, Wnt2+ cells labeled after E12.5 exhibit a significant reduction in their ability to contribute to vascular or airway smooth muscle, the cardiac inflow tract, and proximal vascular endothelium but retain their ability to contribute to the Pdgfrβ+ lung pericyte-like cells in the alveoli (Supplemental Figs. 9 and 10).

To define the clonal relationship of cell lineages generated by CPPs, we performed clonal analysis using limiting amounts of tamoxifen at E8.5 in *Wnt2*^{creERT2}*:R26R*^{mTmG} mice that would reproducibly induce single clones of 1–3 cells at E9.5 within the posterior pole of the heart (Fig. 3a–c, Supplemental Fig. 11, Supplemental Table 1, Supplemental Table 2) [10]. All of the clones with cell clusters in the lung had associated clusters in the sinus venosus and the posterior wall of the atria at E10.5–E11.5, suggesting that CPPs clonally generate cell lineages within the cardiac and pulmonary mesoderm (Fig. 3b and c and Supplemental Table 2). To confirm these results, we performed clonal analysis utilizing *Gli1*^{creERT2}*:R26R*^{confetti} mice [11]. Tamoxifen induction at E8.5 led to well-isolated and same-colored clones located in both the primitive lung bud and sinus venosus/posterior atria (Fig. 3d and e, Supplemental Fig. 11, and Supplemental Table 3). These analyses also show that vascular smooth muscle,

airway smooth muscle, proximal endothelium, and Pdgfrβ+ pericytes-like cells share a common clonal origin in the lung (Fig. 3f–h and Supplemental Table 4).

The expression of Shh in the anterior foregut endoderm adjacent to the cardiac inflow tract during pulmonary vascular initiation (Fig. 4a, [2]), and the co-expression of the hedgehog activated effector Gli1 with Wnt2 and Isl1 in CPPs (Supplemental Fig. 7), suggest that hedgehog signaling plays an important role in regulating CPP development. *Shh*$^{-/-}$ mutants have a disorganized vascular endothelial plexus along the anterior foregut that fails to connect to the inflow and outflow tract of the heart (Fig. 4b and c). Conditional inactivation of smoothened (Smo) within different vascular lineages and in CPPs shows that loss of hedgehog signaling in endothelium and smooth muscle does not affect cardiopulmonary vascular patterning and differentiation (Supplemental Fig. 12,[12,13]). In contrast, Smo inactivation in Isl1+ CPPs reproduced the *Shh*−/− phenotype (Fig. 4d and Supplemental Fig. 13). *Isl1*^{cre}*:Smo*$^{flox/flox}$ embryos exhibit a persistence of an aortopulmonary collateral circulation in addition to the persistent truncus arteriosus previously reported (Supplemental Fig. 13) [14]. *Isl1*^{cre}*:Smo*$^{flox/flox}$ mutants also demonstrate severe inflow tract defects with pulmonary vein atresia (Supplemental Figs. 13 and 14). Lineage tracing analyses revealed a significant reduction of vascular and airway smooth muscle derived from Isl1+ and Gli1+ CPPs lacking Smo expression compared to controls (Fig. 4e–l).

We have used cell lineage tracing coupled with clonal analysis, to identify and characterize a novel population of mesoderm progenitors called CPPs that generate both cardiac inflow tract and pulmonary mesoderm cell lineages (Fig. 4m–p). Furthermore, we show that hedgehog signaling is required for the development of CPPs into the smooth muscle compartment in the lung and promotes the cardiopulmonary connection essential for terrestrial existence.

METHODS

Animals

The *Wnt2*^{creERT2} mouse line was generated by insertion of the creERT2 cDNA downstream of the ATG of the mouse *Wnt2* gene using homologous recombination in mouse embryonic stem cells as previously described [15]. Schematic of targeting construct and Southern blot probes are shown in Supplemental Fig. 4. PCR genotyping primers for *Wnt2*^{creERT2} are: common forward primer: 5′-TGAGTCTCACCACTAGCCGCA-3′; *Wnt2*^{creERT2} WT reverse: 5′-ACTGGGAATCAGCCAGGGAGGGT-3′; *Wnt2*^{creERT2} mutant reverse: 5′-TCCAGGTATGCTCAGAAAACG-3′. Generation and genotyping of the *Gli1*^{creERT2}, *Nkx2.5*^{cre}, *Isl1*^{cre},*R26R*^{confetti}, *Smo*$^{flox/flox}$, *R26R*^{mTmG}, *Tie2*^{cre}, *Sm22*^{cre}, *VE-Cadherin*^{creERT2}, *Shh*^{cre}, *Ctnnb1*$^{flox/flox}$, *Gli1*$^{LacZ/+}$, and *Isl1*^{MerCreMer} lines have been previously described[4,5,9,11,16–21]. All animal experiments were done with approval of the University of Pennsylvania IACUC Committee.

