Engineered Pulmonary Arterial Tissue (EPAT): A novel platform assessing vasoactivity to validate emerging PAH therapies

Submitted by: Kate Quigley
CID: 02179328
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Supervised by:
Dr Beata Wojciak-Stotthard & Dr Adam Fellows

This research project is submitted in partial fulfilment of the requirements for the degree of MRes in Clinical Research: Translational Medicine

Imperial College of Science, Technology and Medicine NHLI, Imperial College London Hammersmith Hospital Campus, London

9th August 2022
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Many aspects of this projects were done collaboratively. Katie is a motivated, focused and hardworking individual, capable of planning and carrying out experiments with a minimal level of assistance. Her project was novel and technically challenging and despite this and the COVID19 pandemic, Katie managed to complete her experiments as planned. The results obtained by Katie will be included in a paper we are currently preparing for publication. It was a pleasure to have her in our laboratory.

Katie has shown an excellent ability to learn new techniques, familiarise herself with the background literature and develop her capacity to independently design and execute experiments. She has also demonstrated critical thinking as well as impressive writing and presentation skills.

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II. Thesis Submission Form

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(Translational Medicine 2021-2022)

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**Thesis Title:** Engineered Pulmonary arterial Tissues (EPATs): A novel platform assessing vasoactivity to validate emerging PAH therapies.

**Supervisor(s):** Dr Beata Wojciak-Stothard & Dr Adam Fellows

**Deadline for submission:** 09.08.2022

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III. Abstract

Pulmonary arterial hypertension (PAH) is a multifactorial disease characterised by progressive narrowing of small intrapulmonary arteries due to excessive vasoconstriction and exuberant proliferation and migration of cells in the arterial intimal, medial, and adventitial layers. PAH is considered an unaddressed global health burden due to the lack of effective treatment. Current therapies provide only a symptomatic relief and many new drug candidates fail in clinical trials. This is due to the lack of accurate preclinical drug testing platforms utilising patient cells which would provide further insights into methods of drug delivery and drug interactions. Human tissue engineering has the ability to address current limitations experienced within the drug development process.

The aim of this study was to develop a new in vitro platform for studying vasoreactivity of human pulmonary vascular smooth muscle cells (PASMCs) in response to drug treatment, to facilitate validation of candidate PAH therapeutics.

Engineered Pulmonary Arterial Tissues (EPAT) were designed and fabricated using human PASMCs and were generated in agarose casting moulds with custom made PDMS racks. EPATs were in culture for up to 30 days with vasoactive properties of EPATs analysed between day 7–28 using brightfield microscopy (HWF1-Zeiss Axio Observer). Vasoreactivity was tested with the use of known vasoconstrictors, Endothein-1 (ET-1), U46619 and Potassium chloride (KCL). Following optimisation of culture conditions and device validation for studying PASMC vasoreactivity, PASMCs responses to approved and new PAH drug candidates was carried out.

Results show the EPAT model successfully validated known vasodilators; bosentan, seleipag and epoprostenol. The EPAT model established a distinct vasodilatory mechanism of imatinib. Imatinib induced vasodilation in response to endothelin-1 and U46619 in both healthy and PAH patient pulmonary arterial smooth muscle cells, with a Donor dependent response observed. Serelaxin also produced a dilatory response to endothelin-1 and U46619, indicating a potential option for repurposing for the treatment of PAH.

In summary the EPAT model was designed to assess vascular smooth muscle contractility in response to vasoactive compounds. This model addresses the need for new, more physiologically relevant in vitro systems utilising human cells to identify novel therapeutic targets and improve clinical translation.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>2D</td>
<td>Two Dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three Dimensional</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BMPR2</td>
<td>Bone Morphogenetic Protein Receptor Type 2</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
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<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<tr>
<td>CCB</td>
<td>Calcium Channel Blockers</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic Guanosine Monophosphate</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cells</td>
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<tr>
<td>EC50</td>
<td>Half Maximal Effective Concentration</td>
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<tr>
<td>EHTs</td>
<td>Engineered Heart Tissues</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>EPATS</td>
<td>Engineered Pulmonary Arterial Tissues</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>IC50</td>
<td>Half Maximal Inhibitory Concentration</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>IP</td>
<td>Prostaglandin I</td>
</tr>
<tr>
<td>KCL</td>
<td>Potassium chloride</td>
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<tr>
<td>MCT</td>
<td>Monocrotaline</td>
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<tr>
<td>MLC</td>
<td>Myosin Light Chain</td>
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<tr>
<td>MLCK</td>
<td>Myosin Light Chain Kinase</td>
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<td>mm</td>
<td>Millimetre</td>
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<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>mPAP</td>
<td>Mean Pulmonary Arterial Pressure</td>
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<td>nM</td>
<td>Nanomolar</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<td>PAH</td>
<td>Pulmonary Arterial Hypertension</td>
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<tr>
<td>PASMC</td>
<td>Pulmonary Arterial Smooth Muscle Cells</td>
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<td>PAWP</td>
<td>Pulmonary Arterial Wedge Pressure</td>
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<tr>
<td>PCH</td>
<td>Pulmonary Capillary Hemangiomatosis</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PDE-5</td>
<td>Phosphodiesterase type 5</td>
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<td>PDGFR</td>
<td>Platelet Derived Growth Factor Receptor</td>
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<td>PDGFRBi</td>
<td>Platelet Derived Growth Factor Receptor Inhibitor</td>
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<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<tr>
<td>PH</td>
<td>Pulmonary Hypertension</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<td>PKG</td>
<td>Protein Kinase G</td>
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<tr>
<td>PVOD</td>
<td>Pulmonary Veno-occlusive Disease</td>
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<td>R&amp;D</td>
<td>Research and Development</td>
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<tr>
<td>RLN2</td>
<td>Serelaxin</td>
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<tr>
<td>sGC</td>
<td>Soluble Guanylate Cyclase</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
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<tr>
<td>SMCBM</td>
<td>Smooth Muscle Cell Basal Medium</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium Nitroprusside</td>
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<tr>
<td>SR</td>
<td>Sarcoplasmic Reticulum</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
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<tr>
<td>TKI</td>
<td>Tyrosine Kinase Inhibitor</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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V. Acknowledgements

Firstly, I would like to thank my supervisor Dr Beata Wojciak-Stothard for allowing me the opportunity to undertake my MRes research project in her lab. I have thoroughly enjoyed working within the lab and greatly appreciate Dr Wojciak-Stothard’s continuous guidance and support. I would also in particular like to thank Dr Adam Fellows for mentoring me throughout my project and allowing me the opportunity to contribute to his research project. It has been a pleasure to work with you both additionally.
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1. Introduction

The focus of this study was the fabrication, optimisation, and validation of a new in vitro model, to facilitate the evaluation of current and emerging PAH therapeutics.

1.1 An Overview of Pulmonary Arterial Hypertension

Pulmonary hypertension (PH) is defined hemodynamically by an increase in mean pulmonary arterial pressure above 20 mm Hg at rest (Simonneau et al., 2019). PH can be classified according to the presence of identified causes or risk factors, into five clinical groups (Table 1).

Pulmonary arterial hypertension (PAH), belonging to PH Group 1, is a complex disease characterised by progressive narrowing of small intrapulmonary arteries due to excessive vasoconstriction and aberrant proliferation and migration of cells in the arterial intimal, medial, and adventitial layers (Tuder et al., 2007; Price et al., 2012). This results in a rise in vascular resistance and blood pressure in the lung, leading to right ventricular hypertrophy and, if left untreated, right heart failure and death. PAH mortality remains high despite advances in the understanding of underlying disease mechanisms, with an average annual survival of 86% (Emmons-Bell et al., 2022). PAH accounts for approximately 10% of pulmonary hypertension cases worldwide and it is estimated that PAH affects 15 per 1 million adults, the majority of whom are women (Humbert et al., 2006), with disease severity increased among elderly men (Hoeper, Huscher, et al., 2013).
Table 1. Clinical Classification of Pulmonary Hypertension according to the 2018 6th World Symposium on Pulmonary Hypertension, Nice, France. Abbreviations: mPAP, mean pulmonary artery pressure; PCH, pulmonary capillary hemangiomatosis; PAWP, pulmonary artery wedge pressure; PVOD, pulmonary veno-occlusive disease. (Hassoun, 2021).

