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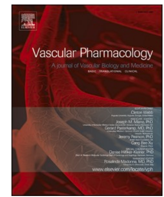

The diverging roles of insulin-like growth factor binding proteins in pulmonary arterial hypertension

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ABSTRACT

Pulmonary hypertension (PH) is a progressive, severe and to date not curable disease of the pulmonary vasculature. Alterations of the insulin-like growth factor 1 (IGF-1) system are known to play a role in vascular pathologies and IGF-binding proteins (IGFBPs) are important regulators of the bioavailability and function of IGFs. In this study, we show that circulating plasma levels of IGFBP-1, IGFBP-2 and IGFBP-3 are increased in idiopathic pulmonary arterial hypertension (IPAH) patients compared to healthy individuals. These binding proteins inhibit the IGF-1 induced IGF-1 receptor (IGF1R) phosphorylation and exhibit diverging effects on the IGF-1 induced signaling pathways in human pulmonary arterial cells (i.e. healthy as well as IPAH-hPASMCs, and healthy hPAECs). Furthermore, IGFBPs are differentially expressed in an experimental mouse model of PH. In hypoxic mouse lungs, IGFBP-1 mRNA expression is decreased whereas the mRNA for IGFBP-2 is increased. In contrast to IGFBP-1, IGFBP-2 shows vaso-constrictive properties in the murine pulmonary vasculature. Our analyses show that IGFBP-1 and IGFBP-2 exhibit diverging effects on IGF-1 signaling and display a unique IGF1R-independent kinase activation pattern in human pulmonary arterial smooth muscle cells (hPASMCs), which represent a major contributor of PAH pathobiology. Furthermore, we could show that IGFBP-2, in contrast to IGFBP-1, induces epidermal growth factor receptor (EGFR) signaling, Stat-3 activation and expression of Stat-3 target genes. Based on our results, we conclude that the IGFBP family, especially IGFBP-1, IGFBP-2 and IGFBP-3, are deregulated in PAH, that they affect IGF signaling and thereby regulate the cellular phenotype in PH.

1. Introduction

Pulmonary hypertension is a progressive disease of the pulmonary vasculature which often leads to right heart failure [1] at the end stage of the disease. The 3 year mortality rate in high-risk PAH patients is up to 55% [2], and to date, there is no treatment available to cure the disease or to reverse the pathological vascular remodeling process. There are different standard treatment options like endothelin receptor antagonists, phosphodiesterase 5 (PDE5) inhibitors, soluble guanylate cyclase activators, prostacyclin derivates, nitric oxide (NO) [3] and different tyrosine kinase inhibitors [4] which only treat the symptoms, but do not resolve the cause of disease.

Many different growth factor pathways are known to be involved in disease progression [5]. One of these growth factor families is the

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insulin-like growth factor family [6,7], which includes the autocrine, paracrine and endocrine acting IGF-1 and IGF-2 [8]. IGF-1 is mainly expressed during postnatal life, whereas IGF-2 is highly expressed in fetuses [9,10]. Alterations of the IGF-1 system are known to play a role in vascular pathologies like atherosclerosis, angiogenesis, restenosis, hypertension [11] and also pulmonary hypertension [12–14]. IGF-1 binds mainly to the insulin-like growth factor 1 receptor (IGF1R), and acts through the RAS/MAP kinase and the PI3K/AKT signaling pathway [11,15,16]. These pathways, and their respective growth factors, are known to control cell proliferation, survival, differentiation and migration [11,17]. An important regulator of IGF-1 and IGF-2 is presented by the insulin-like growth factor binding proteins (IGFBPs), which are predominantly synthesized by the liver and subsequently secreted into the blood stream and act in an autocrine, paracrine and endocrine fashion [18,19]. The IGFBP family consists of six different IGFBPs. All of them share the capability to bind IGF-1 with a higher affinity than the IGF1R, and thereby increase the half-life of IGF-1 and regulate its bioavailability for the receptor [20,21]. The importance of these proteins is underlined by the fact that around 99% of circulating IGF is bound to IGFBPs [21,22]. In this regard, IGFBP-3 is the most important carrier due to its highest abundancy of all the members of the family in the human blood circulation [23,24]. In addition to their binding capacity, some of the IGFBPs can also exhibit IGF-independent effects [21,25]. IGFBP-1 possesses an integrin binding domain (RGD sequence) as well as IGFBP-2 which, in addition to this, also contains a heparin binding domain and a nuclear localization sequence (NLS). Therefore, it can bind to the cellular surface as well as to the extracellular matrix and exert regulatory effects on gene transcription [8,26]. IGFBP expression is linked to many types of cancer [27–32]. Due to the similarities in the pathological events in cancer and PH with respect to the disease onset and progression [4,33–36], we hypothesize that IGFBPs play a role in pulmonary arterial hypertension. Less is known about the implications of IGFBPs in PAH, but for IGFBP-1 and IGFBP-2 increased plasma concentrations in PAH patients [7,37] and an association with disease severity was reported [6,7,37,38]. It has not been addressed whether this family of proteins has any effects on pulmonary vascular cells and on the aberrant signaling pathways that contribute to the onset of the remodeling processes driving PAH. Because of this huge lack of understanding of the role of IGFBPs in PAH, we performed a series of in vitro studies using human pulmonary vascular cells as well as an ex vivo functional analysis using the isolated ventilated and perfused murine lung model. Based on our results, we conclude that IGFBPs, especially IGFBP-1, IGFBP-2 and IGFBP-3, are deregulated in PAH and thereby modulate IGF signaling thereby interfering with the cellular phenotype in this disease.

2. Methods

2.1. Enzyme-linked immunosorbent assay (ELISA)

To measure the IGFBP-1 (#DY871), IGFBP-2 (#DGB200) and IGFBP-3 (DY675) protein concentrations in human plasma, blood plasma samples from IPAH patients and blood plasma samples from healthy volunteers were used and measured by enzyme-linked immunosorbent assays (ELISAs) according to manufacturer's instructions (R&D Systems). For the correlation analyses, the correlation co-efficient (r) and the corresponding probability value (p) between IGFBP-1, IGFBP-2 or IGFBP-3 concentrations and the different measured clinical parameters were determined.

2.2. Cell culture

hPASMCs, IPAH-hPASMCs and hPAECs were cultured at 37 °C under normoxic (21% O_2) or under hypoxic conditions (1% O_2). HPASMCs were cultured in smooth muscle cell growth medium 2 (SmGM2) and starved for 18 h in smooth muscle basal medium (BM, both obtained from Lonza), whereas hPAECs were cultured in endothelial cell growth medium 2 (GM) and starved for 18 h in endothelial cell basal medium (BM, both obtained from Promocell). After starvation, cells were stimulated as specified in Fig. 2a.

2.3. Western-blot analysis

Cells were harvested and lysed in RIPA buffer. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) according to manufacturer's instructions. Electrophoresis was performed and protein expression was determined by using antibodies mentioned in Supplementary Table 1 and 2. The immunoreactive bands were quantified by densitometric analyses.

2.4. Real-time polymerase chain reaction

For RNA expression studies, mRNA of human pulmonary vascular cells, and mRNA of mouse lungs from male C57BL/6 J mice (Charles River Laboratories) kept under normoxic conditions (21% O_2) or under hypoxic conditions (10% O_2) for 3 weeks, respectively, were used. RNA purification was performed using mRNA RNeasy Mini Kit (Qiagen GmbH) according to manufacturer's instructions. Reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories GmbH). Primers used for Real-time PCR are listed in Supplementary Table 3. Data were analyzed using the MxPro and AriaMx Software (Agilent Technologies), with Ct thresholds being set in the linear range.

2.5. Isolated perfused and ventilated murine lung

The isolated perfused and ventilated murine lung experiments were performed as described previously [39]. Lungs were perfused with electrolytic solution containing increasing concentrations of recombinant mouse IGFBP-1 (rm-IGFBP-1) and rm-IGFBP-2, as indicated in Supplementary Fig. 2.

2.6. Knockdown of IGF1R expression using siRNA

To downregulate IGF1R protein expression in healthy hPASMCs, cells were transfected with 100 nM ON-TARGETplus Human IGF1R (3480) siRNA-SMARTpool (*L*−003012−00−0010) and ON-TARGETplus Non-targeting pool (D-001810–10-05) as a control, both purchased by Dharmacon. Cells were cultivated for additional 48 h before cell starvation and subsequent treatment as described in Supplementary Fig. 3.

2.7. Peptide-based kinase activity assay

Protein isolation and peptide-based kinase activity assays for tyrosine as well as serine/threonine kinases (using the PamStation device) were conducted by the manufacturer's instructions as described previously [40] in addition to the Supplementary Methods.

2.8. Statistical analyses

Data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 5 and 7 software (GraphPad), using analysis of variance with post hoc analysis. *P* values of <0.05 were considered statistically significant.

A more detailed description of the performed methods is provided in the Supplementary Methods.

3. Results

3.1. IGFBP-1, IGFBP-2 and IGFBP-3 expression levels in IPAH patients

To gain insights into the role of IGFBPs in PAH and which of the three examined IGFBPs might play a role in the pathophysiological processes,

IGFBP-1, IGFBP-2 and IGFBP-3 blood plasma concentrations in IPAH patients were determined by ELISA measurements. They were compared to the blood plasma concentrations of healthy individuals. The demographic characteristics and clinical parameters of IPAH patients and healthy controls are summarized in Table 1.

The blood plasma concentration analyses revealed elevated levels of IGFBP-1 (Fig. 1a), IGFBP-2 (Fig. 1c) and IGFBP-3 (Fig. 1e) in IPAH patients compared to healthy controls. To test the potential of IGFBPs to serve as a biomarker, or as a marker of disease severity in PAH, correlation analyses between the IGFBPs concentration and determined clinical parameters (Table 1) were performed. The IGFBP-1 concentration does not only show a positive correlation between IGFBP-1 and BNP levels ($r = 0.452$; $p = 0.002$), additionally a negative correlation between the IGFBP-1 concentration and the 6 MWD ($r = -0,341$; $p = 0.024$) could be determined (Fig. 1b). BNP is an approved biomarker for PH and is furthermore of prognostic significance for IPAH [41]. The same significant correlations could be shown for the IGFBP-2 concentration and the BNP levels ($r = 0.531$; $p = 0.0002$), as well as the 6 MWD ($r = -0.374$; $p = 0.012$)(Fig. 1d). Additional positive correlations for IGFBP-2 with NYHA functional class ($r = 0.408$; $p = 0.006$) and the age of patients ($r = 0.383$; $p = 0.010$) could be seen (data not shown). In contrast to this, for IGFBP-3 blood plasma levels no significant correlation with the 6 MWD ($r = -0.073$; $p = 0.640$) or the BNP levels ($r = 0.034$; $p = 0.828$, Fig. 1f) could be seen, neither for any of the other determined clinical parameters (data not shown).