Histological analysis

At least 3 mouse embryos per genotype were collected at each indicated time points and fixed in 2% paraformaldehyde, dehydrated in a series of increasing ethanol concentration

washes, embedded in paraffin and sectioned (except the experiments performed with the *Isl1*^{MerCreMer}:*R26R*^{tdTomato} embryos, which were performed with frozen sectioning as previously described [21]). Antibodies used are anti-smooth muscle actin (mouse anti-SMA 1:200 Abcam), CD31 (rat anti-CD31 1:500 BD Pharmingen), Von Willibrand Factor (rabbit anti-VWF 1:200 Sigma), MF20 (mouse anti-MF20 1:20 Abcam), Isl1 (mouse anti-Isl1 1:10 HybridomaBank), Nkx2.5 (goat anti-Nkx2.5 1:10 Santa Cruz), Sarcomeric α-actinin (mouse anti-SAA 1:100 Sigma), NG2 (rabbit anti-NG2 1:100 Millipore), and GFP (goat anti-GFP 1:100 Abcam). LacZ staining of embyros was performed as previously described [22]. In situ hybridization was performed as previously described [24]. For whole mount immunohistochemistry of embryos, a previously published protocol was employed [25]. Embryos were fixed in 2% paraformaldehyde for 20 min, and washed with cold PBS 3 times for 10 min each. The washed embryos were then washed with blocking solution (0.4% Triton X-100, 5% serum in PBS) for 1 hour on ice, and incubated with primary antibody (rat anti-CD31 1:500 BD Pharmingen, rabbit anti-GFP 1:1000 MBL, goat anti-GFP 1:500 Abcam, rabbit anti-SM22α, 1:250 Abcam) in blocking solution overnight. The next day, embryos were washed in 0.4% Triton x-100 in PBS on ice 3 times for one hour each and incubated with secondary antibody overnight. The next day, the embryos were washed on ice 3 times for one hour each. The stained embryos were then dehydrated in 100% methanol, and then BABB (1 part benzyl alcohol: 2 part benzyl benzoate) and mounted on a slide with Fastwell and sealed with cover slip. The slide was imaged on a Zeiss LSM 710 confocal microscope and analyzed and reconfigured in ImageJ software.

Imaging of *Gli1*^{creERT2}:*R26R*^{confetti} embryos with immunohistochemistry

A previously published protocol for immunostaining of thick tissue sections embedded in agarose was employed [23]. Embryos were fixed in 4 % paraformaldehyde overnight and washed with cold PBS 4 times for 30 minutes each. The embryos was then embedded in 4% agarose and sectioned on a vibratome to obtain 300 μm thick slices. The individual slices embedded in agarose were then incubated in blocking solution (0.4% Triton X-100, 5% serum in PBS) for 1 hour on ice, and incubated with primary antibody (rat anti-CD31 1:500 BD Pharmingen, rabbit anti-SM22α, 1:250 Abcam) in blocking solution overnight. The next day, slices were washed in 0.4% Triton X-100 in PBS on ice three times for one hour each and incubated with secondary antibody overnight. The next day, the slices were washed on ice three times for one hour each, and then mounted on a glass slide with Prolong Gold anti-fade reagent (Molecular Probes) and sealed with cover slip. The slides were imaged on a Zeiss LSM 710 confocal microscope and analyzed in ImageJ software.