The pathophysiological triggers of PAH are variable, due to the complex nature of the disease. Converging effects of hypoxia, shear stress, viral infections, toxins, autoimmune disorders, and various genetic and epigenetic factors are thought to contribute to chronic vasoconstriction and vascular remodelling in PAH (Budhiraja et al., 2004a).

The heightened vasoconstriction observed in PAH, presented at both resting pulmonary arterial tone and reactivity to endogenous vasoconstrictors, plays a fundamental role in this disease (Cahill et al., 2012; Jernigan et al., 2017; Stenmark & Mccurtry, 2005). Persistent vasoconstriction occurs as a result of chronically elevated intracellular Ca\(^{2+}\) levels which promote actin-myosin interactions, via calcium sensitisation, as a result of
the activation of RhoA/Rho kinase signalling in medial pulmonary smooth muscle cells (PASMCs) (Amberg & Navedo, 2013). In addition, dysfunctional pulmonary arterial endothelium produces lower levels of vasorelaxants, such as nitric oxide and prostacyclin and higher levels of vasoconstrictors, such as endothelin-1 and thromboxane (Jernigan et al.; Undem, Luke & Shimoda, 2016) (Budhiraja, Tuder & Hassoun, 2004).

Figure 1. Schematic diagram of vascular remodelling in pulmonary arterial hypertension. Left: A cross-sectional view of the three layers of a healthy pulmonary vessel: the intima, media, and adventitia. Right: Increased proliferation of the intimal ECs, medial SMCs and adventitial fibroblasts resulting in thickening of the pulmonary arterial wall. Figure created using BioRender software.
1.2 Mechanism of Vascular Smooth Muscle Contraction

SMC contraction can be initiated by a range of stimuli, such as mechanical, electrical, and chemical. Additionally, the passive stretching of SMC can initiate constriction which originates directly from the smooth muscle itself, resulting in a myogenic response (Schubert & Mulvany, 1999). Electrical depolarisation, hormones and neurotransmitters also elicit contraction by causing Ca\textsuperscript{2+} to enter the cell via the opening of voltage dependent calcium channels (L-type channels) located in the caveolae of the membrane, causing an increase in intracellular Ca\textsuperscript{2+} (Jackson & Boerman, 2018).

SM contraction is dependent on an increase in intracellular Ca\textsuperscript{2+} level. As seen in Figure 2, calcium ions can enter the cell via L-type calcium channels in the plasma membrane (1.) or alternatively can be released from internal stores, such as the sarcoplasmic reticulum (SR) (2.) (Amberg & Navedo, 2013). Binding of Ca\textsuperscript{2+} to calmodulin activates myosin light chain kinase, which phosphorylates and activates myosin light chain (MLC) in the presence of ATP (5-7). Phosphorylation of MLC results in the binding of myosin heads to actin filaments, followed by contraction, which initiates the shortening of VSMCs (8-10) (Kuo & Ehrlich, 2015).

Relaxation of SMCs occurs as a result of reduced MLC phosphorylation which can be caused by reduced Ca\textsuperscript{2+} entry, diminished Ca\textsuperscript{2+} release from the SR, MLC kinase inhibition and phosphatase-activated MLC dephosphorylation (Figure 2).

These processes are regulated by G-protein coupled signal transduction pathways as well as activation of guanylyl cyclase and formation of cGMP induced by nitric oxide. Vasoconstrictors such as endothelin-1, angiotensin II and vasopressin stimulate G\textsubscript{q} which simultaneously promotes the activation of MLC kinase and inhibition of MLC phosphatase via Ca\textsuperscript{2+} release from the SR and activation of Rho-kinase, respectively. Alternatively, vasodilators such as prostacyclin and adenosine activate G\textsubscript{s} which causes an increase in cAMP thereby inhibiting MLC kinase and reducing MLC phosphorylation (Jin et al., 2006).
Figure 2. Smooth Muscle Contraction Mechanism. (1) Membrane depolarisation, (2) Opening of L-type calcium channels, (3) Release of calcium from sarcoplasmic reticulum, (4) Increase in intracellular calcium, (5) Calcium binds to calmodulin, (6) Activation of myosin light chain, (7) Myosin light chain phosphorylation, (8-10) Myosin ATPase activity, (9) Phosphorylated myosin binds actin, (10) Cross bridging leading to muscle contraction. MLCK; Myosin light chain kinase, MLCP; Myosin light chain, phosphatase, GTP; Guanosine triphosphate, cGMP; Cyclic guanosine monophosphate. Figure adapted from (CV Physiology | Vascular Smooth Muscle Contraction and Relaxation, n.d.).
Introduction

1.3 The Triple Therapy Approach to PAH

Contemporary therapies employed for PAH are predominately vasodilators (Gali et al., 2010; Humbert et al., 2014a; Thenappan et al., 2018a) which target one of the three pathways that contribute to endothelial dysfunction and vasoconstriction: the endothelin pathway, the prostacyclin pathway, and the nitric oxide pathway (Budhiraja et al., 2004a).

**Endothelin-1 pathway.** Endothelin 1 (ET-1) is a potent endothelium-derived vasoconstrictor, significantly upregulated in the vasculature of PAH patients (Thenappan et al., 2018; Humbert et al., 2014). ET-1 binds two types of receptors, ET\textsubscript{A} and ET\textsubscript{B}, members of the G-protein-coupled receptors family (Takigawa et al., 1995). ET\textsubscript{A} receptors are located on SMCs and cardiac myocytes, with ET\textsubscript{B}-receptors localised to SMC and endothelial cells (Seo et al., 1994). Activation of ET\textsubscript{A} receptors triggers vasoconstriction, proliferation, migration, and ROS generation (de Nucci et al., 1988). Activation of ET\textsubscript{B} causes endothelial stimulation and release of NO and prostacyclin (Hirata et al., 1993), preventing apoptosis, inhibiting ECE-1 expression, leading to vasodilation (Clozel et al., 1993). Bosentan, an oral dual endothelin receptor antagonist (ET\textsubscript{A} and ET\textsubscript{B}) commonly used in PAH treatment is used to inhibit both ET\textsubscript{A} and ET\textsubscript{B} receptors (Channick et al., 2001).

**Prostacyclin pathway.** Endothelial cells are the predominant producers of prostacyclin, a potent pulmonary vasodilator (Levin et al., 1984). The prostacyclin I (IP) receptor is specifically confined to VSMCs, with its activation mediating VSMC relaxation (Read et al., 1985). The IP receptor is coupled to G-proteins, which activates adenylyl cyclase, and increases (Coleman et al., 1994; Olschewski et al., 2001) cyclic adenosine monophosphate (cAMP), resulting in protein kinase A (PKA) activation (Billington & Penn, 2003; Chow et al., 2003). Synthetic prostacyclin’s such as epoprostenol and selexipag induce vasodilation and have become routine therapies in the treatment of PAH. Selexipag differs from epoprostenol in that it is a selective non-prostanoid IP receptor agonist, with its mechanism similar to that of endogenous prostacyclin (Gomberg-Maitland & Olschewski, 2008).

