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Original Paper

STATs in Lung Development: Distinct Early and Late Expression, Growth Modulation and Signaling Dysregulation in Congenital Diaphragmatic Hernia

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Key Words
Signal transducer and activator of transcription (STAT) • Suppressor of cytokine signaling (SOCS) • Congenital diaphragmatic hernia (CDH) • Lung development • Nitrofen

Abstract
Background: Congenital diaphragmatic hernia (CDH) is a life-threatening developmental anomaly, intrinsically combining severe pulmonary hypoplasia and hypertension. During development, signal transducers and activators of transcription (STAT) are utilized to elicit cell growth, differentiation, and survival. ***Methods:*** We used the nitrofen-induced CDH rat model. At selected gestational time points, lungs were divided into two experimental groups, i.e., control or CDH. We performed immunohistochemistry and western blotting analysis to investigate the developmental expression profile of the complete family of STATs (STAT1-6), plus specific STATs activation (p-STAT3, p-STAT6) and regulation by SOCS (SOCS3) in normal lungs against those of diseased lungs. The normal fetal lung explants were treated with piceatannol (STAT3 inhibitor) *in vitro* followed by morphometrical analysis. ***Results:*** Molecular profiling of STATs during the lung development revealed distinct early and late expression signatures. Experimental CDH altered the STATs expression, activation, and regulation in the fetal lungs. In particular, STAT3 and STAT6 were persistently over-expressed and early over-activated. Piceatannol treatment dose-dependently stimulated the fetal lung growth. ***Conclusion:*** These findings suggest that STATs play an important role during normal fetal lung development and CDH pathogenesis. Moreover, functionally targeting STAT signaling modulates fetal lung growth, which highlights that STAT3 and STAT6 signaling might be promising therapeutic targets in reducing or preventing pulmonary hypoplasia in CDH.

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Introduction

Congenital diaphragmatic hernia (CDH) is a life-threatening developmental anomaly. Its clinical management highly impacts the outcome of vulnerable patients and still remains challenging for the involved medical specialists [1-3]. In CDH, an incomplete diaphragm fails to separate the thoracic and abdominal cavities during development. This diaphragmatic malformation causes the abdominal viscera to invade the thorax, irretrievably impairing proper growth and development of the lungs. Concomitantly with herniation, severe pulmonary hypoplasia and pulmonary hypertension (PH) develop [4, 5]. In fact, these have proven to be the most complex aspects to therapeutically address in CDH [6, 7]. The nitrofen animal model has contributed extensively to CDH research. It is well-established that administering nitrofen, formerly used as an herbicide, to pregnant rodents at an early gestational time induces fetal defects that closely resemble those observed in human CDH [8-10].

Normal lung development is commanded by complex signaling cascades and rigorous molecular control. Pivotal developmental processes such as temporal-spatial organization, cellular proliferation, differentiation, and death are instructed by transcriptional programs [11-13]. The signal transducer and activator of transcription (STAT) family of transcription factors have a broad range of roles in development. The STAT pathway is the selected signaling route for numerous cytokines and growth factors crucial for proper lung development [14-17]. Classically, when such ligands interact with its receptor, latent STATs residing in the cytoplasm are recruited to the receptor followed by STAT activation that is induced through tyrosine kinase mediated phosphorylation, namely Janus kinases (JAKs). The activated STATs are then released from the receptor to interact with other STATs in order to form homo- or hetero-dimers, which are capable of nuclear translocation and specific binding to DNA. They ultimately activate or repress transcription of targeted genes [18-20]. Normal STAT activation must be maintained as a transient cellular response, therefore it is conveniently controlled by physiological negative regulators, such as the family of suppressors of cytokine signaling (SOCS). These proteins are induced by JAK-STAT activation, and they inhibit the same cascade afterwards thus creating a negative feedback loop [21-23].

Therefore, in this study, a preliminary investigation is conducted to establish the presence and distribution of STAT family during normal fetal lung development, given that thorough evidence in fetal lung models is manifestly lacking. The developmental expression profiles of all the members of the STAT family (STAT 1-6) are addressed in the normal developing lung as well as the pathological setting of CDH. Specific STATs activation and regulation by SOCS were also assessed in both normal and diseased lungs. Finally, the functional significance of STAT signaling in lung development was investigated by assessing the effect of STAT pathway inhibition in normal fetal lung growth.

Materials and Methods

Ethical Approval

This study was carried out in strict accordance with the recommendations in the 'Guide for the Care and Use of Laboratory Animals,' published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). Animal experiments were performed according to the Portuguese law for animal welfare (Diário da República, Portaria 1005/92) and were subjected to internal institutional ethical review (DGV 022162 - 520/000/000/2006). Moreover, all efforts were made to minimize animal suffering.

Animal model and experimental design

Sprague-Dawley female rats (225 g; Charles-River, Spain) were maintained in appropriate cages under controlled conditions and fed with commercial solid food. According to the nitrofen-induced CDH rat model [24, 25], pregnant rats were treated either with 100 mg of nitrofen (2, 4-dichlorophenyl-p-

nitrophenylether) dissolved in olive oil or olive oil alone. At selected time points, i.e., 15.5, 17.5, 19.5, and 21.5 days postconception (dpc), fetuses were delivered by cesarean section to inspect the diaphragm and harvest the lungs. Fetuses were divided into two groups, namely, control group (Control) with fetuses exposed to olive oil alone and CDH group (CDH) with those exposed to nitrofen with CDH. Regarding this experimental design, undoubtedly assessment of CDH by surgical inspection at 15.5 dpc is impractical. Therefore, for this early gestational age, CDH group refers to the fetuses exposed to nitrofen (independently of CDH development) and control group refers to those that are not exposed to nitrofen. Lungs were either snap-frozen in liquid nitrogen for protein extraction or fixed in 4% paraformaldehyde for immunohistochemistry.

Immunohistochemistry

Immunostaining was performed on paraformaldehyde-fixed and paraffin-embedded excised lungs and embryos of different gestational ages (15.5 - 21.5 dpc) as previously described [26-28]. Primary antibodies for STAT1 (1:200, Abcam Inc., UK), STAT2 (1:50, Abcam Inc.), STAT3 (1:50, Cell Signaling Technology Inc., USA), STAT4 (1:50, Cell Signaling Technology Inc.), STAT5 (1:50, Santa Cruz Biotechnology Inc., USA), and STAT6 (1:50, Abcam Inc.) were used. Negative control reactions included omission of the primary antibody and the simultaneous omission of the primary and secondary antibodies. In both the cases, immunoreactive staining was not observed. Sections were incubated with a labeled streptavidin-biotin immunoenzymatic antigen detection system (UltraVision Large Volume Detection System Anti-Polyvalent, Horseradish Peroxidase, Lab Vision Corporation, USA) according to the manufacturer's instructions. At least three independent experiments were performed for each antibody tested. In each experiment, a different set of slides (duplicates included, two sections from the same individual, per slide) comprising the whole range of gestational ages plus the adult were used. Different and unrepeated animal samples were selected for each group (gestational age). Six different animals for each group per studied antibody were examined.

