Pediatric RESEARCH

REVIEW ARTICLE

Pulmonary hypertension secondary to congenital diaphragmatic hernia: factors and pathways involved in pulmonary vascular remodeling

Louise Montalva[1,2], Lina Antounians[1,2] and Augusto Zani[1,2]

Congenital diaphragmatic hernia (CDH) is a severe birth defect that is characterized by pulmonary hypoplasia and pulmonary hypertension (PHTN). PHTN secondary to CDH is a result of vascular remodeling, a structural alteration in the pulmonary vessel wall that occurs in the fetus. Factors involved in vascular remodeling have been reported in several studies, but their interactions remain unclear. To help understand PHTN pathophysiology and design novel preventative and treatment strategies, we have conducted a systematic review of the literature and comprehensively analyzed all factors and pathways involved in the pathogenesis of pulmonary vascular remodeling secondary to CDH in the nitrofen model. Moreover, we have linked the dysregulated factors with pathways involved in human CDH. Of the 358 full-text articles screened, 75 studies reported factors that play a critical role in vascular remodeling secondary to CDH. Overall, the impairment of epithelial homeostasis present in pulmonary hypoplasia results in altered signaling to endothelial cells, leading to endothelial dysfunction. This causes an impairment of the crosstalk between endothelial cells and pulmonary artery smooth muscle cells, resulting in increased smooth muscle cell proliferation, resistance to apoptosis, and vasoconstriction, which clinically translate into PHTN.

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INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a severe birth defect characterized by disrupted lung organogenesis that results in an underdeveloped lung (pulmonary hypoplasia) with structural alterations of the wall of pulmonary vessels, also known as vascular remodeling.[1] The hypermuscularized pulmonary arterial bed, a hallmark of vascular remodeling, leads to a strong vasoconstrictive response, resulting in increased pulmonary vascular resistance.[2] Physiologically, pulmonary arterial vasodilation is necessary at birth for the transition from fetal to neonatal circulation. However, the pulmonary vasoconstriction that occurs in hypoplastic lungs translates clinically into postnatal pulmonary hypertension (PHTN), one of the main determinants of morbidity and mortality in CDH patients.[3] In fact, the mortality rate at discharge in children with CDH is known to be directly related to the severity of PHTN, as shown by a prospective multicenter study.[3] PHTN in neonates with CDH can lead to circulatory shunting, hypoxia, hypercapnia, and cardiac dysfunction.[4] PHTN secondary to CDH is often refractory to treatments, which are effective in PHTN of other etiologies, and survivors often carry a long-term burden into childhood and adulthood.[5]

The pathogenesis of pulmonary vascular remodeling and the link between remodeling and PHTN are still incompletely understood. To investigate PHTN pathogenesis, several experimental models have been developed, including hypoxia models, genetic models, and models based on the administration of drugs, such as monocrotaline.[6] The nitrofen rat model of pulmonary hypoplasia is of particular interest to investigate vascular remodeling secondary to CDH, as it reproduces pulmonary vascular changes similar to those observed in human neonates with CDH.[7] The nitrofen model relies on the administration of a herbicide (2,4-dichlorophenyl-p-nitrophenyl ether) to a pregnant rat at 9 days of gestation (E9), which causes pulmonary hypoplasia in 100% of the offspring and a diaphragmatic defect in 50–60% of the litter.[8] In the nitrofen model, fetuses with CDH have decreased distal vessel density, increased muscularization of small arteries, resulting in increased pulmonary vascular wall thickness.[7,9] In fact, both medial and adventitial layers of the vascular walls are thickened in the experimental and human CDH.[10]

Over the years, the nitrofen model has been extensively employed, but to the best of our knowledge, no study has synthesized the accumulated data on the pathogenesis of PHTN in this experimental model of CDH. The aim of the present study was to comprehensively analyze all factors and pathways involved in vascular remodeling secondary to CDH in the nitrofen rat model and to understand their relevance to the human condition. This study could help researchers better understand PHTN pathophysiology, which is essential to design novel preventative and treatment strategies for these babies.

MATERIALS AND METHODS

A systematic review of the literature was conducted according to the Preferred Reporting Items for Systematic Reviews and

[1]Division of General and Thoracic Surgery, Department of Surgery, The Hospital for Sick Children, Toronto, Canada and [2]Developmental and Stem Cell Biology Program, The Hospital for Sick Children, Toronto, Canada
Correspondence: Augusto Zani (augusto.zani@sickkids.ca)

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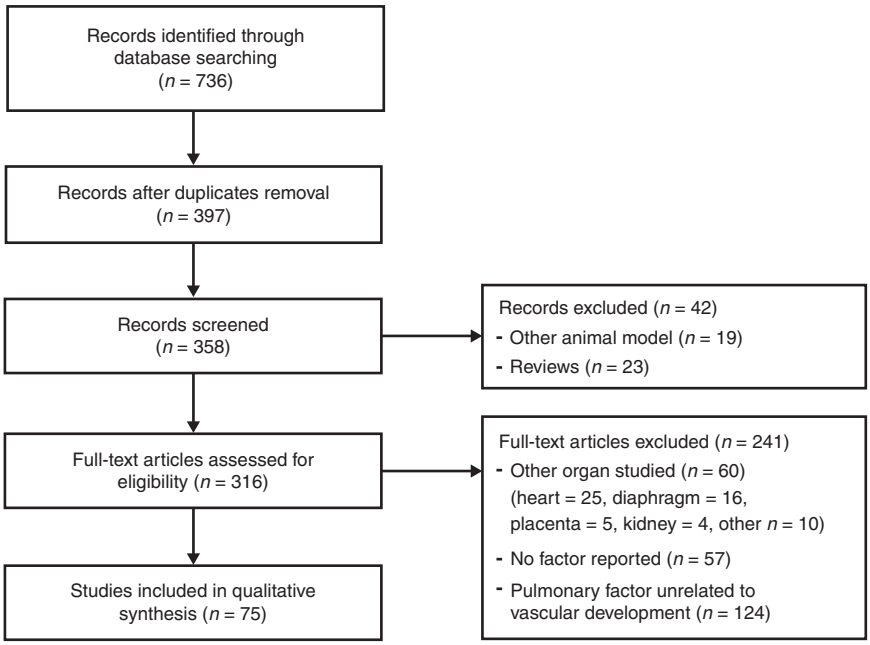

Fig. 1 PRISMA flowchart of search results

Meta-analysis (PRISMA) statement.[11] Eligible studies were identified by searching scientific databases (PubMed, Medline, Cochrane Collaboration, Embase, and Web of Science) involving studies published in English from the first available date in each database to August 2018. The search strategy combined the keywords: "congenital diaphragmatic hernia" and "nitrofen". Reference lists were searched to identify relevant cross-references. Case reports, reviews, and opinion articles were excluded from the review. All gray literature publications (i.e., reports, theses, conference proceedings, bibliographies, commercial documentations, and official documents not published commercially) were excluded. Publications using other models of CDH or the murine nitrofen model were excluded. The full text of potentially eligible studies was retrieved and assessed for eligibility. Inclusion criteria were experimental studies reporting at least one dysregulated factor with a potential role in vascular remodeling.

