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Redirecting TGF- β signaling pathways: advancing targeted therapy for PAH and FOP

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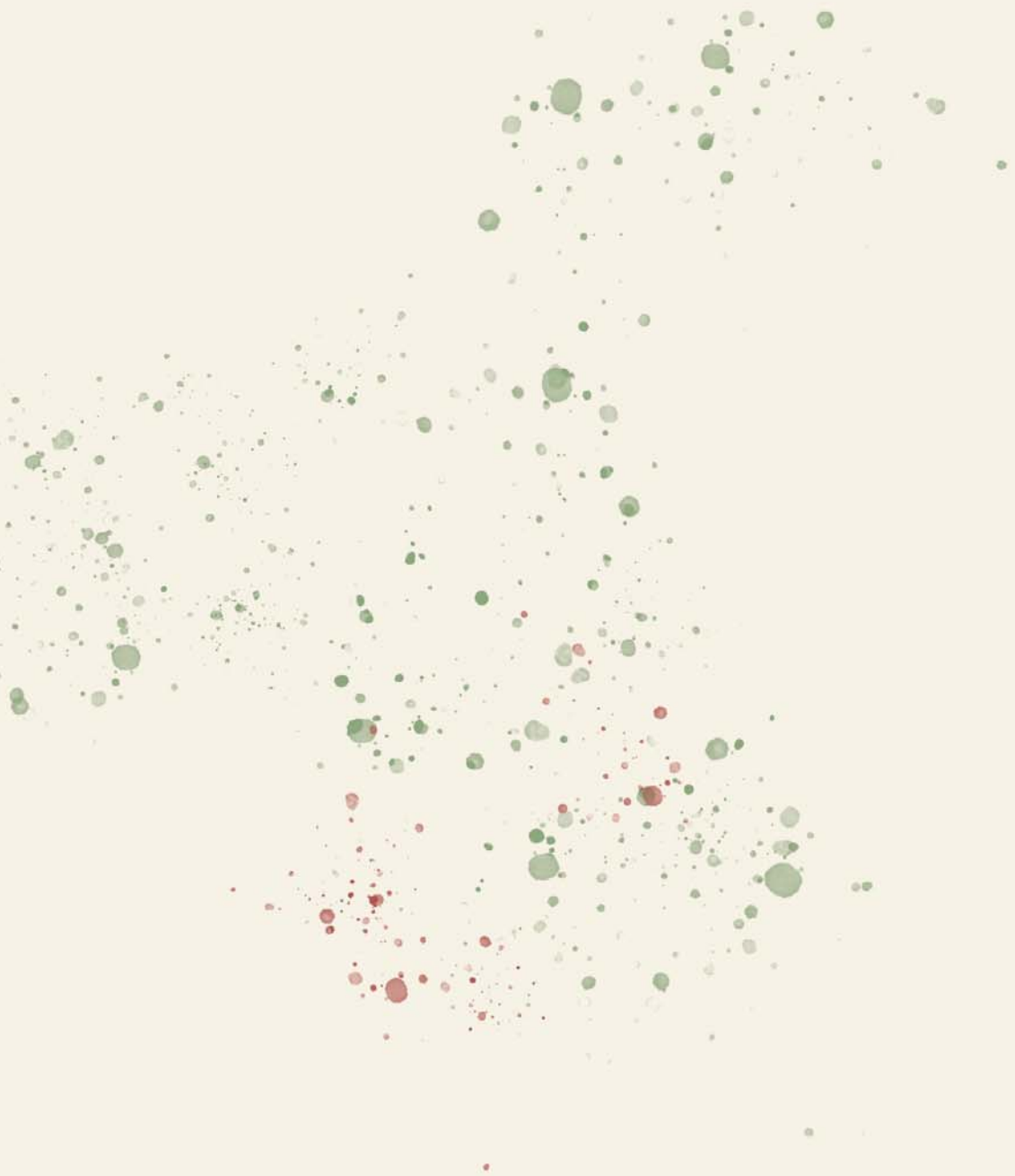
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Chapter 7