Clonal analysis

To assess for clonal relationships in CPPs, the *Wnt2*^{creERT2}:*R26R*^{mTmG} were treated with tamoxifen at E8.5 at a concentration (0.025 mg/g body weight) that was emprically determined to generate small clones or cluster of cells 1–3 in number at 24 hours after treatment. A total of 59 embryos between E10.5–11.5 were determined to contain GFP clusters, and each was imaged per whole mount immunohistochemistry protocol with CD31/GFP double staining described above. Z-stacks were acquired for the entire volume capturing the heart and the lung to include all of the GFP+ cells. From 59 embryos, 22 clusters of GFP+ cells were identified that were spatially segregated with cellular

distribution in the lung mesenchyme. Cells per cluster were counted through all Z-stacks and doubling time was calculated ($DT=(t-t_0)\log_2/(\log N-\log N_0)$). For clonal analysis of *Gli1*^{creERT2}:*R26R*^{confetti} mice, 0.05 mg/g body weight of tamoxifen was injected at E8.5 and tissue was collected at the indicated time points and sectioned at a thickness of 300 μm and imaged per protocol described above with CD31 or SM22α counterstaining. Z-stacks were acquired for the entire volume capturing the entire thickness of the section to include all fluorescently labeled cells. From 23 embryos, 15 clusters of single-colored and spatially segregated cell clusters with distribution in the lung mesenchyme were identified for cellular localization, and 19 clusters for concurrent lineage analysis.

Cell Counting

Sections included in cell count analysis were acquired using confocal microscopy. At least 3 embryos per genotype per time point were used and at least 5 sections were used per embryo at comparable levels. A minimum of 300 cells were counted per embryo. Cell counts were performed on ImageJ using the “Cell Counter” plug-in and performed by two people blinded to the specimen genotype and condition. Results were averaged between the two cell counters and standard deviations were calculated per genotype/time point. 1-tailed paired t-tests were used to determine the p value.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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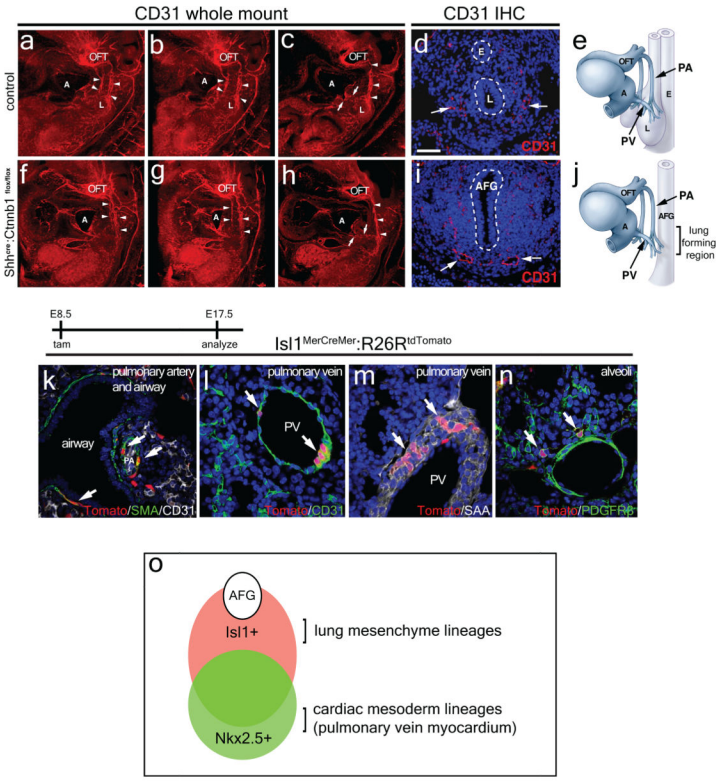

Figure 1. The pulmonary vasculature develops in the absence of lung specification

The paired PAs are observed tracking in a posterior direction in both the control (a–c, arrowheads) and *Shh*^{cre}*:Ctnnb1*$^{flox/flox}$ mutants (f–h, arrowheads) at E10.5 using CD31 whole mount immunostaining and section immunostaining (d and i, arrows). The primitive PVs are observed emerging from the atria and extending towards the region of the foregut where the lung would normally form in both control and *Shh*^{cre}*:Ctnnb1*$^{flox/flox}$ mutants (c and h, arrows). These developmental hallmarks are illustrated in models (e and j). Isl1+ progenitors tagged at E8.5 give rise to the PA and airway smooth muscle (k), endothelial (l), myocardial (m), and Pdgfrβ+ pericyte-like (n) lineages of the pulmonary vasculature and other mesodermal derivatives. This overlapping but distinct origin of lung mesoderm derivatives is diagrammed in (o). PA=pulmonary artery, PV=pulmonary vein, AT=atria, AFG=anterior foregut. OFT=outflow tract, L=lung, T=trachea, E=esophagus. Control for a–h= *Shh*^{cre}*:Ctnnb1*$^{flox/+}$. Scale bar (g and h)=100 μm.