Dysregulation of the factor was defined as a significant decrease or increase of mRNA and/or protein and/or enzyme activity quantification in the lungs of nitrofen-exposed rats with CDH compared with control rats (statistical significance defined as $p < 0.05$). Factors were classified according to whether they were involved in the dysfunction of endothelial cells (ECs) or pulmonary artery smooth muscle cells (PASMCs), or in the crosstalk between these two cell populations. To investigate the potential translational effects of the dysregulated factors, we then searched for reported similarities in human CDH.

RESULTS

Study selection and characteristics

Within the 358 articles screened, 75 reported dysregulated factors with a potential role in pulmonary vascular remodeling and were included in the review (Fig. 1). The articles were published between 1995 and 2018, with an interesting increment in the last decade (Fig. 2).

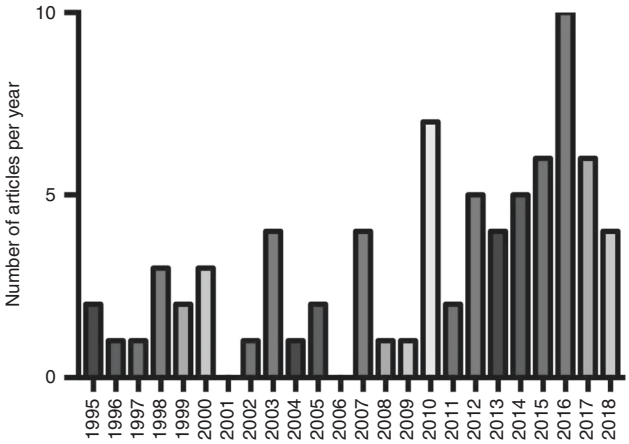

Fig. 2 Date of publication of the articles included in the review

Factors involved in EC dysfunction

During fetal development, the pulmonary vasculature develops in close synergy with the airways in a process regulated by interactions between the developing epithelium and endothelium (Fig. 3 and Table 1). In pulmonary hypoplasia secondary to CDH, the impaired homeostasis of the respiratory epithelium results in decreased signaling to ECs.[9,12]

Vascular endothelial growth factor (VEGF) is a signal protein released by the epithelial cells to regulate differentiation of mesenchymal cells into ECs, EC proliferation, and vascular development.[13] ECs express two receptors for VEGF: VEGFR-2 that is essential for angiogenesis in early stages and has a proliferative role in ECs, and VEGFR-1 that is a negative regulator of EC division in later embryonic stages, and allows for EC maturation, tube formation, and integrity of the vessel wall. VEGF can be upregulated by lung expansion and hypoxia, via hypoxia-inducible factor-1 (HIF-1).[13,14] VEGF and both receptors have been frequently reported as downregulated in the lungs of nitrofen-exposed CDH fetuses throughout development, from the pseudoglandular stage to the saccular stages.[15–24] VEGF production by fetal lung epithelial cells can be upregulated by fibroblast growth factor (FGF)-10/FGFR-2 signaling, which is downstream of canonical Wnt signaling.[25] In nitrofen-exposed lungs of fetuses with CDH, mesenchymal FGF-10 and canonical Wnt signaling are decreased, resulting in a decrease of VEGF production by epithelial cells.[26,27] Ventilation is another regulator of VEGF

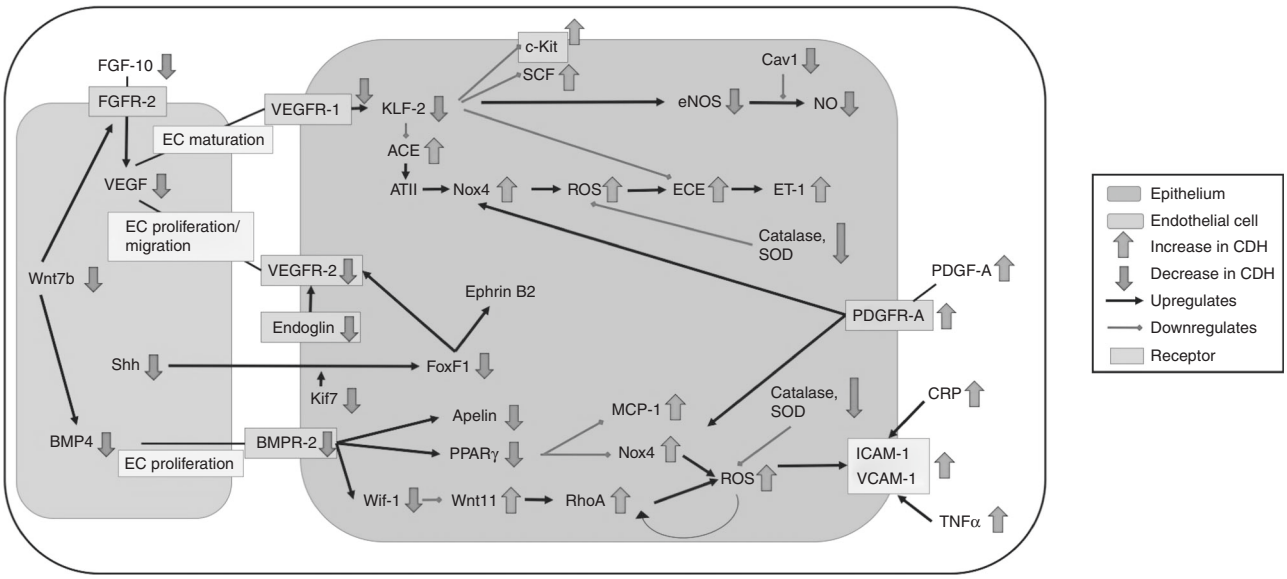

Fig. 3 Factors involved in endothelial cell dysfunction in the nitrofen model of congenital diaphragmatic hernia

signaling. An experimental study where nitrofen pups were ventilated after birth showed that ventilation increased VEGFR-1 and decreased VEGFR-2 expression in lungs of pups with CDH.[28]

In human CDH pregnancies, VEGF levels have been reported to be decreased at amniocentesis, as well as in lungs of CDH fetuses.[29,30] Although pulmonary expression of VEGF is similar between newborns with or without CDH at birth, increased levels of pulmonary VEGF have been reported in newborns with CDH and confirmed PHTN compared with controls.[31–33] Similarly, although plasma levels of VEGF at birth are similar between newborns with or without CDH, increased plasma levels of VEGF in CDH newborns on the third day of life have been reported to be predictive of severe PHTN and mortality.[34,35] Endothelial colony-forming cells derived from cord blood of CDH newborns have blunted responses to VEGF and high levels of nitric oxide production, as well as reduced potential for proliferation and migration, which suggests a reduced number of both VEGF receptors in endothelial cells of CDH newborns.[36]