Western blot analysis

Pooled tissue samples (n=3 subjects from the same litter, per sample) of both experimental groups at the selected gestational ages were processed for Western blot analysis. Proteins were obtained according to Kling et al [29]., and the protocol was performed as previously described [28]. Blots were probed with antibodies to STAT1 (1:2000, Cell Signaling Technology Inc.), STAT2 (1:2000, Abcam Inc.), STAT3 (1:2000, Cell Signaling Technology Inc.), STAT4 (1:1000, Cell Signaling Technology Inc.), STAT5 (1:2000, Cell Signaling Technology Inc.), STAT6 (1:2000, Abcam Inc.), phospho-STAT3 (1:1000, Cell Signaling Technology Inc.), phospho-STAT6 (1:1000, Cell Signaling Technology Inc.), and SOCS3 (1µg/mL; Abcam Inc.) according to the manufacturer's instructions. For loading control, blots were probed with β-tubulin (1:150000, Abcam Inc.), and the quantitative analysis was performed with Quantity One 4.6.5 1-D Analysis Software (Bio-Rad). For analysis of each antibody, at least three independent experiments were performed (n=3) in each experiment an unrepeated pooled sample was used. In total, nine animals were used in each group (gestational age/condition) per antibody.

Fetal lung explant culture

Lungs were removed from 13.5 dpc embryos, harvested, and dissected in DPBS (Lonza, Switzerland) under a stereomicroscope (SZX16, Olympus). Then, the lungs were transferred to porous membranes (Isopore™ membrane filters, Merck Millipore, Germany) and cultured for 4 days as previously described [28, 30]. The culture medium was replaced every 48 hours. Cultures were supplemented daily with several doses of piceatannol (Calbiochem®, Merck Millipore) ranging from 0.1 to 30 ng/mL (n=9 for each dose tested), and 0.1% dimethylsulfoxide (DMSO) was used as control condition (n=12). Three independent experiments were performed and the explants were randomly assigned a culture condition.

Morphometric analysis

Branching morphogenesis was monitored daily by photographing the explants using a stereomicroscope equipped with a camera (DP71, Olympus). At day 0 (D0: 0 h) and day 4 (D4: 96 h) of culture, the total number of peripheral airway buds and the epithelial perimeter in all lung explants were determined using the Image J image processing and analysis software (version 1.44, USA). For all experimental conditions, the results were expressed as D_4/D_0 ratio.

Statistical analysis

All quantitative data are presented as mean ± SEM. The statistical analysis was performed by two-way ANOVA for lung condition (normal and CDH) and gestational age (15.5, 17.5, 19.5, and 21.5 dpc) on protein expression level. One-way ANOVA was performed on the number of peripheral airway buds or epithelial perimeter on piceatannol concentration (0.1, 1, 10, 20, 30 ng/mL). The parametric test assumptions were previously verified, and an additional Holm-Sidak test was used for post-test analysis. Statistical software SigmaStat (version 3.5; Systat Software Inc., USA) was used in all procedures. Statistical significance was set at $p< 0.05$.

Results

STATs expression pattern during rat fetal lung development

Immunohistochemical studies were performed in order to assess the protein presence and tissue distribution of STAT1, 2, 3, 4, 5 and 6 during rat normal pulmonary development at the selected gestational ages, i.e., 15.5, 17.5, 19.5 and 21.5 dpc. Analysis of normal adult tissue was also performed. Immunohistochemistry revealed that all members of this family of proteins are expressed in the developing normal rat lung as well as the adult (Fig. 1). Most STATs, namely STAT1 (Fig. 1A-E), STAT2 (Fig. 1F-J), STAT4 (Fig. 1a-e), and STAT5 (Fig. 1f-j), have early widespread expression in the primitive tissues that constitute the lung at 15.5, 17.5 and 19.5 dpc. Nonetheless, as the tissues progressively differentiate, particularly towards the 21.5 dpc term, their expression becomes predominant in epithelial cells of the airways. Mesenchymal STAT3 expression is more restricted to peri-bronchial regions at early 15.5 dpc. Prominent nuclear staining of STAT3 is observable from early gestation

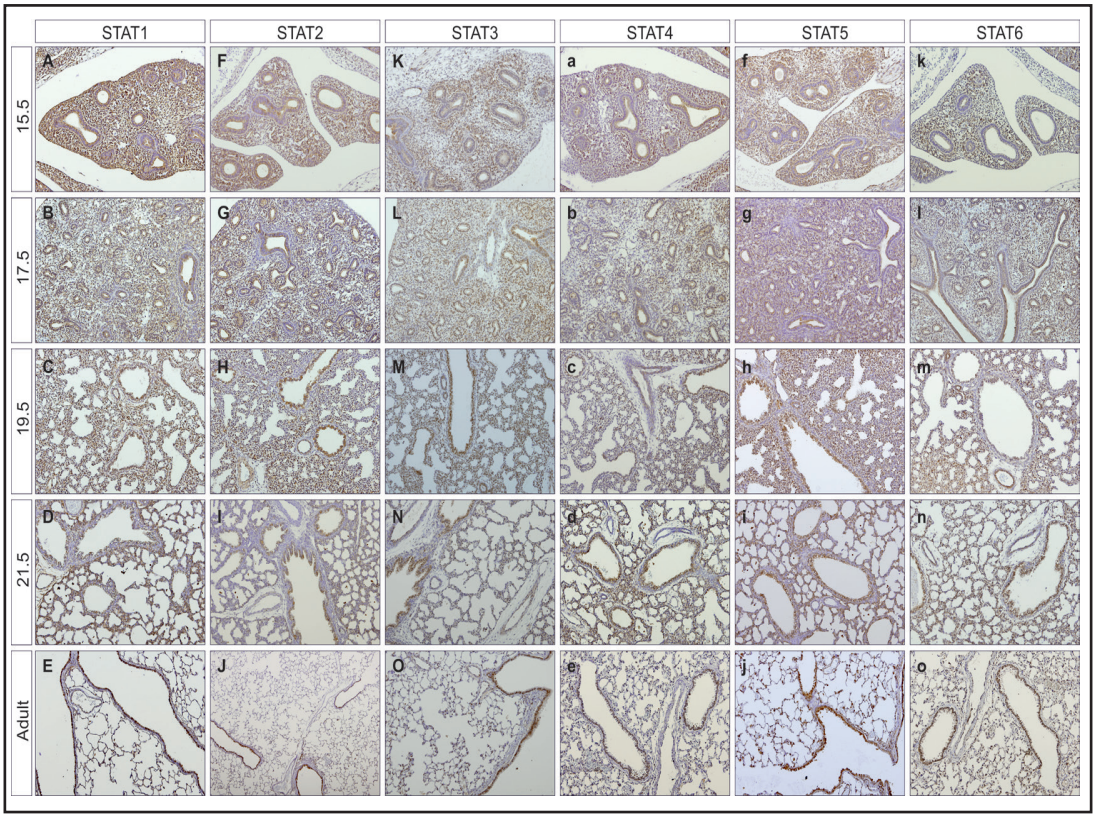

Fig. 1. Protein expression pattern of signal transducers and activators of transcription (STAT) family members (STAT1- 6) during fetal rat lung development. Representative immunohistochemical evidence for the presence of (A-E) STAT1, (F-J) STAT2, (K-O) STAT3, (a-e) STAT4, (f-j) STAT5 and (k-o) STAT6 throughout lung development, from early 15.5 dpc until late 21.5 dpc, and also in the adult. Original magnification 100×.