General discussion and future perspectives

Introduction

Currently, the severe TGF- β -associated genetic disorders pulmonary arterial hypertension (PAH) and fibrodysplasia ossificans progressiva (FOP) remain incurable. The work presented in this thesis aimed to advance future therapies for these cardiovascular (PAH) and musculoskeletal (FOP) diseases. As both conditions are characterized by disturbed TGF- β signaling, we explored various approaches to target TGF- β -induced SMAD and non-SMAD signaling pathways. As such, in PART I, we presented novel in vitro methods to screen and assess TGF- β receptor-selective drugs in a live cell setting. As proof-of-principle, a kinase inhibitor screen was performed. Simultaneously, in PART II, various molecular mechanisms underlying PAH and FOP were profiled and investigated to identify novel druggable targets. Notably, the newly developed methods in PART I were utilized to assess potential off-target effects of a drug in PART II, showing coherence throughout the thesis.¹ This general discussion contextualizes all the presented studies within the broader scientific landscape and outlines plausible directions for future research.

Improved methodology may advance TGF- β -specific drug discovery

This thesis aimed to improve methodologies for measuring TGF- β signaling modulators and to identify novel drugs for TGF- β -associated disorders. Conventional approaches, including in vitro kinase assays, western blotting, surface plasmon resonance (SPR), crosslinking-based protocols, and downstream reporter assays, provide valuable insights but face significant limitations. These include insufficient physiological complexity, the absence of live-cell kinetic readouts, the lack of target specificity or a lack of scalability for high-throughput applications. By employing a complex system with high target specificity, hits are pre-evaluated for their mechanism of action and cellular uptake capabilities, potentially leading to more efficient and safer drug candidates: advancing the drug development process. Accordingly, our objective was to develop novel, complex methods to overcome these methodological limitations and enable the measurement of TGF- β signaling modulators.

In recent years, other groups have also focused on developing methods to enhance the detection of TGF- β signaling. For example, Jatzlau and colleagues introduced a versatile technique using N-terminally HALO- and FLAG-tagged constructs to detect TGF- β ligand-receptor binding in cells through a microscopy-based pipeline.² While this method enables the measurement of receptor-specific complex formation, ligand binding at the cell surface and complex internalization, it does not capture downstream intracellular signaling cascades and has limited scalability, making it less suitable for drug screening applications.

In the studies presented in **chapter 2 and 3**, we utilized nano-Luciferase (NanoLuc) bioluminescence resonance energy transfer (nanoBRET) for an easy live cell molecular detection readout. Choosing bioluminescence over fluorescence (FRET) has advantages mainly in the context of live cells due to a reduced auto-fluorescence and subsequent lower background noise. This small sized (19.1 kDa) luminescent reporter (i.e. NanoLuc) is very stable and has highly increased luminescence output compared to traditional Renilla or Firefly

luciferases (>150 fold).³ By overexpression of TGF- β receptor-NanoLuc fusions we developed and optimized two different nanoBRET protocols: nanoBRET target engagement (TE) and nanoBRET protein-protein interaction (PPI). In terms of nanoBRET TE, a fluorescently tagged small molecule, binding the kinase ATP-pocket, called the 'tracer' was added to the system. By compound and tracer competition we showed functional kinase inhibition.⁴ Most importantly, this assay resembles a complex cellular environment, has a live cell kinetic readout, and can be performed in high-throughput while being able to measure selective kinase inhibitors to the targets of interests. However, after extensive testing, the nanoBRET TE assay was not able to measure ligand-induced kinase activation and therefore is unsuitable to screen for agonists, antibodies, ligand traps or allosteric molecules, at least in the context of TGF- β family receptors. Therefore, we further developed the nanoBRET PPI systems, based on HALO-tagged constructs and nanoBRET acceptor ligand binding, to measure ligand-inducible TGF- β receptor complex formation as surrogate for signaling activation. As described in **chapter 3**, these systems are still very experimental and unfortunately lack consistency. Other groups have successfully utilized this strategy for other non-TGF- β related targets to perform drug screens identifying protein complex modulators, for instance identifying RAS/RAF inhibitors.⁵ Overall, we show that these nanoBRET-based systems may outperform conventional methods by having a TGF- β receptor specific, live-cell kinetic readout in a complex cellular system while being performed in a HTP setting.

