PBX transcription factors drive pulmonary vascular adaptation to birth

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A critical event in the adaptation to extrauterine life is relaxation of the pulmonary vasculature at birth, allowing for a rapid increase in pulmonary blood flow that is essential for efficient gas exchange. Failure of this transition leads to pulmonary hypertension (PH), a major cause of newborn mortality associated with preterm birth, infection, hypoxia, and malformations including congenital diaphragmatic hernia (CDH). While individual vasoconstrictor and dilator genes have been identified, the coordination of their expression is not well understood. Here, we found that lung mesenchyme–specific deletion of CDH-implicated genes encoding pre–B cell leukemia transcription factors (Pbx) led to lethal PH in mice shortly after birth. Loss of Pbx genes resulted in the misexpression of both vasoconstrictors and vasodilators in multiple pathways that converge to increase phosphorylation of myosin in vascular smooth muscle (VSM) cells, causing persistent constriction. While targeting endothelin and angiotensin, which are upstream regulators that promote VSM contraction, was not effective, treatment with the Rho-kinase inhibitor Y-27632 reduced vessel constriction and PH in Pbx-mutant mice. These results demonstrate a lung-intrinsic, herniation-independent cause of PH in CDH. More broadly, our findings indicate that neonatal PH can result from perturbation of multiple pathways and suggest that targeting the downstream common effectors may be a more effective treatment for neonatal PH.

Introduction

Early fetal detection and improvements in medical and surgical management of complex developmental defects have resulted in increased survival among infants born with previously lethal anomalies. Congenital diaphragmatic hernia (CDH) is a common congenital anomaly that occurs in 1 in 3,500 to 5,000 live births (1, 2). Despite improvements in the care of infants with CDH, the mortality rate associated with the disease continues to be 20% in isolated cases and 50% in complex cases (3–5). The primary cause of the high mortality rate is a lethal combination of pulmonary hypoplasia and pulmonary hypertension (PH) (6, 7). The severity of these defects is highly variable among patients. Furthermore, the PH that frequently leads to lethality in patients with CDH is often not responsive to available medications (8–11).

The longstanding model of CDH is one that originates from malformation of the diaphragm, and, secondarily, herniation of the abdominal organs into the chest causes compression of the lungs and pulmonary vasculature, preventing their normal development. However, the severity of lung hypoplasia and PH and the rate of mortality in patients with CDH exceed what is observed in infants with other malformations, such as congenital pulmonary airway malformation or omphalocele, which similarly compromise lung development due to mechanical compression (12, 13). These findings raise the possibility that there may be diaphragm-independent causes of lung abnormalities in CDH. Indeed, lung growth and patterning defects have been observed in animal models of CDH prior to normal diaphragm closure (14–19). However, there has been no clear in vivo demonstration as yet that PH can arise independently of the diaphragmatic defect and herniation seen in CDH.

Recent genomic sequence analysis of patients with CDH, correlated with gene expression studies in the developing mouse diaphragm, has identified a growing list of candidate genes implicated in the pathogenesis of CDH (14, 16, 20–23). Among them are Pbx genes that encode homeodomain-containing transcription factors best known to act as Hox cofactors (24, 25). Global inactivation of Pbx1 in mice led to diaphragm malformation, supporting its involvement in CDH (26). In this study, we set out to address the role of Pbx in pulmonary vascular development, a process that is frequently impaired in patients with CDH. Our findings support the model that mutation of CDH candidate genes can act within the developing lung to directly impair lung and pulmonary vascular development and function, independently of their role in diaphragm formation. Furthermore, our data illustrate the importance of identifying the causative genetic defect in patients with CDH and demonstrate how, in the mouse model, pharmacological manipulation of the altered downstream signaling cascades can be explored to reverse the lethal PH that affects infants with the disease.

Conflict of interest: The authors have declared that no conflict of interest exists.
Reference information: J Clin Invest. https://doi.org/10.1172/JCI93395.
vascular smooth muscle (VSM) cells, alveolar myofibroblasts, and lipofibroblasts, as indicated by costaining with cell-specific markers (Figure 1, C–F). Both the level and pattern of PBX1 expression are maintained in the late embryonic and early postnatal lung. PBX1 is not expressed in epithelial type 1 or type 2 cells (Supplemental Figure 1, A and B; supplemental material available online with this article; https://doi.org/10.1172/JCI93395DS1).

Lung mesenchymal deletion of Pbx1/2 results in postnatal lethality, lung simplification, and cardiac hypertrophy. To determine whether Pbx genes play a cell-autonomous role in the lung, we applied a tissue-specific deletion approach, using a Tbx4 Cre allele that has broad lung mesenchymal activity but no activity in the diaphragm (28). We found that Tbx4Cre induced widespread recombination in the lung mesenchyme by E12.5 (Figure 1G). This overlapped with the pattern of PBX1 expression in multiple cell types including VSM cells and alveolar myofibroblasts (Figure 1H, and Supplemental Figure 1, C–E). Notably, Tbx4Cre was not active within the vascular endothelial cells where PBX1 was also expressed (Supplemental Figure 1F).

As it has been reported that the PBX2 expression pattern is similar to that of PBX1 in a number of tissues including the lung mesenchyme (29), we inactivated Pbx2 in addition to Pbx1, generating Tbx44Cre Pbx1fl/fl Pbx2–/– mutant mice (hereafter referred to as Pbx1/2-CKO mice; CKO, conditional knockout) (Supplemental Figure 1G). Pbx1/2-CKO mice showed more complete deletion of PBX1 expression in the alveolar mesenchyme than in the peribronchial region (Figure 1I). As predicted from Cre activity, the mutant lungs retained PBX1 expression in vWF-expressing vascular endothelium but not in SM22-expressing VSM cells or alveolar myofibroblasts (Figure 1J, and Supplemental Figure 1, C–E). Notably, Tbx44Cre was not active within the vascular endothelial cells where PBX1 was also expressed (Supplemental Figure 1F).