**Nitric oxide and cGMP pathway.** Nitric oxide is produced from L-arginine by nitric oxide synthases (eNOS, iNOS and nNOS). The endothelial NOS (eNOS) plays the main role in physiological regulation of VSMC contraction (Michelakis, 2003). NO diffuses into SMCs and stimulates soluble guanylyl cyclase (sGC) to produce cyclic GMP (cGMP), activating protein kinase GI (PKGI), thus causing vasodilation (Sausbier et al., 2000;
Introduction

Surks, 2007). Apart from NO, other vasodilators such as natriuretic peptides (A, B and C) can produce cGMP via the activation of particulate guanylate cyclases (Boerrigter et al., 2009). Phosphodiesterase type 5 (PDE-5) is the main enzyme responsible for cGMP degradation in the lung. In PH, the levels of NO, natriuretic peptides and cGMP are reduced and the levels of PDE5 are increased, contributing to chronic vasoconstriction (Baliga et al., 2008).

Therefore, PDE-5 inhibitors, such as sildenafil have the ability to maintain and regulate cGMP levels within pulmonary vasculature while also stimulating the vasodilatory effects of endogenous NO. Riociguat, which directly stimulates sGC, generates cGMP independently of NO release (Schermuly et al., 2008).

![Figure 3. The Classic pathways of targeted therapy for PAH. Current PAH-specific therapies target one of three pathways: the endothelin pathway, the prostacyclin pathway, and the nitric oxide pathways. Adapted from (Hassoun, 2021).](image)

Upon early examination, a small portion of PAH patients are considered to be vasoactive and are eligible for treatment with calcium channel blockers (CCB). CCBs inhibit the calcium influx into vascular cells, leading to relaxation of smooth muscle cells and vasodilatation (Lin & Ma, 2018). Because CCBs have a widespread effect on the body, in order to benefit from this therapy, PAH patients are required to meet a predefined hemodynamic criterion. If considered a ‘responder’, CCBs are therefore a viable treatment option (Rich et al., 1992)
Introduction

Certain recent therapies have been demonstrated to improve pulmonary haemodynamics and survival, but none are curative (Humbert et al., 2010). Contributing to this is the lack of comparative studies involving different vasodilators, applied alone or in combination with other drugs. Therefore, a viable *in vitro* platform is needed to facilitate measurements of PASMC vasoreactivity in response to single and combination therapies.
Introduction

1.4 Established Drug Screening Models

One of the major issues hindering the development of new and better therapies for PAH has been the lack of appropriate pre-clinical models that capture the full phenotype of human disease (Stenmark et al., 2009). Thus far, no single animal model has accurately reproduced the complex and multifactorial pathobiological spectrum observed in PAH. This, combined with a lack of an accurate platform to measure vasoactive properties in vitro, has limited the ability to evaluate potential vasoactive therapies on human cells or tissues, further widening the translational gap seen between bench and beside.

Currently a wide range of models are used to reproduce PH, the most commonly used are rat and mouse chronic hypoxia models, which produce mild and reversible disease, respectively. The Sugen/Hypoxia rat and mouse and inflammatory monocrotaline (MCT) rat models produce a more pronounced haemodynamic phenotype (Heath & Kay, 1967; Smith et al., 1970). The Sugen/Hypoxia model, combine the effects of hypoxia with the VEGF receptor blocker, causing a more pronounced PH phenotype than hypoxia alone and has the ability to reproduce severe and irreversible PH with angio-obliterative lesions (Toba et al., 2014; Abe et al., 2010). Genetically modified models are also available to provide an insight into specific mutations implicated with PH (Toba et al., 2014).

The drug development process is slow and costly, with total R&D costs varying from $161 million to $4.54 billion (Schlander et al., 2021). Moreover, these costs fail to address current and emerging trends in pharmaceutical R&D. Although animal models work well to verify therapies against clinically validated targets, they are often inaccurate in evaluation of novel targets and therapies (van Norman, 2019). New, more physiologically relevant in vitro systems utilising human cells are required to identify novel therapeutic targets and improve clinical translation.
Introduction

1.5 Measurement of Vasoactivity \textit{in vitro}

Measurement of pulmonary vasoactivity is crucial in understanding both the prognoses and potential therapeutic options available for PAH patients. The current methods employ two-dimensional cell culture to study vasoactive responses of single VSMCs, cell monolayers or myography, to evaluate vasoreactivity of isolated blood vessels.

Two-dimensional cell culture. Cell based assays have been fundamental in drug discovery, with two-dimensional cell monolayers being the most convenient platforms to discover potential therapeutic candidates. The 2-D cellular models are limited by their physiological relevance which fails to replicate interactions between cells and the extracellular matrix \textit{in vivo}. In failing to replicate the orientation and phenotypic maturity observed in native SMCs, evaluation of vasoactivity cannot be accurately carried out (Bonnans et al., 2014; Sebens & Schafer, 2012). Vasoreactivity in 2-D cell cultures can be evaluated by measurement of the intracellular calcium release using fluorescent indicators (Kao et al., 2010), measurement of MLC phosphorylation via western blotting and immunofluorescence or morphometric analysis of VSMC shape.

Myography. Vasoreactivity of small resistance arteries isolated from animal or human tissues can be carried out with the use of myography. In this technique, blood vessels are excised from animal or human tissue, cleaned, and mounted onto a myograph under isometric conditions (Spiers & Padmanabhan, 2005). Wire myography involves mounting a blood vessel on fine, steel wires and recording the tension generated by the vascular wall in response to different treatments (Wenceslau et al., 2021). Pressure myography involves inserting a perfusable cannula at one or both ends of the blood vessel, with the intraluminal pressure set at a physiologically relevant value. While useful at testing drug effects, these techniques are contained by limited access to human material and, in case of animal models, differences between human and animal physiology (Pound et al., 2004). Myography is low through put, labour intensive and requires specialised equipment and a high level of skill and training.

Therefore, there is a considerable need for new preclinical models that contain the elements to preform accurate therapeutic evaluations of vasoactivity \textit{in vitro} using human cells.
Introduction

1.6 Tissue Engineered Models of Smooth Muscle

Tissue engineering aims to create and construct bioartificial tissues in vitro in order to improve the development and translation of new therapeutic options. Over the last decade, a broad range of platforms have been developed to support the range of biochemical and mechanical cues observed in vivo.

Miniaturised force generating engineered heart tissues (EHTs) have previously been developed to produce spontaneously beating three-dimensional heart muscle constructs in vitro. Hansen et al., refined and developed a robust, reproducible, and automated protocol to measure contractility of EHTs in a 24-well format with a limited number of cells. EHTs are fabricated using a fibrin hydrogel combined with isolated cardiomyocytes in casting moulds. Remodelling of the hydrogel occurs, resulting in realignment of cardiomyocytes, leading to the formation of coherently beating cardiomyocytes. The EHT platform provides an accurate system for pre-clinical drug development as it allows for in vitro monitoring of cardiac functions such pace making activity and contraction, as well as responses to drug treatments while resembling in vivo cardiac physiology. We developed protocols to form Engineered Pulmonary Arterial Tissues (EPATs) using human pulmonary arterial smooth muscle cells, following principles from EHT fabrication.

Isolated cells combined with synthetic materials such as hydrogels, a cross-linked polymeric system, allow for the recreation of the extracellular matrix found in vivo to produce 3D living tissue. Hydrogels can be prepared from a wide range of natural biomaterials and synthetic polymers (Thiele et al., 2014; Huang et al., 2017), for example hydrogels composed of compounds such as fibrin, which is inherently biocompatible and bioactive (Daley et al., 2008).