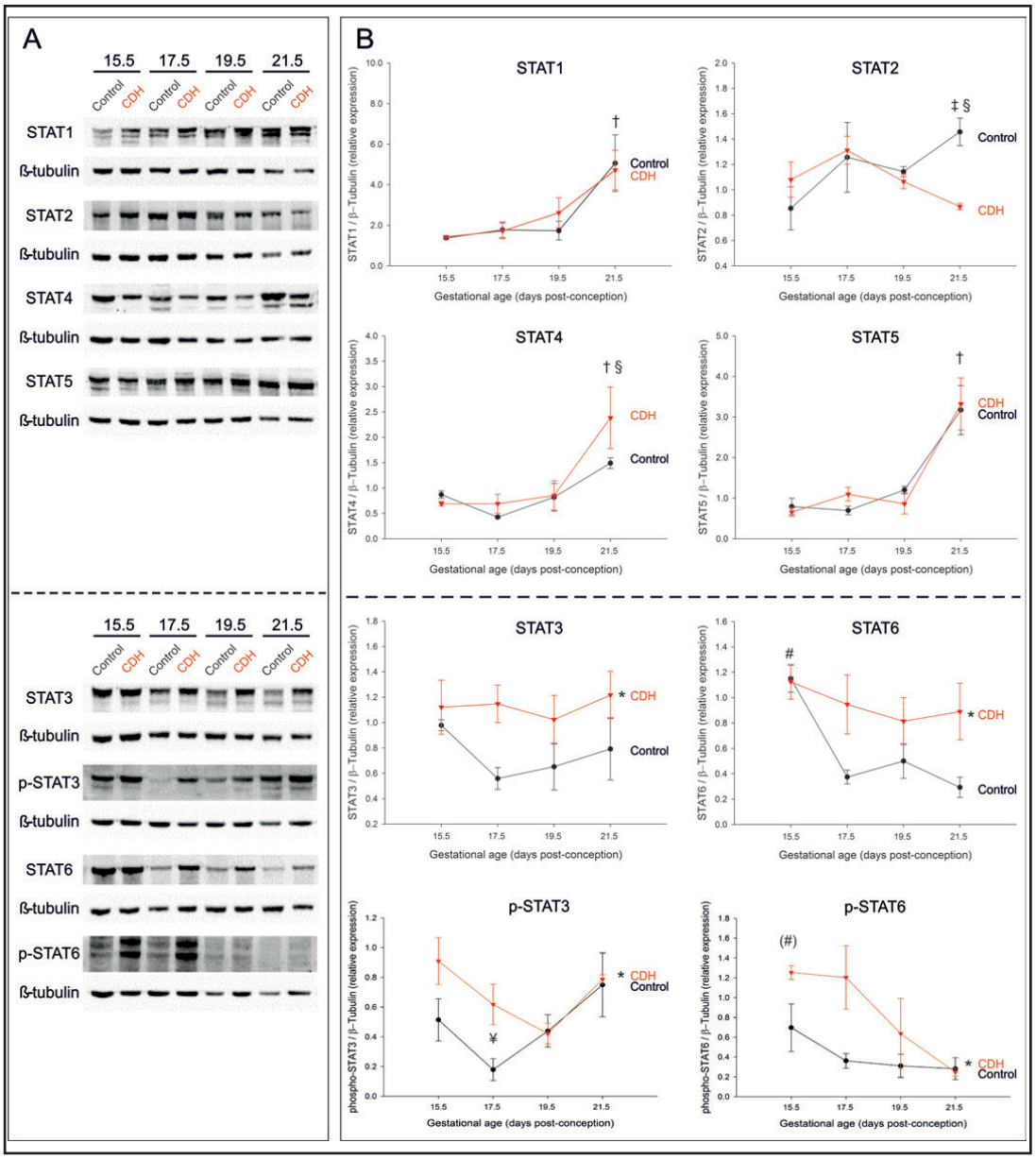

Fig. 2. Experimental CDH alters STATs protein expression and induces early activation of STAT3 and STAT6 in fetal lung development. Western blot analysis of STAT1-6 and phosphorylated STAT3 and STAT6 protein levels in normal fetal lungs (Control) and experimental CDH fetal lungs (CDH) at selected gestational ages from 15.5 to 21.5 dpc. (A) Representative immunoblots are shown. Each lane represents a pooled-tissue sample, and relative expression was determined against β-tubulin. (B) Semi-quantitative analysis of three independent experiments for each STAT is plotted. Results are presented as mean ± SE. Symbols indicate main effects and non-redundant interactions of the factorial ANOVA. $p < 0.05$. * CDH vs. Control; # 15.5 dpc vs. 17.5/19.5/21.5 dpc; (#) 15.5 dpc vs. 21.5 dpc; ¥ 17.5 dpc Control vs. 21.5 dpc Control; † 21.5 dpc vs. 15.5/17.5/19.5 dpc; ‡ 21.5 dpc Control vs. 15.5 dpc Control; § 21.5 dpc CDH vs. 21.5 dpc Control.

until term and is particularly associated with the epithelial tissue at late gestational ages (Fig. 1K-O). Lastly, STAT6 is detected early at 15.5 dpc mainly in the undifferentiated mesenchyme. Its expression remains extensively noticeable in this tissue and also in the epithelium as the pulmonary development progresses (Fig. 1k-o). Concerning the adult rat lung tissue, the immunostaining is mostly restricted to the epithelial lining of the bronchial

airways and alveoli, with predominant nuclear localization. This revealed to be a consistent observable feature in all the members of this transcription factors family.

Experimental CDH alters STATs protein expression in fetal lung development

STATs protein expression profiling in lung development was assessed by Western blot in normal and nitrofen-induced CDH rat lungs at the aforementioned gestational ages (Fig. 2). Forthwith analysis mainly revealed two distinct expression profiles for these proteins in lung development (Fig. 2B). On one hand, some STATs presented a low level of expression at an early gestation of 15.5 dpc, which remains stable until the later gestational age studied (21.5 dpc), that corresponds to an observable peak of expression. This is found to be the case of STAT1, STAT4, and STAT5 expression levels in both normal and experimental CDH fetal lungs throughout development (Fig. 2B, top left and central plots). During development of both normal and CDH-induced lungs, STAT1, STAT4 and STAT5 expression levels at late gestation (21.5 dpc) are significantly higher than all the other gestational ages ($p<0.001$). Exceptionally for STAT4, at 21.5 dpc, CDH lungs exhibit even higher expression than the already observed high expression in normal lungs at this gestational age, being such levels significantly different ($p=0.032$). On the other hand, a couple of STATs presented the highest level of expression at a very early gestational age, 15.5 dpc, which immediately drops to lower levels and continues to remain low until term, 21.5 dpc. This is observed in STAT3 and STAT6, interestingly only in normal fetal lungs. In experimental CDH, the expression levels of both the proteins are elevated throughout all the studied gestational ages and are significantly different compared to the normal fetal lungs (Fig. 2B, plots below dashed line). The effect of experimental CDH in STAT3 and STAT6 expression is, therefore, statistically significant ($p\leq0.01$). Regarding STAT6 expression at 15.5 dpc, protein levels are significantly higher than those in any other studied gestational age ($p=0.011$) (Fig. 2B, below dashed line, top right). Furthermore, STAT2 normal protein expression during development resembles STAT1, STAT4, and STAT5, which is characterized by low expression levels at early gestation and high levels at term. It is further substantiated by the significantly higher expression at 21.5 dpc compared to that at 15.5 dpc ($p=0.005$). Conversely, in CDH lungs at 21.5 dpc, STAT2 expression decreases significantly in comparison to normal lungs ($p=0.009$) (Fig. 2B, top right).