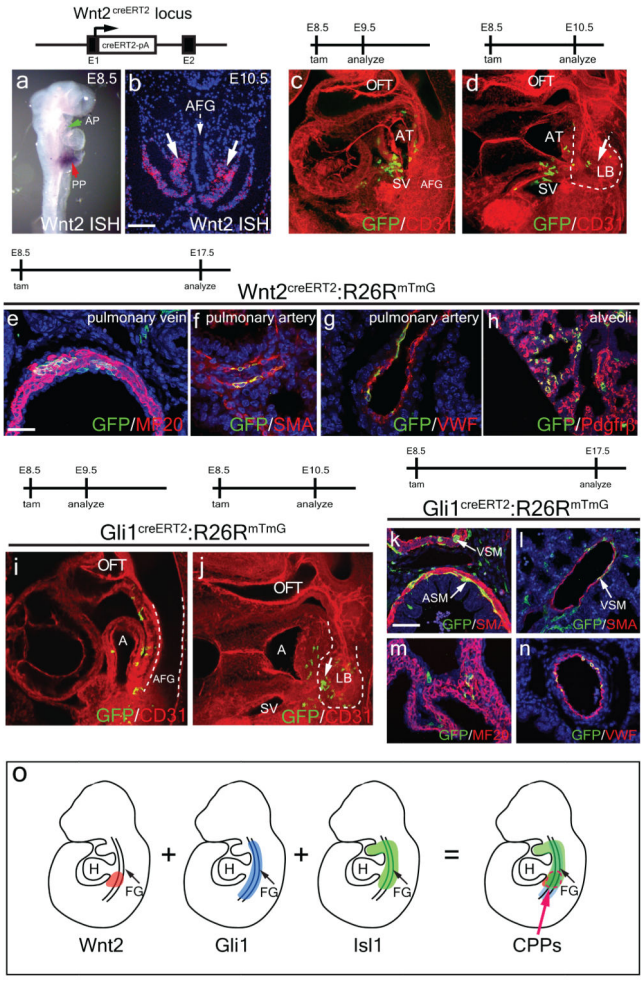

Figure 2. Wnt2+ and Gli1+ cells define a cardiopulmonary progenitor (CPP) and generate mesoderm derivatives of the lung and cardiac inflow tract

Wnt2 is expressed in the ventral mesoderm surrounding the anterior foregut and overlapping the posterior pole of the heart (a and b, arrows). Wnt2+ cells tagged at E8.5 can generate cells within the cardiac inflow tract as well as in the lung mesoderm (c and d). Wnt2+ cells tagged at E8.5 generate myocardium of the PV, smooth muscle of the PA, endothelium of the proximal PA, and Pdgfrβ+ pericyte-like cells in the lung by E17.5 (e–h). Gli1+ cells generate derivatives within the inflow tract and early lung mesoderm similar to Wnt2+ cells (i and j) and generate mesoderm lineages within the lung including airway and vascular smooth muscle (k and l), and endothelium of the proximal pulmonary vessels (n) as well as the myocardium of the atria (m). CPPs are located in a region of overlapping Wnt2, Gli1, and Isl1 expression between the developing heart and the anterior foregut (o). AP=anterior pole of the heart, PP=posterior pole of the heart, AFG=anterior foregut, AT=atria, OFT=outflow tract, PA=pulmonary artery, PV=pulmonary vein, LB=lung bud, ASM=airway smooth muscle, VSM=vascular smooth muscle, SMA=smooth muscle actin, SAA= sarcomeric α-actinin, VWF=von Willibrand factor. Scale bars b=100 μm, e–h=50 μm, k–n=50 μm.