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**Figure 2.** *Pbx1/2*-CKO mice exhibit abnormal postnatal lung development, progressive cardiac hypertrophy, and PH. (A–C) At birth, *Pbx1/2*-CKO mice had normal-appearing lungs and hearts relative to those of controls. (D–F) At P3, *Pbx1/2*-CKO mice had evidence of lung simplification compared with controls but continued to have normal-appearing heart morphology. (G–I) At P28, in the few surviving *Pbx1/2*-CKO mice, there was severe alveolar simplification and cardiac hypertrophy relative to controls. No consistent hemorrhaging of the heart was observed. (J) Echocardiographic measurements of the RV wall thickness (RVWT) and LV wall thickness (LVWT) revealed that RV hypertrophy was present in *Pbx1/2*-CKO mice relative to control mice at P10 (*P = 0.03), while the left ventricle had normal thickness (P = 0.48). (K) The PAAT/PAET ratio was used as an estimate for the pulmonary artery pressure and RV function. (L) The PAAT/PAET ratio was similar in *Pbx1/2*-CKO mice and controls at P3 (P = 0.45) but significantly different at P7 (**P = 0.004), P10, (**P = 0.008), and P14 (**P = 0.001). For all statistical analyses, 4 samples were included from each group. Comparisons were made using a Student’s t test. Data represent the mean ± SEM. Scale bars: 200 μm.

*Pbx1/2*-CKO mice survived birth at the expected Mendelian ratio (Supplemental Figure 2A) and had no change in alveolar surfacetant C (SPC) expression (Supplemental Figure 1, I and J). At P0, the lungs and hearts of *Pbx1/2*-CKO mice resembled those of their littermate controls (Figure 2, A–C). Despite their normal appearance at birth, *Pbx1/2*-CKO mice failed to thrive, had tachypnea, were underweight, and started to die during the second week of life, with only rare survivors beyond 3 weeks of age (Supplemental Figure 2, A and B). Histological sections of *Pbx1/2*-CKO lungs showed alveolar simplification that was first evident at P3, as quantified by mean linear intercept (MLI) analysis (Figure 2, D and E, and Supplemental Figure 2, C and D). While grossly normal at P3 (Figure 2F), the hearts of *Pbx1/2*-CKO mice became progressively enlarged, with profound hypertrophy evident in surviving mice at P28 (Figure 2I). The majority of *Pbx1/2*-CKO mice died between 2 and 3 weeks of age, with lung simplification, cardiac hypertrophy, and right atrial enlargement (Figure 2, G–I).
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A  Control    Pbx1/2-CKO

B  P7  VWF

C  Control    Pbx1/2-CKO

D  P7  SMA

E  P7  VWF

F  Control    Pbx1/2-CKO

G  P3  PECAM

H  P3  PECAM

I  Control    Pbx1/2-CKO

J  Control    Pbx1/2-CKO    Pbx-iCKO

K  P14  ICAM2

L  P14  ICAM2

M  P14  ICAM2

N  P14  ICAM2

O  P14  ICAM2

P  Control    Pbx1/2-CKO    Pbx-iCKO

Q  Control    Pbx1/2-CKO    Pbx-iCKO

R  Control    Pbx1/2-CKO    Pbx-iCKO

S  VSM relaxation

Embryonic  Postnatal

T  Smooth muscle cell size = Vessel circumference + SM22 nuclei number

U  Control    Pbx1/2-CKO

E18  P3  P7
Figure 3. PH in Pbx1/2–CKO mice is associated with failed VSM relaxation after birth. (A–C) At P7, when hypertension was apparent, vWF staining of proximal vascular endothelial cells showed no significant difference in vessel numbers (P = 0.19). (D–F) At P7, SMA staining revealed no change in pattern and no smooth muscle hypertrophy (P = 0.44). (G–I) At P3, 3D reconstruction and quantification of PECAM-positive alveolar capillaries showed reduced PECAM density and volume (**P = 0.02). (J–L) At P14, H&E staining showed alveolar simplification in Pbx1/2-CKO and Pbx-iCKO mice relative to controls. Tbx4−/− Tbx2−/− and Tbx4rtTA Tet-O-Cre Pbx1−/− Tbx2−/− mice were normal in terms of morphology, physiology, and capillary quantification and were used as a combined control. (P) MLI analysis revealed significant alveolar simplification in Pbx1/2-CKO (**P < 0.0001) and Pbx-iCKO mice (**P = 0.0002) relative to controls (n = 3 in each group). Pbx1/2-CKO and Pbx-iCKO mice had similar alveolar simplification (P = 0.26). (M–O) ICAM2 staining shows reduced capillary endothelial density in Pbx1/2-CKO and Pbx-iCKO mice (**P = 0.0007) relative to controls (n = 3 in each group). Capillary density was similar in Pbx1/2-CKO and Pbx-iCKO mice (P = 0.99). (R) The PAAT/PAET ratio was reduced in Pbx1/2-CKO mice (P = 0.07) but not in Pbx-iCKO mice (P = 0.82) compared with controls. (S) Illustration showing that VSM cells elongate after birth. (T) To estimate the average VSM cell size, the vessel perimeter was divided by the number of SM22-stained VSM cell nuclei. Asterisks indicate nuclei of SM22-positive cells. (U) Pbx1/2-CKO VSM cells were similar in size to control VSM cells prior to birth (E18, P = 0.92) but did not increase in size after birth compared with controls (P3, 1P = 0.02; P7, 1P = 0.04). For all statistical analyses, except MLI and ICAM2 staining density, 4 samples were included from each group. Comparisons between 2 groups were made using a Student’s t test or 1-way ANOVA for comparisons among 3 groups. Data represent the mean ± SEM. Scale bars: 200 μm. Original magnification, ×40 (D and E, insets).