To further develop our nanoBRET protocols, we propose implementing various adjustments (Figure 1). For instance, it would be promising to create novel tracer molecules that might discriminate between active and inactive TGF- β receptors. As such, utilizing kinase inhibitors binding the active/open ATP pocket may form the basis of such activity responsive tracers. One potential approach involves leveraging the flexible Asp-Phe-Gly (DFG) motif within the ATP cleft of the kinase; the DFG-in conformation corresponds to an open ATP pocket, whereas the DFG-out state represents a closed conformation.⁶ Inhibitors specifically binding the DFG-in state may only recognize an active kinase (also known as type I inhibitors), and are therefore promising molecules for tracer development.^{7,8} Suppose these tracers can only bind to active TGF- β receptors, one might use the nanoBRET TE assay for drug screening libraries including agonists, antibodies and ligand traps. Further, combining tracers with separate excitation spectra allows the detection of multiple targets of interest in one cellular system (multiplexing).

Another approach involves optimizing the nanoBRET PPI systems, such as integrating the NanoBiT system. NanoBiT relies on two fragments of the NanoLuc enzyme (the Large/LgBiT is 18 kDa, and Small/smBiT only 1.3 kDa) that combine to form a functional reporter molecule when being in close proximity.⁹ Compared to conventional NanoLuc-HALO PPI, NanoBiT offers several advantages, including smaller tags, a readout limited to luciferase activity, and eliminating the need for additional nanoBRET ligands. Also, recent developments using other reporter systems may offer solutions for the PPI experiments, like implementing the proximity assisted photo-activation (PAPA) system.¹⁰ This technology allows the reporter to be activated (and remains active) following a brief interaction, minimizing false-negative results caused by rapid hit-and-run interactions, which may have been the reason for failures in detecting TGF- β receptor – SMAD interactions. If such a system is implemented for TGF- β receptor

interactions, the sensitivity of activation may be much stronger, leading to better detection of TGF- β modulators like agonists, antibodies and ligand traps. Further, developing more stable nanoBRET PPI assays using other TGF- β targets, such as the SMAD1/2-SMAD4 interaction, could significantly enhance the method sensitivity and robustness.

Additionally, future research incorporating these next-generation nanoBRET methods could focus on increasing the complexity of cellular model systems. For example, the NanoBRET/NanoBiT protocols can be adapted for multiplexing, including functional readouts such as cytotoxicity. Distinct excitation spectra or sequential fluorescent and luminescent readouts technically allow the combination of multiple assays. For instance, NanoBRET methods can be combined with the CellTox Green cytotoxicity assay, enabling measurement of target engagement and cytotoxicity within the same well to identify more safe hits. Further, developing complex culture systems could better mimic physiological conditions while measuring live-cell TGF- β activity, like creating a nanoBRET-on-chip platform. As such, it is conceivable to model 3D vessels for PAH or 3D muscle organoids/myotubes for FOP, utilizing cells compatible with the nanoBRET system through stable expression constructs. Correspondingly, current nanoBRET cell lines rely on overexpression systems, whereas endogenously tagged NanoBRET/NanoBiT cell lines may enhance their physiological relevance: especially when studying disease-relevant TGF- β signaling through mutant receptors.

Novel methods to detect TGF- β signaling, like the techniques presented in this thesis, are upcoming and promising to advance novel drug detection studies. The ideal high-throughput kinetic methods that resemble complex cellular environments are expected to yield more relevant hits and accelerate the drug development process, as they can account for critical factors such as cytotoxicity, target engagement, and drug uptake early on. Moreover, new systems may also drive fundamental discoveries about TGF- β signaling mechanics and could consequently push translational solutions forward.