Krüppel-like factor 2 (KLF-2), a central regulator of endothelial function, is known to mediate the VEGF-induced EC maturation.[37] In lungs of nitrofen-exposed fetuses with CDH, KLF-2 is down-regulated.[38] As a result, the transmembrane tyrosine kinase receptor c-Kit, and its ligand stem cell factor (SCF) have an increased expression from E15 to E21 in ECs of nitrofen-exposed rat lungs compared with controls.[39] c-Kit$^+$ and SCF$^+$ cells in developing lungs are markers of endothelial cell progenitors, and the increase in their expression suggests a delay in differentiation of the ECs, which remain immature and dysfunctional.[40] KLF-2 is also known for regulating the crosstalk with PASMCs by upregulating endothelial nitric oxide synthase (eNOS) and down-regulating endothelin-1 (ET-1) and angiotensin-converting enzyme (ACE).[41]

Forkhead box F1 (FoxF1) is a transcription factor essential for pulmonary angiogenesis, required for VEGF signaling in ECs, and is reported as downregulated in lungs of nitrofen-exposed CDH pups.[42,43] A decrease in FoxF1 results in decreased expression of EC genes essential for vascular development, such as VEGF receptors and Ephrin B2.[44] FoxF1 acts downstream of epithelial sonic hedgehog signaling, which is downregulated during late gestation in nitrofen-exposed lungs of rats with CDH, as is kinesin family member 7 (Kif-7), an essential component of sonic hedgehog signaling to ECs.[45–47]

Bone morphogenetic protein (BMP) signaling is crucial for lung angiogenesis, and loss of the receptor BMPR-II in ECs has been shown to lead to PHTN.[48] During lung development, BMPR-II is mainly expressed in ECs, and its activation by BMP-2 and BMP-4 results in EC proliferation and migration through phosphorylation of Smad 1 and 5.[49] In lungs of nitrofen-exposed rats with CDH, BMP4/BMPR-II signaling is downregulated from E17 to E21, as well as several downstream targets of BMP signaling in ECs, such as phosphorylated Smad 1/5/8 and Apelin (APLN).[27,50–54] APLN is essential for EC homeostasis and attenuates the response of SMCs to growth factors, through its receptors that are present on ECs and PASMCs. APLN and APLN receptors are both downregulated in nitrofen CDH lungs at E21 (Figs. 3 and 5).[54]

The loss of BMPR-II signaling results in the production of reactive oxygen species (ROS) in ECs through the activation of a RhoA/ROCK1 pathway, which causes EC injury and enhances ROS production by inhibiting eNOS. Moreover, ROS production can result from the decrease of peroxisome proliferator-activated receptor-γ (PPAR-γ), which protects against oxidative stress and inflammation by inhibiting monocyte chemoattractant protein-1 (MCP-1) and NADPH oxidase (Nox)-4.[55,56] Nox has been reported to be a major source of hydrogen superoxide in the vasculature, contributing to EC dysfunction and PASMC proliferation. Hydrogen superoxide reacts with nitric oxide (NO) to form the ROS peroxynitrite. The activation of oxidative stress through these pathways is present in lungs of nitrofen-exposed rats with CDH, as shown by the increase in RhoA and its activator Wnt11 in the endothelial layer and the decrease in PPAR-γ associated with an increase in Nox-4 and MCP-1 in the vascular wall.[57–61] Nox-4 can be activated by PDGF-A which is increased in the lungs of nitrofen-exposed CDH fetuses.[62] As a result, increased levels of hydrogen superoxide have been reported in the lungs of nitrofen-exposed CDH fetuses, as well as in the increased number of oxidative-damaged proteins.[62,63] Moreover, expression and activities of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, are decreased in lungs of nitrofen-exposed CDH fetuses, suggesting that the negative feedback regulatory loop is affected.[60,64] These changes are observed also in PASMCs (Fig. 5), where a cyclic stretch due to excessive vasoconstriction further increases Nox4 and the production of ROS in fetal pulmonary arteries.[65]

Endoglin (ENG) is a transmembrane accessory receptor for transforming growth factor (TGF)-β signaling in ECs, and it is

Table 1. Factors involved in endothelial cell (EC) dysfunction

Factor	Change in nitrofen model	Location	Role in angiogenesis/vasculature	Human congenital diaphragmatic hernia (CDH)
Vascular endothelial growth factor (VEGF)	Decreased E20–22[15–21] and in E13.5 + 3-day culture explants[22] No difference E11–21 (in situ hybridization)[144] and E21[28] Increased E21 (medial and adventitial layers of pulmonary arteries)[145]	Epithelial cells	Proliferation and differentiation of endothelial cells (EC) VEGF production can be upregulated by hypoxia and tracheal occlusion	Decreased in amniotic fluid[30] and lungs of fetuses[29] No difference in lungs of newborns[33] or in umbilical cord plasma[35] Increased in lungs of CDH fetuses[101] and in lungs of newborns with CDH and confirmed pulmonary hypertension (PHTN)[31,32] Decreased plasma levels of VEGF in CDH infants under ECMO,[35] and increased plasmatic VEGF-A at 3–4 days in non-survivors with PHTN[34]
VEGFR-1(Flt-1)	Decreased E18.5–21[16,18,23,24] No difference[13] Increases after ventilation[28]	Endothelial cells	VEGF receptor Negatively regulates EC division, role in later stages: maturation, tubule formation, and integrity of the vessel wall	Not reported
VEGFR-2(Flk-1)	Decreased E18.5–21[16,18,23,24] No difference[13] Decreases after ventilation[28]	Endothelial cells	VEGF receptor Mitogenic for ECs Role in early stages: activation of angiogenesis	Decreased lung levels of VEGFR-2[29] No difference in lung levels of VEGFR-2 protein[33]
Fibroblast growth factor (FGF-10)	Decreased E21[26]	Mesenchyme	Increases expression of VEGF Controls branching morphogenesis, epithelial cell proliferation, and differentiation	Decreased in amniotic fluid[146]
Wnt-2	Decreased E15[27]	Mesenchymal cells	Upstream of FGFR2 and BMP4	Not reported
Wnt-7b	Decreased E15[27]	Epithelial cells	Upstream of FGFR2 and BMP4	Not reported
Krüppel-like factor 2 (KLF-2 = lung KLF)	Decreased E18–21[38]	Endothelial cells	Mediates VEGF-induced EC maturation Increases expression of eNOS Decreases expression of ET1 and ACE	Not reported
Stem cell factor (SCF)	Increased E15–21[39]	Endothelial cells	Marker of endothelial progenitor cell	Not reported
c-Kit	Increased E15–21[39]	Endothelial cells	Marker of endothelial progenitor cell	Not reported
Forkhead box F1 (FoxF1)	Decreased E21[43]	Endothelial cells	Promotes VEGF signaling	Not reported
Sonic hedgehog	Decreased E12–22[46]	Epithelial cells	Regulates FoxF1	Expression delayed in CDH lungs[46]
Kinesin family member -7 (Kif7)	Decreased E15–18[47]	Mesenchymal cells	Essential for sonic hedgehog signaling	Not reported
Bone morphogenetic protein (BMP) 4	Decreased E15[27]	Epithelial cells	Mediates BMP/BMPR signaling	Not reported
BMPR-II	Decreased E17–21[50–52]	Endothelial and smooth muscle cells	EC proliferation and migration negatively regulates SMC proliferation induced by growth factors—protects against vascular remodeling induced by MCP1	Not reported
Gremlin-1	Increased E17–21[50,51]	Epithelial and vascular cells	BMPR-II antagonist	Not reported
Id1	Decreased E21[51]		Downstream of BMPR-II marker of BMP activity	Not reported
p-SMAD1/5/8	Decreased E21[50,53]		Activated by BMPR-II	Not reported
Apelin	Decreased E21[54]	Endothelial cells	Target gene of BMPR-II in ECs EC homeostasis Attenuates the response of PA smooth muscle cells to growth factors	Not reported