mark cells undergoing apoptosis, we found no change in the rate of cell proliferation or cell death (Supplemental Figure 3, A–F). Previous data from us and others showed that proper organization of the extracellular matrix, especially the Elastin matrix, is essential for normal alveolar septal formation (31–33). To determine whether loss of PBX1/2 is associated with defective extracellular matrix organization, we assayed for Elastin patterning and Supplemental Figure 2D) was accompanied by decreased capillary density relative to controls (Figure 3, J–Q). Interestingly, in contrast to the Pbx1/2-CKO mice, which rarely survived beyond weaning, the Pbx-iCKO mice were healthy at weaning and survived normally into adulthood, with no apparent increase in mortality (Supplemental Figure 4G). Using transthoracic echocardiography, we found that, despite alveolar simplification and decreased capillary density, Pbx-iCKO mice, unlike Pbx1/2-CKO mice, showed no evidence of PH, suggesting that decreased capillary density was not sufficient to cause PH in the Pbx mutants (Figure 3R).

Prior to birth, normal pulmonary VSM cells are contracted to observed in control mice, while the left ventricle was similar in thickness at that stage (Figure 2J).

To determine the pulmonary vascular resistance (PVR), we attempted direct measurement of the RV pressure at early postnatal stages before death in Pbx1/2-CKO mice. While we were able to obtain consistent measurements in the control mice, the Pbx1/2-CKO mice were too fragile and died during this procedure. Thus, we used transthoracic echocardiography to estimate the PVR. We calculated the ratio of the pulmonary artery acceleration time (PAAT) to the pulmonary artery ejection time (PAET) that is commonly used as a proxy for pulmonary artery pressure and RV function (Figure 2K) (34, 35). Using this approach, we found that control mice had a clear increase in the PAAT/PAET ratio between P3 and P7 and a more gradual increase between P7 and P14, indicating the expected reduction in pulmonary artery pressure after birth (Figure 2L). In contrast, Pbx1/2-CKO mice had a gradual decline in the PAAT/PAET ratio after P3, suggesting that the pulmonary artery pressure did not decrease normally and that the right heart function may worsen with age (Figure 2L). The combination of findings including the decreased PAAT/PAET ratio and RV hypertrophy (Figure 2, J and I) is consistent with the presence of PH in the Pbx1/2-CKO mice. As PH is a major cause of lethality in patients with CDH, the Pbx-mutant mouse represents the first CDH model to our knowledge to mimic this key aspect of the disease, independent of the diaphragmatic defect.

Pbx1/2-CKO mutants develop PH as a result of failed pulmonary VSM relaxation after birth. To determine the mechanism underlying the severe PH observed in Pbx1/2-CKO mutants, we investigated possible causes including decreased lung vessel number and size, VSM hypertrophy, and ectopic placement of VSM on distal pulmonary arterioles (36–38). At P7, when PH was detected by the PAAT/PAET ratio, we detected no statistically significant reduction in the number of vessels outlined by vWF staining (Figure 3, A–C). Similarly, we observed no difference in the pattern of vascular smooth muscle actin (SMA) staining (Figure 3, D and E) and no quantitative difference in the width of the VSM layer (Figure 3F), suggesting an absence of ectopic or increased VSM.

The alveolar simplification evident by P3 (Figure 2, D and E, and Supplemental Figure 2D) was accompanied by decreased capillary density (Figure 3, G and H) and loss of capillary volume (Figure 3D). To determine whether this decrease in capillary volume is sufficient to cause PH, we used doxycycline to induce Tbx4rtTA TetO-Cre–mediated deletion of Pbx1/2 at E17 (referred to hereafter as Pbx-iCKO mice), which resulted in loss of PBX1 expression in the distal mesenchyme but not in the proximal VSM (Supplemental Figure 4, A–F) (39). As was observed in Pbx1/2-CKO mice, the Pbx-iCKO mutants had lung simplification and decreased capillary density relative to controls (Figure 3, J–Q). Interestingly, in contrast to the Pbx1/2-CKO mice, which rarely survived beyond weaning, the Pbx-iCKO mice were healthy at weaning and survived normally into adulthood, with no apparent increase in mortality (Supplemental Figure 4G). Using transthoracic echocardiography, we found that, despite alveolar simplification and decreased capillary density, Pbx-iCKO mice, unlike Pbx1/2-CKO mice, showed no evidence of PH, suggesting that decreased capillary density was not sufficient to cause PH in the Pbx mutants (Figure 3R).
Limit blood flow through the lungs. After birth, VSM cells relax and elongate to allow for increased pulmonary blood flow (Figure 3S). Patients with CDH often have constricted pulmonary vessels that do not relax normally after birth (8–11, 40). We hypothesized that in the Pbx-mutant model of CDH, deletion of Pbx1/2 might impair the normal VSM relaxation process. To test this, we measured the circumference of lung vessels and divided this by the number of SM22-stained VSM cells surrounding each vessel to generate the average size of individual smooth muscle cells (Figure 3T). We found that prior to birth, the Pbx1/2-CKO mice had VSM cells that were similar in size compared with those of their littermate controls (Figure 3U). After birth, in control mice, we found that the VSM cells elongated from E18 to P3 and further elongated out to P7. In contrast, the VSM cells of Pbx1/2-CKO mice remained contracted, with no significant change in cell size from E18 to P3 or P7, and were statistically different when compared with controls at P3 and P7 (Figure 3U). These results indicate that in Pbx1/2-CKO mutants, VSM cells do not relax after birth, resulting in persistent constriction of the pulmonary vessels.