Tissue engineering has the potential to further enhance and develop our understanding of disease mechanisms. The development of an accurate preclinical model with the use of primary cells will provide further insight into methods of drug delivery, mechanisms, and interactions. This study aims to develop a new platform utilising human PASMCs, which would allow for the measurement of PASMC contractility and validation of candidate PAH therapeutics. Despite the advances in drug development, a cure for PAH has yet to be developed and therefore continues to be an unmet clinical need.
1.7 Hypotheses and Objectives

Hypotheses


b. EPATs can be used to assess vasoactive effects of drugs in current and emerging PAH therapies.

Objectives

a. To optimise production of EPAT model and conditions of cell culture. EPATs will be constructed and adapted for long-term (up to 4 weeks) smooth muscle cell culture.

b. To validate EPATs for the measurement of SMC contractility in vitro. EPATs will be treated with known vasoconstrictors and vasodilators to assess SMC vasoreactivity.

c. To evaluate vasoactive effects of selected current and emerging PAH therapeutics. EPATs will be implemented for medium-throughput screening, to evaluate vasoactive properties of selected approved and emerging PAH therapeutic agents, such as serelaxin and imatinib.
2. **Materials and Methods**

2.1 **Manufacturing PDMS Posts and Teflon Spacers**

Custom made cell racks were produced from PDMS, using a 3-D printed resin mould designed using computer-aided software and printed at Advanced Hackspace, Imperial College London (Figure 4a, b, c). Silicone racks were composed of 4 pairs of posts and had the following geometry (Figure 4d): length/width of rack: 79 x 18.5 mm, length of posts 12 mm, diameter 1mm. Teflon spacers (EHT Technologies) for producing the casting moulds (Figure 4e) had the following geometry: length 12 mm, width 3 mm, height 13.5 mm. Teflon spacers were boiled twice in H₂O for 5 minutes each and autoclaved prior to use. PDMS were cleaned thoroughly with dH₂O and autoclaved prior to EPAT fabrication.

![Figure 4](image)

**Figure 4.** (A-C) 3-D printed resin mould designed using computer-aided software, printed at Advanced Hackspace, Imperial College London; (D). Custom made silicone racks composed of 4 posts: (E). Teflon spacers used to produce casting moulds.

2.2 **Cell Culture Conditions**

Human pulmonary arterial smooth muscle cells (HPASMCs; Lonza) were cultured in smooth muscle cell basal medium containing supplements (SMBM; Lonza) as well as penicillin (100U/ml; Gibco) streptomycin (0.1mg/ml; Gibco) and L-glutamine (200mM; Gibco). HPASMCs were maintained in SMBM in a 37°C humified cell culture incubator supplied with 5% CO₂. For HPASMC subculture, cells were incubated with trypsin-EDTA
Materials and Methods

(0.05%, Life Technologies) for 5 min at 37°C, followed by the addition of complete Dulbecco’s Modified Eagle’s Medium (DMEM containing 4.5g/L glucose; Gibco) to inactivate trypsin. Cell suspension was centrifuged at 1,500 rpm for 5 minutes, followed by resuspension SMBM. HPASMCs were seeded onto tissue culture flasks coated with 0.1% gelatin. Cells were used up to passage 15. Only cells with population doubling time<48h and displaying "hill and valley" morphology, typical of VSMCs, were used for experimentation (Figure 5a-d). Five distinct donors of HPASMCs were cultured and used to fabricate EPATs (Table 2). Donors 1-3 were obtained commercially and each set of HPASMCs were isolated from different individuals. HPASMCs from two separate patients with idiopathic PAH were gifted from Prof Nicholas Morell, University of Cambridge (Donor 4&5).

![Figure 5](image_url). Human Pulmonary Arterial Vascular Smooth Muscle cells in 2D culture. Cells are at 90% confluency and 'hill-and-valley' shape is observed using an EVOS XL microscope with a 10X objective (A) Donor 1 p10 (B) Donor 2 p9 (C) Donor 4 p8 (D) Donor 5 p5.
Table 2: HPASMC donor information. Information regarding lot number, sex, age and race unavailable for Donor 1. Information regarding race unavailable for Donor 4 & 5.

2.3 Generation of EPATs

With sufficient numbers of desired cells at 90% confluency, HPASMCs were deemed eligible for EPAT fabrication and cells were imaged to confirm morphology and phenotype (Figure 5a-d). A single cell suspension was prepared using trypsin-EDTA (see 2.2) and cells were carefully counted manually using a haemocytometer.

Cell count was determined, and a master-mix was prepared based on $1 \times 10^6$ cells with the following volumes per EPAT: 2.75µl fibrinogen (Sigma A1153), 101.2µl SMBM, 6.05µl 2X DMEM (26.8mg/ml DMEM powder (Gibco) plus 20% horse serum (Thermo Scientific) in dH$_2$O). Once master-mix was prepared, cells were centrifuged at 1,000 rpm for 5 minutes. Following centrifugation, supernatant was aspirated to obtain pellet. HPASMCs pellet was resuspended in the master-mix and triturated thoroughly before EPATs were casted.

EPATs were generated in agarose casting moulds with custom made PDMS racks, as previously described (Schaaf et al., 2014; Hansen et al., 2010). Casting moulds were prepared by placing the Teflon spacer in 24-well culture dishes and adding 1.6ml of warm 2% agarose in PBS (Invitrogen) per well. After agarose solidification (approximately 15 minutes) the spacers were removed, and silicon racks were placed into the plate with pairs of posts reaching into each casting mould (Figure 6a).
Materials and Methods

For each EPAT, 97µl master-mix was mixed with 3µl thrombin (100U/ml; Sigma) and pipetted into the agarose slot. EPATs were placed in a cell culture incubator for 90 minutes to allow fibrinogen polymerisation to occur. Next, 500µl of DMEM was added to each well to aid in the removal of the EPATs from the agarose casting moulds. The EPATs were then transferred into a new 24-well culture dish containing 1.5ml of EPAT medium per well.

EPATs were maintained in a standard cell culture incubator, media was changed every Monday, Wednesday and Friday and tissues were monitored daily. EPAT media consisted of DMEM (Gibco), 10% foetal bovine serum (Gibco), penicillin (100U/ml; Gibco), streptomycin (0.1mg/ml; Gibco), L-glutamine (200mM; Gibco), amphotericin B (250ng/ml; Sigma) and aprotinin (0.1%; Sigma). EPAT tissues were given 7-10 days to reach phenotypic maturity before vasoactivity was measured and were generally used between day 7-28.

2.4 Measurement of Contractility

Vasoactive measurements of EPATs were performed on 7–28-day old EPATs using time-lapse brightfield microscopy (Zeiss Axio Observer). The setup for video-microscopy included a cell incubator with temperature control and an automated stage for rapid imaging. For all experiments, EPATs were placed in “starvation” media (serum-free DMEM containing 2.5 mM CaCl₂) for a minimum of 90 minutes prior to baseline recordings to allow for pre-equilibration. Baseline images were taken every 5 minutes.
Materials and Methods

for 15 minutes to confirm EPAT equilibration to starvation media and microscope conditions.

For optimisation studies EPATs were imaged in baseline media every 5 minutes for 15 minutes. EPATs were then treated with 75mM KCL for 30 minutes with images taken every 5 minutes to confirm functionality. For functionality studies, EPATs were placed in 100nM nifedipine, 10µM sildenafil and 50µM SNP once maximum constriction was achieved following 30 minutes of KCL treatment.

For vasoactivity studies, EPATs were treated with U46619 (1µM) or ET-1 (100nM) for 1 hour to reach maximum constriction following baseline recordings. A vasodilator relevant to PAH (Table 3) was then applied at increasing concentrations every 30 minutes for 2 hours, always in the presence of the initial vasoconstrictor. Images were captured every 5 minutes for analysis (Zen Pro 3.3). Contractility measurements were performed a minimum of 48 hours apart to give EPATs sufficient time to recover.