3.2. Effects of IGFBP-1, IGFBP-2 and IGFBP-3 on IGF-1 signaling

Cell culture experiments using pulmonary vascular cells were performed to determine the impact of IGFBP-1, IGFBP-2 and IGFBP-3 on IGF-1 induced signaling events. In addition, cells were also treated with the IGFBP inhibitor NBI-31772 to analyze the functional consequences of IGFBP loss of function. NBI-31772 displaces biologically active IGF-1 from the complex with IGFBP-1 (3.36 ± 1.82 nm), IGFBP-2 (1.18 ± 0.27 nm) and IGFBP-3 (5.64 ± 0.47 nm) at concentrations on a low nanomolar range [42]. Therefore, hPASMCs and hPAECs were stimulated with IGF-1 to induce the intracellular signaling cascade. To study the effect of IGFBPs, IGF-1 was preincubated for two hours with IGFBP-1, IGFBP-2 or IGFBP-3 before adding the protein complexes to the final cell culture media. To see the effect of IGFBP inhibition, the cells were stimulated with a mixture of IGF-1 which was again preincubated with IGFBP-1, IGFBP-2 or IGFBP-3 and the IGFBP inhibitor NBI-31772 (Fig. 2a). Western-blot analyses of hPASMCs clearly showed the phosphorylation of IGF1R upon IGF-1 stimulation (Fig. 2b). An inhibitory effect of all three binding proteins on the IGF-1 induced IGF1R phosphorylation could clearly be seen (Fig. 2b). The same effect of IGF-1 and IGFBP-1, IGFBP-2 and IGFBP-3 on the IGF-1 induced IGF1R phosphorylation was observed in hPAECs (Fig. 2d), this was also confirmed by densitometric analyses of the Western-blots (Fig. 2c + Fig. 2e). Interestingly, the effects of the IGFBPs on the IGF-1 induced activation of the downstream signaling pathways, i.e. AKT and ERK, were different between the two vascular cell types. In hPASMCs, IGFBP-1, IGFBP-2 and IGFBP-3 showed a reduced AKT phosphorylation pattern (Fig. 2b) and a trend to a decreased relative expression, represented by densitometric analyses (Fig. 2c). The inhibition of IGFBP-1, IGFBP-2 and IGFBP-3 by NBI-31772 could restore the effects of IGF-1 on AKT phosphorylation (Fig. 2b). The IGF-1 induced phosphorylation of ERK could be reduced by IGFBP-1 and IGFBP-3 but interestingly, IGFBP-2 could not reduce the IGF-1 induced ERK phosphorylation (Fig. 2b). Unfortunately, this clear picture could not be validated by densitometric analyses, which combine the investigation of three different donors in both cases, for hPASMCs (Fig. 2c) as well as for hPAECs (Fig. 2e). In hPAECs however, AKT is permanently phosphorylated independent of the stimulation condition (Fig. 2d). The phosphorylation pattern of ERK seemed to be similarly regulated and increased by IGFBP-2 and IGFBP-3 compared to IGFBP-1 (Fig. 2d), but the densitometric analyses showed no significant differences (Fig. 2e).

Table 1
Demographic characteristics and clinical parameters of IPAH patients and healthy controls enrolled in the IGFBP-1, IGFBP-2 and IGFBP-3 blood plasma analyses. NYHA: New York Heart Association, 6 MWD: 6-min walk distance, BNP: Bone morphogenetic protein, PAP: Pulmonary arterial pressure, CI: Cardiac index, PVR: Pulmonary vascular resistance, PAWP: Pulmonary arterial wedge pressure. Data are presented as mean ± SEM.

	IPAH	Healthy
Patients [n]	44	6
Age [years]	55.5 ± 18.21	33.83 ± 8.54
Female sex [%]	65.91	50.00
NYHA functional class [%]		
II	18.2	0
III	72.7	0
IV	9.1	0
6 MWD [m]	313.8 ± 127.7	–
BNP [pg ml $^{-1}$]	331.1 ± 308.9	–
Mean PAP [mmHg]	47.1 ± 13.8	–
CI [l/min/m^2]	2.2 ± 0.5	–
PVR [dyn*s/cm5]	888.6 ± 482.9	–
PAWP [mmHg]	7.55 ± 3.11	–

3.3. Impact of hypoxia (i.e. a trigger of PH) on the IGF-1/IGFBP signaling axis

Group three PH is classified as PH caused by lung diseases and/or hypoxia [43]. Cultivation of pulmonary vascular cells under hypoxic conditions is a common cell culture model used to mimic the pathobiological phenotype including the respective signaling mechanisms that pulmonary vascular cells experience in PAH. To investigate the effect of the IGF/IGFBP-system in the context of PH, we cultured healthy hPASMCs and hPAECs under hypoxic conditions (1% instead of the standard 21% O_2 in normoxia) and stimulated as described in Fig. 2a. Western-blot analysis revealed that IGF1R expression was increased in hPASMCs under hypoxic conditions (Hox), compared to normoxic conditions (Nox, Fig. 3a). Similar to the normoxic conditions, IGF-1 induced IGF1R phosphorylation in hypoxic hPASMCs, but to a lesser extent than under normoxic conditions (Fig. 3a). This increased phosphorylation could be prevented by IGFBP-1, IGFBP-2 and IGFBP-3 (Fig. 3a). Densitometric analysis of three different donors in total confirmed these results (Fig. 3b). This effect could again be also seen in hPAECs, but in these cells hypoxia had no influence on IGF1R expression (Fig. 3d + Fig. 3e). In hypoxic hPASMCs and hPAECs, IGFBP inhibition by NBI-31772 couldn't restore the IGF-1 induced effect on IGF1R phorphorylation (Fig. 3a + Fig. 3d) and the activation of the following signaling cascade in hPAECs (Fig. 3d). As under normoxic conditions, AKT was phosphorylated upon IGF-1 stimulation and this effect was reduced by simultaneous administration of IGFBP-1, IGFBP-2 or IGFBP-3 in hypoxic hPASMCs (Fig. 3a). Densitometric analysis revealed that only when IGFBP-3 or IGFBP-2 with NBI-31772 were added, the relative expression of P-AKT was significantly decreased compared to IGF-1 stimulation in both oxygen conditions (Fig. 3b). Hypoxia lead to a slight increase in the phosphorylation pattern of ERK (Fig. 3a). In hPAECs, the densitometric analysis showed no influence of hypoxia on the relative expression neither of AKT nor ERK phosphorylation, independent of the stimulation (Fig. 3e). But the representative Western-blot showed under hypoxic conditions (similar to the normoxic conditions), a higher ERK phosphorylation pattern after stimulation of the cells with IGF-1 and IGFBP-2 as well as IGFBP-3, and the general phosphorylation intensity under hypoxic conditions is reduced (Fig. 3d). In hPASMCs as well as in hPAECs, independent of the oxygen concentration or the IGFBP which was added, NBI-31772 could not restore the effect of IGF-1 (Fig. 3a + Fig. 3d).

To confirm the results obtained from the hypoxic studies, we wanted to have a look on the effect of IGFBP-1, IGFBP-2 and IGFBP-3 on IGF-1

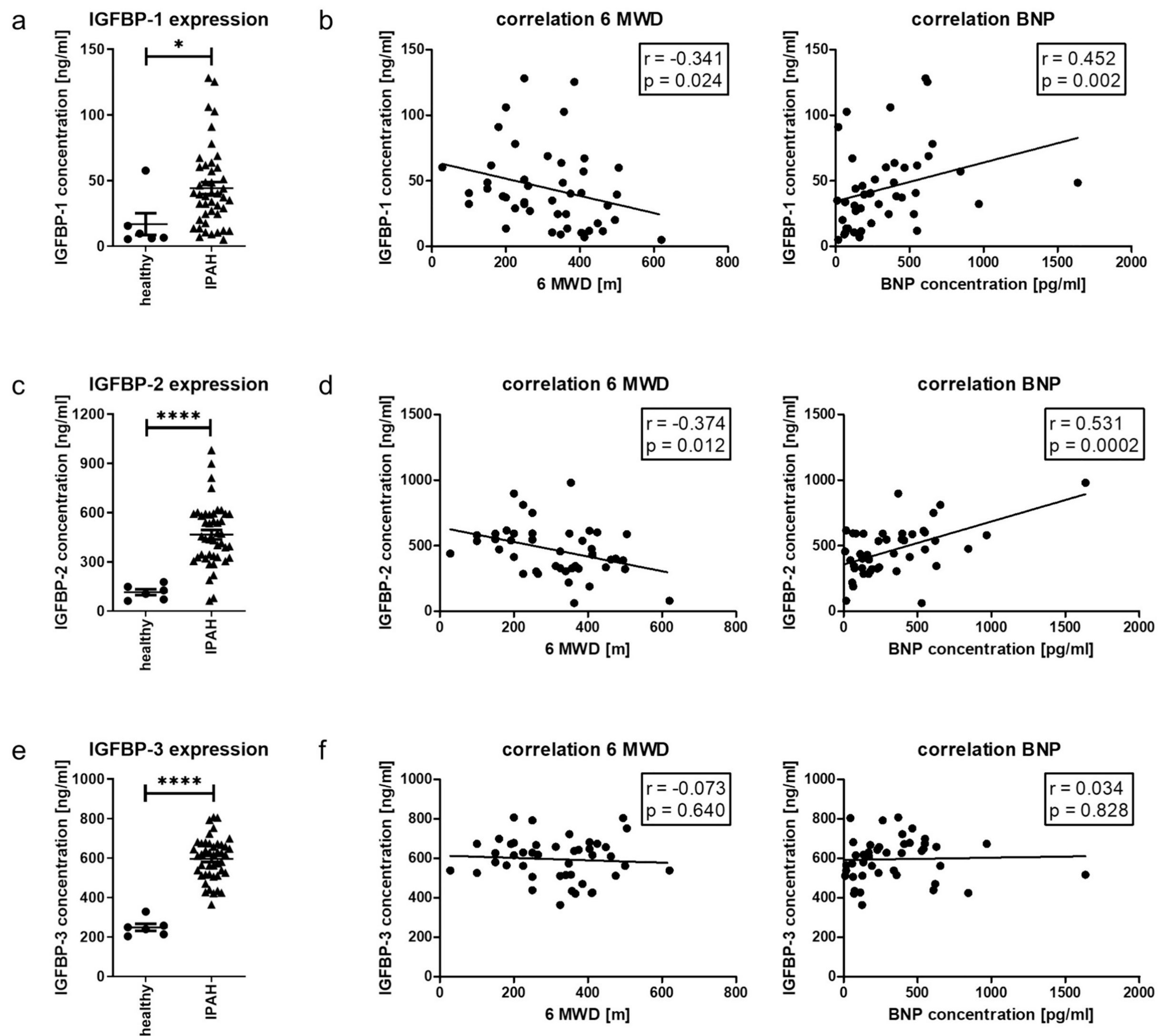

Fig. 1. ELISA measurements of IGFBP-1, IGFBP-2 and IGFBP-3 plasma levels in IPAH patients and correlations with disease associated clinical parameters. IGFBP-1 (a), IGFBP-2 (c) and IGFBP-3 (e) blood plasma concentrations in healthy individuals ($n = 6$) and IPAH patients ($n = 44$) were measured by ELISA. Samples were measured in duplicates and are presented as mean ± SEM of the duplicates of each subject. Optical density was determined at 450 nm wavelength, with a wavelength correction measurement at 540 nm. Statistical analysis was performed using Mann-Whitney test for IGFBP-1 and students *t*-test for IGFBP-2 and IGFBP-3; $^{*}p < 0.05$, $^{****}p < 0.0001$