Table 1 continued

Factor	Change in nitrofen model	Location	Role in angiogenesis/vasculature	Human congenital diaphragmatic hernia (CDH)
Apelin receptor	Decreased E21[54]	Endothelial cells and PASMCs	Regulated by BMPR-II via PPARg	Not reported
Peroxisome proliferator-activated receptor γ (PPAR-γ)	Decreased E21[58,59]	Endothelial cells and PASMCs	Expression regulated in EC by BMPR-2 Reduces Nox-4 and MCP-1 expression	Not reported
NADPH oxidase (Nox)	Nox 1,2,4: increased E21[59,60]	Endothelial cells and PASMCs	Generates reactive oxygen species	Not reported
Monocyte chemoattractant protein 1 (MCP1)	Increased E19[58,61]	Endothelial cells	Recruitment of monocytes inhibited by PPAR-γ	Plasmatic levels increased in CDH newborns with severe PHTN[61]
Wnt inhibitor factor-1	Decreased E19–21[53]		Inhibits Wnt11	Not reported
Wnt11	Increased E21[57]	Endothelial layer of peripheral vessels	Activates RhoA	Not reported
RhoA	Increased E21[57]	Endothelial layer of peripheral vessels	Downstream of Wnt11 Mediates PASMC contraction	Not reported
Platelet-derived growth factor (PDGF)-A	Increased E18, no change at E15 or 21[62]		Induces hydrogen peroxide production by activating Nox4	Not reported
PDGFR-α	Increased E15, no change at E18–E21[62,146]		PDGF receptor	Not reported
Hydrogen peroxide	Increased E15–18[62]		Reacts with NO to form reactive oxygen species	Not reported
Oxidative-damaged proteins	Increased E21[63]		Reflects oxidative stress	Not reported
Antioxidant enzymes	Decreased E21[60,64] (superoxide dismutase 1 and 2; catalase)		Eliminates excess reactive oxygen species	Not reported
Endoglin	Decreased E21[68]	Endothelial cells	Promotes EC proliferation via TGFβ/ALK-1 signaling	Not reported
C-reactive protein (CRP)	Increased E21[9]	Pulmonary artery	Induces expression of adhesion molecules	Not reported
Tumor necrosis factor (TNF-α)	Increased E21[70]	Epithelial cells and macrophages	Induces expression of adhesion molecules	mRNA expression increased in epithelial cells of CDH newborns and stillborns[71]
Adhesion molecules (ICAM-1, VCAM-1)	Increased E21[69]	Pulmonary vessels	Adherence of leukocytes to the endothelium	Increased levels in pulmonary artery ECs and plasma of CDH newborns with PHTN[72]

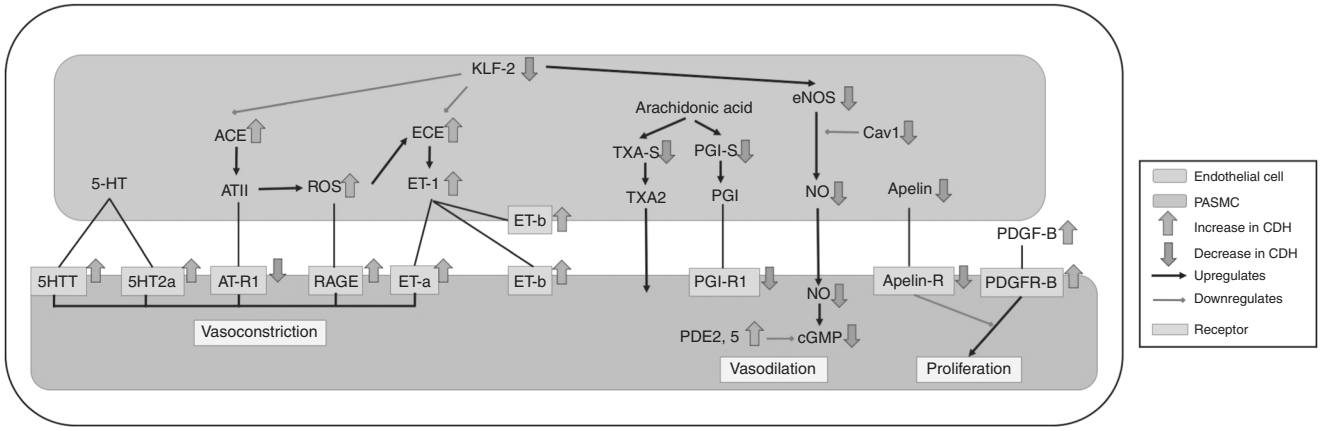

Fig. 4 Factors involved in the disrupted crosstalk between endothelial cells and pulmonary artery smooth muscle cells in the nitrofen model of congenital diaphragmatic hernia

crucial for the activation of EC proliferation and migration through activin receptor-like kinase-1 (ALK1)/Smad1/5.[66] This accessory receptor also interacts with VEGFR2 to promote VEGF-A-induced angiogenesis and VEGF signaling.[67] ENG has been reported as downregulated in the lungs of CDH pups.[68]

The C-reactive protein (CRP) is a well-known marker of inflammation and a risk factor for endothelial dysfunction and, when present in the vessel wall, induces the expression of the vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) by ECs and serves as a chemoattractant for monocytes.[9] CRP and both adhesion molecules were reported as significantly increased in pulmonary arteries of newborn pups with CDH.[9,69] Another trigger for expression of these adhesion molecules by ECs is tumor necrosis factor-α (TNFα), which was increased in the lungs of nitrofen-exposed pups with CDH.[70] In human CDH, inflammation seems to play a role in endothelial dysfunction, with increased levels of adhesion markers in pulmonary artery ECs and in the plasma of CDH newborns and stillborns, and increased TNF-α expression in pulmonary epithelial cells.[71,72]

Disrupted crosstalk between ECs and PASMCs

As ECs modulate PASMC proliferation, apoptosis, and contraction, EC dysfunction results in a disrupted secretion of paracrine factors and PASMC dysfunction (Fig. 4 and Table 2).