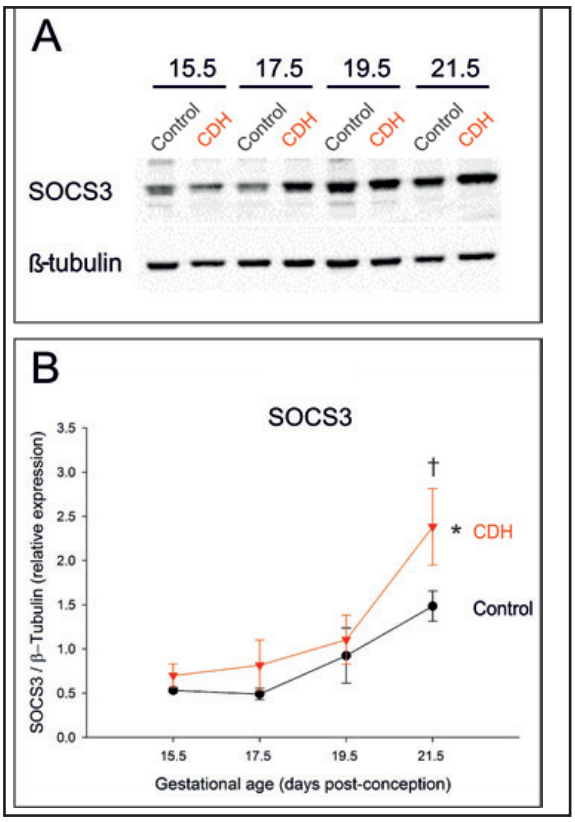

Fig. 3. Experimental CDH induced overexpression of SOCS3 in fetal lung development. Western blot analysis of SOCS3 protein levels in normal fetal lungs (Control) and experimental CDH fetal lungs (CDH) at selected gestational ages from 15.5 to 21.5 dpc. (A) Representative immunoblots are shown. Each lane represents a pooled-tissue sample and relative expression was determined against β-tubulin. (B) Semi-quantitative analysis of three independent experiments for SOCS3 is plotted. Results are presented as mean ± SE. Symbols indicate main effects and non-redundant interactions of the factorial ANOVA. $p< 0.05$. * CDH vs. Control; † 21.5 dpc vs. 15.5/17.5/19.5 dpc.

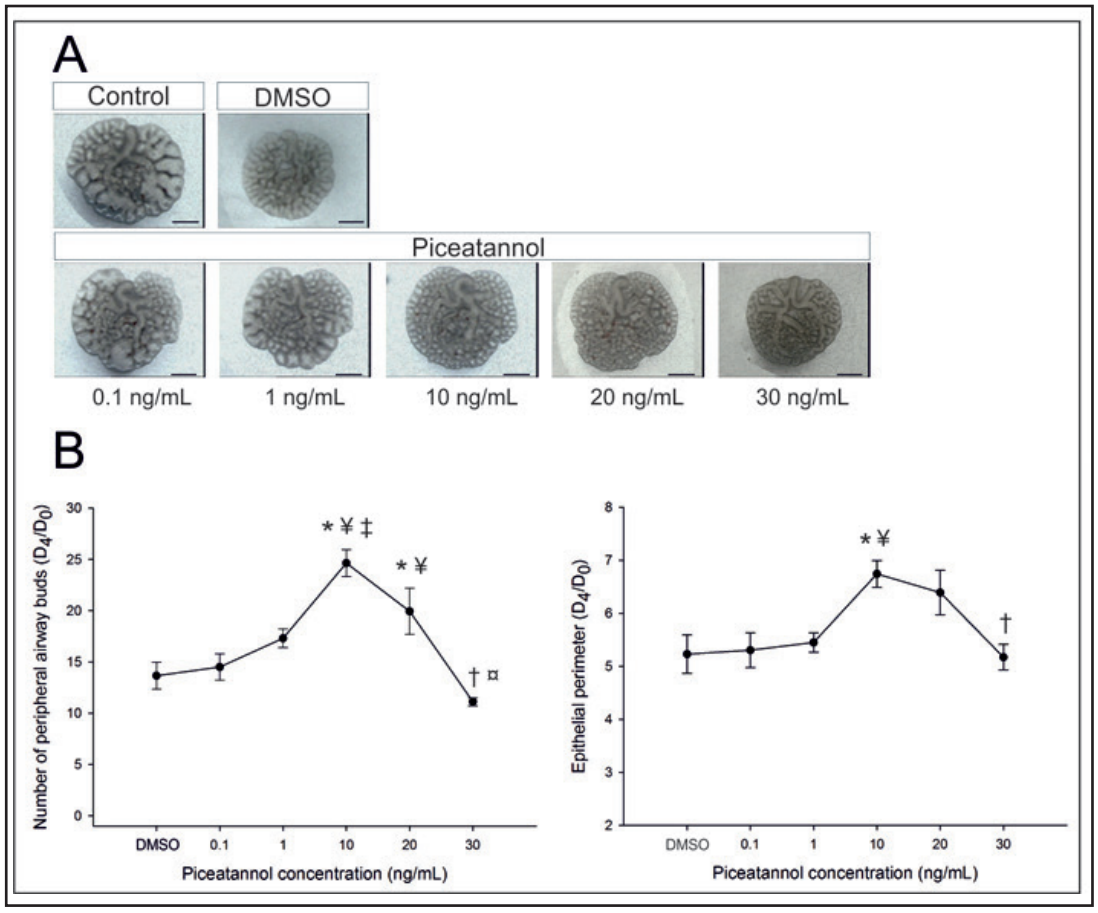

Fig. 4. STAT inhibition, by piceatannol, dose-dependently increased fetal lung growth. (A) Upper panel is representative of untreated lung explants (Control) and lung explants treated with vehicle (DMSO) at day 4 (D4); the bottom panel represents lung explants treated with several piceatannol concentrations (PIC) on day 4 (D4). All images are at same magnification. Scale bar = 6349 μm. (B) Morphometric analysis of the number of peripheral airway buds and epithelial perimeter of fetal rat lung explants supplemented with increasing concentrations of piceatannol. Results are expressed as the D_4/D_0 ratio. $p< 0.05$. * vs. DMSO; ¥ vs. 0.1; ‡ vs. 1; † vs. 10; ¤ vs. 20 ng/mL.

Experimental CDH induced early activation of STAT3 and STAT6 in fetal lung development

Since overexpression of STAT3 and STAT6 was observed in CDH-induced fetal lungs, the activation status of both transcription factors was investigated in this condition and also in normal developing lungs (Fig. 2). Immunoblot analysis of the levels of phosphorylated STAT3 (p-STAT3) indicated that there is a significant difference in CDH-induced lungs during development compared to normal ones (p=0.032) (Fig. 2B, below dashed line, bottom left plot). Likewise, the same significant difference was observed regarding phosphorylated STAT6 (p-STAT6) levels (p=0.009) (Fig. 2B, below dashed line, bottom right plot). Experimental CDH induces activation of these two transcription factors since increased phosphorylation of STAT3 and STAT6 is largely observed during gestation and is particularly significant in early gestational ages of pulmonary development.