Mesenchymal deletion of Pbx1/2 alters the expression of multiple genes involved in vascular contraction. To determine the molecular basis for the impact of loss of Pbx, we performed unbiased transcriptome analysis of whole lungs from Pbx1/2-CKO and littermate control mice at E14, P0, and P3 and found that, among the differentially expressed transcripts, there was a significant enrichment of genes that regulate VSM contraction (Figure 4A and Supplemental Tables 1–3). Focusing on this class of genes, quantitative real-time PCR (qRT-PCR) analysis at P3, prior to
Figure 5. Abnormal VSM contraction in Pbx1/2-CKO mice is reversed by treatment with a Rho-kinase inhibitor. (A) Treatment of P0 to P7 mice with captopril or the endothelin receptor-A antagonist BQ-123 did not reduce the PVR of Pbx1/2-CKO mice compared with controls (untreated, *P = 0.004; captopril-treated, **P = 0.003; BQ-123–treated, ***P = 0.0004). (B) Acute treatment with BQ-123 at P7 increased the PAAT/PAET ratio in control (†P = 0.03) but not Pbx1/2-CKO (P = 0.80) mice. (C–E) Phosphorylated MLC-S20 staining was increased in VSM cells from Pbx1/2-CKO mice (§P = 0.013). (F–H) Lung perfusion with the Rho-kinase inhibitor Y-27632 decreased MLC-S20 staining compared with PBS in Pbx1/2-CKO mice (‡P = 0.03). (I) Y-27632 perfusion at P3 increased VSM cell size in Pbx1/2-CKO (‡‡P = 0.001) but not control (P = 0.14) mice. VSM cell size was similar following Y-27632 perfusion in control and Pbx1/2-CKO lungs (P = 0.37). VSM cell size was similar between Y-27632–treated Pbx1/2-CKO and PBS–treated control (P = 0.07) mice. (J) PVR computed from the histological small artery size revealed increased resistance in Pbx1/2-CKO mice relative to controls (‡‡‡P = 0.008). Perfusion with Y-27632 significantly decreased the PVR in Pbx1/2-CKO lungs (‡‡‡P = 0.02) but not in control lungs (P = 0.36). The PVR of Y-27632–treated Pbx1/2-CKO mice was similar to that of untreated controls (P = 0.96). (K) Measurement at P14 showed increased RVSP following central venous injection of PBS (‡‡‡P = 0.0006) and decreased RVSP following injection into lungs of Y-27632 compared with the RVSP before treatment (§§P = 0.008) and after treatment with PBS (‡‡P < 0.0001). (L) Acute treatment with Y-27632 increased the PAAT/PAET ratio in Pbx1/2-CKO mice (‡‡‡P = 0.04) but not in controls (P = 0.08) and restored the PAAT/PAET ratio in Pbx1/2-CKO mice to that of the untreated controls (P = 0.22). For all statistical analyses, 4 samples were included from each group. Statistical analysis was performed using a Student’s t test for comparisons between 2 groups, 1-way ANOVA with Tukey’s method for comparisons among 3 groups, or 2-way ANOVA with Tukey’s method for comparisons between 2 groups under 2 or more conditions. Data are presented as the mean ± SEM. Scale bars: 200 μm.
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PAET ratio measurement was repeated 2 hours after treatment (Supplemental Figure 5B). Using this approach, we found that, although BQ-123 induced an increase in the PAAT/PAET ratio in control mice, we did not observe a statistically significant increase in Pbx1/2-CKO mice (Figure 5B). These data suggest that blocking signals that promote vasoconstriction at the level of cell-surface receptors is not sufficient to reverse PH in Pbx1/2-CKO mice, possibly because of PBX regulation of other factors that act epistatically to these signals.

Because several downstream factors that act within the VSM cells to promote constriction are altered, we hypothesized that a common downstream effect could be an imbalance of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), causing increased phosphorylation of myosin light chain (MLC) and subsequent increased VSM contraction. Using phosphorylated smooth muscle MLC (Ser20) (MLC-S20) as an indicator, we found that Pbx1/2-CKO mice had increased MLC-S20 staining in VSM compared with that in controls, supporting this hypothesis (Figure 5, C–E).

We then tested whether an agent that acts directly on the balance of MLCK/MLCP activity, such as the Rho-kinase inhibitor Y-27632, might be effective at inducing VSM relaxation and reducing PH in Pbx1/2-CKO mice. Lung vascular perfusion with Y-27632 resulted in decreased MLC-S20 staining of VSM in Pbx1/2-CKO mice at P3 compared with that observed in Pbx1/2-CKO mice perfused with PBS (Figure 5, F–H). In addition, perfusion with Y-27632 resulted in increased VSM cell size in Pbx1/2-CKO mice compared with VSM cell size in lungs perfused with PBS (Figure 5I). These data indicate that treatment with Y-27632 promotes VSM relaxation in Pbx1/2-CKO mice.

To estimate the effect of Y-27632–induced smooth muscle relaxation on relative PVR following treatment with Y-27632, we...
used a mathematical model to compute changes in resistance to blood flow through the lung based on the measured small artery size. The results showed that prior to treatment, Pbx1/2-CKO mice had increased PVR relative to controls (Figure 5J). Following lung perfusion with Y-27632, the model predicted a decrease in PVR in Pbx1/2-CKO mice but not in control mice (Figure 5J). Furthermore, treatment of Pbx1/2-CKO mice with Y-27632 was estimated to reduce the PVR to that observed in PBS-treated control mice (Figure 5J).