induced signaling in hPASMCs from IPAH patients. These cells were isolated from the lungs of IPAH patients, cultured under normoxic conditions, and stimulated as described in Fig. 2a. In contrast to healthy hPASMCs cultured under hypoxic conditions, IPAH-hPASMCs showed no increase in IGF1R expression (Fig. 3g). The IGF1R phosphorylation pattern per se was decreased in IPAH-hPASMCs compared to healthy hPASMCs, which was shown by the representative Western-blot (Fig. 3g). The relative IGF1R phosphorylation after IGF-1 stimulation was significantly decreased in IPAH-hPASMCs and IGFBP-1, IGFBP-2, as well as IGFBP-3 could further reduce this IGF-1 induced IGF1R phosphorylation (Fig. 3h). With respect to the IGF-1 downstream signaling, neither the relative P-AKT nor the relative P-ERK expression was significantly altered between healthy and IPAH-hPASMCs (Fig. 3g + Fig. 3h). But the representative Western-blot showed, that the phosphorylation pattern of ERK was rather increased in IPAH-hPASMCs and the phosphorylation could not be inhibited by IGFBP-2 in contrast to IGFBP-1 or IGFBP-3 exposure (Fig. 3g). Similar to healthy hPASMCs and hPAECs under normoxic and hypoxic conditions, NBI-31772 could not restore IGF-1 induced signaling in IPAH-hPASMCs (Fig. 3g). For gene expression analyses of IGFBP-1, IGFBP-2 and IGFBP-3, mRNA was isolated from the cells mentioned above and real-time PCR was performed. In hPASMCs, hypoxia led to an increase in IGFBP-1 and IGFBP-3 mRNA expression under serum-free basal media (BM) conditions (Fig. 3c). For the IGFBP-2 mRNA expression under hypoxic conditions, only a trend in increased IGFBP-2 mRNA expression could be seen (Fig. 3c). Likewise to hPASMCs, the IGFBP-3 mRNA expression was significantly increased under hypoxic conditions in hPAECs (Fig. 3f). Hypoxia led to a trend in increasing IGFBP-1 and a trend in reduced IGFBP-2 mRNA expression levels in hPAECs (Fig. 3f). The mRNA expression levels in IPAH-hPASMCs under BM conditions showed, that IGFBP-1 mRNA expression was significantly decreased in IPAH-hPASMCs, whereas IGFBP-2 and IGFBP-3 mRNA expression was significantly increased in IPAH-hPASMCs compared to healthy hPASMCs (Fig. 3i). Further analyses revealed that in hPASMCs neither IGFBP-1 nor IGFBP-2 mRNA levels were significantly altered, independent of the stimulation or the oxygen concentration (Supplementary Fig. 1a). IGFBP-3 mRNA expression was significantly increased under hypoxic conditions after IGF-1 stimulation, as well as for IGF-1 complexed with IGFBP-1 and IGFBP-3, compared to normoxic condition (Supplementary Fig. 1a). IGFBP-3 expression under hypoxic conditions was decreased in

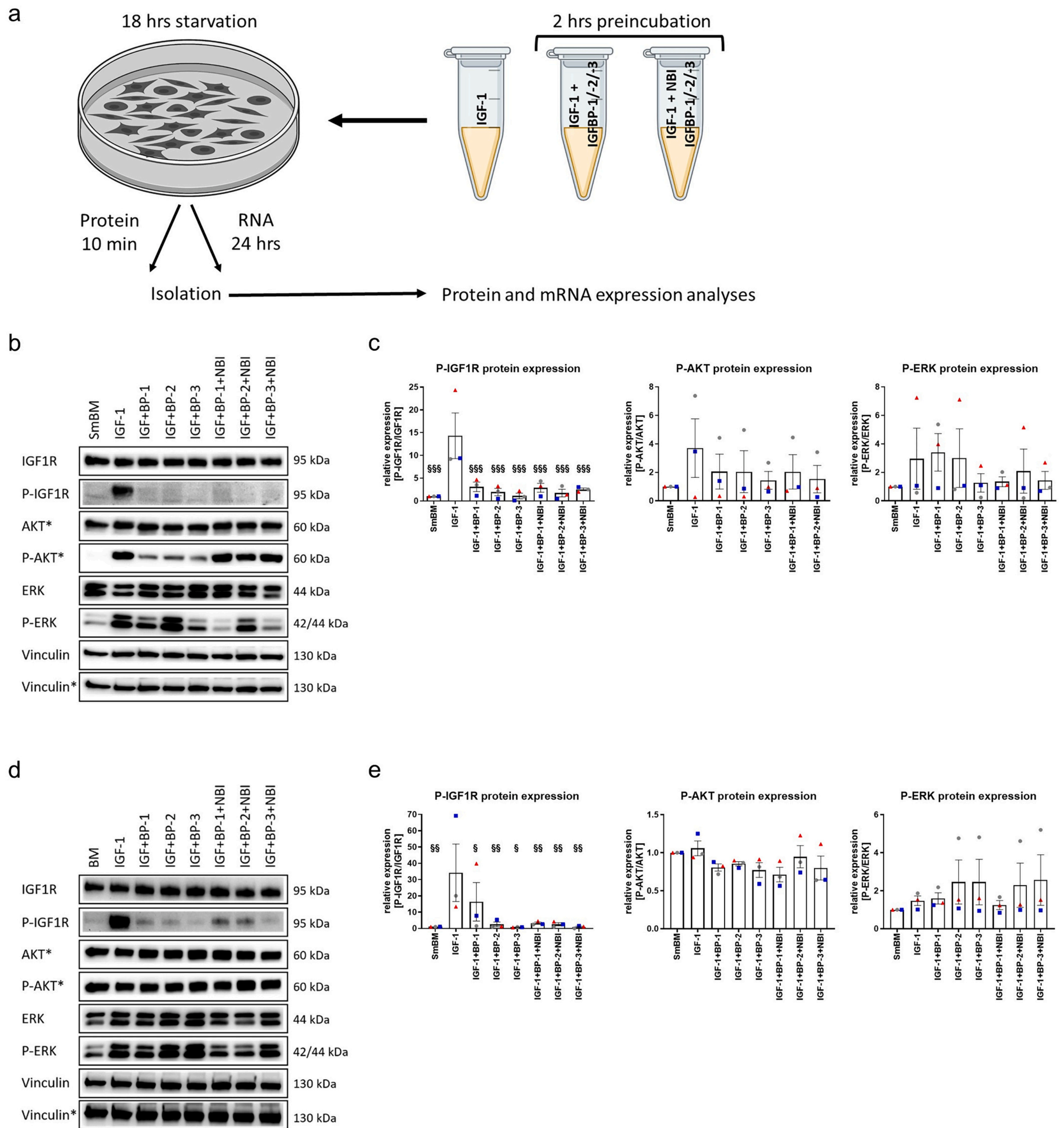

Fig. 2. Effects of IGFBPs on IGF-1 induced signaling in hPASMCs and hPAECs and consequences of IGFBP inhibition by NBI-31772. Human pulmonary vascular cells were stimulated with IGF-1 and with preincubated IGF-1, IGFBP-1, IGFBP-2 or IGFBP-3 and NBI-31772 for the indicated time points (a, created with BioRender.com). Representative Western-blots of hPASMCs (b) and hPAECs (d) with regard to (P-)IGF1R expression and the activation of the IGF-1 induced signaling pathway, represented by P-(AKT) and (P-)ERK. One representative experiment of three independent repetitions is shown. Expression of vinculin protein is shown as a loading control. SmBM: Smooth muscle basal medium; BM: Basal medium, NBI: NBI-31772, * antibodies incubated on the same membrane. Densitometric analysis of Western-blots for hPASMCs (c, $n = 3$) and hPAECs (e, n = 3) was performed using ImageJ software. The expression levels of the proteins were normalized to BM and then normalized to the expression of vinculin. Afterwards the values of phosphoproteins were normalized to respective total protein. All data represent the mean ± SEM of relative expression compared to IGF-1 stimulation. Each primary cell isolate, i.e. from donor 1 (grey circle), donor 2 (red triangle), donor 3 (blue box), can be distinguished due to a unique colour and a respective symbol. Statistical analysis was performed by one-way ANOVA with Newman-Keuls multiple comparison test, § $p < 0.05$; §§ $p < 0.01$; §§§ $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