**Figure 7.** Measurement of EPAT Contractility *in vitro*. Images are taken of EPATs at 5-minute intervals using brightfield microscopy. EPATS are placed in starvation medium for 15 minutes (Blue) followed by 30 minutes period in 75mM KCL (Red) then 30 minutes in 100nM nifedipine, 10µM sildenafil and 50µM SNP (Green).
Materials and Methods

<table>
<thead>
<tr>
<th>Vasodilator</th>
<th>Mechanism</th>
<th>Dose 1</th>
<th>Dose 2</th>
<th>Dose 3</th>
<th>Dose 4</th>
</tr>
</thead>
<tbody>
<tr>
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<td>10nM</td>
<td>100nM</td>
<td>1µM</td>
</tr>
<tr>
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<td>10µM</td>
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<tr>
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<td>Beta-Blocker</td>
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<td>10µM</td>
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<td>10ng/ml</td>
<td>50ng/ml</td>
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<td>Calcineurin inhibitor/BMPR2</td>
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<td>10nM</td>
<td>100nM</td>
<td>1µM</td>
</tr>
<tr>
<td></td>
<td>Activator</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>1µM</td>
<td>10µl</td>
<td>100µM</td>
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<td>1nM</td>
<td>10nM</td>
<td>100nM</td>
</tr>
<tr>
<td>Imatinib</td>
<td>PDGFRβ Inhibitor</td>
<td>100nM</td>
<td>1µM</td>
<td>10µl</td>
<td>100µM</td>
</tr>
</tbody>
</table>

Table 3. **PAH-relevant vasodilators used in contractility experiments.** (IP, prostanoid receptor I; ETR, endothelin receptor; BMPR2, bone morphogenic protein receptor 2; RXFP, relaxin receptor; PDGFRβ, platelet-derived growth factor receptor β).

2.5  **Data Analysis and Sample Size Calculation**

The distance between the posts is inversely proportional to the degree of contractility being exerted (Hansen et al. 2010). Images were exported into TIFF files using Zen Blue software, the distance between the posts was measured using the line function on Image J as seen in Figure 8. These values were copied into Microsoft Excel for further processing.

Baseline recordings were examined to ensure EPATs has established equilibrium in starvation media and contamination via background noise was not present. All recordings were normalised to maximal contractile responses produced to ET-1 or U46619 respectively. EPATs that failed to meet the 5% minimum threshold of contraction when treated with ET-1 or U46619 were excluded from the study. EPAT responses were analysed for any tissues that failed to produce minimum contraction threshold, an adequate dilatory response to vasodilators or failed to complete testing i.e., broke and were excluded from analysis.

Graphs were generated using GraphPad Prism (Version 9.4). GraphPad Prism log (inhibitor) vs response (three parameter) non-linear regression analysis was used to determine IC\(_{50}\) of vasodilators tested. Graphs are presented as mean ± SEM. Biological
replicates are limited by the number of donors currently available from commercial sources and available patient samples.

**Figure 8.** Measurement of change in distance between posts using Image J using the line of function command. Distance measure is indicated by arrows shown.
3 Results

3.1 Custom made PDMS racks improve the evaluation of vasoactive responses, compared to commercial racks.

Custom made silicone racks were produced, using a 3-D printed resin mould designed using computer-aided software and printed at Advanced Hackspace, Imperial College London. To determine if custom-made silicone racks produce comparable results to commercial racks, vasoactive testing was conducted using KCl (75mM). Following the application of KCl for 30 minutes, EPATs on homemade posts demonstrated a -6.73% peak change in length compared to a -5.64% change in length observed using the commercial racks. Custom-made racks produced results comparable to the commercially sourced racks (Figure 9a, b, c).

![Figure 9](image)

**Figure 9.** Validation of custom made PDMS racks compared to commercially available racks. (A). Peak change in length between the two EPAT models, (B). Custom made EPAT as seen under a light microscope, (C). Commercial rack as seen under a light microscope (Biological replicate n=1, technical replicates n=2, independent experiments n=1)

3.2 EPATs are contractile over long term culture.

To investigate the optimal number of PASMCs required for EPATs, the percentage of peak change in length between models containing 500,000 and 1,000,000 cells per tissue, were compared. EPATs were tested on day 7, 14 and 21 using KCl (75mM) as a vasoconstrictor followed by a combination of nifedipine (100nM), sildenafil (10µM) and sodium nitroprusside (SNP; 50µM) to induce vasodilation (Figure 7). Overall, EPATs
**Results**

containing 500,000 cells exhibited diminished vasoactivity over time whereas those with 1,000,000 SMCs maintained contractile function much better over the 28-day period (Figure 10a, b). However, there was a clear reduction in vasodilation from day 14 to day 28 (Figure 10a, b). As this was not significant, future experiments were conducted within a 7-to-28-day period following EPAT fabrication.

![Figure 10](image.png)

**Figure 10. Contractility of EPATs in long term culture. Vasoactivity was measured at Day, 7, 14 and 21. (A). The peak change in length (%) resulting in contraction when EPATs contained 500,000 and 1,000,000 HPASMCs. (B). The peak change in length (%) resulting in dilation when EPATs contained 500,000 and 1,000,000 HPASMCs. (Biological replicate n=1, technical replicates n=2-3, independent experiments n=1)**

### 3.3 Calibration of EPAT model using known vasoconstrictors.

After demonstrating that EPATs are capable of responding to maximal doses of various vasoactive drugs, we tested different doses of three vasoconstrictors (ET-1, U46619 and KCl) to determine which was the most potent (Figure 11a-c). KCl induced a peak change in length around 15-20% (Figure 11a), whereas U46619 and ET-1 caused a 20% and 25% change in peak length, respectively (Figure 11b, c). In addition to the validation of the model, the EC$_{50}$ (half-maximal concentration) of each compound was determined for future experimentation. The EC$_{50}$ of KCl, U46619 and ET-1 acting on SMC within the EPAT model was 16mM, 41µM and 9nM respectively. Establishing the EC$_{50}$ of each agonist's potency within the model aided in both calibrating the model and establishing its translational abilities.
Results

Figure 11. Calibration of EPAT model using known vasoconstrictors. Dose-response curve curves were fitted to obtain the absolute IC50 of each compound. (A) KCL, (B) Endothelin-1, (C) U46619. (Biological replicate n=1, technical replicates n=2-5, independent experiments n=1).

3.4 Evaluation of clinically approved therapies

Therapies for PAH currently used in the clinic were tested in the EPAT model using the protocol previously described (Section 2.4).

Bosentan, an established ET-1 antagonist (Channick et al., 2001), produced a standard dose-response curve in the presence of ET-1. A maximum change in length of 112.4% was observed at Dose 4 of 100µM bosentan (Figure 12a). Bosentan failed to induce vasodilation in a dose responsive manner to U46619. The final concentration of 100µM reduced constriction to 1.7% but prior doses failed to initiate a response in the presence of U46619. Bosentan produced an IC50 of 710nM to ET-1 but failed to generate a proper dose-response curve to U46619 (Table 4). The vasoactive response of bosentan observed in the presence of ET-1 compared to that of a prostanoid both further verified the model’s ability to accurately establish a vasoreactivity profile in vitro.
Results

Propranolol (1µM-1mM), a non-selective beta-adrenergic receptor antagonist was implemented as a negative control given that it should not elicit any vasodilation. As predicted, no dilation was observed in response to ET-1 or U46619 for the lowest three concentrations (Figure 12d). The highest concentration of propranolol (1mM) produced a supramaximal dilatory response of 168.8% and 148.0% to ET-1 and U46619 respectively. The sharp increase in dilation observed in response to the final concentration in the presence of both ET-1 and U46619 indicates potential toxicity.