Distinctly, p-STAT6 expression levels in both normal and CDH-lungs are significantly higher at the earliest studied gestational age, 15.5 dpc (p=0.003), compared to the latest gestational age at 21.5 dpc, which presents the lowest levels of expression, indicating an effect of gestation time on the phosphorylation status of this transcription factor.

Normal developing lungs present relatively low levels of phosphorylation of these two STATs, particularly residual in some gestational ages. For instance, the p-STAT3 expression

levels drop considerably at a specific gestational age of 17.5 dpc, being significantly lower (p= 0.006) than the gestational age with the highest expression level detected at 21.5 dpc.

Experimental CDH induced over-expression of SOCS3 in fetal lung development

To further explore our findings on STATs signaling in experimental CDH, the contribution of SOCS3 to STATs regulation during lung development was determined by Western blot analysis (Fig. 3). In both CDH-induced and normal lungs, a gradual increase in SOCS3 expression is detected, as lung development progresses, that culminates in a late gestational peak of expression. Consequently, SOCS3 expression levels at 21.5 dpc are significantly higher than all the other gestational ages (p<0.001). Also, despite sharing a similar SOCS3 expression trend, CDH lungs significantly differed from normal lungs (p=0.04), and experimental CDH induced an increase of SOCS3 expression (Fig. 3B).

STAT inhibition, by piceatannol, increased fetal lung growth in a dose-dependent manner

We functionally assessed the effect of STAT pathway inhibition on fetal lung growth by supplementing 13.5 dpc fetal lung explant cultures with piceatannol (STAT3 inhibitor). When added to the culture medium, piceatannol significantly stimulated fetal lung growth in a dose dependent manner (Fig. 4). Lower doses of piceatannol at 1 and 10 ng/mL have an enhancing effect on lung growth in a dose-dependent manner, with 10 ng/mL having the most significant growth stimulatory effect. Morphometric analysis of lung explants, supplemented with 10 ng/mL, showed a significantly higher number of peripheral airway buds compared to both DMSO control (p< 0.001) and the lower doses of 0.1 and 1 ng/mL (p< 0.001 and p= 0.002, respectively). It also showed a significantly higher epithelial perimeter compared to both DMSO control (p= 0.028) and the lowest dose tested (0.1 ng/mL) (p= 0.019). In these culturing conditions, the optimum concentration of the compound is 10 ng/mL. At higher doses, the piceatannol effect on lung explant growth is comparatively more moderate. Particularly at 30 ng/mL, the number of peripheral airway buds and the epithelial perimeter are significantly reduced compared to the optimal dose of 10 ng/mL (p< 0.001, p= 0.034, respectively). However, no morphometric values significantly lower than DMSO were observed.

Discussion

STAT proteins instruct key cellular processes, namely growth, differentiation, and survival [18, 20]. Despite our broad understanding of the transcriptional control of lung branching morphogenesis by other families of transcription factors [11, 13], both expression and role of STATs remain unclear in lung development.

Our study provides evidence for a developmental expression pattern of all the members of STAT family of transcription factors in the lung. All studied members of this family of proteins are herein described to be constitutively expressed in fetal pulmonary tissues since early development. We also detail STATs tissue distribution in the developing lung. In general, STAT family members have diffuse protein expression in the primitive pulmonary tissue; however, as development progresses, these proteins gradually display expression specificity towards the pulmonary epithelium (Fig. 1). Therefore, by the end of gestation, fetal STATs distribution resembles the tissue distribution observed in the adult lung, mainly restricted to the epithelial lining of the bronchi and alveoli, with predominant nuclear distribution. The results shown in the present study contribute to the demonstration of the early and continuous presence as well as a dynamic distribution pattern of STAT members throughout the developmental stages of the lung, suggesting that this family of transcription factors has multiple functions in lung development. In agreement with it, previous findings also support diverse roles for STATs in the normal developmental processes of different organs, including branching organs [14, 31-35].

We also detected a particular immunolocalization pattern regarding STAT3 at 15.5 dpc; this pattern is noticeable for differing from the widespread expression observed for most STATs at early gestation. Specifically, at this gestational age, STAT3 expression is restricted to the mesenchyme surrounding the primitive respiratory tract, including lung bud tips. In the rat, 15.5 dpc is representative of the pseudoglandular stage, during which the embryonic lung essentially undergoes branching morphogenesis, conducted by signaling interactions that occur locally at the distal lung buds. STAT3 distribution in the lung, during the pseudoglandular stage, is compatible with this distal bud signaling center suggesting an underlying role of STAT3 in early branching morphogenesis. Recently, Carraro et al. suggested that stabilization of STAT3 RNA expression during the pseudoglandular stage may be important for correct branching morphogenesis [36]. Previous studies have also implicated STAT3 in lung branching morphogenesis [26], and a consistent downstream of STAT3 activation was observed in response to the inhibitory actions of cytokines on fetal lung branching [27].

Several families of transcriptional factors have been strongly implicated in the etiology of congenital diaphragmatic hernia [37, 38]. In the present study, we investigated the pulmonary protein expression levels of the STAT family members in both normal and nitrofen-induced CDH rat lungs during representative stages of lung development (Fig. 2). Firstly, our observations evidenced a dynamic temporal expression pattern of STAT proteins during normal fetal lung development. Most members of this family share a similar expression pattern in normal lungs. STAT1, STAT2, STAT4, and STAT5 have their lowest expression at early gestation and the highest expression by the end of gestation, indicating an increasing requirement of these transcription factors as pulmonary development progresses. Normal mammalian lung development proceeds towards increasing pulmonary epithelial cell proliferation and differentiation. This underlines that the current findings on STATs expression pattern are in agreement with our previous observation, i.e., STATs distribution is more restricted to epithelial pulmonary tissue at the latest studied gestational ages.

Similarly to normal fetal lungs, some STATs in CDH lungs, namely STAT1, STAT4, and STAT5, present a low level of expression at early gestation (15.5 dpc), which remains stable until the later studied gestational age (21.5 dpc), where a peak of expression is observed. On the contrary, STAT2 in CDH lungs strongly differs from normal lungs at 21.5 dpc since its expression is markedly decreased. This observation at late-gestation most likely represents a mechanistic-side effect of disease progression. It is worth noting that STAT2 is a pivotal component of the IFN-induced transcriptional machinery and mediates anti-growth and apoptotic effects [39, 40]. Moreover, IFN-α was demonstrated to be significantly increased in newborns with CDH [41]. Considering all these observations, one question that remains unexplored is whether CDH progression causes disturbances in the IFN-α -STAT2 signaling axis. Interestingly, prior studies have evidenced that persistent exposure to IFN-α led to resistance to type I IFN–induced apoptosis due to loss of STAT2 expression [42].