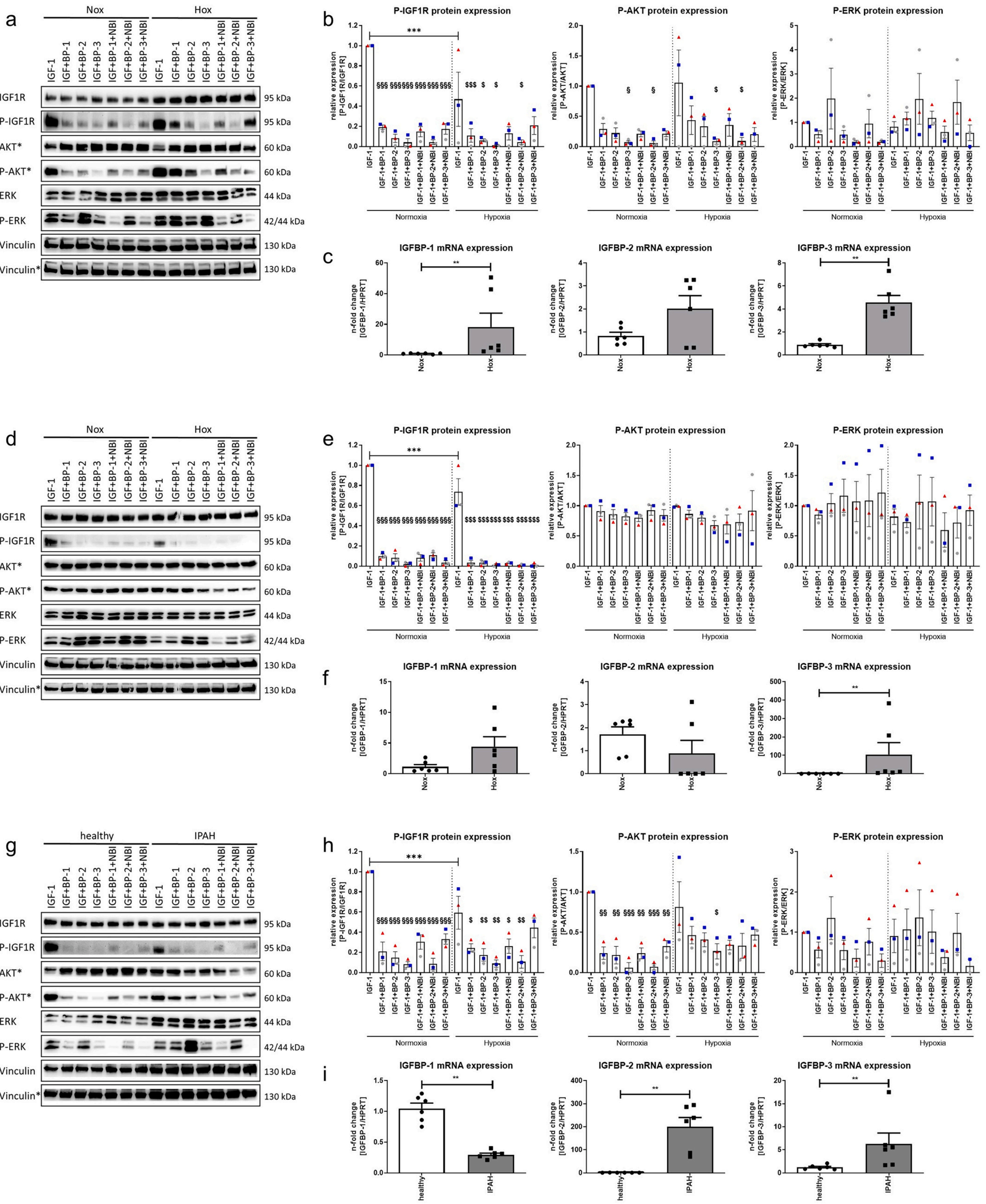

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Fig. 3. Impact of hypoxia and of the IPAH phenotype on the IGF-1 induced signaling pathways. Human pulmonary vascular cells were stimulated with IGF-1 and with preincubated IGF-1, IGFBP-1, IGFBP-2 or IGFBP-3 and NBI-31772. Representative Western-blots of hPASMCs (a) and hPAECs (d) cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions and IPAH-hPASMCs (g) with regard to (P-)IGF1R expression and the activation of the IGF-1 induced signaling pathways, represented by P-(AKT) and (P-)ERK. One representative experiment of three independent repetitions is shown. Expression of vinculin protein is shown as a loading control. Densitometric analysis of Western-blots for hPASMCs (b, $n = 3$), hPAECs (e, n = 3) and IPAH-hPASMCs (h, n = 3) was performed. All data represent the mean ± SEM of relative expression. The expression levels of the proteins were normalized to IGF-1 Nox or to healthy condition, respectively, and these values were normalized to the expression of corresponding vinculin. Then, the values of phosphoproteins under Nox and Hox conditions (healthy and IPAH, respectively) were each normalized to respective total protein. Each primary cell isolate, i.e. from donor 1 (grey circle), donor 2 (red triangle), donor 3 (blue box), can be distinguished due to a unique colour and a respective symbol. * antibodies incubated at the same membrane. Statistical analysis was performed by one-way ANOVA with Newman-Keuls multiple comparison test. IGFBP-1, IGFBP-2 and IGFBP-3 mRNA expression in hPASMCs (c, normalized to HPRT as reference gene, n = 3), in hPAECs (f, normalized to GAPDH as reference gene, n = 3) cultured under normoxic and hypoxic conditions and IGFBP-1, IGFBP-2 and IGFBP-3 mRNA expression in healthy and IPAH-hPASMCs (i, normalized to HPRT as reference gene, n = 3) in BM. The different cell lots were measured in duplicates. All data are presented as mean ± SEM of the *n*-fold change ($2^{-\Delta\Delta Ct}$) and analyzed statistically using a Mann–Whitney test. SmBM: Smooth muscle basal medium; BM: Basal medium, Nox: Normoxia, Hox: Hypoxia, NBI: NBI-31772. § $p < 0.05$; §§ $p < 0.01$; §§§ $p < 0.001$ comparison within Nox or healthy conditions, \$ $p < 0.05$; \$\$ $p < 0.01$; \$\$\$ $p < 0.001$ comparison within Hox or IPAH condition, ** $p < 0.01$, *** $p < 0.001$ comparison between Nox and Hox or healthy and IPAH condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unstimulated control settings (i.e. BM) and under the stimulation of IGF-1 complexed with IGFBP-2 (Supplementary Fig. 1a). In hPAECs, neither a reduced oxygen concentration nor the different stimulations led to an alteration in IGFBP-1, IGFBP-2 or IGFBP-3 mRNA expression (Supplementary Fig. 1b). In IPAH-hPASMCs, all conditions led to a significant decrease in IGFBP-1 mRNA expression and to a tremendous increase in IGFBP-2 mRNA expression (Supplementary Fig. 1c). IGFBP-2 mRNA expression was significantly increased in IPAH-hPASMCs in unstimulated control setting (SmBM), compared to IGF-1 stimulation under hypoxic conditions (Supplementary Fig. 1c). IGFBP-3 mRNA expression was not altered between healthy and IPAH-hPASMCs (Supplementary Fig. 1c). Although IGFBP-3 is the most common IGFBP in the blood circulation in terms of quantity, the results indicate a stronger local role for IGFBP-1 and IGFBP-2. Of particular interest are the different effects of IGFBP-1 and IGFBP-2 on the IGF-1 induced signaling and the oppositional mRNA expression in IPAH-hPASMCs. Therefore, we have focused on IGFBP-1 and IGFBP-2 with regard to the local expression and the physiological function of IGFBPs.

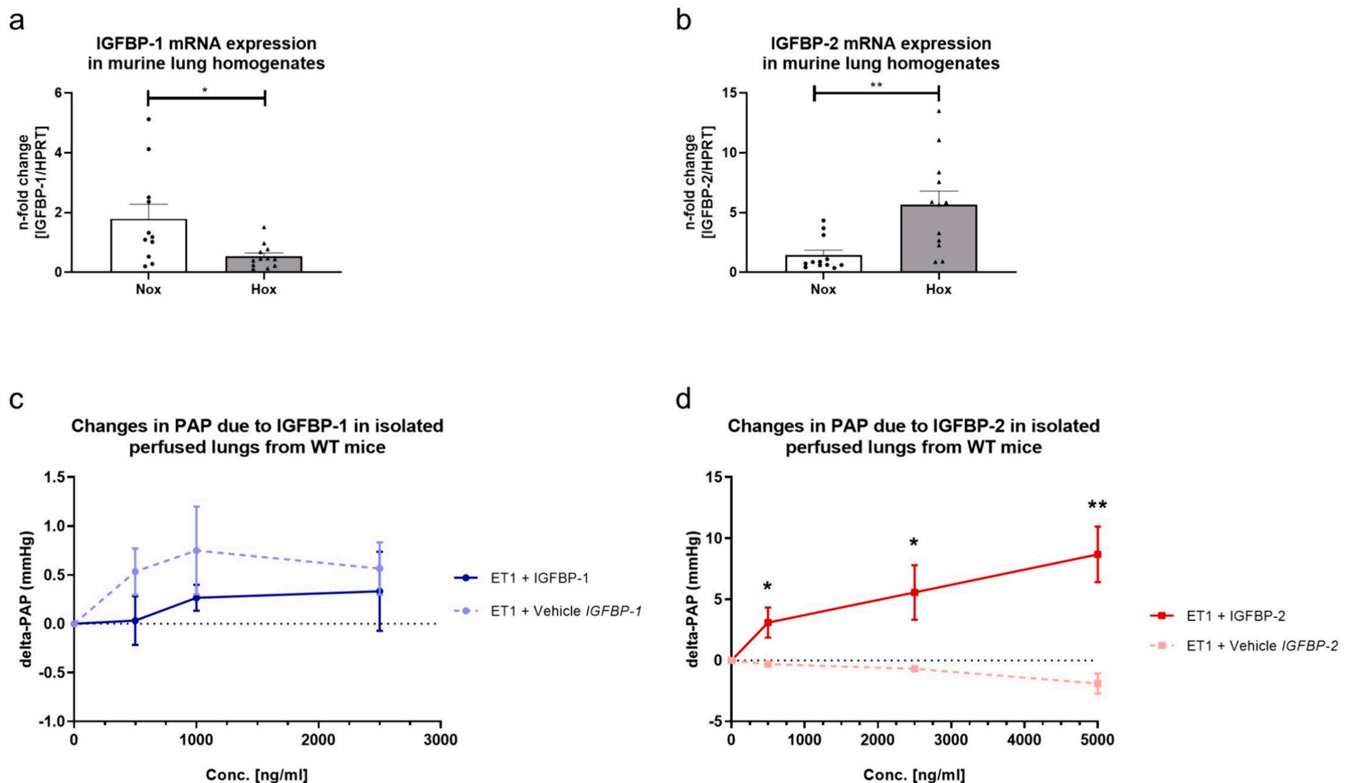

Fig. 4. Expression profile and physiological effects of IGFBP-1 and IGFBP-2 on vasoconstriction in mouse lungs. Analysis of IGFBP-1 (a, Nox $n = 11$, Hox $n = 12$) and IGFBP-2 (b, Nox and Hox n = 12) mRNA expression, normalized to HPRT as reference gene, in male C57BL/6 J mouse lung homogenates. Mice were kept under normoxic or hypoxic conditions for 3 weeks. Data from all individual animals are presented as mean ± SEM of the *n*-fold change ($2^{-\Delta\Delta Ct}$). Statistical analysis was performed by using unpaired *t*-test. To test the effects of IGFBP-1 and IGFBP-2 on vasoconstriction, the isolated ventilated and perfused mouse lung model was used, where mouse lungs of C57BL/6 J mice were normoxic ventilated and perfused with increasing concentrations of rm-IGFBP-1 or rm-IGFBP-2. The effect of IGFBP-1 (c, $n = 6$) and IGFBP-2 (d, $n = 5$) on vasoconstriction was examined by adding the indicated concentrations of IGFBP-1 and IGFBP-2 and the following determination of the delta-PAP. A mixture of acetonitrile (30%) and TFA (0.1%) was used as a control for IGFBP-1, for IGFBP-2 PBS was used as a control. Statistical analysis was performed by multiple t-test using the Holm-Sidak method where each row (i.e. IGFBP vs. control at a defined concentration) was analyzed individually, without assuming a consistent SD. * $p < 0.05$; ** $p < 0.01$

3.4. Local IGFBP-1 and IGFBP-2 expression in mouse lungs and their influence on pulmonary arterial pressure

Next, we wanted to investigate if IGFBPs not only function as secretory circulating proteins in the blood circulation regulating general IGF bioavailability but also play a role based on their local expression in the pulmonary vasculature. For this purpose, we first kept C57BL/6 J mice for three weeks under normoxic (21% O_2), or under hypoxic (10% O_2) conditions to induce PH. Interestingly, hypoxia led to a decrease in IGFBP-1 mRNA expression (Fig. 4a), whereas IGFBP-2 mRNA expression was increased (Fig. 4b) in mouse lung homogenates. These opposing directions in IGFBP-1 and IGFBP-2 mRNA expression in the lungs of mice points towards fundamental differences in the physiological effects of these IGF-binding proteins. Therefore, we used the isolated perfused and ventilated mouse lung model and measured the effects of these two IGF-binding proteins on the pulmonary artery pressure (PAP) directly in the lung. Here, ex vivo perfusion of the pulmonary unit (consisting of the lung and the heart of normoxic C57BL/6 J mice) with buffer and subsequent addition of 20 nM endothelin-1 (ET-1) were carried out to induce vasoconstriction. This was followed by administration of increasing concentrations of rm-IGFBP-1 or rm-IGFBP-2 (Supplementary Fig. 2a). Perfusion of the lungs with ET-1 resulted in a significant increase in PAP, compared to buffer alone which serves as a negative control (Supplementary Fig. 2b). By addition of IGFBP-1 or IGFBP-2 to the perfusion buffer containing ET-1, we hypothesized that the PAP would either decrease (i.e. demonstrating a vaso-dilatatory effect) or rise (i.e. demonstrating a vaso-constrictive mode of action). The solvent for reconstitution of each protein was used as a negative vehicle control. For IGFBP-1, a trend in decreasing delta-PAP values could be examined, compared to the indicated vehicle control (i.e. buffer containing acetonitrile (30%) and TFA (0.1%)) (Fig. 4c). But at 2.500 ng/ml IGFBP-1, no difference in delta-PAP between IGFBP-1 and the vehicle control could be seen (Fig. 4c). In contrast, IGFBP-2 led to a significant and concentration dependent increase in delta-PAP compared to the vehicle control (i.e. buffer with PBS) (Fig. 4d) which demonstrates that IGFBP-2 exerts vaso-constrictive effects on the lung vasculature in mice.