Both selexipag and epoprostenol, selective prostacyclin receptor agonists were examined using the EPAT model. Selexipag (100nM-100µM) reduced the degree of constriction to 47.9% and 63.3% in the presence of ET-1 and U46619 respectively (Figure 12c). The IC<sub>50</sub> of selexipag was determined as 202nM and 228nM to ET-1 and U46619 respectively (Table 4). A dose-response curve relationship was observed when EPATs were treated with epoprostenol, (1nM-1µM) in response to both ET-1 and U46619 (Figure 12b). Maximum vasoconstriction was reduced to 34.1% and 43.1% in response to ET-1 and U46619 respectively. The IC<sub>50</sub> of epoprostenol varied among the vasoconstrictor present, 17nM and 805pM in response to ET-1 and U46619 respectively. Epoprostenol produced similar maximal responses but different IC<sub>50</sub> values in the presence of both ET-1 and U46619 in the EPAT model. Of the licenced therapies tested in EPAT model, bosentan was the most effective but only against ET-1. Both epoprostenol and selexipag produced modest results in comparison to ET-1 and U46619 but overall, their vasoactive effects were more reproducible in both ET-1 and U46619.

<table>
<thead>
<tr>
<th>Vasodilator</th>
<th>IC50</th>
<th>Maximal Response (Vasoconstriction %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epoprostenol</td>
<td>17nM, 805pM</td>
<td>ET-1: 34.11, U46619: 43.13</td>
</tr>
<tr>
<td>Bosentan</td>
<td>710nM *</td>
<td>ET-1: -7.542, U46619: -</td>
</tr>
<tr>
<td>Propranolol</td>
<td>*</td>
<td>ET-1: -286, U46619: -</td>
</tr>
<tr>
<td>Selexipag</td>
<td>202nM, 228nM</td>
<td>ET-1: 47.91, U46619: 63.33</td>
</tr>
</tbody>
</table>

Table 4. The IC 50 values of vasodilators tested against ET-1 and U46619 calculated by a nonlinear regression analysis. * Did not produce typical sigmoidal shape of a dose-response curve curve.
Results

Figure 12. Clinically licenced therapies for the treatment of PAH validated using EPAT model. Each therapy was tested at 4 concentrations against 10nM ET-1 and 1µM U46619. Each therapy is represented in as a concentration response curve. (A) Bosentan, (B) Epoprostenol, (C) Selexipag, (D) Propranolol. (Biological replicates n= 1, technical replicates n= 4, independent experiments n=1)

3.5 Imatinib: a vasoactive profile observed in healthy patients’ cells.

Imatinib is a tyrosine kinase inhibitor which has been shown to be beneficial in clinical trials for patients with PAH (Solinc et al., 2022) presumably due to its ability to reduce PASMC proliferation. The vasodilatory effects of imatinib are not completely understood and therefore we tested whether imatinib can also affect contractility in EPATs. Imatinib was tested on EPATs composed of HPASMCs from 3 healthy donors. To gain insight into the vasoactive properties of imatinib, 4 concentrations were tested (100Nm-100µM) against ET-1 (10nM) and U46619 (1µM) (Figure 13a, b).

With respect to vasoconstrictors, each donor displayed a varied vasoactive profile. A dose response relationship was observed in Donor 1 to ET-1, with a maximal response of 163.9%. observed (Figure 14a). Donor 1 failed to elicit a dose response in the presence of U46619. Both Donor 2 and 3 failed to exhibit a dose-response relationship.
Results

when treated with Imatinib in the presence of ET-1 and U46619 (Figure 13b & 14b, c). No vasodilatory effects were observed at the 3 lowest concentrations but a sharp increase in dilation is seen at the highest concentration (Figure 13b). Of the 3 healthy donors tested, Donor 1 was determined as a responder to imatinib treatment.

**Figure 13.** Vasoactive response of Imatinib to ET-1 and U46619. Each Donor was tested against 4 concentrations of imatinib against 10nM ET-1 and 1µM U46619. Drug response observed is represented as a concentration response curve (A) Imatinib versus ET-1, as tested on healthy HPASMC Donors 1, 2 and 3. (B) Imatinib versus U46619, as tested on healthy HPASMC Donors 1, 2 and 3 (Biological replicates n= 3, technical replicates n= 14, independent experiments n=3)

**Figure 14.** Donor-dependent response of Imatinib to ET-1 and U46619 in EPATs using healthy HPASMCs. Each donor was tested against 4 concentrations of imatinib against 10nM ET-1 and 1µM U46619. Drug
Results

response observed is represented as a concentration response curve. (A) Donor 1, (B) Donor 2, (C) Donor 3 (Technical replicates n=3-8, independent experiments n=1-2)

3.6 Imatinib on EPATs derived from iPAH-SMCs

Further to the previous testing in the EPAT model, the vasoactive response of imatinib in iPAH-SMCs was examined. Like healthy patient cells, varied vasoactivity was observed in iPAH patient cells dependent on donor. Donor 4 responded well to imatinib treatment in the presence of ET-1. A maximal response of 151.7% was observed with an IC$_{50}$ of 970nM (Figure 15a). Donor 4 failed to produce a dose-response curve in the presence of U46619 (Figure 15b). Additionally, Donor 5 produced a dose-response relationship to both ET-1 and U46619. In the presence of ET-1 Donor 5 produced a maximal response of 112.19% (Figure 5a). Similar to ET-1, a dose-response was also observed in the presence of U46619, but the magnitude of the response was substantial in comparison of previous results obtained. Donor 5 tissues extended beyond their original length, with a maximal response of 302.4% observed (Figure 15b). Both Donor 4 and 5 were determined as responders imatinib as a result of producing at least one dose-dependent response. A clear donor-dependent variability in vasoreactivity to imatinib is observed, with Donors 1, 4 and 5 producing at least one dose-dependent response, with Donors 2 and 3 failing to respond at all.

![Graph A. Imatinib v ET-1](image1)

![Graph B. Imatinib v U46619](image2)

**Figure 15.** Vasoactive response of imatinib to ET-1 and U46619 in iPAH-SMCs. Each Donor was tested against 4 concentrations of imatinib against 10nM ET-1 and 1µM U46619. Donor 1 HPASMCs were implemented as a control. Drug response observed is represented as a concentration response curve. (A) Imatinib in the presence of 1µM U46619, using EPATs fabricated of Donor 1, Donor 4 and Donor 5 (B) (Biological replicates n= 3 technical replicates n=3-4, independent experiments n=1).
Results

![Graphs showing vasoconstriction (%)](image)

**Figure 16.** *Donor-dependent response of Imatinib to ET-1 and U46619 in EPATs using iPAH-SMCs.* Each Donor was tested against 4 concentrations of imatinib against 10nM ET-1 and 1µM U46619. (A) Direct comparison of vasoconstriction observed in Donor 4 treated with imatinib in the presence of 100nM ET-1 and 1µM U46619. (B) Direct comparison of vasoconstriction observed in Donor 5 treated with imatinib in the presence of 100nM ET-1 and 1µM U46619. (Biological replicates n=3 technical replicates n=3-4, independent experiments n=1)

### 3.7 Vasoactive Profile of Serelaxin

Serelaxin is a recombinant form of human relaxin-2 and was assessed as a potential PAH therapeutic using EPATs. To determine the vasoactive profile of serelaxin, 4 concentrations of serelaxin (100pM-100nM) were tested against 10nM ET-1 and 1µM U46619. Distinct results can be observed among donors and vasoconstrictors used.