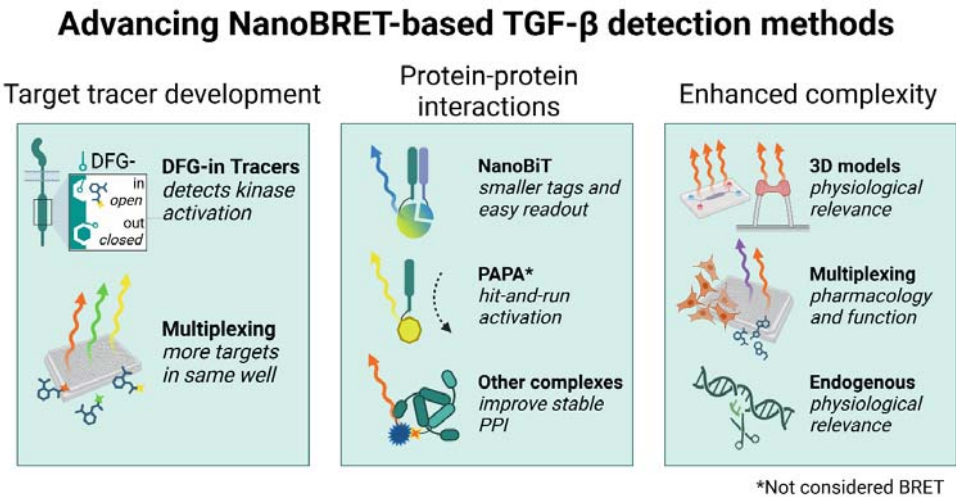


Figure 1. (continue on next page)

Figure 1. A schematic overview of the suggested advancements to further develop nanoBRET-based TGF- β detection methods. Novel tracers can be developed to measure kinase activity or multiplex for different drug targets simultaneously. The nanoBRET protein-protein interaction systems can be improved by implementing smaller tags, changing to hit-and-run activation technologies or developing PPI in more stable TGF- β related complexes, like SMAD-SMAD interactions. The complexity of the models can be enhanced for more physiologically relevant studies, including 3D culture models, functional multiplexing to pre-evaluate efficient and safe therapeutics, and apply endogenous tagging for more relevant TGF- β signaling detection.

Paving the way for enhanced therapeutics: targeting TGF- β signaling has significant momentum in the clinic

In line with the aim of this thesis, other groups also sought to find novel TGF- β therapeutics to treat PAH and FOP. In the past years, highly promising clinical results have been presented using therapeutics like Fc-fusion proteins, blocking antibodies and small molecule kinase inhibitors.

For instance, Sotatercept, an ACTR2A-Fc ligand trap, has shown promising clinical results and may become the first drug addressing the underlying cause of PAH pathobiology.¹¹ In combination with baseline therapy, this novel drug rebalances the disturbed TGF- β signaling pathway in PAH by sequestering multiple ligands including Activin A, Activin B, GDF8/11, BMP5, BMP6, BMP7 and BMP10 with different binding affinities.¹² Clinical improvements have been observed within weeks of administration, suggesting a vasodilatory effect.¹¹ Additionally, studies in PH animal models indicate that Sotatercept partially reverses pulmonary vascular remodeling.¹³ Unsurprisingly, side effects such as anemia and telangiectasias have been described in the clinic. The latter may result from BMP10 inhibition, which impairs endothelial cell quiescence and contribute to vascular dysfunction.¹⁴ The long-term effects of Sotatercept on organs such as the heart remain to be elucidated. Sotatercept's ambiguous role in trapping not only Activins, but also BMPs makes it a non-specific treatment resulting in side-effects. Currently, some companies like Keros are producing modified ACTR2-Fc ligand traps to make them more specific targeting SMAD2/3 signaling while permitting SMAD1/5/8 signaling.¹⁵

Sotatercept's clinical efficacy underscores the central role of TGF- β signaling in PAH and highlights the potential of targeting this pathway as a therapeutic strategy. In alignment with this, other pre-clinical drugs directly or indirectly targeting the TGF- β signaling pathway have been used to treat (pre-clinical) PH, like TGF- β R2-Fc ligand traps, FK506 (Tacrolimus) or SMURF1 inhibitors (nicely reviewed in ¹⁶). Alternatively, in **chapter 2 and 3** of this thesis, we presented new screening methods for ALK4 and ALK5 kinase inhibitors, which theoretically can result in a more selective approach to target Activin-induced signaling in PAH.

Also for FOP, several potentially promising therapeutics targeting dysregulated TGF- β signaling entered clinical evaluation since the initiation of this thesis. In 2023, the FDA and Canada approved Palovarotene (i.e., brand-name Sohonos) for the treatment of HO in FOP patients older than 8 years for females or 10 years for males.¹⁷ This retinoic acid receptor gamma (RAR γ) agonist reduces HO formation by activating retinoid signaling and indirectly inhibiting BMP signaling. However, lesion formation still occurs.^{18,19} All patients exhibited adverse events upon

Palovarotene administration. Due to these safety concerns and insufficient evidence, the EMA did not approve Palovarotene.¹⁷

