The Role of Connexin 43 in the Function of Systemic and Pulmonary Blood Vessels

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Abstract

Background: Connexins are membrane channel-forming proteins that play a pivotal role in direct intercellular communication within the vasculature. They are crucial in mediating various aspects of vascular physiology such as vasodilation and vasoconstriction, and also play a significant role in vascular pathology. The aim of this research is to elucidate the roles of connexins, particularly connexin 43 (Cx43), in the regulation of systemic and pulmonary circulations. The hypothesis is that Cx43 critically influences vascular reactivity and remodelling, which is particularly evident in pathological conditions like pulmonary hypertension. By investigating these roles, this study seeks to deepen our understanding of Cx43's multifaceted functions in both physiological and pathophysiological contexts, including its involvement in disorders such as atherosclerosis, systemic hypertension, and pulmonary hypertension (PH).

Introduction: Connexin 43 (Cx43) is the most abundant isoform within the vasculature, and this thesis will examine its role in regulation of both the systemic and pulmonary circulation. In chapter 3 and 4, the role of Cx43 will be investigated in the systemic and pulmonary circulations respectively using mouse thoracic aortae, mouse intra-lobar pulmonary arteries (IPAs) and lungs. Pulmonary hypertension is a severe condition characterised by both pulmonary vascular remodelling and abnormal vasoconstriction, resulting in increased pulmonary vascular resistance and eventually leading to right-sided heart failure and death. Given the significant contribution of pulmonary artery fibroblasts (PAFs) to pulmonary vascular remodelling, chapter 5 will focus on examining the role of connexin 43 in proliferation and migration of mouse PAFs (MPAFs).

Methods: Thoracic aortae from 4- to 6-month-old mice and lungs and IPAs from 2- to 3-month-old mice were dissected free from age-matched female and male wild-type (WT) mice and connexin 43 heterozygous ($Cx43^{+/-}$) mice from a C57BL/6 background. Using pentobarbital sodium (60mg/kg) and lidocaine (4mg/kg), mice were euthanized by intraperitoneal injection (i.p.). In all experiments, the role of Cx43 in the systemic and pulmonary vasculature was investigated using both pharmacological and genetic approaches. The gap junction blocker ^{37,43}Gap27 was employed to pharmacologically inhibit Cx43 function. Additionally, $Cx43^{+/-}$ mice were used, wherein Cx43 expression is genetically reduced, not completely

inhibited, providing a model to understand the impact of lower Cx43 levels on vascular function. The use of $Cx43^{-/-}$ mice, which completely lack Cx43, was not feasible due to lethal developmental defects that these mice experience, making them unsuitable for such studies. In the first part of this study, wire myography was used to assess both systemic and pulmonary vascular reactivity of thoracic aortae and IPAs. NO production was then investigated in whole thoracic aortae and lung tissues using a Sievers 280 analyser. Western blot was also used to investigate the protein expression of Cx43 and eNOS in the thoracic aortae and lungs. Cell culture was then used to examine the role of Cx43 in MPAFs. MPAFs were explanted from main and branch pulmonary arteries and were exposed to normoxic or hypoxic (5% O_2) conditions for 24h. Proliferation and migration were assessed using an automated cell counter or a scratch assay respectively.

Results: Pharmacological inhibition and genetic reduction of Cx43 had effects on vascular reactivity and NO production in both the systemic and pulmonary circulations. In all of the experiments presented in this thesis on systemic and pulmonary vasoreactivity and NO assays, no significant differences between sexes were found. This study found that Cx43 associates with endothelial nitric oxide synthase and exists in the same protein complexes within whole thoracic aortae and lungs from both genotypes. This study further revealed that Cx43 significantly promotes the proliferation and migration of MPAFs under hypoxic conditions. This could ultimately lead to the remodelling of the pulmonary vasculature and subsequently leading to pulmonary hypertension.

Conclusion: This study has shown that Cx43 plays a role in systemic and pulmonary vasoreactivity and interacts with NO signalling pathways, indicating its potential as a pharmacological target. It is further demonstrated in this study that Cx43 is involved in hypoxic-induced cell proliferation and migration. Thus, Cx43 may be involved in the pulmonary vascular remodelling in response to hypoxia. This cellular process is of significant interest in the context of pulmonary vascular changes, and these findings should be seen as a foundation for further understanding the complex role of Cx43 in conditions like PH. Future research should investigate deeper into elucidating the specific molecular pathways involved and assessing the implications of targeting Cx43 therapeutically.

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Author’s Declaration

I declare that the work presented in this thesis has been carried out by me unless otherwise stated. It is completely of my own composition and has not in whole or in part been submitted for any other degree.