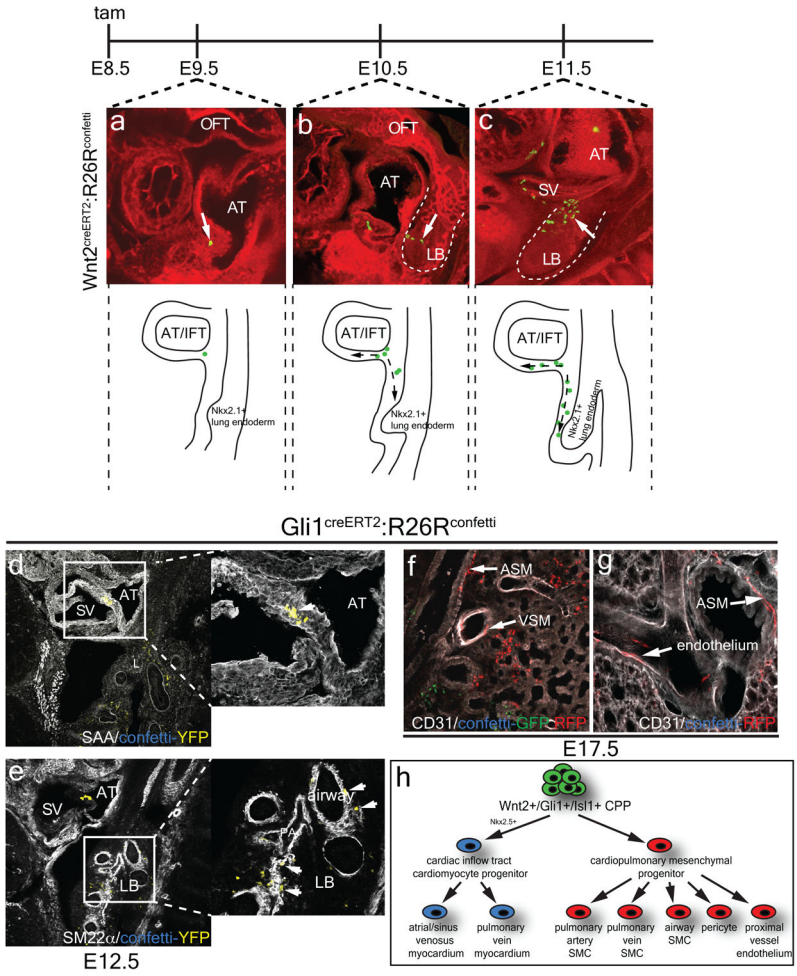

Figure 3. Clonal analysis reveals that CPPs generate related lineages within the cardiopulmonary system

Single clones of Wnt2+ CPPs contribute to both the developing cardiac inflow tract as well as the mesoderm of the lung (a–c). Clonal analysis with the *Gli1*^{creERT2}*:R26R*^{confetti} line shows that a single CPP clone (YFP+) can generate myocardium (SAA+) within the cardiac inflow tract (d, arrow) as well as lung mesodermal lineages such as smooth muscle (SM22α +) around the airway and PA (e, arrows). Clonal analysis of *Gli1*^{creERT2}*:R26R*^{confetti} lungs show that vascular and airway smooth muscle and endothelium of the proximal vessels share a common Gli1+ progenitor at E8.5 (f and g). A cell lineage tree showing the relationship of CPPs to differentiated lineages within the cardiopulmonary system (h). AT=atria, OFT=outflow tract, LB=lung bud, SV=sinus venosus, ASM=airway smooth muscle, VSM=vascular smooth muscle, SAA= sarcomeric α-actinin.