New drugs are emerging for FOP treatment that target Activin A-induced signaling directly. Among these, the Activin A blocking antibody Garetosmab has shown promising results in earlier trials and is currently in phase 3 clinical testing (NCT05394116).²⁰ Additionally, ALK2 receptor kinase inhibitors like Zilurgesitib (INCB000928), Fidrisertib (IPN60130), and Saracatinib (AZD0530) have demonstrated ALK2 specificity and are undergoing clinical evaluation.²¹ However, the structural similarities between ALK2 and other BMP signaling receptors present a challenge for small molecule inhibitors, as off-target effects may occur. To help find selective and mutation-specific kinase inhibitors, this thesis presented an optimized nanoBRET TE assay incorporating the FOP mutant ALK2^{R206H} receptor in **chapter 2**. Further, these strategies target systemic Activin A/ALK2 signaling upstream in the pathway, but carry risks of side effects in off-target tissues due to the widespread expression of Activin A and ALK2. Given the context-dependent nature of non-SMAD signaling pathways and the necessity of ALK2-induced signaling for normal tissue homeostasis, targeting downstream ALK2-induced non-SMAD signaling pathways might be a good approach for FOP treatment.

Indeed, some pre-clinical treatment strategies focus on targeting downstream non-SMAD signaling pathways in FOP, for instance Rapamycin²² and BYL719²³, a mammalian target of rapamycin (mTOR) and PI3K α inhibitor, respectively. In **chapter 5** of this thesis, we explored non-SMAD signaling routes to identify novel druggable targets for FOP. We found multiple upregulated non-SMAD signaling mediators, including various MAP Kinases, YAP1, Piezo1, mTOR, RUNX2, Activator Protein-1 (AP-1) and RHOA-associated factors.²⁴ Some of these, like mTOR^{22,25}, RHOA²⁶, and YAP1²⁶ have previously been described as upregulated in FOP. Others, like AP-1, are novel targets in FOP and seem good disease- and tissue-specific candidates with therapeutic potential in vitro.²⁴ Ongoing studies by our group are currently translating AP-1 targeting therapeutics to validate efficacy in vivo. Furthermore, in **chapter 6**, we have further delved into the working mechanisms and therapeutic potential of PI3K α inhibition (using BYL719) and therefore contributed towards an alternative therapy for FOP.¹ As PI3K is an upstream kinase of mTOR, this approach reduces increased FOP-specific non-SMAD signaling pathways.

After identifying effective molecules, and to reduce possible side effects in non-diseased tissues, one might consider targeted delivery approaches such as using antibody drug-conjugates (ADCs). These ADCs typically consist of a monoclonal antibody guiding the drug towards the cells of interest, a small linker and the small molecule warhead eliciting its pharmacological effect after cellular uptake.²⁷ In the context of PAH, endothelial-targeted therapies could be explored, such as using ALK1-specific antibodies conjugated with small-molecule inhibitors (e.g. ALK4 inhibitors). For FOP, to target the fibro-adipogenic progenitor cells (FAPs), antibodies targeting the PDGFR α or TIE2 receptors (or bispecific) might be used to deliver the drug (e.g. AP-1 inhibitors or BYL719) more specifically. Tissues lacking the expression of the targeted receptor will consequently not be affected by the drug, resulting in reduced potential side effects. A suitable model to test such ADCs may be the nanoBRET assays presented in **chapter 2 and 3**, as it's efficacy depends on complex cellular dynamics

evaluated in live cells, including cellular uptake (i.e. targeted receptor expression), intracellular drug release, and drug target kinase availability.

In summary, the current field of targeting TGF- β signaling for PAH and FOP shows significant potential in the clinic, which further paves the way for more selective and novel TGF- β associated modulators like those presented in this thesis.

Bridging PAH and FOP through overlapping cellular and molecular determinants

While PAH and FOP are clinically distinct diseases, we hypothesize that meaningful comparisons can be drawn between their underlying mechanisms. Phenotypically, PAH patients may develop vascular calcifications^{28,29} while FOP patients can develop severe pulmonary hypertension^{30,31}, suggesting a convergence of pathogenic mechanisms. Both diseases are characterized by disturbed TGF- β signaling, which play central roles in their pathobiology. Mutations in *BMPR2* are a well-established driver of PAH. *BMPR2* haploinsufficiency promotes Activin-induced phosphorylation of SMAD1/5/8,^{32,33} a hallmark of disturbed signaling also observed in FOP.³⁴ Activin signaling, therefore, emerges as a druggable target in both diseases, targeted by Garetosmab in FOP²⁰ and Sotatercept in PAH.¹¹ Notably, as presented at the IFOPA Drug Development Forum 2024, a recent case of a FOP-like patient with a gain-of-function mutation in *BMPR2*³⁵ underscores the genetic links shared between the two diseases. Taken together, this suggests that despite differences in mutations and tissue contexts, the signaling mechanisms exhibit significant similarities.