Overall, all 3 donors had a response to U46619 when treated with serelaxin (Figure 17b), with a dose response produced across the three donors. In response to ET-1 and U46619, a maximal change in length of 84.69% and 122% was observed respectively. In Donor 2, serelaxin resulted in a total vasoconstriction being reduced to 15.9% and 33.0% in response to ET-1 and U46619 respectively. Overall, Donor 3 produced more modest vasodilatory effects. Serelaxin caused a change in length of 84.1% and 81.8% in response ET-1 and U46619 respectively. All three donors responded well to serelaxin in the presence of both vasoconstrictors, producing dose-response curves. These results offer a potential opportunity for the repurposing of serelaxin for the treatment of PAH.
Results

Figure 17. Vasoactive response of serelaxin to ET-1 and U46619. Each donor was tested against 4 concentrations of serelaxin against 10nM ET-1 and 1µM U46619. Drug response observed is represented as a concentration response curve (A) serelaxin versus ET-1, as tested on healthy HPASMC Donor 1, 2 and 3. (B) serelaxin versus U46619, as tested on healthy HPASMC Donor 1, 2 and 3. (Biological replicates n= 3 technical replicates n=2-6, independent experiments n=1-2)

Figure 18. Donor-dependent response of Serelaxin to ET-1 and U46619 in EPATs using healthy HPASMCs. Each donor was tested against 4 concentrations of serelaxin against 10nM ET-1 and 1µM U46619. Drug response observed is represented as a concentration response curve. (A) Donor 1, (B) Donor 2, (C) Donor 3 (Biological replicates n= 3 technical replicates n=2-6, independent experiments n=1-2)
Discussion

4. Discussion

The aim of this study was to (1) optimise production of EPAT model and conditions of cell culture, (2) validate EPATs for the measurement of SMC contractility in vitro and (3) evaluate vasoactive effects of selected current and emerging PAH therapeutics.

4.1 Key Findings:

1. EPATs were optimised for \textit{in vitro} modelling of PASMC responses.
2. EPATs can be used to evaluate vasoactive properties of PAH therapies.
3. Imatinib shows a donor-dependent response.
4. Serelaxin has the potential to be repurposed for the treatment of PAH.

4.2 Optimisation of EPATs for \textit{in vitro} modelling

Established vasoactive compounds were tested on both custom made and commercial racks, with custom made racks producing results comparable to that of the commercially sourced racks. SMCs cultured in custom made racks produced a contractile phenotype, as previously observed in cells cultured in commercial racks (Hansen et al., 2010). The use of custom-made racks allowed for increased oversight into the design of the racks and flexibility for modifications.

Further optimisation of the model included verifying the long-term functionality of EPATs. At Day 14 and 21 EPATs maintained their contractile phenotype and produced a notably greater contractile and dilatory response with 1,000,000 cells compared to that of 500,000 cells. In demonstrating the ability to remain in culture for up to 30 days, a high volume of repeat testing could be achieved on EPATs. When compared to alternative \textit{in vitro} models available, EPATs offer a higher throughput of drug screening in addition to the direct comparison of individual EPAT responses under identical conditions.
4.3 **EPATs are an accurate model to evaluate vasoactive properties of PAH therapies**

Current therapies administered in the clinic for the treatment of PAH were tested in the EPAT model against ET-1 and U46619, allowing for a baseline response to be established to known vasodilators. Overall, the model successfully mimicked the vasodilatory action of several clinically validated therapies: bosentan, selexipag and epoprostenol. Propranolol, a non-selective beta-adrenergic receptor antagonist was implemented as a negative control given that it should not elicit any vasodilation – which was also the case our results. This study indicates that of the 3 clinically validated vasodilators tested, bosentan was the most effective but only against ET-1. Both epoprostenol and selexipag produced modest results in comparison to ET-1 and U46619 but overall, their vasoactive effects were more consistent in response to both ET-1 and U46619.

Further to the therapies analysed in this study, emerging therapies are potential candidates for vasoactive profiling in the EPAT model, to better understand their pharmacological impact. Novel mechanisms targeted by drug trials in pulmonary hypertension offer a new avenue for drug development and in turn will require comprehensive profiling regarding vasoactive properties.

Therapies such as sotatercept, would benefit from testing using the EPAT model. Gaining a comprehensive understanding of new therapies will allow for a quicker development process. Sotatercept functions as a ligand trap for TGF-β superfamily members; regulating the balance between growth promoting activin differentiation factor pathway and growth inhibiting BMP pathway (Yung et al., 2020). Sotatercept has undergone phase II clinical trials and has demonstrated a reduction in pulmonary vascular resistance. Exposing the EPAT model to PAH therapies with alternative mechanisms will allow further insight into these novel therapies.

4.4 **Imatinib: A potential therapeutic to target vascular constriction**

Imatinib is an orally active tyrosine kinase inhibitor (TKI), that inhibits platelet-derived growth factor receptors (PDGFR) (Capdeville et al., 2002). TKIs such as imatinib are approved for the treatment of malignant diseases (Curran et al., 2004; Demetri et al.,
Discussion

2002). PDGF has also been shown to play a key role in the progression of pulmonary hypertension, limiting vascular smooth muscle cell proliferation (Schermuly et al., 2011).

Previous studies carried out in animal models have demonstrated the efficacy of imatinib in reversing pulmonary hypertension via inhibition of cell-growth related kinases and mitigation of vascular remodelling (Schermuly et al., 2005), which had been supported by two-placebo controlled randomised clinical trials (Ghofrani et al., 2010; Hoeper, Barst, et al., 2013). Results indicated clinical efficacy in the treatment of PAH, but concerns were raised following the Phase III IMPRES trial (Hoeper, Barst, et al., 2013), where eight patients developed a subdural haematoma while taking imatinib, resulting in two deaths.

Despite safety concerns, imatinib has attracted considerable interest. Due to current available therapies lacking the ability to halt disease progression, there is a clinical need for PAH-specific therapies that target the genetic and molecular mechanisms behind small-vessel loss and obstructive vascular remodelling. Imatinib remains a viable PAH candidate, since it has been suggested that it may act as a vasodilator (Abe et al., 2011; Rieg et al., 2019) in addition to its role in inhibiting vascular remodelling.

The EPAT model was implemented to assess potential vasoactive effects of imatinib in response to ET-1 and U46619. Both healthy and idiopathic PAH patient cells were used to assess imatinib. Interestingly, there was a clear donor-dependent variability in vasoreactivity to imatinib, with Donors 1, 4 and 5 producing at least one dose-dependent response, with Donors 2 and 3 failing to respond at all. The donor-dependent response of imatinib would indicate a genetic component to the variation in responses observed – despite our small sample size of 5 donors. Previous studies have suggested as a result of PDGFRβ being an important target of imatinib in PAH, the PDGFR genotype may influence therapeutic response, or the dose required to elicit a response in patients (Wilkins et al., 2021).

The mechanism in which imatinib causes vascular dilation is yet to be verified. Interestingly, in preliminary experiments using the EPAT model, imatinib failed to elicit vasoconstrictor effects in the absence of a vasoconstrictor (data not shown). There are several potential mechanisms by which imatinib may elicit pulmonary vasodilation in vascular smooth muscle cells. These include: (1) PDGF receptor mediated inhibition via elevation of intracellular Ca²⁺ levels. The activation of MLCP via Ca²⁺ desensitisation results in endothelium independent vascular smooth muscle relaxation (Hughes, 1995),
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(2) inhibition of alternative off target protein kinases; epidermal growth factor receptor, Src and protein kinase C (Fernandez-Patron, 2007) and (3) inhibition of c-Abl-mediated actin polymerisation (Tang & Anfinogenova, 2008). The precise mechanism of dilatory effects of imatinib remains to be established but our data clearly indicates a direct effect of the drug on PASMC vasoreactivity.

Following on from imatinib studies using the EPAT model, novel approaches utilising imatinib data may be investigated. The further development of a highly specific tyrosine kinase inhibitor such as seralutinib offers a potential alternative for imatinib. Seralutinib is a potent tyrosine kinase inhibitor that targets the colony stimulating factor 1 receptor, c-Kit, PDGFR as well as increasing bone morphogenic protein receptor type 2 (BMPR2) signalling in the pulmonary vasculature (Galkin et al., n.d.). The EPAT model has the ability to be implemented for testing which will allow for a greater insight into the therapeutic potential of this compound.