Endothelin-1 (ET-1) is produced by pulmonary artery ECs and is known to be proliferative and vasoactive in PASMCs.[73] ET-1 acts through two different receptors: ET-a that is present on PASMCs and mediates vasoconstriction by stimulating cytosolic calcium release, and ET-b that is present on PASMCs and ECs, and mediates vasodilation by stimulating the release of prostacyclin and NO from ECs. Both receptors mediate PASMC proliferation. After stimulation by angiotensin II or ROS, ET-1 is converted from its precursor into its active form by endothelin-converting enzymes (ECE). Lungs of nitrofen-exposed rats with CDH had increased levels of ET-1, ECE, and both receptors as early as E15.[9,51,73–78] The increase in receptors was confirmed in a study showing that arterioles from nitrofen-exposed CDH lungs constricted more than those in controls in response to ET-1.[79] Interestingly, administering an inhibitor of ECE prenatally to dams exposed to nitrofen was reported to increase the survival rate of pups with CDH.[80]

Human babies with CDH have been reported to have increased plasma levels of ET-1 and pulmonary levels of both ET-1 receptors.[77,81–84] Moreover, lungs from babies who died with CDH have been reported to have ET-a receptors in very small capillaries (<25 µm), whereas these receptors are limited to small and large capillaries in control lungs.[77] Finally, babies with CDH had increased levels of ECE during fetal life, whereas in the lungs of control fetuses, ECE expression is low during fetal life and increases just before birth.[77]

Angiotensin-converting enzyme (ACE) is produced by the pulmonary vascular endothelium and converts angiotensin I into angiotensin II, a potent vasoconstrictor. Along with angiotensin II receptors, AT-R1 and AT-R2, ACE is expressed in the developing lung as early as the pseudoglandular stage.[85] In lungs of nitrofen-exposed rats with CDH, ACE had an increased activity, whereas the expression of angiotensin receptors was decreased, possibly due to a negative regulatory feedback loop.[21,86,87] Angiotensin II enhances PASMC migration and proliferation through AT-R1.[88,89] Angiotensin II also has autocrine effects on ECs, mediating endothelial dysfunction by increasing the production of ROS, which in turn induce endothelial production of ET-1, ICAM-1, and VCAM-1.[90]

Nitric oxide (NO), produced by nitric oxide synthase (NOS) in pulmonary ECs, is a potent vasodilator, whose production is stimulated by oxygenation, ventilation, and shear stress at birth. ECs produce the constitutive endothelial isoform, eNOS, and an inducible isoform, iNOS. eNOS expression is increased in normal lung development in late gestation and maximal levels are reported in the near term. Caveolin-1 is a scaffolding protein of caveolae, which are invaginations of the cell surface plasma membrane in ECs and PASMCs, allowing and regulating crosstalk between these cell populations. Caveolin-1 stabilizes eNOS, and regulates its activity, resulting in decreased NO synthesis. Loss of caveolin-1 has been shown to result in endothelial dysfunction.[91] In the nitrofen CDH rat model, studies on NOS have yielded conflicting results. NO production and eNOS expression have been mainly reported as decreased during the saccular stage in nitrofen-exposed fetuses with CDH.[9,19,21,51,92–94] eNOS expression has also been reported as increased at E21 in CDH lungs, with a concomitant decrease of caveolin-1.[77,91,95] A possible explanation for the increased level of eNOS at E21 in these three studies could be found in the different fetal harvesting techniques. In these studies, pups were delivered by C-section and then euthanized,[77,91,95] whereas in the other studies, the dam was euthanized and the pups were harvested after the dam's death, preventing any breathing for the pups.[9,19,21,51,92–94] Indeed, ventilation has been shown to increase eNOS expression and decrease iNOS in pups with CDH.[28,96,97]

High levels of endothelial NO have anti-proliferative effects on PASMCs and cause vasodilation through the activation of soluble guanylate cyclase (sGC) that generates cyclic guanosine monophosphate (cGMP).[98] The level of cGMP is controlled by a family of catabolic enzymes, cyclic nucleotide phosphodiesterases (PDEs). Several PDEs are found expressed in fetal rat lungs, but only PDE2

Table 2. Factors involved in disrupted crosstalk between endothelial cells (ECs) and pulmonary artery smooth muscle cells (PASMCs)

Factor	Change in nitrofen model	Location	Role in angiogenesis/vasculature	Human congenital diaphragmatic hernia (CDH)
Endothelin-1 (ET-1)	Increased E21–22[9,51,73–76] Increased after 1 and 6 h of ventilation[97] No difference in E21[77]	Endothelial cells and leukocytes	Vasoconstrictor, a mitogen for PASMC	Increased plasmatic levels in newborns[81] and at 1–3 weeks in infants with CDH and severe PHTN[82,84] Increased in pulmonary arteries of newborns with CDH and PHTN[81] No difference in lungs of newborns[77]
Preproendothelin-1 (PPET-1)	Increased E21[51]	Endothelial cells	Precursor of ET-1	Not reported
Endothelin receptor A	Increased E15–22[9,51,73,74,76–78]	PASMCs	Vasoconstriction PASMC proliferation	Increased in lungs of newborns[77,83]
Endothelin receptor B	Increased E15–E21[9,74,77,78] No change in E21–22[51,73]	PASMCs and endothelial cells	Vasodilation ± vasoconstriction PASMC proliferation	Increased in lungs of newborns[77,83]
Endothelin-converting enzyme (ECE)-1	Increased E21[77] No change in E21[51]	Endothelial cells	Converts PPET-1 into active ET-1	Increased in lungs of fetuses and newborns[77]
Angiotensin-converting enzyme	Increased activity in E21[86,87]	Vascular endothelium	Converts angiotensin I into vasoconstrictor angiotensin II and inactivates vasodilator bradykinin	Not reported
Angiotensin receptors	Decreased E21[21]		Mediates vasoconstriction	Not reported
Phosphorylated eNOS	Decreased in pulmonary arteries at E21[9]	Endothelial cells	Synthetizes NO in ECs Active form	Not reported
Endothelial nitric oxide synthase (eNOS = NOS3)	Decreased E20–22[19,21,51,92–94] Increased E21[77,91,95] Decreased E21, increased after > 30mn of ventilation[28,96,97] No difference in E15–E19[77]	Endothelial cells	Synthetizes NO in ECs Vasodilator	Decreased in lungs of newborns and of fetuses terminated[101,102] Increased in pulmonary endothelium in newborns with CDH[103–105]
Inducible NOS (iNOS = NOS2)	Increased E21, decreased after 30mn ventilation (back to control levels)[28,96] Decreased E21[51]	Endothelial cells	Synthetizes NO in ECs Vasodilator	Decreased in CDH newborns, increased to normal levels with ECMO[103]
Nitric oxide (NO)	Decreased E21[51]	Endothelial cells and PASMCs	Vasodilation Antiproliferative effects on SMCs and anti-inflammatory	Not reported
Cyclic guanosine monophosphate (cGMP)	Decreased E21[19]	PASMCs	Vasodilation Downstream of NO in PASMCs	Not reported
Phosphodiesterase (PDE 1, 2, 3, 4, 5, and 9)	No change in mRNA but increased PDE2 protein[99] Increased PDE5 mRNA[95] No change in mRNA but increased p-PDE5 protein[19]	Pulmonary arteries	Decreases cellular cGMP by hydroxylation (inhibits NO vasodilation)	Not reported
Prkg2	Increased E21[95]	PASMCs	Downstream of PDE5	Not reported
Caveolin-1	Decreased E21[9,91]	Endothelial cells	Scaffold protein essential for crosstalk	Not reported
5-HT2a	Increased E21[106]	PASMCs	Serotonin receptor on PASMC—induces proliferation and contraction of PASMC	Not reported
5-HTT	Increased E21[106]	PASMCs	Internalizes serotonin into PASMC—initiates the proliferation of PASMC	Not reported
Prostacyclin (PGI) receptors (PTGIR, PTGER1)	Increased mRNA and decreased protein E21[77,95]		Vasodilation	Decreased in the lungs of fetuses and newborns[77]
PGI synthase			Generates prostaglandin	Not reported