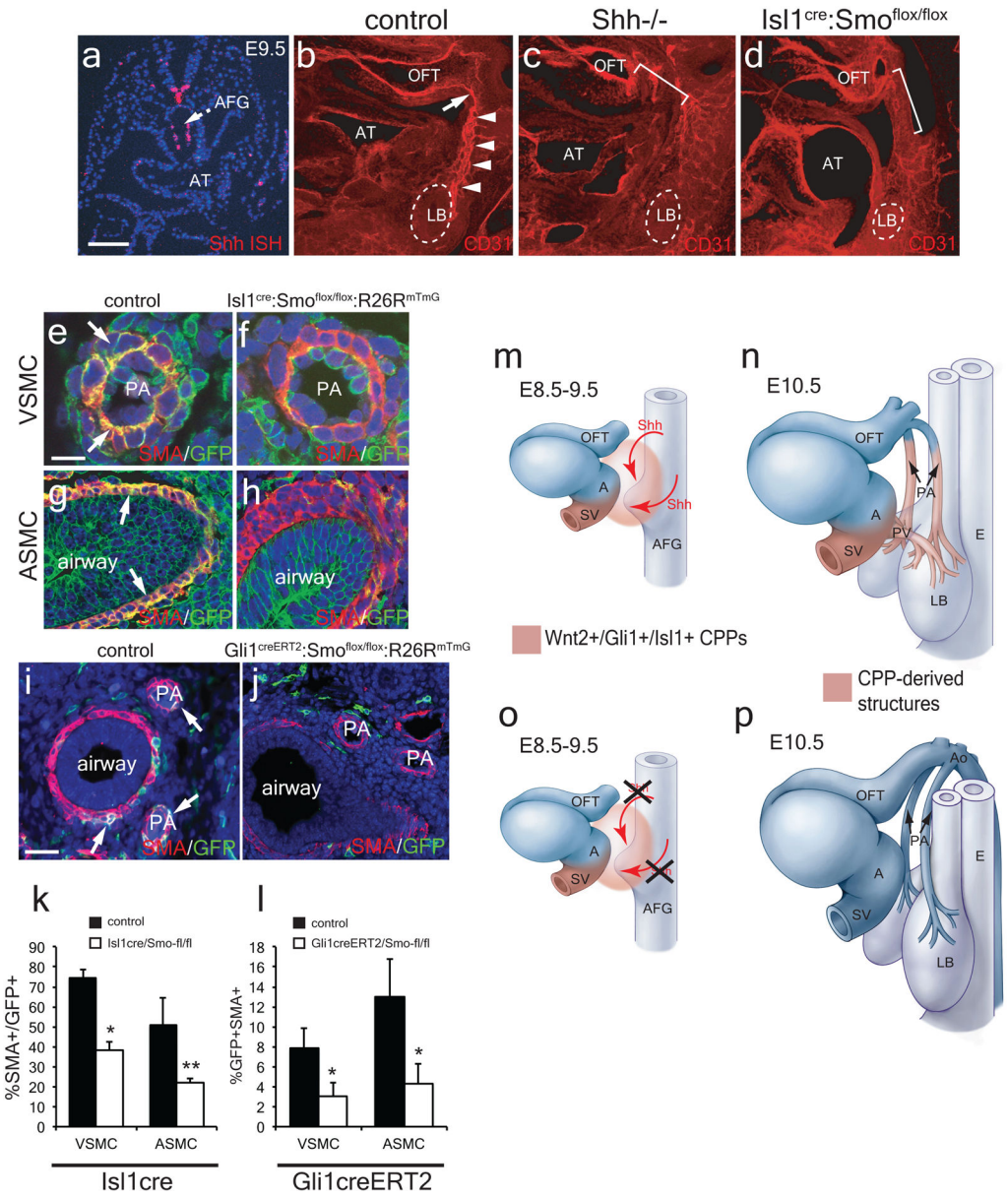

Figure 4. Hedgehog signaling is required in CPPs to coordinate the vascular connection between the heart and lung

Shh expression at E9.5 in the anterior foregut endoderm by in situ hybridization (a). *Shh*−/− mutants and *Isl1*^{cre}:*Smo*$^{flox/flox}$ mutants display a disrupted vascular plexus between the developing heart and lung at E10.5 (b–d, brackets, control=*Shh* +/−). Inactivation of Smo within Isl1+ CPPs inhibits their contribution to airway and vascular smooth muscle at E13.5 (e–h, k, control= *Isl1*^{cre}:*Smo*$^{flox/+}$:*R26R*^{mTmG}). Inactivation of Smo within Gli1+ CPPs inhibits their contribution to airway and vascular smooth muscle at E13.5 (i, j, l, control= *Gli1*^{creERT2}:*Smo*$^{flox/+}$:*R26R*^{mTmG}). CPPs orchestrate lung and cardiac co-development and are regulated in turn by Shh expression from the anterior foregut endoderm (m–p). AT=atria, AFG=anterior foregut, OFT=outflow tract, LB=lung bud, PA=pulmonary artery, Ao=aorta,

PV=pulmonary vein, SV=sinus venosus, SVC and IVC=superior and inferior vena cava. *p<0.01, **p<0.05. Error bars=S.D. Scale bars A=50 μm, E-h=20 μm, i and j=50 μm.