In the present study, we also found that STAT3 and STAT6 are expressed differently. It is noteworthy that our results show a specific modulation of STAT3 and STAT6 expression in the context of CDH since these proteins are persistently overexpressed during the development of diseased lungs. However, the other STATs expression levels remain unchanged or only are mildly modified compared to normal lungs. These findings highlight a conditional association between STAT3 and STAT6 persistent overexpression in CDH. It also uncovers an important role for of these two transcription factors in the pathogenic events of this congenital lung disease. In addition to the overexpression of these STATs, a concomitant CDH-induced hyperactivation of these two transcription factors was observed in the developing lung evident mostly in the early gestational ages (15.5 and 17.5 dpc). Thus, reinforcing that STAT3 and STAT6 signaling may contribute to the modifications of gene expression underlying the CDH pathogenesis (Fig. 2). In agreement with our findings, a recent report provided evidence of an increased STAT3 activation in CDH, particularly in pulmonary vasculature [43]. Furthermore, dysregulated activation of STAT proteins is implicated in several pathological conditions, particularly STAT3 and STAT6 [44-48], which makes the prospect of targeting the

JAK–STAT pathway therapeutically appealing.

Our results also evidenced that the expression of SOCS3, a negative feedback inhibitor of STAT signaling, is higher in CDH subjects (Fig. 3). Given that SOCS protein expression has been shown to be STAT-dependent, and SOCS genes are target genes of STATs and get induced by active JAK-STAT signaling [21], altered SOCS3 expression was expected and is indicative of a regulatory role of SOCS3 in CDH. In many pathological circumstances, increased expression of SOCS proteins coincides with the activation of JAK/STAT pathways [21, 49-51]. Collectively, our data provide the first evidence for a previously unappreciated regulatory mechanism occurring in the pathological development of experimental CDH.

Taken together, these observations urged us to investigate the functional outcome of STAT pathway inhibition in the developing lung and assess its effect on normal fetal lung growth *in vitro*. Rat fetal lung explants at 13.5 dpc cultured in the presence of piceatannol, a known STAT3 signaling inhibitor [52, 53], exhibited a clear increase in growth in a dose-dependent manner (Fig. 4). Piceatannol has been widely used to effectively inhibit STAT3 phosphorylation [54-56]; nevertheless, it is possible that the effects of piceatannol other than STAT3 inhibition may have contributed to its ability to interfere with fetal lung growth. For example, besides JAK1 kinase, piceatannol is also reported to inhibit SYK kinase, as well as other transcription factors, namely nuclear factor-kB (NF-kB). Therefore, further studies are necessary to support the pharmacological approach of STAT3 inhibition. Herein, it is demonstrated that precise regulation of STAT signaling is necessary for proper fetal lung growth during early development, revealing an unanticipated role of STAT signaling during lung branching morphogenesis. Even though STAT transcription factors have been largely overlooked in lung development, STAT3 has been previously implicated in the regulation of developmental processes [14-17], namely lung branching [26, 27, 36], which is consistent with our findings. More importantly, we show that modulation of fetal lung growth can be achieved by conditionally targeting STAT signaling, though the detailed mechanistic interactions are yet to be fully defined.

Till date the pathogenesis of CDH has not been fully established; nonetheless, the current expertise consensually regards the retinoid-signaling disruption as a contributing factor [57-59]. Strikingly, previous observations suggest that there may be a significant functional cross-talk between STAT and retinoic acid receptor (RAR) families of transcription factors. For example, a fusion protein STAT5-RARalpha was identified and demonstrated to have an enhancing effect on STAT3 transcriptional activity in promyelocytic leukemia [60]. The same authors presented a model of enhancement of STAT3 transcriptional activity and its effect on apoptosis being modulated by the presence/absence of retinoic acid (RA) [61]. In normal hematopoiesis, STAT5 and RAR associate *in vivo* in a cytokine-dependent manner [62]. Glioma cells also display an increased STAT3 activation upon RA treatment [63]. More importantly, in respiratory epithelial cells, besides the protein-protein interaction, STAT3 and RAR were found to act synergistically in regulating surfactant protein B [64, 65]. It would be important in pursuing a more comprehensive perspective on CDH pathogenesis to understand the nature of the molecular interaction between STAT and RAR family members as well as their functional outcomes. An intriguing possibility is that retinoid signaling disruption could lead to inappropriate STAT activation in CDH, particularly STAT3 and STAT6. Following this line of reasoning, these transcription factors could play a role in the primary events determining the onset of the disease. Lung development is complex, and highly dependent on the timely interplay of molecular signals and their tight regulation. The identification of important regulators, such as transcription factors, and how their pathways are disrupted in lung diseases like CDH are essential for the development of successful therapies.

Importantly, the novel data presented herein provides direct evidence that all STAT proteins are constitutively expressed in fetal pulmonary tissue since early development. Experimental CDH alters STATs protein expression, activation, and regulation in the fetal lung, as demonstrated by the persistent overexpression of STAT3 and STAT6 and their early overactivation combined with SOCS3 overexpression in diseased fetal lungs (Fig. 5). These findings stress previously unappreciated disturbances of pulmonary developmental

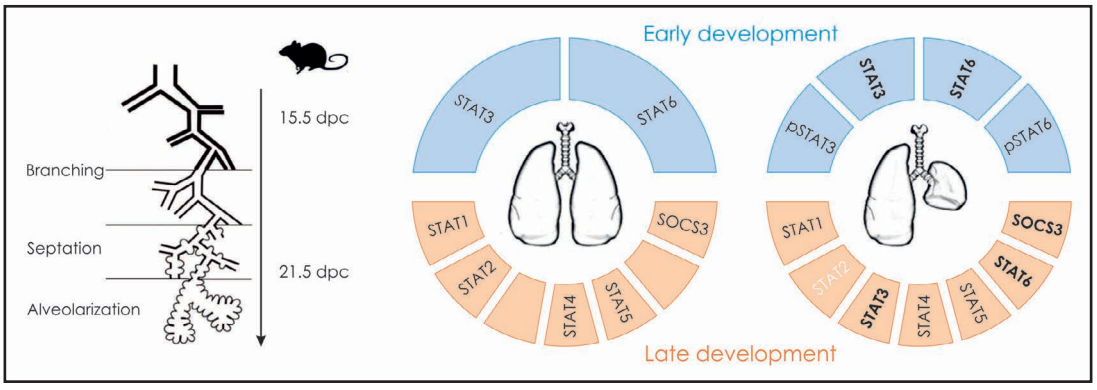

Fig. 5. Comparative summary of the studied signals in normal lung development and in congenital diaphragmatic hernia. Schematic representation of the rat lung airways development in the left, and signaling differences between normal and CDH lungs in the right. Bold font represents increased expression of the signal; fainted color font represents decreased expression of the signal.

signaling pathways, such as STAT, in CDH. Moreover, compelling evidence is presented that targeting STAT signaling through STAT3 inhibition impacts the fetal lung growth *in vitro.* This suggests that precise STAT signaling regulation may be important during normal fetal lung development. While presented evidence is still far from providing any efficient lung growth modulation strategy, further functional studies would greatly aid in addressing the therapeutic potential of targeting active STATs. Clearly, this work supports a role of these specific molecular mediators in the complex pathophysiological aspects of CDH development.

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Disclosure Statement

The authors have declared that no competing interest exists.