Other disease triggers are also overlapping in PAH and FOP. As such, inflammation plays a crucial role in driving HO upon flare-ups^{36–38} while increased pro-inflammatory stimuli trigger vascular dysfunction and remodeling in PAH.^{39,40} Furthermore, a key characteristic in diseased tissue environments in PAH and FOP is hypoxia. It's a known driver in PH development^{41,42} and hypoxia has shown to promote HO by amplifying BMP signaling in FOP.⁴³ Another shared trigger might be disrupted mechanotransduction. In FOP, as concluded in **chapter 5** and corroborated by others²⁶, *ALK2*^{R206H}-expressing cells exhibit increased YAP1, Piezo1, and RhoA activation in response to mechanical stimuli. Similarly, in PAH, altered blood flow and elevated arterial pressures due to vascular remodeling and constriction exacerbates mechanotransduction-related processes (including YAP), further driving the progression of cardiovascular disease.^{44,45} All of these three triggers (inflammation, hypoxia, and mechanotransduction) have shown to cross-talk with TGF- β signaling,^{40,43,46,47} which may further underlie PAH and FOP disease progression.

While the affected tissues differ between PAH and FOP, certain cell-types involved are shared among both pathologies. For instance, activated macrophages and mast-cells play important secretory (e.g., IL-1, IL-6, TNF α , Activin A) roles enhancing pro-inflammatory environments.^{39,40,48,49} Also endothelial cells play an important role in driving endochondral ossification; for instance, besides FAPs, other MSCs within HO lesions may be derived from endothelial origin (found through TIE2⁺-lineage tracing) via endothelial-to-mesenchymal transition (EndMT).^{50,51} EndoMT is also an important process mediating vascular remodeling

in PAH.⁵² In that regard, induced-MSCs and other mesenchymal cells (e.g. pericytes and adventitial fibroblasts) play a role in matrix remodeling in the pulmonary vasculature in PAH,^{53,54} while MSCs and related derivatives mediate lesion formation in FOP.⁵⁵

Unsurprisingly, following the fact that many disease triggers and related cell-types are overlapping in FOP and PAH, various treatment options have been investigated using similar or the same downstream targets. For instance, Imatinib has been used to treat both PAH and FOP patients.^{56,57} Further, as mentioned earlier, Activin A has become a shared treatment target using Sotatercept in PAH and Garetosmab in FOP. Interestingly, as detailed in **chapter 5**, Activator Protein-1 (AP-1) is dysregulated in FOP and emerges as a promising therapeutic target, with evidence from other studies showing that AP-1 inhibition exhibits clear efficacy in preclinical models of PH.⁵⁸ As further discussed in **chapter 5**, RUNX2 has been recognized as driver for HO in FOP,²⁴ while others depict RUNX2 as a key mediator of calcified lesion formation in PAH.²⁸ Also, in **chapter 6** we showed again the efficacy of PI3K α inhibition in preventing HO in FOP, while other groups have shown a similar effective approach using the same target in preclinical PH.⁵⁹

Not only can we bridge PAH and FOP, our findings might be extended to other diseases too. Clinical overlap has been observed between PAH and hereditary hemorrhagic telangiectasia (HHT), with HHT patients presenting PAH-like phenotypes and vice versa.⁶⁰ This overlap is likely attributable to shared mutations and disruptions in BMP signaling, despite each condition primarily affecting distinct vascular beds. Accordingly, a common adverse event of Sotatercept treatment in PAH is telangiectasia development, therefore resulting in PAH patients with HHT-like symptoms.¹¹ Interestingly, gain-of-function mutations in ALK2 can lead to both FOP and the rare brain cancer diffuse intrinsic pontine glioma (DIPG), with FOP arising from germline mutations and the same mutation occurring somatically in DIPG.⁶¹ Therefore, careful translation of new therapeutics in one disease might be applicable to related disorders.