Table 2 continued

Factor	Change in nitrofen model	Location	Role in angiogenesis/vasculature	Human congenital diaphragmatic hernia (CDH)
	Decreased E21[95]			
	No difference in E21[77]			
Thromboxane synthase 1 (Tbxas1)	Decreased E21[77]		Converts prostaglandin H2 into thromboxane A2 → vasoconstriction	Plasmatic level of TXB2 (stable metabolite of TXA2) increased in CDH neonates with postductal arterial hypoxemia[147]
Thromboxane receptor (Tbxa2r)	No change in E21[77]		Vasoconstriction	Not reported

and PDE5 levels were increased in lungs of nitrofen-exposed rats with CDH, resulting in decreased levels of cGMP.[19,95,99] Pulmonary arteries from lungs of nitrofen-exposed CDH pups have blunted responses to NO and GMP activators, but these are restored back to normal by the administration of a PDE5 inhibitor.[100] This suggests the absence of a physiological downregulation of PDE5, which is seen postnatally in lungs of control rats.[100] In lungs of nitrofen-exposed CDH pups, an increased expression of the protein kinase Prkg2, downstream of PDE5, confirms the upregulation of PDE5 expression, resulting in increased elimination of cGMP by PDE5, which participates in the lack of vasodilation seen in these lungs.[95]

In human CDH, eNOS expression has been reported as decreased at birth and earlier in CDH fetuses,[101,102] whereas increased levels of eNOS have been reported in neonates with CDH after a few days of life or when treated with extracorporeal membrane oxygenation (ECMO).[103–105]

Serotonin (or 5-hydroxytryptamine, 5-HT) can be produced by pulmonary vascular ECs, neuroendocrine cells, and neuroepithelial bodies in response to hypoxia. Released 5-HT can bind to serotonin receptors and transporters on PASMCs, such as 5-HTT and 5-HT2a, inducing PASMC proliferation and contraction (Fig. 5). In lungs of nitrofen-exposed rats with CDH, the expression of 5-HTT and 5-HT2a is increased compared with that of controls during the canalicular stage of lung development.[106]

Two studies have investigated the role of the prostaglandin pathway in experimental and human hypoplastic lungs secondary to CDH. A decrease in prostaglandin synthase and thromboxane synthase was reported in experimental CDH, and a decrease in the prostaglandin receptor has been found in human CDH newborns.[77,95]

Factors involved in PASMC dysfunction

Several studies have reported an increase in proliferation of PASMCs in the small pulmonary vessels of CDH pups at E21 through several mechanisms (Fig. 5 and Table 3).[95,107,108] One of these mechanisms involves the activation of signal transducer and activator of transcription 3 (STAT-3) signaling.[109] STAT-3 is increased in the vasculature of lungs of nitrofen-exposed fetuses with CDH from E17.5 to E21, and results in the upregulation of Pim-1, KLF-5, and Survivin, transcription factors involved in PASMC proliferation and resistance to apoptosis.[110–113] Pim-1 is a target of the micro-RNA miR-33, which is downregulated in fetal lungs of nitrofen-induced CDH rats.[114] STAT-3 is activated by phosphorylation in response to vasoconstrictive agents (such as endothelin-1 and angiotensin-II), growth factors, and cytokines.[115] In PASMCs of nitrofen-exposed lungs of rats with CDH, the increased expression of the receptor for advanced glycation end products (RAGE) strongly activates STAT-3 but also induces BMPR-II and PPARγ downregulation.[50–52,58,111,116,117] Loss of BMPR-II in PASMCs has been shown to result in phosphorylation of the anti-apoptotic p38 MAPK, which is increased in lungs of nitrofen-exposed pups with CDH, resulting in a reduced PASMC apoptosis rate.[50,118]

STAT3 also upregulates NFATc2 (nuclear factor of activated T-cell, cytoplasmic, calcineurine-dependent-2), a transcription factor that is upregulated in lungs of nitrofen-exposed pups with CDH and that plays an important role in modulating vascular tone response.[110,111,119] NFATc2 actively suppresses the expression of voltage-gated potassium channels, which are important regulators of calcium channels.[119] In nitrofen-exposed lungs of rats with CDH, the inhibition of potassium channels in the vascular smooth muscle layer depolarizes PASMCs to a threshold that opens voltage-gated calcium channels, increasing cytosolic-free calcium concentration and resulting in PASMC vasoconstriction and proliferation.[119–121] In parallel, an increase in canonical transient receptor channel 6 (TRPC6) and calcium-sensing receptor (CaSR), which interact together to increase the cytosolic concentration in calcium, has been shown.[122] Finally, impaired vascular tone in the

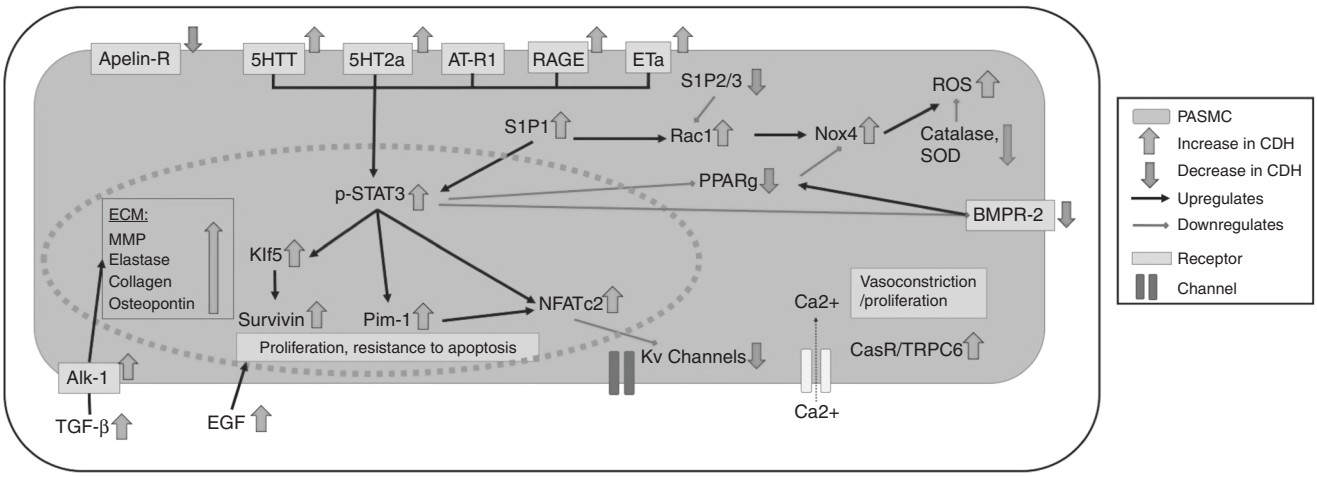

Fig. 5 Factors involved in pulmonary artery smooth muscle cell dysfunction in the nitrofen model of congenital diaphragmatic hernia

nitrofen model has been confirmed, with an overconstriction of pulmonary arterioles in response to endothelin-1, a blunted vasodilation in response to oxygen, and a blunted vasoconstrictive response to hypoxia.[79,123,124]