3.5. IGF-1 receptor dependent as wells as IGF-1 receptor independent effects of IGFBP-1 and IGFBP-2

To fully reveal all yet hidden effects of both IGFBPs on cellular signaling in an IGF1R-dependent as well as an independent manner, we conducted a peptide-based kinase activity profiling. Therefore, we cultured healthy hPASMCs under normoxic conditions and transfected them with siRNA against the IGF1R or a negative control siRNA. We decided to only use hPASMCs, because the effects of the IGFBPs on the IGF-1 induced activation of the downstream signaling pathways in hPAECs were comparably weak. We transfected healthy hPASMCs with 100 nM IGF1R siRNA (si-IGF1R) or negative control siRNA (si-NC) and stimulated the cells for 2 h with IGF-1, or with IGF-1 complexed with rh-IGFBP-1 or rh-IGFBP-2 (Supplementary Fig. 3a and 3b). Heat maps for tyrosine (Supplementary Fig. 4a) and serine/threonine (Supplementary Fig. 4b) kinase mediated phosphorylation as the primary result of the peptide-based kinome profiling revealed a distinct pattern of substrate peptide phosphorylation. Upstream kinase analyses based on the individual phospho-peptide signatures led to the identification of kinases which are predicted to be either affected by IGFBP-1 (13 hits, all down-regulated upon IGFBP-1 incubation) or IGFBP-2 (17 hits in total: 5 down-regulated and 12 over-activated by IGFBP-2 incubation) compared to IGF-1 stimulation alone in the presence of the IGF1 receptor (Fig. 5a, si-NC). In the absence of the IGF-1 receptor, both IGFBPs still interfered with intracellular signaling. In case of IGFBP-1, only 2 kinases were affected (1 up- and 1 down-regulated), and 5 kinaseś activity were solely down-regulated by IGFBP-2 (Fig. 5b, si-IGF1R). Interestingly, 2 kinases were modified in the same manner by both IGFBPs – 1 each in both directions. Complete tables including the statistics of all kinases referred to in Fig. 5a and Fig. 5b are provided in the supplement (Supplementary Tables 4–8). Prior to the in-depth analysis of the differential kinase activity of newly identified candidates, we performed Western-blot analysis to verify the knock-down of IGF1R and the associated loss of AKT phosphorylation (in the case of IGFBP-1) and ERK phosphorylation (in the case of IGFBP-2) (Fig. 5c). Next, according to the String protein database network analyses, clusters of kinases could be detected that clearly demonstrate the different mode of action on IGF-induced signaling events on two levels - firstly dependent on the IGFBP, i.e. IGFBP-1 (Supplementary Fig. 5a) versus IGFBP-2 (Supplementary Fig. 5b), and furthermore with respect to the presence or absence of the IGF1R (Supplementary Fig. 5c). The Top 10 deregulated pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) classification uncovers the diverse and opposing effects of both IGFBPs on kinase-mediated signaling pathways (Supplementary Fig. 5d). A complete description of all results from the String database query can be found in the supplement (Supplementary Table 9–11). Amongst those pathways governed by IGFBP-2 in contrast to IGFBP-1, the kinome profiling predicts a unique activation of the ErbB signaling especially EGFR (also termed ErbB1) as well as chemokine signaling and general cancer pathways (Fig. 5d). This led us to the further investigation of such signaling events in the respective conditions where we could indeed confirm the phosphorylation of EGFR as well as of Stat-3 via IGFBP-2 (but not IGFBP-1) with subsequent downstream target gene expression namely Cyclin-D3 and c-Myc (Fig. 5e, si-NC). These effects were only partially reversible in the absence of the IGF1 receptor (Fig. 5e, si-IGF1R) again supporting the hypothesis of yet unknown IGF1R independent effects of IGFBP-2 clearly distinguishable from the role of IGFBP-1. The main findings and conclusions of the presented study concerning the diverse functions of IGFBP-1 and IGFBP-2 on IGF-induced signaling in hPASMCs in the context of pulmonary arterial hypertension are summarized in Fig. 5f

4. Discussion

In this study, for the very first time, the role of IGFBPs in the cellular context of vascular cells, their function in cell signaling and the effects on pulmonary vascular tone with respect to the disease of pulmonary hypertension, was explored. It was shown that circulating levels of IGFBP-1, IGFBP-2 and IGFBP-3 are increased in IPAH patients and these IGF-binding proteins exhibit diverging effects on the IGF-1 induced signaling pathway activation in human pulmonary arterial cells. Furthermore, IGFBP-1 and IGFBP-2 were differentially expressed in hypoxic mouse lungs and, in contrast to IGFBP-1, IGFBP-2 shows vaso-constrictive properties in the murine pulmonary vasculature.

The circulating levels of IGFBPs in PAH have come into increasing focus in the recent years. Several investigations revealed significantly increased IGFBP-1 [7] and IGFBP-2 levels in PAH patients [7,37]. The IGFBP expression levels could be correlated to important determined clinical parameters in PAH, i.e. NT-proBNP and 6 MWD [6,7], as well as with disease severity [6,38,44]. As presented here, not only IGFBP-1 and IGFBP-2, but also IGFBP-3 plasma concentration was significantly increased in IPAH patients, compared to healthy controls. In our study, IGFBP-1 and IGFBP-2 plasma levels were positively correlated with BNP levels and negatively correlated with the 6 MWD. Interestingly we could also observe a correlation of IGFBP-2 with age and disease severity (NYHA functional class). However, a clear liming factor of this study was the small cohort size of 44 IPAH patients and 6 healthy controls.

IGFBPs are mainly produced by the liver but they are also expressed in a widespread manner, which led to the assumption, that the binding proteins also regulate IGF-1 activity locally acting on a particular cell type or tissue [45]. In many diseases, autocrine, paracrine as well as endocrine actions of IGFBPs are involved [18,19] and a link between the IGF-1 system and vascular pathologies was pointed out [11]. On the basis of upregulated systemic IGFBP levels the question arose as to the effects of IGFBP-1, IGFBP-2 and IGFBP-3 on the IGF-1 induced signaling