To determine whether the prediction based on artery size reflects PVR in vivo, we measured the RV peak systolic pressure (RVSP) in control mice before and after treatment with Y-27632 (Figure 5K). Treatment with Y-27632 decreased the RVSP relative to baseline, however the effect was transient, lasting only 3 to 5 minutes (Figure 5K). To determine whether the Y-27632-induced increase in small artery size, the decrease in the predicted PVR, and the decrease in the RVSP translate to decreased PH in Pbx1/2-CKO mice in vivo, we measured the PAAT/PAET ratio of P7 Pbx1/2-CKO and control mice before and 2 hours after Y-27632 treatment (Supplemental Figure 5B). Treatment with Y-27632 led to an increase in the PAAT/PAET ratio in Pbx1/2-CKO mice but not in controls (Figure 5L). Furthermore, the PAAT/PAET ratio in the treated Pbx1/2-CKO mice was similar to that in the untreated control mice. These data suggest, as predicted by both the change in VSM cell size and PVR modeling, that treatment with the Rho-kinase inhibitor was effective at normalizing the PVR in Pbx1/2-CKO mice to that of controls (Figure 5L). We note that prolonged treatment with i.p. injection of Y-27632 led to lethality in both control and mutant mice, possibly because of the pleiotropic effects of Rho-kinase inhibition, including that of systemic hypertension (41, 42). This suggests that targeted delivery and timed release of the agent to lung VSMs will be required to successfully prevent lethal PH in this genetic model.

Discussion

A drastic increase in pulmonary blood flow in the perinatal period is critical for survival at birth (43). This transition relies on relaxation of pulmonary VSMs. Multiple factors have been demonstrated to control this process, including both non–cell-autonomous, upstream mechanisms such as oxygen, NO signaling, and endothelin-1 (ET-1), as well as cell-autonomous, downstream mechanisms including cGMP and PDE5 (44–46). However, how these factors are coordinated in a short amount of time to allow the necessary abrupt increase in blood flow is not known. Here, we identify PBX transcription factors as a principal controller of this process in the lungs. PBX regulates the expression of both vasoconstrictors and vasodilators that culminates in the reduction of PVR. Failure of this reduction in Pbx1/2-CKO mutants resulted in a genetic model of lethal PH in mice (Figure 6).

In patients with CDH, PH has been postulated to arise from several causes including mechanical compression by herniated organs, decreased vessel numbers, decreased capillary density, and decreased vessel size caused by smooth muscle hypertrophy or persistent smooth muscle contraction (6–9, 47–49). An improved understanding of the primary causes of PH in individual CDH patients will help direct effective treatment. Our approach has been to use a mouse model of CDH to determine the degree to which each of these factors contributes to the severity of PH.

All infants with CDH have some degree of mechanical compression of the lung that contributes to PH after birth. This direct, causal relationship has been challenged, for example, in cases of congenital pulmonary airway malformation (CPAM), in which mechanical compression of the lung does not lead to elevated PVR (12). Our findings from the lung mesenchymal deletion of Pbx1/2 demonstrate for the first time to our knowledge that molecular defects intrinsic to the lung are sufficient to cause PH independently of the diaphragmatic defect and mechanical compression.

Abnormal muscularization of distal, intra-acinar arteries and failure of perinatal pulmonary VSM relaxation are both common features in CDH patients with severe PH (37). In the Pbx1/2 deletion model of CDH, there is no ectopic VSM on distal arterioles, so this model cannot be used to assess the impact of excess smooth muscle. Instead, our data demonstrating failed smooth muscle cell expansion and increased MLC phosphorylation indicate persistent smooth muscle contraction. Reversal of these defects relieved VSM contraction and normalized the PAAT/PAET ratio, suggesting that smooth muscle contraction is a major contributor to lethal PH in this model.

In patients with CDH, direct measurement of PVR can be done in the cardiac catheterization laboratory. However, because of the invasive nature of this approach, patients are routinely monitored by echocardiography to estimate the pulmonary vascular pressures and response to PH medications. In this study, while we were able to obtain a direct measurement of RV pressure in control mice, none of the Pbx1/2-CKO mice survived the procedure to yield data. In comparison, transthoracic echocardiography provided consistent and longitudinal data in both mutant and control mice. Changes in the PAAT/PAET ratio measured by echocardiography have been shown to sensitively reflect fluctuations in pulmonary artery pressure, PVR, and RV function (34, 35). In this study, we were able to use transthoracic echocardiography and the PAAT/PAET ratio to link physiology to cellular mechanisms. Our data suggest that the persistence of smooth muscle contraction after birth significantly changes the PAAT/PAET ratio and is the primary contributor to lethal PH in Pbx mutants.

The abnormal expression of genes that cause persistent VSM contraction in Pbx1/2-CKO mutant mice is consistent with reports on patients with CDH. Several proteins that control VSM tone, including ET-1 and prostacyclin (PGI2), have been found to be present in abnormal concentrations in patients with CDH (49–51). The documented abnormal expression of these factors has been associated with more severe PH and worse clinical outcome in these patients. Interestingly, even though the gene expression and physiological abnormalities responsible for PH in Pbx1-mutant mice are not evident until after birth, the level and pattern of PBX1 expression does not appear to change in the late embryonic and early postnatal period (Figure 1). These data suggest that the developmental and physiological roles played by PBX in the lungs are modulated by its posttranslational modification, transcriptional partners, and/or downstream mediators that act in a chronological and cell-type–specific manner.