4.5 Serelaxin has the potential to be repurposed as a treatment for PAH.

Serelaxin is a recombinant form of human relaxin 2, a vasodilator hormone that is known to contribute to both cardiovascular and renal adaptions during pregnancy (Bathgate et al., 2013; Du et al., 2010). The vasodilatory effects of serelaxin have raised interest due several mechanisms that could potentially influence the cardiovascular system.

Studies have been carried out to investigate the potential use of serelaxin in the treatment of acute heart failure. The Relax-AF-2 trial was conducted (Metra et al., 2019), where serelaxin significantly reduced blood pressure but failed to lower cardiovascular mortality within the desired timeframe and in some patients resulted in worsening of heart failure. In summary, it was determined that serelaxin would not be suitable for the treatment of acute heart failure following a failed phase III study (Teerlink et al., 2013). As serelaxin has undergone multiple phases of the clinical trial process, high quality safety and tolerability data have been obtained. The lack of efficacy in treating acute heart failure, allows for the potential repurposing of serelaxin for alternative cardiovascular diseases such as PAH. The EPAT model provides a potential platform to re-enter the compound into the drug development pipeline.

The relaxins induce vasodilatory effects via their interaction with the nitric oxide pathway, mediating nitric oxide production via endothelial nitric oxide synthase (eNOS) (Nistri &
Discussion

Bani, 2003) and inducible nitric oxide synthase (iNOS) stimulation (Li et al., 2014). Additionally, they increase vascular endothelial growth factor (VEGF) (Li et al., 2014) and the matrix metalloproteinases, MMP-2 and MMP-9. VEGF is a known contributor to vasodilation mediation (Ho et al., 2007). Further vasodilatory mechanisms are initiated via relaxins, through the inhibition of ET-1 production. The ET$_B$ receptor is involved in the mediation of ET-1 removal and release of nitric oxide (NO) from the endothelium. By acting as an ET$_B$ agonist, this process is upregulated by relaxin which plays a major role in its function (Dschietzig et al., 2003)

Repurposing serelaxin offers several advantages over developing a novel therapy. It reduces research and development costs significantly. The measures taken to reposition a therapy have the potential to reduce high attrition rates associated with R&D. Additionally, compounds such as serelaxin have demonstrated tolerability and safety in humans and therefore do not require Phase I clinical trials. The EPAT model has the potential to be a fundamental tool in identifying potential candidates to reposition for the treatment of PAH. A vasoactive profile of these compounds will offer insight into their potential as new therapies.

4.6 Study Limitations

The EPAT model does not reflect the cellular environment observed in pulmonary vasculature as it bypasses potential influence of endothelial cells and fibroblasts. Additionally, the model is not perfusable and fails to consider external influences such as flow, pressure, and wall shear stress on vascular smooth muscle cell remodelling.

Another limitation of EPATs is that they require relatively high numbers of cells (1,000,000 cells per tissue), which precludes the use of less abundant cell sources such as blood-derived SMC (BOSMCs) (Ahmetaj-Shala et al., 2021). A further limitation of the model is its ability to accurately represent the structure of a blood vessel.

Currently, organ-on-a-chip models combat some of these limitations seen in the EPAT model, but ultimately fail to measure vasoactive properties. At the current stage in EPAT development, the organ-on-chip model provides insight in mechanisms and properties which EPATs are unable to explore, such as EC-SMC crosstalk and cell proliferation. It should be noted that the benefit of the EPAT model is its ability to measure vasoactivity in vitro, which organ-on-chip fails to achieve. In vitro models should be implemented in
Discussion

a collaborative manner to utilise the resources available and gain further insight into the mechanisms underlying molecular and cellular changes observed in disease.

The limited number of biological and technical replicates obtained throughout this study occurred as a result of restricted access to patient cells and low commercial donor availability. Currently the EPAT model lacks scalability and is considered a medium throughput system that allows for the identification of novel therapies and mechanisms. The viability of cells in the EPAT model is yet to be assessed. Therefore, proliferation and apoptosis have not been monitored. As a result, optimal culture conditions for EPATs may not be in use. It has previously been shown that approximately 20-30% of cells are lost while during the fabrication of these tissues (Hansen et al., 2010). Phenotypic characterisation of HPASMCs has not been carried out to compare cells in 2D culture to those in EPATs.

4.7 Future Studies

Next steps will include the introduction of multiple cell types into the EPAT model, including pulmonary artery endothelial cells and fibroblasts. This will allow for a more comprehensive model that would more closely represent cell-cell interactions seen in the pulmonary vasculature in vivo. Additionally, growth factors such as IGF-1 and substrates such as collagen, fibronectin and proteoglycan may be introduced to aid extracellular matrix composition.

To gain further insight into the observed donor-dependent vasodilatory effects of imatinib and/or serelaxin, genomic, transcriptomic, and proteomic analyses of donor cells may be carried out to identify potential mediators of this response. In addition, it would be interesting to explore the differences in vasoactive responses according to sex, as PAH predominantly affects females (Humbert et al., 2006).

Future work will include repeat testing of therapies from this study and the acquisition of more biological donors, to increase the statistical power of the observed effects. Furthermore, EPATs could theoretically be fabricated using HPASMCs derived from induced pluripotent stem cells (iPSCs), which could also be obtained from PAH patients. The use of iPSCs would allow for patient specific vasoactive profiling and in turn allow for the testing of patient specific treatments.
Discussion

4.8 Summary of Main Findings

- The aim of this study was to fabricate, optimise and validate an engineered pulmonary arterial tissue model to evaluate the vasoactive effects of current and emerging PAH therapies.
- EPATs were functionally validated and shown to survive in culture for up to 30 days, maintaining their vasoactive properties.
- Calibration of the model using known vasoconstrictors provided insight into EPAT activity. Further testing using clinically approved therapies allowed for baseline responses to be established.
- Imatinib, a tyrosine kinase inhibitor, produced a donor-dependent vasodilatory response in the EPAT model. Potential mechanisms of imatinib dilation are yet to be explored. Imatinib demonstrated vasodilation in both healthy and pulmonary hypertension patient cells. Donor-dependent responses suggest a genetic component may be affecting the therapeutic potential of this drugs.
- Serelaxin, a recombinant form of human relaxin 2, demonstrated potential vasoactive properties within the EPAT model, suggesting that the drug may potentially be considered for repositioning.

Overall, the successful fabrication and validation of the EPAT allowed for the evaluation of vasoactive properties of selected current and emerging PAH therapeutics.
Conclusion

5. Conclusions

In summary this study developed a new *in vitro* model using vascular smooth muscle cells to which allowed for the measurement of smooth muscle cell contractility. As a result, both established and potential therapies for the treatment of PAH were validated and profiled with regards to their vasoactive properties.

Despite the advances in drug development, a cure for PAH has yet to be developed and therefore continues to be an unmet clinical need. PAH-specific therapies targeting the genetic and molecular mechanisms behind small-vessel loss and obstructive vascular remodelling are required to progress the treatment of this disease. With the availability of omics, molecular tools, and *in vitro* modelling, resources are available to carry out comprehensive and collaborative preclinical studies. Despite these advancements the translational gap remains high and contributes greatly to the elevated attrition rates associated with drug development.

The development of accurate and representative preclinical *in vitro* models will reduce Phase II and Phase III attrition rates during drug development and promote more sustainable R&D costs. New, more physiologically relevant *in vitro* systems are required to identify novel therapeutic targets and improve a drugs ability to be clinically translatable.
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