The sphingosine-1-phosphate receptor 1 (S1P1) activates STAT-3 and increases the expression of Ras-related C3 botulinum toxin substrate 1 (Rac1), which is an important mediator of pulmonary vascular remodeling by promoting PASMC proliferation. In normal conditions, PASMC proliferation is negatively regulated by a decrease in expression of S1P1 and an increase in S1P2 and S1P3.[125] In lungs of nitrofen-exposed pups with CDH, there is an increase in S1P1 and a decrease in S1P2 and S1P3 compared with those of control lungs.[126] This is confirmed by the upregulation of downstream targets of S1P1, Rac1, and STAT3, participating in the excessive proliferation of PASMCs.[126]

In the developing lung, it has recently been shown that TGF-β can stimulate PASMCs through ALK-1/Smad 1,5 signaling.[104] In nitrofen CDH lungs, expression of ALK-1 is upregulated at E21 in the pulmonary vasculature.[127] The expression of TGF-β1 has been reported as increased in PASMCs of newborn pups with CDH compared with those of controls.[128] Elastin microfibril interface-located protein 1 (Emilin-1), expressed by PASMCs, is essential for elastogenesis and inhibits TGF-β signaling by binding to its precursor.[129] In nitrofen CDH lungs, Emilin-1 is downregulated.[130] Taken together, these results could suggest an increased autocrine TGF-β signaling in PASMCs, resulting in PASMC proliferation through ALK-1 and increased deposition of collagen.[131]

In human CDH pregnancies, levels of TGF-β are decreased at amniocentesis, whereas TGF-β3 and its regulator microRNA 200b have been reported as increased in pulmonary arteries and lungs of CDH newborn lungs.[132,133] An increased TGF-β signaling to PASMCs results in an altered extracellular matrix gene expression, such as collagen, elastase, and osteopontin, which can lead to thickening of the medial and adventitial layers of pulmonary arteries. Osteopontin is highly expressed by PASMCs from patients with idiopathic pulmonary hypertension, and mediates signals of proliferation to other PASMCs.[134] Pulmonary arteries from rat pups with CDH present an extracellular matrix with an increased proteolytic activity (serine elastase and matrix metalloproteinase activity) and enriched in growth factors, such as epidermal growth factor and osteopontin, enhancing PASMC proliferation and muscularization of pulmonary arteries.[75,107,135] In parallel, another growth factor mitogenic for PASMCs and fibroblasts, PDGF-B is increased in the medial layer of pulmonary arteries of lungs of nitrofen-exposed pups with CDH.[136]

Embryonic essential myosin light-chain (MLC)-1a and regulatory MLC (MLC-2), which are normally expressed in PASMCs and parabronchial smooth muscle cells, are absent in lungs of nitrofen-exposed pups during the pseudoglandular stage, while their expression is recovered during late stages.[137] The absence of these proteins suggests an early impairment in PASMCs during the pseudoglandular stage, before the establishment of vascular remodeling (late canicular and saccular stages).

DISCUSSION

This systematic review confirms that in experimental CDH, the impaired homeostasis of the respiratory epithelium, which is a hallmark of pulmonary hypoplasia, leads to pulmonary vascular remodeling (Fig. 6). The main signaling pathways disrupted in the epithelium, such as BMP, Shh, Wnt, and VEGF, are at the origin of the pulmonary endothelial dysfunction, thus confirming the close relationship between epithelial and vascular development during fetal lung morphogenesis. The resulting endothelial dysfunction is characterized by the immaturity of the EC, the decreased expression of transcription factors essential for EC function in angiogenesis (e.g., KLF-2, Apelin, and FoxF1), and the imbalanced signaling to PASMCs. This impaired crosstalk between ECs and PASMCs leads to an increase in PASMC proliferation rate, resistance to apoptosis, and constriction, leading to increased medial wall thickness. Moreover, the abnormal secretion of factors, such as collagen, by PASMCs into the extracellular matrix allows PASMCs to create their own favorable extracellular environment to maintain further proliferation along with autocrine stimulation and increased adventitial wall thickness. Eventually, all these modifications to normal lung growth lead to the postnatal development of PHTN.

Interestingly, several studies have shown that the pathways leading to vascular remodeling were reported to be predominantly disrupted in nitrofen-exposed pups that developed CDH.[7,17,22,138] In these studies, in fact, control pups and pups exposed to nitrofen that did not have CDH had normal levels of factors, such as VEGF and eNOS. Nonetheless, we acknowledge that most studies included in this systematic review focused only on nitrofen-exposed pups, which developed CDH, thus making it challenging to draw conclusions on this observation.

Over the years, many studies have reported a number of factors and signaling pathways that are affected in CDH and result in vascular remodeling. The present systematic review of the literature shows for the first time in a comprehensive manner how these factors and pathways are affected. For this reason, the nitrofen-induced model of CDH has great value not just for the study of pulmonary hypoplasia, but also for the understanding of PHTN pathogenesis. In the literature, there are mainly two other

Table 3. Factors involved in pulmonary artery smooth muscle cell (PASMC) dysfunction