Another question that remains a key challenge in CDH is why some patients with PH do not respond to vasodilating med-
ications. Many of the available medications to treat PH address upstream vasorelaxation either via receptor-mediated effects (oxygen, inhaled NO [iNO], prostacyclin analogs, and endothelin receptor antagonists), or at the cyclic GMP or cyclic AMP (cAMP) second messenger level (inhibitors of phosphodiesterase 5 and 3). We show here that loss of Pbx1/2 is associated with misregulation of multiple factors that culminate downstream to control VSM tone. Even though loss of PBX transcripational repression resulted in increased expression of Edn1 and Agrp, targeting these upstream factors was not effective at reducing PH. Our data suggest that PBX1/2 transcriptional regulation is required for maintaining the balance of gene expression necessary for normal postnatal pulmonary VSM relaxation. Targeting the common downstream effectors, such as Rho-kinase, was more effective at reversing smooth muscle contraction and reducing PH. It should be noted that the serial systemic administration of the Rho-kinase inhibitor resulted in lethality in both control and Pbx1/2-CKO mice, probably due to the systemic hypotension that has been reported previously following systemic treatment (41, 42). Targeted and time-restricted delivery of agents like these to the pulmonary VSM is critical to safely take advantage of their therapeutic benefit. Nevertheless, these results raise the possibility that some patients with CDH may have defects in the downstream regulators of VSM contraction, which would explain the observed lack of response to drugs that target upstream factors. In these patients, targeting the downstream regulators may prove more effective.

Genetic evaluation of patients with CDH is now standard, with fetal microarray analysis being offered to families. Whole-exome and whole-genome sequencing is being performed on an increasing number of patient and family trios and has led to the identification of a number of single-gene variants that are associated with CDH (52–54). Our research highlights the opportunity to link individual gene mutations to prognosis and indications for specific therapeutic approaches. As whole-genome sequencing becomes more available and the diverse mechanisms linked to individual mutations associated with CDH become clear, these will greatly improve the accuracy and resolution of prenatal testing offered to families. The data obtained will help to identify fetuses that have an increased risk of lethal PH and also provide clinicians with a targeted and effective treatment strategy.

Methods

Animal handling and genetics. All mice were bred on a mixed genetic background, and age-matched littermates were used for comparison. Pbx1-Boxed and Pbx2-null alleles were previously described (29, 55). Recombination of the conditional Pbx1 allele was achieved by crossing with the Tbx4-rtTA TetO-Cre transgenes, as previously described (28, 39). Tbx4-rtTA was induced by feeding doxycycline food (Harlan Laboratories) to pregnant females starting on E17. Cre-positive, Pbx1-heterozygous, and Pbx2-null (Tbx4-rtTA Pbx1+/−, Pbx2−/−) littermates were used as controls.

Histology. Mice were euthanized and lungs were inflated with 4% paraformaldehyde (PFA) by gravity inflation with a pressure of 20 cm H2O. Lungs were then prepared for paraffin (8 μm), or vibratome (100 μm) sectioning. For each histological assessment, a minimum of 4 mice from each group were analyzed using matched sections from the apex, middle, and base of the lungs of each mouse.

MLI analysis. The MLI of control, Pbx1/2-CKO, and Pbx-iCKO lungs was calculated using >20 H&E-stained images. Three mice from each group were analyzed, with three images taken from sections of the apex, middle, and base of the lungs of each mouse. The MLI was calculated as previously described using a 12 × 8, 50-μm grid (56). The average MLI was calculated for each mouse and then averaged within each group. The data are reported as the MLI ± SEM, with statistical analysis performed using a Student’s t test for comparisons between 2 groups or 1-way ANOVA with Tukey’s method for comparisons among 3 groups.

Immunofluorescence staining. The following primary antibodies were used for immunofluorescence: mouse anti-PBX1b, 1:100 (Santa Cruz Biotechnology; catalog sc-101852, clone 41.1); rabbit anti-SM22a, 1:200 (Abcam; catalog ab14106); mouse anti-SMA-Cy3, 1:200 (Sigma-Aldrich; catalog C6198, clone 1A4); rabbit anti-VWF, 1:50 (Sigma-Aldrich; F3520); rat anti-PECAM (CD31), 1:100 (BD Biosciences; catalog 551262, clone MECL3.3); rabbit anti-ADRP, 1:100 (Abcam; catalog ab52356); rabbit anti-MLC20, 1:100 (Abcam; catalog ab2480); rabbit anti-GFP, 1:100 (Synaptic Systems; catalog 132002); rabbit anti-PDGFRβ, 1:100 (Cell Signaling Technology; catalog 3169P, clone 2E1); anti-ICAM2, 1:100 (BD Biosciences; catalog 553325, clone 3C4); rabbit anti-tropoelastin, 1:100 (Abcam; catalog ab21600); rabbit anti–cleaved caspase-3, 1:100 (Cell Signaling Technology; catalog 9661S). The following secondary antibodies were used: goat anti-rabbit CY3, 1:200 (Jackson ImmunoResearch; catalog 111-165-144); goat anti–rabbit FITC, 1:200 (Jackson ImmunoResearch; catalog 111-095-144); goat anti–mouse CY3, 1:200 (Jackson ImmunoResearch; catalog 115-165-003); goat anti–rat CY3, 1:200 (Jackson ImmunoResearch; catalog 112-165-003); and goat anti–rabbit CYS, 1:200 (Jackson ImmunoResearch; catalog 111-175-144).

Cell proliferation and apoptosis assays. EdU was used to label newly synthesized DNA according to the protocol provided with the Click-iT Edu Alexa Fluor 488 Imaging Kit (Invitrogen, Thermo Fisher Scientific). Mice were injected with Edu 60 minutes prior to euthanasia and tissue collection. Immunostaining for cleaved caspase-3 was used to label cells undergoing apoptosis. Paraffin-embedded lung sections (8-μm) from the apex, middle, and base of equivalent lung regions from 4 control mice and 4 Pbx1/2-CKO littermates were stained and imaged using a ×40 objective. The rate of cell proliferation or cell death in the alveolar region of the lung was determined by dividing the number of EdU- or cleaved caspase-3–positive cells by the total number of nuclei stained by DAPI, respectively. Data are presented as the mean rate of cell proliferation (percentage of EdU-positive cells) or cell death (percentage of cleaved caspase-3–positive cells) ± SEM, with statistical comparison made using a Student’s t test.