In summary, although clinically PAH and FOP are two worlds apart, in this section we argue that many cellular and molecular determinants do overlap in both pathologies. This suggests that findings (including effective therapies) in one disease may be thoughtfully translated towards the other, although tissue-specific cues should be taken into account. Accordingly, systemic reviewing of the current literature may provide valuable insights into therapies and drug targets extending from PAH or FOP to the other condition. This thesis primarily focused on PAH and FOP; however, the findings may also be relevant to other genetic disorders associated with disrupted TGF- β signaling, such as HHT and DIPG.

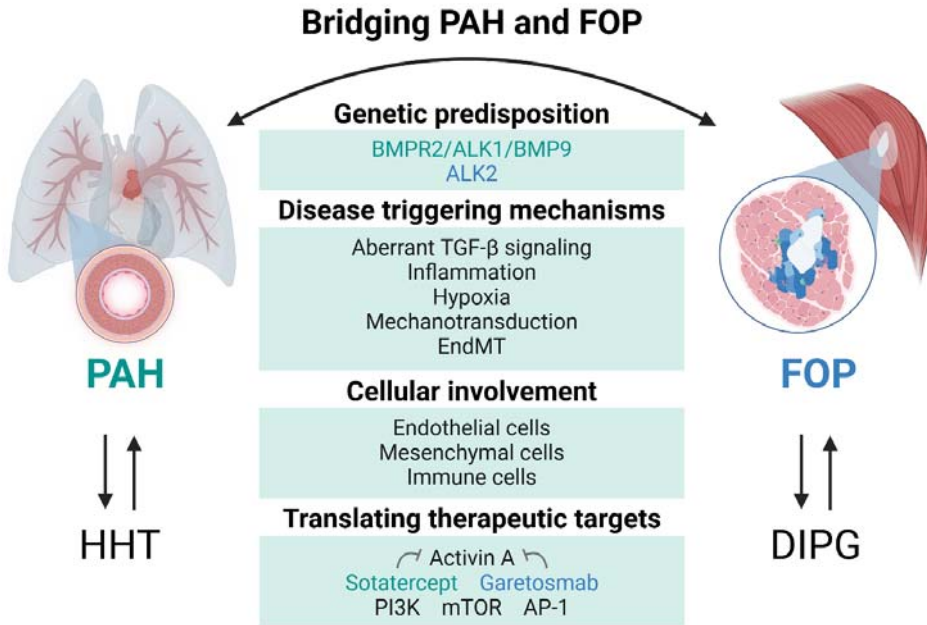


Figure 2. Overlapping molecular and cellular determinants may bridge the two clinically distinct diseases PAH and FOP. Mutations in genes within the TGF- β /BMP signaling pathways cause genetic predisposition in PAH and FOP. Consequently, aberrant TGF- β signaling underlies disease development in both diseases. Other disease-triggering mechanisms overlap the two pathologies, including inflammation, hypoxia, altered mechanotransduction and endothelial-to-mesenchymal transition (EndMT). While the tissue contexts are different, the cells involved in the pathobiology show overlapping phenotypes, including endothelial cells, mesenchymal cells and immune cells. These overlapping mechanisms and target cells may result in therapies targeting the same target in PAH and FOP. PAH patients can have disease phenotypes corresponding to hereditary hemorrhagic telangiectasia (HHT) patients, and vice versa. Likewise, FOP and the rare brain cancer diffuse intrinsic pontine glioma (DIPG) arise from the same mutations. This enables the translation of novel findings from one disease to the other.

Concluding remarks

The studies presented in this thesis aimed to advance novel therapies targeting TGF- β -induced SMAD and non-SMAD signaling pathways to treat the detrimental TGF- β associated genetic disorders PAH and FOP. We contributed to this objective by improving the current state-of-the-art drug screening methodologies and by deciphering important downstream molecular mechanisms and related targets. Future studies may further enhance the newly presented methods and translate the presented novel compounds and targets towards the clinic. Not only could our findings contribute to new therapies for PAH and FOP, these studies may also be applied to other TGF- β associated disorders including cancer, fibrosis, cardiovascular disorders, and musculoskeletal diseases. All in all, in this thesis we have established new research lines bringing novel therapeutic solutions closer to the patient.

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