Factor	Change in nitrofen model	Location	Role in angiogenesis/vasculature	Human congenital diaphragmatic hernia (CDH)
pSTAT3	Increased E17.5–21[110,111]	PASMCs	Increases expression of Pim-1	Not reported
SOCS3	Increased E21[110]		Induced by pSTAT3, a negative feedback inhibitor of STAT signaling	Not reported
Pim-1	Increased E21[111]	PASMCs	Proliferation and resistance to apoptosis in PASMC	Not reported
miR-33	Decreased E21[114]		Targets Pim-1	Not reported
KLF-5	Increased E21[113]	PASMCs	Transcription factor activated by STAT3 Induces survivin	Not reported
Survivin	Increased E21[113]	PASMCs	Induces proliferation and decreases apoptosis	Not reported
RAGE	Increased E21[116]	PASMCs	Mediates PASMC proliferation Inhibits PPARg/BMPR pathway	Not reported
Phosphorylated p38-MAKP	Increased E21[52]		Anti-apoptotic in PASMCs	Not reported
NFATc2	Increased E21[119]	PASMCs	Promotes Ca-dependent PASMC proliferation Downregulates potassium channels Increased expression by STAT-3	Not reported
Potassium channels	Decreased E21: KCNQ5,[121] Kv1.2, Kv2.1, Kca,[120] Kv1.5,[119] Kcnc3, Kcnj8, and Kcnj15[21]	PASMC and endothelial cells	Inhibition opens calcium channels and increases cytosolic Ca and constriction	Not reported
CaSR	Increased E21[122]	PASMCs	Activated by calcium, interacts with TRPC6 to increase extracellular Ca influx and cytosolic Ca	Not reported
TRPC6	Increased E21[122]	PASMCs	Increases intracytosolic Ca	Not reported
S1P1	Increased E21[126]	Vasculature	Leads to activation of STAT3—increases Rac1	Not reported
Rac1	Increased E21[126]	Vasculature	Proliferation of PASMC via PAI-1	Not reported
S1P2	Decreased E21[126]	Vasculature	Downregulates Rac1	Not reported
S1P3	Decreased E21[126]	Vasculature	Downregulates Rac1	Not reported
Alk-1	Increased E21[127]	PASMC and endothelial cells	Receptor for TGFb family, signals through Smad 1/5 in PASMCs	Not reported
Transforming growth factor (TGF)-β	TGFβ1 increased E21 in peripheral lung and PASMCs[128,148] TGFβ3 decreased E21[21] TGFβ1 decreased E21[21,149] TGFβ1 decreased E17.5[137]	PASMCs	PASMC proliferation Signals through smad proteins Target of miR-200b Enhances miR-200 expression (negative feedback loop)	Increased TGFβ3 in the adventitial layer of pulmonary arteries in newborns with CDH and PHTN[132] Umbilical cord TGFβ2 increased in CDH neonates with liver up or ECMO[35] No change in pulmonary TGFβ[150]
Emilin-1	Decreased E21[130]	PASMCs and endothelial cells	Inhibits TGFβ by binding to a precursor Role in elastogenesis	Not reported
TGFβR1	Decreased E21[68] Increased E21 (peripheral lung)[128]		TGF-β receptor	Not reported
TGFβR2	Decreased E21[21,68] No differences in E21[128]		TGF-β receptor	Not reported
TGFβR3	Decreased E21[21]		TGF-β receptor	Not reported
Osteopontin	Increased E21[107]	Pulmonary arteries	PASMC proliferation expressed by PASMCs in response to TNFα, elastase, and MMP	Not reported

Table 3 continued

Factor	Change in nitrofen model	Location	Role in angiogenesis/vasculature	Human congenital diaphragmatic hernia (CDH)
Serine elastase	Activity increased E21 in PA[107]	Pulmonary arteries	Elastase produced by PASMC	Not reported
Matrix metalloproteinase (MMP)	Activity increased E21 in PAs[107] Increased MMP9/TIMP1 ratio E21[135]	Pulmonary arteries	Role in primary PH, increased by oxidative stress	Not reported
EGF	Increased E21[75,107]	PASMC and bronchiolar epithelium	PASMC proliferation and role in primary PH	Upregulated in the epithelium of CDH newborns[151]
Procollagen	Increased E21[152]	Whole lung	Major component of the extracellular matrix—provides rigidity in the lung tissue	Increased procollagen synthesis in PA of newborns with CDH and PH[132]
PDGF-B and PDGFR-β	Increased E21[136]	Medial layer of pulmonary arteries	Stimulates migration and proliferation of SMCs	Not reported
p-MLC	Increased E21 in PA[9] Decreased MLC1a E17.5, which was then unchanged[136]	Pulmonary arteries	Vasoconstriction, downstream of Et-a Possibly regulated by retinoic acid	Not reported

Fig. 6 Pathogenesis of pulmonary hypertension secondary to congenital diaphragmatic hernia

models of CDH, which are both based on the surgical creation of the diaphragmatic defect in the rabbit and in the lamb. Although these two models have been used for preclinical studies, there are only a few articles that have reported disrupted factors and pathways involved in vascular remodeling in these experimental animals. In fact, in the rabbit model, only two studies have been published and have reported a decrease in eNOS in CDH lungs compared with controls, but no disruption in VEGF or ET pathways.[139,140] In the sheep model of CDH, there are a few more studies, indicating similar findings to those observed in the nitrofen model: an altered interaction between dysfunctional EC and PASMCs via increased expression of ET-1 and decreased eNOS.[141–143] However, contrarily to what has been reported in the nitrofen model, ECs of CDH lamb lungs had a higher expression of VEGF and its receptors compared with those of controls.[141] These differences with the nitrofen model can be explained by the fact that the diaphragmatic defect is created late in gestation. As these studies on surgical models were not sufficient to investigate the pathogenesis of vascular remodeling in CDH, we focused on the nitrofen model of CDH in this review.

The literature reported in the nitrofen model of CDH seems to be relevant for potential translation into clinical practice, as the observations made mirror the pathway disruption reported in human fetuses with CDH. As these changes occur in utero, fetuses with CDH could benefit from an antenatal treatment that induces epithelial maturation prenatally, to prevent the establishment or to reverse the development of vascular remodeling. Although this seems to be a promising strategy, one needs to remember that, at present, translating novel fetal therapies into clinical application remains difficult. In fact, the fetus is a particularly challenging patient that responds to interventions in a distinctive way. Challenges include addressing several dysfunctional pathways at the same time with one single treatment, risk of side effects due to unintended targeting of other organs, treatment delivery route, and differences in lung development between experimental models and human fetuses. For the latter, lung development stages in rat and human fetuses occur at different time points during gestation. The present review has shown that the changes observed in vascular remodeling occur at the same stage of development in both rat and human fetuses, and that is the saccular stage. This stage has a different length as it occurs at the end of pregnancy between E20 and term in rats, and from 24 weeks of gestation to birth in human fetuses. Despite these

differences, the saccular stage seems to be the ideal time frame for a potential translation of antenatal therapies in human fetuses.

We acknowledge the limitations of this systematic review, whose quality is dependent on the quality of the papers published. For instance, within the same model, we have observed discordant findings on some factors or pathways at the same time point. The possible explanation for this could be due to study design, variations in assays employed, and influence of the size and/or side of the diaphragmatic defect that may affect the severity of vascular remodeling. Furthermore, the studies included in this review were focused only on factors that were already known to be dysfunctional in other models of PHTN associated to other diseases. Unbiased investigations were limited to the whole-lung RNA sequencing, and thus were not informative on pathways that might be disrupted at the cellular level at different developmental time points. To minimize the differences and improve the quality of the results, the authors of future studies designed to address the changes in vascular remodeling in experimental CDH should systematically verify and report the presence and laterality of the diaphragmatic defect.

In conclusion, this systematic review has shown that, over the years, there has been an increasing interest in vascular remodeling secondary to CDH, with exponentially more research articles dedicated to this important aspect of the disease. However, a full understanding of the pathogenesis of vascular remodeling in CDH fetuses has yet to be achieved. For this reason, there is sparse literature on novel potential strategies to prevent or treat vascular remodeling in utero. Further studies are required to better explore the pathophysiology and possible translational strategies for PHTN, which still remains a critical determinant of morbidity and mortality in CDH infants.

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ADDITIONAL INFORMATION

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