Transthoracic echocardiography. Transthoracic echocardiography was performed by the Cardiovascular Physiology Core Facility at the University of Wisconsin using a VisualSonics Vevo 770 ultrasonograph with a 40-MHz transducer (57).

End-diastolic and end-systolic RV and LV diameter as well as anterior wall and posterior wall thickness were measured online from M-mode images using the leading edge–to–leading edge convention. These measurements were normalized to mouse body weight. The data are reported as the average normalized RV and LV wall thickness, with statistical comparison made using a Student’s t test. As a proxy for PVR, we measured the PAAT/PAET ratio. PAAT and PAET were
measured directly, and the PAAT/PAET ratio was calculated for each mouse (34, 35). The data are reported as the average PAAT/PAET ratio ± SEM for each group, with statistical comparisons made using a Student’s t test for comparisons between 2 groups, 1-way ANOVA with Tukey’s method for comparisons among 3 groups, or 2-way ANOVA with Tukey’s method for comparisons between 2 groups under 2 or more conditions. Mice older than P7 were anesthetized with isoflurane during transthoracic echocardiography.

3D Elastin fiber staining and capillary volume measurement. Vibratome-cut sections (100-μm) of equivalent alveolar regions from 4 control and 4 Pbx1/2-CKO mice were stained by immunofluorescence for tropoelastin or PECAM and imaged using an LSM 510 confocal laser scanning microscope (Carl Zeiss) equipped with an argon laser (excitation 488 nm) and a DPS laser (excitation 561 nm). Serial optical sections (80 μm total depth, 1 μm Z-step) were used to generate a surface rendering of the tropoelastin-stained Elastin fibers or the PECAM-stained alveolar capillaries of each vibratome section. Imaris software (Bitplane, Oxford Instruments) was used to calculate the volume of each PECAM surface rendering. The average PECAM volume was calculated for each mouse and then averaged within each group. The data are presented as the average PECAM volume normalized to the control samples ± SEM, with statistical comparison made using a Student’s t test.

ICAM2-stained area percentage. Paraffin-embedded sections (8-μm) from the apex, middle, and base of lungs from 3 control, 3 Pbx1/2-CKO, and 3 Pbx1/iCKO mice were stained by immunofluorescence for ICAM2 and imaged using a ×20 objective. The percentage of area covered by ICAM2 staining was determined by counting pixels using ImageJ software (NIH). The average percentage of ICAM2-stained area was calculated for each mouse and then averaged within each group. The data are presented as the average percentage of ICAM2-stained area ± SEM, with statistical comparisons made using 1-way ANOVA with Tukey’s method to adjust for multiple comparisons.

Smooth muscle cells size analysis. Paraffin-embedded sections (8-μm) from the apex, middle, and base of lungs from 4 control, 4 Pbx1/2-CKO, and 4 Pbx1/iCKO mice were stained for SM22 by immunofluorescence and imaged using a ×20 objective (see Figure 5T). The inner circumference (green line) of each SM22-positive vessel was measured using ImageJ software. The nuclei of SM22-positive cells (indicated by asterisks) were counted. The circumference was then divided by the number of nuclei, resulting in an average SM22-positive VSM cell length per vessel. The average smooth muscle cell size was calculated for each mouse and then averaged within each group. The data are presented as the average smooth muscle cell size (μm) ± SEM using a Student’s t test for comparisons between 2 groups or 2-way ANOVA with Tukey’s method for comparisons between 2 groups under 2 or more conditions.

qRT-PCR and microarray analysis. All qRT-PCR experiments were conducted using 3 biological and 3 technical replicates. Lungs from Pbx1/2-CKO and control mice were homogenized in TRIzol (Thermo Fisher Scientific), and RNA was isolated using an RNeasy Plus Mini Kit (QIAGEN). cDNA was generated using a Superscript III First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific). qRT-PCR was quantified using SYBR green (Applied Biosystems) and run on a LightCycler 480 (Roche). Gene expression was normalized to β-actin. The data are presented as the relative expression normalized to controls ± SEM, with statistical comparison made using a Student’s t test. The primer sets used are listed in Supplemental Table 4.

For microarray, lungs from 3 Pbx1/2-CKO or 3 control mice were pooled at each stage. RNA (1 μg) was analyzed from each pooled sample at the University of Wisconsin – Madison Gene Expression Center using a Mouse Gene 1.0 Array (Affymetrix). GeneSifter software (Geospiza) was used to analyze differential gene expression. The gene expression microarray data are deposited in the NCBI’s Gene Expression Omnibus (GEO) database (GEO GSE106399; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106399).

Drug treatments. Captopril (Sigma-Aldrich), BQ-123 (Sigma-Aldrich), and Y-27632 (Tocris) were used to treat Pbx1/2-CKO and control mice by i.p. injection. Mice that did not receive drug treatment were given PBS by i.p. injection. For P0 to P7 daily treatment (Supplemental Figure 5A), mice were treated once a day with PBS, captopril (0.5 mg/kg), or BQ-123 (10 mg/kg) and underwent transthoracic echocardiography on P7. For acute treatment (Supplemental Figure 5B), P7 mice were analyzed by transthoracic echocardiography, treated with BQ-123 (10 mg/kg), Y-27632 (50 mg/kg), or PBS and then reanalyzed by transthoracic echocardiography 2 hours after treatment. The average PAAT/PAET ratio was calculated for each group before and after treatment. The data are presented as the average PAAT/PAET ratio ± SEM, with statistical comparison made using a Student’s t test.

For ex vivo Y-27632 treatment of control and Pbx1/2-CKO mice, the mice were euthanized and the right ventricle immediately perfused with Y-27632 (10 μM) or PBS. Immediately following treatment, the lungs were inflated with 4% PFA by gravity inflation with a pressure of 20 cm H2O.