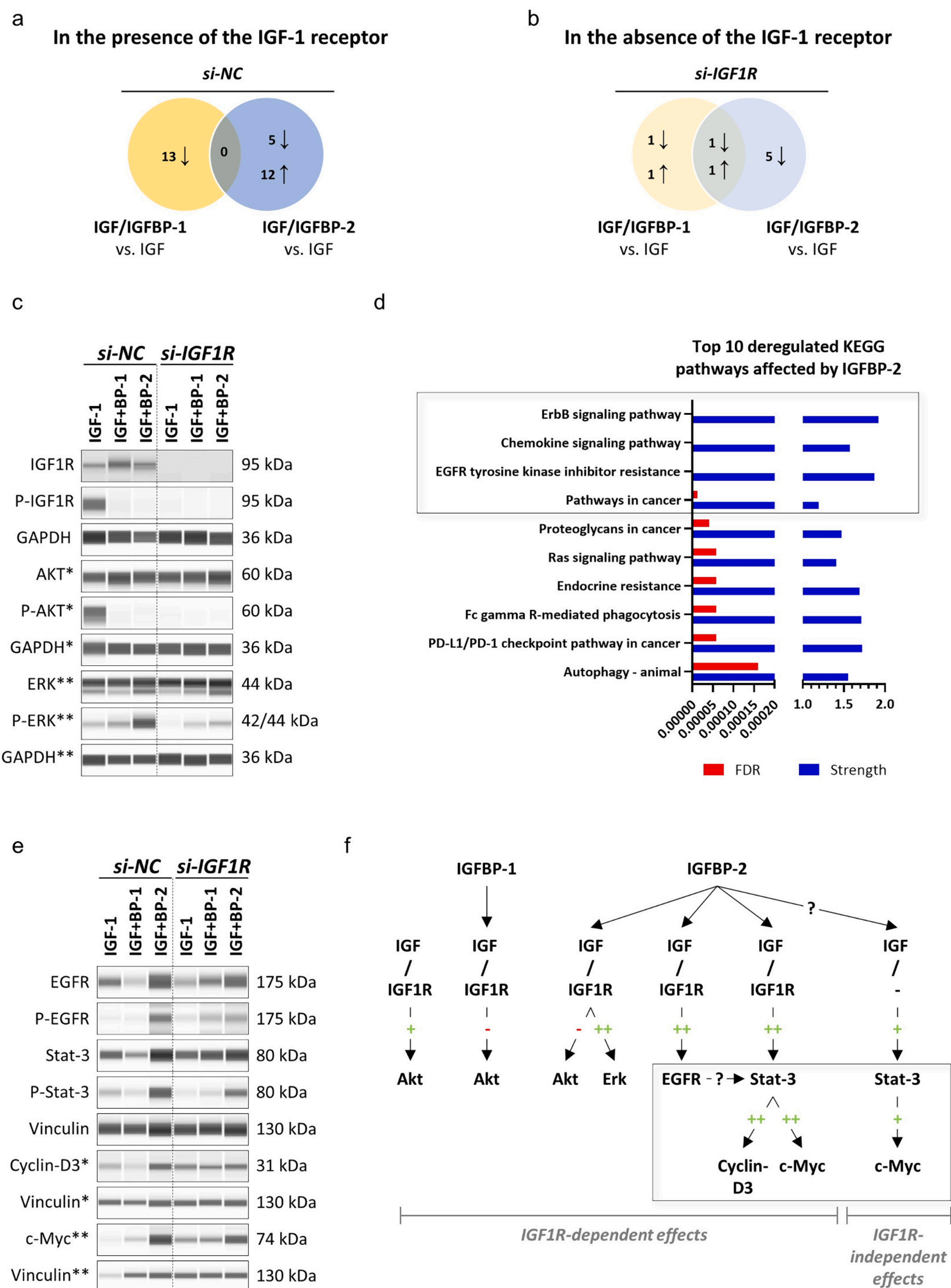

Fig. 5. IGF1R-dependent and IGF1R-independent effects of IGFBP-1 and IGFBP-2 on IGF-induced kinase driven signaling pathways in pulmonary vascular cells. For the peptide-based kinase activity assay, hPASMCs from 3 different donors (each measured in duplicates) were transfected with either 100 nM of a siRNA targeting IGF1R (si-IGF1R) or with a negative control siRNA (si-NC) prior the stimulation with IGF-1, or with a preincubated complex of IGF/IGFBP-1, or IGF/IGFBP-2, respectively. In the presence of IGF1R (si-NC), no overlap of the resulting kinases could be observed in the Venn diagram (a), while in the absence of IGF1R (si-IGF1R), two kinases are shared by both IGFBPs (b). Western-blot analyses with lysates used for the kinome profiling were conducted to verify the IGF1R knockdown as well as its effect on AKT and ERK activation (c). Top 10 pathways according to the KEGG classification which are deregulated upon IGF/IGFBP-2 exposure in the presence of IGF1R are described by two parameters, i.e. the FDR (false discovery rate, red) and the strength (blue). FDR: This measure describes how significant the enrichment is. Shown are *p*-values corrected for multiple testing within each category using the Benjamini–Hochberg procedure. Strength: Log_{10} (observed/expected). This measure describes how large the enrichment effect is. It's the ratio between i) the number of proteins in the network that are annotated with a term and ii) the number of proteins that are expected to be annotated with this term in a random network of the same size (d). Western-blot analyses demonstrating the activation of the Stat-3 signaling pathway including downstream target genes, i.e. c-Myc and Cyclin-D3, together with the EGFR activation in response to IGFBP-2 stimulation in si-NC as well as si-IGF1R conditions (e). Schematic overview about the findings of this study with respect to the diverging roles if IGFBP-1 and IGFBP-2 (f). Final outcomes due to IGFBPs are highlighted in green for the case of an activation of the respective pathway or in red for an inhibitory mode of action. The question mark refers to the yet unknown mechanistic details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pathway in pulmonary arterial cells, more precisely in healthy and IPAH-hPASMCs and in healthy hPAECs. In the in vitro studies we had a focus on IGFBP-1, IGFBP-2 and IGFBP-3 because these three proteins emerged in our blood plasma analyses, they differ in their binding domains and IGFBP-3 is the most abundant in human circulation and builds, in contrast to IGFBP-1 and IGFBP-2, a complex with IGF-1 and the acid-labile subunit (ALS, reviewed in e.g. [23]). Protein analyses revealed a clear inhibitory effect of all three binding proteins on the IGF-1 induced IGF1R phosphorylation, independent of the analyzed pulmonary arterial cell type. Previously, it was shown that several IGFBPs inhibit IGF induced signaling pathway activation by binding to IGF and preventing the binding of IGF to the IGF-1 receptor [22,46]. By having a look at the subsequent signaling pathway activation, different effects on PASMCs and PAECs could be revealed, as well as a diverging mode of action amongst the three IGFBPs on ERK activation. Multiple mechanisms, i.e. IGFBP phosphorylation, proteolysis and binding to other cell surface receptors, alter the affinity of IGFBPs to IGF-1 and thereby modulate IGF-1 induced signaling in a tissue or cell type specific manner [22]. We observed a lack of a reproducible, homogenous activation (or inhibition, respectively) of the downstream signaling proteins (as for P-ERK and P-AKT) for the different cells which were isolated from three individual human donors. This may explain the varying efficacy of the IGFBP effects on the level of AKT and ERK phosphorylation by examining the three unique donor or PAH patient derived pulmonary vascular cells, each. Furthermore the IGF/IGFBP-system is, amongst other influences, highly dependent on the nutritional status [47,48]. This aspect could not be taken into account in our investigations. An additional factor that needs to be considered, is a detail in the experimental cell culture setting which deals with the application of the IGFBPs and the stimulation with IGF-1. It has been shown that coincubation of IGF and IGFBP-3 (i.e. a simultaneous administration of both molecules) leads to inhibitory growth effects of the binding protein, whereas a pre-incubation of both prior to the exposure of the cells with this IGF/IGFBP-2 complex led to increased proliferation [49].

It is known that alterations in the IGF-system are associated with many different malignancies [50]. But apart from this, IGF-1 exerts multiple physiologic effects on the vasculature, including proliferative, hypertrophic, survival, vasomotor, and metabolic effects, too. For instance, IGF-1 depletion in hypoxic neonatal PH models may lead to vascular remodeling and could lead to reduced right ventricular hypertrophy [13]. Additionally, hypoxia leads to increased IGF1R expression in lungs and the right ventricle in mice, as well as to an increase in IGF1R expression in lungs of PH patients [14]. On mRNA expression levels, different expression patterns of the three IGFBPs using different cell types could be shown in relation to the oxygen concentration, independent of the stimuli (i.e. basal medium condition). In particular, in IPAH-hPASMCs IGFBP-2 and IGFBP-3 mRNA expression is significantly upregulated under hypoxic conditions, whereas IGFBP-1 mRNA expression is significantly repressed. This indicates, at least in part, a contribution to the disease phenotype. The IGFBP-2 expression is induced by the transcription factor hypoxia-inducible factor 1 α (Hif1α) [30,51]. In line with this, also in the case of IGFBP-3, hypoxia exposure results in its increased expression in hPASMCs [52]. This all underlines the importance of this investigated cell type, as also in our study, we could show that hypoxia led to an increase in IGFBP-1 mRNA expression, whereas IPAH-hPASMCs show a decrease in IGFBP-1 mRNA expression. And more important hypoxia led to a significant decrease in IPAH-hPASMC IGFBP-1 mRNA expression and to a tremendous increase in IGFBP-2 mRNA expression, independent of the stimulus. Whereas this phenomenon could not be detected in healthy hPASMCs. This again gives a hint, that IGFBPs are important factors in PAH pathophysiology and disease. It was demonstrated that the fetal adaptive response to hypoxia is a trigger of IGFBP-1 hyper-phosphorylation [53] which leads to a 6-fold reduced affinity for IGF-1 [54].

As hypoxia has a strong impact on the IGFBP-system, we analyzed the IGFBP expression in lungs from mice which developed pulmonary hypertension in a time course of three weeks due to hypoxia exposure. Likewise to our observation carried out by cell culture experiments using both primary pulmonary vascular cell types, we again confirmed the diverging roles of IGFBPs: IGFBP-1 mRNA expression is significantly downregulated in hypoxic mouse lungs, whereas IGFBP-2 mRNA expression is significantly increased. Intriguingly, the up-regulation of IGFBP-2 in lung tissue in human PH was demonstrated by other scientists, further supporting a crucial role of IGFBP-2 in this disease [37]. By investigating the impact of IGFBP-1 and IGFBP-2 on the pulmonary arterial pressure in the ex vivo perfused and ventilated mouse lung, we could clearly show that IGFBP-2 exerts a strong vaso-constrictive phenotype while IGFBP-1 seems to be ineffective with respect to the pulmonary vascular tone. Thereby, we could not confirm the postulated vaso-dilatative properties of IGFBP-1 [55] but this might be due to a different experimental design (aortic rings in contrast to mouse lungs, i. e. the whole pulmonary system) or the buffer containing residual amounts of acetonitrile and PFA which was used to dissolve the recombinant IGFBP-1 protein. To gain further insights into the IGF/IGFBP-system it would be of interest what consequences the inhibition of IGFBPs by an IGFBP inhibitor would have relating to the PH disease mouse model and the vascular tone.

IGFBPs exert multiple functions which are altogether dependent on the respective tissue as well as on the distinct cell type. This implies that the final mode of action of a particular IGFBP is dependent on the presence of its protein interaction partners. The latter ones not only consist of the ligand, i.e. IGF-1, but also of cell surface as well as intracellular molecules. Thereby the differences between IGFBP-1 and IGFBP-2 (with regard to their expression and their consequences on cell signaling) could result from their unique protein domain structure with distinct functional motifs. IGFBP-1 is composed of an integrin binding RGD sequence [8], whereas IGFBP-2 contains, in addition to this, also a Heparin binding domain. Therefore, IGFBP-2 is much more equipped to bind to the cell surface or extracellular matrix components. Furthermore, it carries a nuclear localization sequence (NLS) and even shows transactivational activity [8]. IGFBP-2 can for example co-localize with epidermal growth factor receptor (EGFR), translocate to the nucleus via it's NLS and increase nuclear EGFR activation and thereby the activation of EGFR/Stat-3 signaling pathway [56–58].

This led us to the assumption, that IGFBPs, in particular IGFBP-2, have various IGF-1 as well as IGF1R-dependent but also independent effects in general. To reveal the opposing effects of IGFBP-1 and IGFBP-2 in the context of pulmonary hypertension, we focused on PASMCs as those cells represent a major contributor to PH pathobiology. Furthermore, our results showed low or no significant changes in the IGF/IGFBP-system in hPAECs. Therefore, we conclude that hPAECs do not have as great a contribution as hPASMCs and hence we performed the peptide-based kinase activity assay with healthy hPASMCs. To delineate the cellular consequences in the absence of the IGF/IGF1R-system, we included an experimental condition where the IGF-1 receptor was knocked down via siRNA. We identified numerous candidates that are differentially activated (or inhibited) by IGFBP-1 or IGFBP-2 in the presence of IGF1R demonstrating the global effects of those to IGFBPs on IGF-1 stimulated PASMCs. Interestingly, no overlapping signatures in terms of shared signaling pathways that are regulated by both IGFBPs in the presence of IGF-1 could be detected. In contrast to this, upon loss of IGF1R, less downstream effector kinases are affected by the treatment with both IGFBPs in general, highlighting again, that the majority of the cellular mechanisms are mediated by the IGF/IGF1R-system. But interestingly, in the absence of IGF1R, the activity of two kinases was affected by IGFBP-1 as well as by IGFBP-2, which points to the hypothesis that the mode of action of both IGFBPs can quickly adapt to the physiological conditions as by the presence or the absence of a single protein binding structure like the IGF-1 receptor. In line with previous publications (comprehensively reviewed by Baxter [57]) dealing with the wider sphere of influence of IGFBP-2, we observed a distinct set of kinases which are deregulated in their activity by IGFBP-2 compared to IGFBP-1

(with IGF-1 treatment alone considered as a baseline, each). We could show that in pulmonary arterial smooth muscle cells IGFBP-2 stabilizes and activates EGFR as well as Stat-3 signaling leading to the expression of well-known downstream target proteins c-Myc and Cyclin-D3. Still, the exact mechanistic details and the implications for the disease of pulmonary arterial hypertension remain to be elusive. But in glioma cells (amongst other cancer entities) the same finding was presented as to the phosphorylation of Stat-3, in parallel with an increase in total and P-EGFR. The authors also noted an induction of two different Stat-3 transcriptional targets namely the survival protein Bcl-xL and the cell cycle stimulator Cyclin D1 [58]. Similar to our study, these authors admit that it needs to be clarified how IGFBP-2 promotes the nuclear translocation of EGFR. In another publication, similar findings in melanoma were demonstrated including the induction of the immune checkpoint regulator PD-L1 [59] which we could not confirm in our study so far (data not shown). With respect to the cancer-like phenotype of several pulmonary vascular cell-types in PAH, these similarities in the aberrant pathological signaling pathways support the importance of our results. The role and the potential of therapeutic exploitation of the EGFR (exemplarily [60]) as well as of the Stat-3 (exemplarily [61]) signaling pathway in PAH is well described but never in the context of IGFBPs where we now provide a novel piece of knowledge.