References

1 Puligandla PS, Grabowski J, Austin M, Hedrick H, Renaud E, Arnold M, Williams RF, Graziano K, Dasgupta R, McKee M, Lopez ME, Jancelewicz T, Goldin A, Downard CD, Islam S: Management of congenital diaphragmatic hernia: A systematic review from the APSA outcomes and evidence based practice committee. J Pediatr Surg 2015;50:1958-1970.

2 Sluiter I, van de Ven CP, Wijnen RM, Tibboel D: Congenital diaphragmatic hernia: still a moving target. Semin Fetal Neonatal Med 2011;16:139-144.

3 Kotecha S, Barbato A, Bush A, Claus F, Davenport M, Delacourt C, Deprest J, Eber E, Frenckner B, Greenough A, Nicholson AG, Anton-Pacheco JL, Midulla F: Congenital diaphragmatic hernia. Eur Respir J 2012;39:820-829.
4 Greer JJ: Current concepts on the pathogenesis and etiology of congenital diaphragmatic hernia. Respir Physiol Neurobiol 2013;189:232-240.
5 Kluth D, Tenbrinck R, von Ekesparre M, Kangah R, Reich P, Brandsma A, Tibboel D, Lambrecht W: The natural history of congenital diaphragmatic hernia and pulmonary hypoplasia in the embryo. J Pediatr Surg 1993;28:456-462.
6 Wynn J, Krishnan U, Aspelund G, Zhang Y, Duong J, Stolar CJ, Hahn E, Pietsch J, Chung D, Moore D, Austin E, Mychaliska G, Gajarski R, Foong YL, Michelfelder E, Potolka D, Bucher B, Warner B, Grady M, Azarow K, Fletcher SE, Kutty S, Delaney J, Crombleholme T, Rosenzweig E, Chung W, Arkovitz MS: Outcomes of congenital diaphragmatic hernia in the modern era of management. J Pediatr 2013;163:114-119.
7 Deprest J, De Coppi P: Antenatal management of isolated congenital diaphragmatic hernia today and tomorrow: ongoing collaborative research and development. Journal of Pediatric Surgery Lecture. J Pediatr Surg 2012;47:282-290.
8 Beurskens N, Klaassens M, Rottier R, de Klein A, Tibboel D: Linking animal models to human congenital diaphragmatic hernia. Birth Defects Res A Clin Mol Teratol 2007;79:565-572.
9 Guilbert TW, Gebb SA, Shannon JM: Lung hypoplasia in the nitrofen model of congenital diaphragmatic hernia occurs early in development. Am J Physiol Lung Cell Mol Physiol 2000;279:L1159-1171.
10 van Loenhout RB, Tibboel D, Post M, Keijzer R: Congenital diaphragmatic hernia: comparison of animal models and relevance to the human situation. Neonatology 2009;96:137-149.
11 Morrisey EE, Hogan BL: Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell 2010;18:8-23.
12 Warburton D, El-Hashash A, Carraro G, Tiozzo C, Sala F, Rogers O, De Langhe S, Kemp PJ, Riccardi D, Torday J, Bellusci S, Shi W, Lubkin SR, Jesudason E: Lung organogenesis. Curr Top Dev Biol 2010;90:73-158.
13 Maeda Y, Dave V, Whitsett JA: Transcriptional control of lung morphogenesis. Physiol Rev 2007;87:219-244.
14 Hughes K, Watson CJ: The spectrum of STAT functions in mammary gland development. Jakstat 2012;1:151-158.
15 Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S: Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proc Natl Acad Sci U S A 1997;94:3801-3804.
16 Clarkson RW, Boland MP, Kritikou EA, Lee JM, Freeman TC, Tiffen PG, Watson CJ: The genes induced by signal transducer and activators of transcription (STAT)3 and STAT5 in mammary epithelial cells define the roles of these STATs in mammary development. Mol Endocrinol 2006;20:675-685.
17 Yang X, Qiao D, Meyer K, Friedl A: Signal transducers and activators of transcription mediate fibroblast growth factor-induced vascular endothelial morphogenesis. Cancer Res 2009;69:1668-1677.
18 Kiu H, Nicholson SE: Biology and significance of the JAK/STAT signalling pathways. Growth Factors 2012;30:88-106.
19 Sehgal PB: Paradigm shifts in the cell biology of STAT signaling. Semin Cell Dev Biol 2008;19:329-340.
20 Mohr A, Chatain N, Domoszlai T, Rinis N, Sommerauer M, Vogt M, Muller-Newen G: Dynamics and non-canonical aspects of JAK/STAT signalling. Eur J Cell Biol 2012;91:524-532.
21 Croker BA, Kiu H, Nicholson SE: SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol 2008;19:414-422.
22 Kershaw NJ, Murphy JM, Lucet IS, Nicola NA, Babon JJ: Regulation of Janus kinases by SOCS proteins. Biochem Soc Trans 2013;41:1042-1047.
23 Larsen L, Ropke C: Suppressors of cytokine signalling: SOCS. Apmis 2002;110:833-844.
24 Tenbrinck R, Tibboel D, Gaillard JL, Kluth D, Bos AP, Lachmann B, Molenaar JC: Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 1990;25:426-429.
25 Nogueira-Silva C, Carvalho-Dias E, Piairo P, Nunes S, Baptista MJ, Moura RS, Correia-Pinto J: Local fetal lung renin-angiotensin system as a target to treat congenital diaphragmatic hernia. Mol Med 2012;18:231-243.
26 Nogueira-Silva C, Piairo P, Carvalho-Dias E, Peixoto FO, Moura RS, Correia-Pinto J: Leukemia inhibitory factor in rat fetal lung development: expression and functional studies. PLoS One 2012;7:e30517.
27 Nogueira-Silva C, Piairo P, Carvalho-Dias E, Veiga C, Moura RS, Correia-Pinto J: The role of glycoprotein 130 family of cytokines in fetal rat lung development. PLoS One 2013;8:e67607.