MLC-S20 fluorescence analysis. Paraffin-embedded sections (8-μm) from the apex, middle, and base of lungs from 4 control and 4 Pbx1/2-CKO mice were stained by immunofluorescence for phosphorylated MLC-S20, imaged using a ×20 objective, and analyzed with ImageJ. The VSM ring area was determined by measuring the difference between the area of the outer circumference (solid line) and the inner circumference (dashed line) of the MLC-S20-stained VSM cells (Supplemental Figure 6). The fluorescence intensity of each MLC-S20–positive VSM ring was determined by measuring the difference in fluorescence intensity between the outer and inner rings, divided by the area of that ring. The average MLC-S20 fluorescence intensity was calculated for each mouse and then averaged within each group. Data are presented as the average fluorescence intensity for each group, normalized to the control values ± SEM, with statistical comparisons made using a Student’s t test. Because fluorescence intensity varies significantly between experiments, each comparison experiment was performed simultaneously, with all antibody incubations, washes, and imaging conducted in a single batch by a single investigator. The results presented for each comparison represent a single, complete experiment. Each experiment was repeated a minimum of 3 times so as to confirm each result.

Computation of PVR. The predicted PVR response to treatment with the Rho-kinase inhibitor Y-27632 relative to PBS-treated control mice (PVR/PVR_CTL) was calculated using histological vessel size data from control mice treated with Y-27632, Pbx1/2-CKO mice treated with PBS, and Pbx1/2-CKO mice treated with Y-27632. The PVR was assumed to be partitioned in the arterial, venous, and microcirculatory compartments in control conditions according to prior literature (58). Assuming that Y-27632 acts to dilate small arteries and that loss
of PBX does not alter the number of small arteries, the PVR relative to the control in each condition can be calculated according to Poiseuille’s law: PVR/PVR\text{CTL} = \left[2+\left(\frac{D_{\text{avg}}}{D_{\text{avg}}^\text{CTL}}\right)^4\right]/3, where \(D_{\text{avg}}\) is the mean diameter of small arteries in each sample (small arteries were those with 10 or fewer nuclei). The average predicted PVR was calculated for each mouse and then averaged within the groups. Data are presented as the average predicted PVR normalized to control values ± SEM, with statistical analysis done using a Student’s t test for comparisons between 2 groups or 2-way ANOVA with Tukey’s method for comparisons between 2 groups under 2 or more conditions.

In vivo pulmonary artery pressure measurement. Four control mice were anesthetized with urethane (1 g/kg), intubated, and placed on a ventilator (Harvard Apparatus) on a heated pad. A bilateral thoracotomy was performed, and a 1.0-Fr high-fidelity pressure catheter (Millar Inc.) was inserted into the right ventricle. The pressure tracing was recorded and analyzed on commercially available software (Noto- cord). After establishing the baseline recordings, 30 μl PBS was inject- ed into the superior vena cava. The subsequent changes were record- ed 30 seconds after this injection. Mice were then administered an identical volume of Y-27632 (50 mg/kg) by injection into the superior vena cava. The subsequent changes were recorded 30 seconds after this injection, and data were averaged over a 30-second period for each condition. The average RVSP was calculated for each mouse at baseline, after treatment with PBS, and after treatment with Y-27632. The data are reported as the average RVSP ± SEM, with statistical com- parisons made using 1-way ANOVA with Tukey’s method to adjust for multiple comparisons.

Statistics. Statistical comparisons were made for each experiment using a minimum of 3 samples for each group. Comparisons between 2 groups were made using a 2-tailed Student’s t test. Comparisons among 3 groups were made using 1-way ANOVA with Tukey’s meth- od to adjust for multiple comparisons. Comparisons between 2 groups under 2 or more conditions were made using a 2-way ANOVA with Tukey’s method to adjust for multiple comparisons. All data are re- presented as the mean ± SEM. A P value of 0.05 or less was considered statistically significant.

Study approval. Mice were housed and all experimental proced- ures were performed in an American Association for Accreditation of Laboratory Animal Care–accredited laboratory animal facility at the University of Wisconsin – Madison. This study was approved by the University of Wisconsin – Madison Institutional Animal Care and Use Committee and adhered to the NIH’s Guide for the Care and Use of Lab- oratory Animals (National Academies Press, 2011).

Author contributions

DJM devised and performed the experiments, composed the figures, and wrote the manuscript. MDW helped devise and perform the experiments and contributed to the preparation of the figures. EAH conducted the initial Pbxi/2-CKO genetic cross. TAH and AR performed the echocardiographic measurements. RJP and NCC contributed to the mathematical modeling of pulmonary pressure based on vessel size. RZ and LS provided the Pbxi1-floxed and Pbxi2-null mice and contributed to the experimental design and writing of the manuscript. XS is the senior author and contributed to the experimental design and completion of the manuscript.

Acknowledgments

We would like to thank Wei Shi (Keck School of Medicine, USC) and Mark Lewandoski (Center for Cancer Research, National Cancer Institute) for sharing mouse strains; the Laboratory for Optical and Computational Instrumentation (LOCI) at the University of Wisconsin – Madison for assistance with image recon- struction; Jens Eickhoff for review of the statistical analyses; Ellen Wald, Akhiro Ikeda, Marlowe Eldridge, De-Ann Pillers, Jim Gern, Jill Wildonger (all from the University of Wisconsin – Madison), and members of the Sun laboratory for thoughtful discussions and review of the manuscript. This work was supported by a Transla- tional Science Career Development Award (KL2TR000428, to DJM); a subaward from the National Center for Advancing Translational Sciences (NCATS) grant to the University of Wis- consin – Madison Institute for Clinical and Translational Research (UL1TR000427); NIH grants (OT2OD023857, NHLBI RO1 HL113870, HL097134, and HL122406, to XS); and a grant from the Wisconsin Partnership Program (2897, to XS and DJM).

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