Finally, we can provide the novel finding on the existence of diverging pathways that are governed by IGFBP-1 and IGFBP-2 in the presence as well as in the absence of the IGF-1 receptor. We would like to emphasize that especially in the context of pulmonary hypertension, this has never been investigated so far further underscoring the relevance of our data. Further studies are underway to fully decipher the mechanisms of how exactly IGFBPs regulate the pathobiological processes in pulmonary hypertension via interaction with other cell surface, i.e. EGFR, and/or intracellular molecules like Stat-3 also on the level of different cell types within the pulmonary vasculature.

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CRediT authorship contribution statement

Beate Christiane Schlueter: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Validation, Visualization. **Karin Quanz:** Investigation, Methodology. **Julia Baldauf:** Formal analysis, Investigation, Methodology. **Aleksandar Petrovic:** Methodology, Resources. **Clemens Ruppert:** Methodology, Resources. **Andreas Guenther:** Methodology, Resources. **Henning Gall:** Methodology, Resources. **Khodr Tello:** Methodology, Resources. **Friedrich Grimminger:** Funding acquisition, Project administration. **Hossein-Ardeschir Ghofrani:** Funding acquisition, Project administration. **Norbert Weissmann:** Conceptualization, Methodology, Resources. **Werner Seeger:** Funding acquisition, Supervision. **Ralph Theo Schermuly:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Astrid Weiss:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The following authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Werner Seeger received consulting fees from United Therapeutics, Tiakis Biotech AG, Liquidia, Pieris Pharmaceuticalsm Abivax, Pfitzer, Medspray BV.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2024.107379.

References

[1] T. Thenappan, M.L. Ormiston, J.J. Ryan, S.L. Archer, Pulmonary arterial hypertension: pathogenesis and clinical management, BMJ 360 (2018) j5492, https://doi.org/10.1136/bmj.j5492.

[2] K.Y. Chang, S. Duval, D.B. Badesch, T.M. Bull, M.M. Chakinala, T. de Marco, R. P. Frantz, A. Hemnes, S.C. Mathai, E.B. Rosenzweig, J.J. Ryan, T. Thenappan, Mortality in pulmonary arterial hypertension in the modern era: early insights from the Pulmonary Hypertension Association registry, J. Am. Heart Assoc. 11 (2022) e024969, https://doi.org/10.1161/JAHA.121.024969.

[3] M. Humbert, O. Sitbon, G. Simonneau, Treatment of pulmonary arterial hypertension, N. Engl. J. Med. 351 (2004) 1425–1436, https://doi.org/10.1056/NEJMra040291.

[4] S.S. Pullamsetti, R. Savai, W. Seeger, E.A. Goncharova, Translational advances in the field of pulmonary hypertension. from cancer biology to new pulmonary arterial hypertension therapeutics. targeting cell growth and proliferation signaling hubs, Am. J. Respir. Crit. Care Med. 195 (2017) 425–437, https://doi.org/10.1164/rccm.201606-1226PP.

[5] R.T. Schermuly, H.A. Ghofrani, M.R. Wilkins, F. Grimminger, Mechanisms of disease: pulmonary arterial hypertension, Nat. Rev. Cardiol. 8 (2011) 443–455, https://doi.org/10.1038/nrcardio.2011.87.

[6] M.K. Nies, J. Yang, M. Griffiths, R. Damico, J. Zhu, D. Vaydia, Z. Fu, S. Brandal, E. D. Austin, D.D. Ivy, P.M. Hassoun, J.E. van Eyk, A.D. Everett, Proteomics discovery of pulmonary hypertension biomarkers: insulin-like growth factor binding proteins are associated with disease severity, Pulm. Circ. 12 (2022) e12039, https://doi.org/10.1002/pul2.12039.

[7] H. Bouzina, R. Hesselstrand, G. Rådegran, Plasma insulin-like growth factor binding protein 1 in pulmonary arterial hypertension, Scand. Cardiovasc. J. 55 (2021) 35–42, https://doi.org/10.1080/14017431.2020.1782977.

[8] J.B. Allard, C. Duan, IGF-binding proteins: why do they exist and why are there so many? Front. Endocrinol. (Lausanne) 9 (2018) 117, https://doi.org/10.3389/fendo.2018.00117.

[9] W.H. Daughaday, P. Rotwein, Insulin-like growth factors I and II peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations, Endocr. rev. 10 (1989) 68–91, https://doi.org/10.1210/edrv-10-1-68.

[10] V.K. Han, A.J. D'Ercole, P.K. Lund, Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus, Science 236 (1987) 193–197, https://doi.org/10.1126/science.3563497.

[11] L.J. Murphy, G.I. Bell, H.G. Friesen, Tissue distribution of insulin-like growth factor I and II messenger ribonucleic acid in the adult rat, Endocrinology 120 (1987) 1279–1282, https://doi.org/10.1210/endo-120-4-1279.

[12] P. Delafontaine, Y.-H. Song, Y. Li, Expression, regulation, and function of IGF-1, IGF-1R, and IGF-1 binding proteins in blood vessels, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 435–444, https://doi.org/10.1161/01.ATV.0000105902.89459.09.

[13] M. Connolly, B.E. Garfield, A. Crosby, N.W. Morrell, S.J. Wort, P.R. Kemp, miR-322-5p targets IGF-1 and is suppressed in the heart of rats with pulmonary hypertension, FEBS Open Bio 8 (2018) 339–348, https://doi.org/10.1002/2211-5463.12369.

[14] M. Sun, R. Ramchandran, J. Chen, Q. Yang, J.U. Raj, Smooth muscle insulin-like growth factor-1 mediates hypoxia-induced pulmonary hypertension in neonatal mice, Am. J. Respir. Cell Mol. Biol. 55 (2016) 779–791, https://doi.org/10.1165/rcmb.2015-0388OC.

[15] L. Shi, B. Kojonazarov, A. Elgheznawy, R. Popp, B.K. Dahal, M. Böhm, S. S. Pullamsetti, H.-A. Ghofrani, A. Gödecke, A. Jungmann, H.A. Katus, O.J. Müller, R.T. Schermuly, B. Fisslthaler, W. Seeger, I. Fleming, miR-223-IGF-IR signalling in hypoxia- and load-induced right-ventricular failure: a novel therapeutic approach, Cardiovasc. Res. 111 (2016) 184–193, https://doi.org/10.1093/cvr/cvw065.

[16] D. LeRoith, H. Werner, D. Beitner-Johnson, C.T. Roberts, Molecular and cellular aspects of the insulin-like growth factor I receptor, Endocr. Rev. 16 (1995) 143–163, https://doi.org/10.1210/edrv-16-2-143.

[17] A.R. Saltiel, C.R. Kahn, Insulin signalling and the regulation of glucose and lipid metabolism, Nature 414 (2001) 799–806, https://doi.org/10.1038/414799a.

[18] T.E. Adams, V.C. Epa, T.P. Garrett, C.W. Ward, Structure and function of the type 1 insulin-like growth factor receptor, Cell. Mol. Life Sci. 57 (2000) 1050–1093, https://doi.org/10.1007/PL00000744.

[19] C.E. Stewart, P. Rotwein, Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors, Physiol. Rev. 76 (1996) 1005–1026, https://doi.org/10.1152/physrev.1996.76.4.1005.

[20] D.R. Clemmons, Modifying IGF1 activity: an approach to treat endocrine disorders, atherosclerosis and cancer, Nat. Rev. Drug Discov. 6 (2007) 821–833, https://doi.org/10.1038/nrd2359.

[21] C. Livingstone, IGF2 and cancer, Endocr. Relat. Cancer 20 (2013) R321–R339, https://doi.org/10.1530/ERC-13-0231.

[22] J.I. Jones, D.R. Clemmons, Insulin-like growth factors and their binding proteins: biological actions, Endocr. Rev. 16 (1995) 3–34, https://doi.org/10.1210/edrv-16-1-3.

[23] S.M. Firth, R.C. Baxter, Cellular actions of the insulin-like growth factor binding proteins, Endocr. Rev. 23 (2002) 824–854, https://doi.org/10.1210/er.2001-0033.

[24] S. Rajaram, D.J. Baylink, S. Mohan, Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions, Endocr. Rev. 18 (1997) 801–831, https://doi.org/10.1210/edrv.18.6.0321.

[25] R.C. Baxter, J.L. Martin, M.I. Tyler, M.E. Howden, Growth hormone-dependent insulin-like growth factor (IGF) binding protein from human plasma differs from other human IGF binding proteins, Biochem. Biophys. Res. Commun. 139 (1986) 1256–1261, https://doi.org/10.1016/s0006-291x(86)80313-8.

[26] R.C. Baxter, Insulin-like growth factor (IGF) binding proteins: the role of serum IGFBPs in regulating IGF availability, Acta Paediatr. Scand.Suppl. 372 (1991) 107–114, discussion 115, https://doi.org/10.1111/j.1651-2227.1991.tb17983.x.

[27] R.C. Baxter, IGF binding proteins in cancer: mechanistic and clinical insights, Nat. Rev. Cancer 14 (2014) 329–341, https://doi.org/10.1038/nrc3720.

[28] A. Hoeflich, V.C. Russo, Physiology and pathophysiology of IGFBP-1 and IGFBP-2 - consensus and dissent on metabolic control and malignant potential, Best Pract. Res. Clin. Endocrinol. Metab. 29 (2015) 685–700, https://doi.org/10.1016/j.beem.2015.07.002.

[29] V.C. Russo, W.J. Azar, S.W. Yau, M.A. Sabin, G.A. Werther, IGFBP-2: the dark horse in metabolism and cancer, Cytokine Growth Factor Rev. 26 (2015) 329–346, https://doi.org/10.1016/j.cytogfr.2014.12.001.

[30] Y.-W. Lin, X.-F. Weng, B.-L. Huang, H.-P. Guo, Y.-W. Xu, Y.-H. Peng, IGFBP-1 in cancer: expression, molecular mechanisms, and potential clinical implications, Am. J. Transl. Res. 13 (2021) 813–832.

[31] G. Fürstenberger, H.-J. Senn, Insulin-like growth factors and cancer, Lancet Oncol. 3 (2002) 298–302, https://doi.org/10.1016/s1470-2045(02)00731-3.

[32] S.W. Yau, W.J. Azar, M.A. Sabin, G.A. Werther, V.C. Russo, IGFBP-2 - taking the lead in growth, metabolism and cancer, J. Cell Commun. Signal. 9 (2015) 125–142, https://doi.org/10.1007/s12079-015-0261-2.

[33] S.S. Pullamsetti, S. Nayakanti, P. Chelladurai, A. Mamazhakypov, S. Mansouri, R. Savai, W. Seeger, Cancer and pulmonary hypertension: learning lessons and real-life interplay, Glob. Cardiol. Sci. Pract. 2020 (2020) e202010, https://doi.org/10.21542/gcsp.2020.10.