28 Peixoto FO, Pereira-Terra P, Moura RS, Carvalho-Dias E, Correia-Pinto J, Nogueira-Silva C: The role of ephrins-B1 and -B2 during fetal rat lung development. Cell Physiol Biochem 2015;35:104-115.
29 Kling DE, Lorenzo HK, Trbovich AM, Kinane TB, Donahoe PK, Schnitzer JJ: MEK-1/2 inhibition reduces branching morphogenesis and causes mesenchymal cell apoptosis in fetal rat lungs. Am J Physiol Lung Cell Mol Physiol 2002;282:L370-378.
30 Piairo P, Moura RS, Nogueira-Silva C, Correia-Pinto J: The apelinergic system in the developing lung: expression and signaling. Peptides 2011;32:2474-2483.
31 Zhang SS, Wei JY, Li C, Barnstable CJ, Fu XY: Expression and activation of STAT proteins during mouse retina development. Exp Eye Res 2003;76:421-431.
32 Cattaneo E, Conti L, De-Fraja C: Signalling through the JAK-STAT pathway in the developing brain. Trends Neurosci 1999;22:365-369.
33 Murphy K, Carvajal L, Medico L, Pepling M: Expression of Stat3 in germ cells of developing and adult mouse ovaries and testes. Gene Expr Patterns 2005;5:475-482.
34 Kim M, O'Brien LE, Kwon SH, Mostov KE: STAT1 is required for redifferentiation during Madin-Darby canine kidney tubulogenesis. Mol Biol Cell 2010;21:3926-3933.
35 Talbot JJ, Shillingford JM, Vasanth S, Doerr N, Mukherjee S, Kinter MT, Watnick T, Weimbs T: Polycystin-1 regulates STAT activity by a dual mechanism. Proc Natl Acad Sci U S A 2011;108:7985-7990.
36 Carraro G, El-Hashash A, Guidolin D, Tiozzo C, Turcatel G, Young BM, De Langhe SP, Bellusci S, Shi W, Parnigotto PP, Warburton D: miR-17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution. Dev Biol 2009;333:238-250.
37 Chinoy MR, Nielsen HC, Volpe MV: Mesenchymal nuclear transcription factors in nitrofen-induced hypoplastic lung. J Surg Res 2002;108:203-211.
38 Bielinska M, Jay PY, Erlich JM, Mannisto S, Urban Z, Heikinheimo M, Wilson DB: Molecular genetics of congenital diaphragmatic defects. Ann Med 2007;39:261-274.
39 Steen HC, Gamero AM: The role of signal transducer and activator of transcription-2 in the interferon response. J Interferon Cytokine Res 2012;32:103-110.
40 Steen HC, Gamero AM: STAT2 phosphorylation and signaling. Jakstat 2013;2:e25790.
41 Schaible T, Veit M, Tautz J, Kehl S, Busing K, Monz D, Gortner L, Tutdibi E: Serum cytokine levels in neonates with congenital diaphragmatic hernia. Klin Padiatr 2011;223:414-418.
42 Romero-Weaver AL, Wang HW, Steen HC, Scarzello AJ, Hall VL, Sheikh F, Donnelly RP, Gamero AM: Resistance to IFN-alpha-induced apoptosis is linked to a loss of STAT2. Mol Cancer Res 2010;8:80-92.
43 Hofmann AD, Takahashi T, Duess J, Gosemann JH, Puri P: Increased expression of activated pSTAT3 and PIM-1 in the pulmonary vasculature of experimental congenital diaphragmatic hernia. J Pediatr Surg 2015;50:908-911.
44 Yu H, Pardoll D, Jove R: STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 2009;9:798-809.
45 Li Y, Du H, Qin Y, Roberts J, Cummings OW, Yan C: Activation of the signal transducers and activators of the transcription 3 pathway in alveolar epithelial cells induces inflammation and adenocarcinomas in mouse lung. Cancer Res 2007;67:8494-8503.
46 Goenka S, Kaplan MH: Transcriptional regulation by STAT6. Immunol Res 2011;50:87-96.
47 Mullings RE, Wilson SJ, Puddicombe SM, Lordan JL, Bucchieri F, Djukanovic R, Howarth PH, Harper S, Holgate ST, Davies DE: Signal transducer and activator of transcription 6 (STAT-6) expression and function in asthmatic bronchial epithelium. J Allergy Clin Immunol 2001;108:832-838.
48 Seki Y, Inoue H, Nagata N, Hayashi K, Fukuyama S, Matsumoto K, Komine O, Hamano S, Himeno K, Inagaki-Ohara K, Cacalano N, O'Garra A, Oshida T, Saito H, Johnston JA, Yoshimura A, Kubo M: SOCS-3 regulates onset and maintenance of T(H)2-mediated allergic responses. Nat Med 2003;9:1047-1054.
49 Shouda T, Yoshida T, Hanada T, Wakioka T, Oishi M, Miyoshi K, Komiya S, Kosai K, Hanakawa Y, Hashimoto K, Nagata K, Yoshimura A: Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis. J Clin Invest 2001;108:1781-1788.
50 Inagaki-Ohara K, Kondo T, Ito M, Yoshimura A: SOCS, inflammation, and cancer. Jakstat 2013;2:e24053.
51 Lee JH, Kaminski N, Dolganov G, Grunig G, Koth L, Solomon C, Erle DJ, Sheppard D: Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types. Am J Respir Cell Mol Biol 2001;25:474-485.

52 Piotrowska H, Kucinska M, Murias M: Biological activity of piceatannol: leaving the shadow of resveratrol. Mutat Res 2012;750:60-82.
53 Seyed MA, Jantan I, Bukhari SN, Vijayaraghavan K: A Comprehensive Review on the Chemotherapeutic Potential of Piceatannol for Cancer Treatment, with Mechanistic Insights. J Agric Food Chem 2016;64:725-737.
54 Faffe DS, Flynt L, Mellema M, Whitehead TR, Bourgeois K, Panettieri RA, Jr., Silverman ES, Shore SA: Oncostatin M causes VEGF release from human airway smooth muscle: synergy with IL-1beta. Am J Physiol Lung Cell Mol Physiol 2005;288:L1040-1048.
55 Shi X, Jin L, Dang E, Chang T, Feng Z, Liu Y, Wang G: IL-17A upregulates keratin 17 expression in keratinocytes through STAT1- and STAT3-dependent mechanisms. J Invest Dermatol 2011;131:2401-2408.
56 McWhinnie R, Pechkovsky DV, Zhou D, Lane D, Halayko AJ, Knight DA, Bai TR: Endothelin-1 induces hypertrophy and inhibits apoptosis in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2007;292:L278-286.
57 Clugston RD, Zhang W, Alvarez S, de Lera AR, Greer JJ: Understanding abnormal retinoid signaling as a causative mechanism in congenital diaphragmatic hernia. Am J Respir Cell Mol Biol 2010;42:276-285.
58 Montedonico S, Nakazawa N, Puri P: Congenital diaphragmatic hernia and retinoids: searching for an etiology. Pediatr Surg Int 2008;24:755-761.
59 Veenma DC, de Klein A, Tibboel D: Developmental and genetic aspects of congenital diaphragmatic hernia. Pediatr Pulmonol 2012;47:534-545.
60 Dong S, Tweardy DJ: Interactions of STAT5b-RARalpha, a novel acute promyelocytic leukemia fusion protein, with retinoic acid receptor and STAT3 signaling pathways. Blood 2002;99:2637-2646.
61 Dong S, Chen SJ, Tweardy DJ: Cross-talk between retinoic acid and STAT3 signaling pathways in acute promyelocytic leukemia. Leuk Lymphoma 2003;44:2023-2029.
62 Si J, Collins SJ: IL-3-induced enhancement of retinoic acid receptor activity is mediated through Stat5, which physically associates with retinoic acid receptors in an IL-3-dependent manner. Blood 2002;100:4401-4409.
63 Paillaud E, Costa S, Fages C, Plassat JL, Rochette-Egly C, Monville C, Tardy M: Retinoic acid increases proliferation rate of GL-15 glioma cells, involving activation of STAT-3 transcription factor. J Neurosci Res 2002;67:670-679.
64 Yang L, Lian X, Cowen A, Xu H, Du H, Yan C: Synergy between signal transducer and activator of transcription 3 and retinoic acid receptor-alpha in regulation of the surfactant protein B gene in the lung. Mol Endocrinol 2004;18:1520-1532.
65 Yan C, Naltner A, Martin M, Naltner M, Fangman JM, Gurel O: Transcriptional stimulation of the surfactant protein B gene by STAT3 in respiratory epithelial cells. J Biol Chem 2002;277:10967-10972.