[34] P.R. Rai, C.D. Cool, J.A.C. King, T. Stevens, N. Burns, R.A. Winn, M. Kasper, N. F. Voelkel, The cancer paradigm of severe pulmonary arterial hypertension, Am. J. Respir. Crit. Care Med. 178 (2008) 558–564, https://doi.org/10.1164/rccm.200709-1369PP.

[35] O. Boucherat, G. Vitry, I. Trinh, R. Paulin, S. Provencher, S. Bonnet, The cancer theory of pulmonary arterial hypertension, Pulm. Circ. 7 (2017) 285–299, https://doi.org/10.1177/2045893217701438.

[36] C. Guignabert, L. Tu, M. Le Hiress, N. Ricard, C. Sattler, A. Seferian, A. Huertas, M. Humbert, D. Montani, Pathogenesis of pulmonary arterial hypertension: lessons from cancer, Eur. Respir. Rev. 22 (2013) 543–551, https://doi.org/10.1183/09059180.00007513.

[37] J. Yang, M. Griffiths, M.K. Nies, S. Brandal, R. Damico, D. Vaidya, X. Tao, C. E. Simpson, T.M. Kolb, S.C. Mathai, M.W. Pauciulo, W.C. Nichols, D.D. Ivy, E. D. Austin, P.M. Hassoun, A.D. Everett, Insulin-like growth factor binding protein-2: a new circulating indicator of pulmonary arterial hypertension severity and survival, BMC Med. 18 (2020) 268, https://doi.org/10.1186/s12916-020-01734-3.

[38] C.J. Rhodes, J. Wharton, P. Ghataorhe, G. Watson, B. Girerd, L.S. Howard, J.S. R. Gibbs, R. Condliffe, C.A. Elliot, D.G. Kiely, G. Simonneau, D. Montani, O. Sitbon, H. Gall, R.T. Schermuly, H.A. Ghofrani, A. Lawrie, M. Humbert, M.R. Wilkins, Plasma proteome analysis in patients with pulmonary arterial hypertension: an observational cohort study, Lancet Respir. Med. 5 (2017) 717–726, https://doi.org/10.1016/S2213-2600(17)30161-3.

[39] N. Weissmann, E. Akkayagil, K. Quanz, R.T. Schermuly, H.A. Ghofrani, L. Fink, J. Hänze, F. Rose, W. Seeger, F. Grimminger, Basic features of hypoxic pulmonary vasoconstriction in mice, Respir. Physiol. Neurobiol. 139 (2004) 191–202, https://doi.org/10.1016/j.resp.2003.10.003.

[40] A. Weiss, M.C. Neubauer, D. Yerabolu, B. Kojonazarov, B.C. Schlueter, L. Neubert, D. Jonigk, N. Baal, C. Ruppert, P. Dorfmuller, S.S. Pullamsetti, N. Weissmann, H.-A. Ghofrani, F. Grimminger, W. Seeger, R.T. Schermuly, Targeting cyclin-dependent kinases for the treatment of pulmonary arterial hypertension, Nat. Commun. 10 (2019) 2204, https://doi.org/10.1038/s41467-019-10135-x.

[41] G. Warwick, P.S. Thomas, D.H. Yates, Biomarkers in pulmonary hypertension, Eur. Respir. J. 32 (2008) 503–512, https://doi.org/10.1183/09031936.00160307.

[42] X.J. Liu, Q. Xie, Y.F. Zhu, C. Chen, N. Ling, Identification of a nonpeptide ligand that releases bioactive insulin-like growth factor-I from its binding protein complex, J. Biol. Chem. 276 (2001) 32419–32422, https://doi.org/10.1074/jbc.C100299200.

[43] M. Humbert, G. Kovacs, M.M. Hoeper, R. Badagliacca, R.M.F. Berger, M. Brida, J. Carlsen, A.J.S. Coats, P. Escribano-Subias, P. Ferrari, D.S. Ferreira, H. A. Ghofrani, G. Giannakoulas, D.G. Kiely, E. Mayer, G. Meszaros, B. Nagavci, K. M. Olsson, J. Pepke-Zaba, J.K. Quint, G. Rådegran, G. Simonneau, O. Sitbon, T. Tonia, M. Toshner, J.-L. Vachiery, A. Vonk Noordegraaf, M. Delcroix, S. Rosenkranz, 2022 ESC/ERS guidelines for the diagnosis and treatment of pulmonary hypertension, Eur. Respir. J. 61 (2023), https://doi.org/10.1183/13993003.00879-2022.

[44] M. Griffiths, J. Yang, M. Nies, D. Vaidya, S. Brandal, M. Williams, E.C. Matsui, T. Grant, R. Damico, D. Ivy, E.D. Austin, W.C. Nichols, M.W. Pauciulo, K. Lutz, E. B. Rosenzweig, R. Hirsch, D. Yung, A.D. Everett, Pediatric pulmonary hypertension: insulin-like growth factor-binding protein 2 is a novel marker associated with disease severity and survival, Pediatr. Res. 88 (2020) 850–856, https://doi.org/10.1038/s41390-020-01113-x.

[45] L.A. Bach, What happened to the IGF binding proteins? Endocrinology 159 (2018) 570–578, https://doi.org/10.1210/en.2017-00908.

[46] M.M. Rechler, Insulin-like growth factor binding proteins, Vitam. Horm. 47 (1993) 1–114, https://doi.org/10.1016/s0083-6729(08)60444-6.

[47] J.P. Thissen, J.M. Ketelslegers, L.E. Underwood, Nutritional regulation of the insulin-like growth factors, Endocr. Rev. 15 (1994) 80–101, https://doi.org/10.1210/edrv-15-1-80.

[48] M. Dencker, Y. Gårdinger, O. Björgell, J. Hlebowicz, Effect of food intake on 92 biomarkers for cardiovascular disease, PloS One 12 (2017) e0178656, https://doi.org/10.1371/journal.pone.0178656.

[49] J.S. de Mellow, R.C. Baxter, Growth hormone-dependent insulin-like growth factor (IGF) binding protein both inhibits and potentiates IGF-I-stimulated DNA synthesis in human skin fibroblasts, Biochem. Biophys. Res. Commun. 156 (1988) 199–204, https://doi.org/10.1016/s0006-291x(88)80824-6.

[50] A. Kasprzak, W. Kwasniewski, A. Adamek, A. Gozdzicka-Jozefiak, Insulin-like growth factor (IGF) axis in cancerogenesis, Mutat. Res. Rev. Mutat. Res. 772 (2017) 78–104, https://doi.org/10.1016/j.mrrev.2016.08.007.

[51] D. Feldser, F. Agani, N.V. Iyer, B. Pak, G. Ferreira, G.L. Semenza, Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2, Cancer Res. 59 (1999) 3915–3918.

[52] S. Ismail, A. Sturrock, P. Wu, B. Cahill, K. Norman, T. Huecksteadt, K. Sanders, T. Kennedy, J. Hoidal, NOX4 mediates hypoxia-induced proliferation of human pulmonary artery smooth muscle cells: the role of autocrine production of transforming growth factor-{beta}1 and insulin-like growth factor binding protein-3, Am. J. Physiol. Lung Cell. Mol. Physiol. 296 (2009) L489–L499, https://doi.org/10.1152/ajplung.90488.2008.

[53] M.D. Seferovic, R. Ali, H. Kamei, S. Liu, J.M. Khosravi, S. Nazarian, V.K.M. Han, C. Duan, M.B. Gupta, Hypoxia and leucine deprivation induce human insulin-like growth factor binding protein-1 hyperphosphorylation and increase its biological activity, Endocrinology 150 (2009) 220–231, https://doi.org/10.1210/en.2008-0657.

[54] J.I. Jones, W.H. Busby, G. Wright, C.E. Smith, N.M. Kimack, D.R. Clemmons, Identification of the sites of phosphorylation in insulin-like growth factor binding protein-1. Regulation of its affinity by phosphorylation of serine 101, J. Biol. Chem. 268 (1993) 1125–1131.

[55] A. Rajwani, V. Ezzat, J. Smith, N.Y. Yuldasheva, E.R. Duncan, M. Gage, R. M. Cubbon, M.B. Kahn, H. Imrie, A. Abbas, H. Viswambharan, A. Aziz, P. Sukumar, A. Vidal-Puig, J.K. Sethi, S. Xuan, A.M. Shah, P.J. Grant, K.E. Porter, M.T. Kearney, S.B. Wheatcroft, Increasing circulating IGFBP1 levels improves insulin sensitivity, promotes nitric oxide production, lowers blood pressure, and protects against atherosclerosis, Diabetes 61 (2012) 915–924, https://doi.org/10.2337/db11-0963.

[56] H. Huynh, J. Zheng, M. Umikawa, C. Zhang, R. Silvany, S. Iizuka, M. Holzenberger, W. Zhang, C.C. Zhang, IGF binding protein 2 supports the survival and cycling of hematopoietic stem cells, Blood 118 (2011) 3236–3243, https://doi.org/10.1182/blood-2011-01-331876.

[57] R.C. Baxter, Signaling pathways of the insulin-like growth factor binding proteins, Endocr. Rev. 44 (2023) 753–778, https://doi.org/10.1210/endrev/bnad008.

[58] C.Y. Chua, Y. Liu, K.J. Granberg, L. Hu, H. Haapasalo, M.J. Annala, D.E. Cogdell, M. Verploegen, L.M. Moore, G.N. Fuller, M. Nykter, W.K. Cavenee, W. Zhang, IGFBP2 potentiates nuclear EGFR-STAT3 signaling, Oncogene 35 (2016) 738–747, https://doi.org/10.1038/onc.2015.131.

[59] T. Li, C. Zhang, G. Zhao, X. Zhang, M. Hao, S. Hassan, M. Zhang, H. Zheng, L. Liu Da Yang, F. Mehraein-Ghomi, X. Bai, K. Chen, W. Zhang, J. Yang, IGFBP2 regulates PD-L1 expression by activating the EGFR-STAT3 signaling pathway in malignant melanoma, Cancer Lett. 477 (2020) 19–30, https://doi.org/10.1016/j.canlet.2020.02.036.

[60] B.K. Dahal, T. Cornitescu, A. Tretyn, S.S. Pullamsetti, D. Kosanovic, R. Dumitrascu, H.A. Ghofrani, N. Weissmann, R. Voswinckel, G.-A. Banat, W. Seeger, F. Grimminger, R.T. Schermuly, Role of epidermal growth factor inhibition in experimental pulmonary hypertension, Am. J. Respir. Crit. Care Med. 181 (2010) 158–167, https://doi.org/10.1164/rccm.200811-1682OC.

[61] D. Yerabolu, A. Weiss, B. Kojonazarov, M. Boehm, B.C. Schlueter, C. Ruppert, A. Günther, D. Jonigk, F. Grimminger, H.-A. Ghofrani, W. Seeger, N. Weissmann, R. T. Schermuly, Targeting jak-stat signaling in experimental pulmonary hypertension, Am. J. Respir. Cell Mol. Biol. 64 (2021) 100–114, https://doi.org/10.1165/rcmb.2019-0431OC.