Saad Mohammednoor M Wali

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Abbreviations

ACh: Acetylcholine

ADMA: Asymmetric dimethyl arginine

ANOVA: Analysis of variance

BCA: Bicinchoninic acid

BMPR-II: Bone morphogenetic protein receptor type II

BSA: Bovine serum albumin

$[Ca^{2+}]_i$: Intracellular calcium

cGMP: Cyclic guanosine monophosphate

CH: Chronic hypoxia

co-IP: Co-immunoprecipitation

CRCs: Concentration-response curves

Cx(s): Connexin(s)

$Cx43^{-/-}$ mouse: Cx43 homozygous mouse (full knockout)

$Cx43^{+/-}$ mouse: Cx43 heterozygous mouse (partial knockout)

DMEM: Dulbecco's modification of Eagle's medium

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

EDHF: Endothelium-derived hyperpolarizing factor

E_{max}: Maximal contractile response

ERK1/2: Extracellular signal-regulated kinase 1/2

ET: Endothelin

FBS: Foetal bovine serum

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GJs: Gap Junctions

GJA1: Gap Junction Protein Alpha 1

GTP: Guanosine triphosphate

HCs: Hemichannels

hPAH: Heritable PAH

HPV: Hypoxic pulmonary vasoconstriction

HUVECs: Human umbilical vein endothelial cells

iPAH: Idiopathic PAH

IPAs: Intra-lobar pulmonary arteries

KCl: Potassium chloride

LDS: Lithium dodecyl sulfate

Log EC_{50}: Logarithm of median effective concentration

MAPK: Mitogen-activated protein kinase

MCh: Methacholine

MCT: Monocrotaline

MEJs: Myoendothelial gap junctions

miR-1: MicroRNA-1

MPAFs: Mouse pulmonary arterial fibroblasts

MW: Molecular weight

N_2: Nitrogen

NaCl: Sodium chloride

NaI: Sodium iodide

$NaNO_2$: Sodium nitrite

neo^r: Neomycin resistance gene

NO: Nitric oxide

NO_2^-: Nitrite

O_3: Ozone

PAECs: Pulmonary arterial endothelial cells

PAFs: Pulmonary artery fibroblasts

PAGE: Polyacrylamide gel by electrophoresis

PAH: Pulmonary arterial hypertension

PASMCs: Pulmonary arterial smooth muscle cells

PBS: Phosphate buffered saline

p-Cx43: Phospho-connexin 43

PGI_2: Prostacyclin

PH: Pulmonary hypertension

PSS: Physiological salt solution

PTMs: Post-translational modifications

PVAT: Perivascular adipose tissue

PVDF: Polyvinylidene fluoride

PVR: Pulmonary vascular remodelling

RIPA: Radio-Immune Precipitation Assay

R_{max}: Maximal relaxation response

SDS: Sodium dodecyl sulfate

5-HT: Serotonin

SFM: Serum-free media

SHRs: Spontaneously hypertensive rats

siRNA: Small interfering RNA

SNAP: S-Nitroso-N-acetylpenicillamine

TAE buffer: Tris-Acetate-EDTA buffer

TBS: Tris buffered saline

TBS-T: Tris buffered saline-Tween 20

TGF-ß: transforming growth factor-ß

t-Cx43: Total connexin 43

t-eNOS: Total endothelial nitric oxide synthase

WT mouse: Wild-type mouse

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Chapter 1 General Introduction

1.1 Cardiovascular System

The cardiovascular system consists of the heart, blood vessels and blood. The heart is the pump that circulates the conducting medium, the blood, through the vasculature, which is a network of conduits for movements of blood. The circulatory or vascular system not only circulates blood cells, but it is also the transport medium for oxygen, carbon dioxide, hormones, immune cells and nutrients such as amino acids and electrolytes. These are carried to and from the cells, thereby helping to combat disease, provide nourishment and maintain homeostasis, temperature and pH (Hoar et al., 1992). The cardiovascular system comprises two distinct circulations - the systemic and the pulmonary circulations. Cardiovascular disease describes multiple conditions affecting the heart or circulation, such as atherosclerosis, stroke and pulmonary hypertension.

1.1.1 Systemic Circulation

As the blood travels from the heart to all parts of the body, excluding the lungs, it is called systemic circulation. It comprises the left side of the heart and the oxygenated blood that transports oxygen and nutrients to all cells in the body. This blood leaves the left ventricle through the aorta, which branches off into various large arteries including the carotid artery, which supply blood to the brain, and renal arteries, which supply blood to the kidneys. Oxygen and other nutrients in the blood are delivered to cells and carbon dioxide and other waste materials are picked up by the blood and carried to the veins. From the veins, blood returns to the right atrium of the heart via the largest vein, the vena cava (Hoar et al., 1992) (Figure 1-1).

1.1.2 Pulmonary Circulation

The pulmonary circulation is responsible for moving blood between the lungs and the heart. It starts from the right side of the heart, where de-oxygenated blood is pumped from the right ventricle into the pulmonary artery for transport to the lungs. The lungs have a rich network of capillaries which surround the alveoli and are the cavities in which gas exchange takes place. The carbon dioxide is removed, and the blood is re-oxygenated before being returned through the pulmonary veins to the left atrium of the heart (Richardson, 1987) (Figure 1-1).

The pulmonary circulation's role is oxygenation of the blood whereas the systemic circulation transfers oxygen and nutrients to tissues and removes the metabolic waste. Structurally, the walls of the pulmonary circulation system are substantially thinner than correspondingly-sized vessels in the systemic circulation. As it branches, the pulmonary artery splits quickly into branches with thin walls, and consequently larger internal diameters than similarly sized systemic arteries. The pulmonary vessels comprise of substantially less vascular smooth muscle than that of the systemic vessels, and there are no muscular vessels equivalent to the systemic arterioles in the pulmonary system (Hughes and Morrell, 2001) (Table 1-1).

Both the pulmonary and systemic circulatory systems manage an equivalent cardiac output of around 5L/min. However, they operate under markedly different pressure conditions. The pulmonary arteries function within a normal mean pressure range of 9-16 mmHg, which is significantly lower compared to the typical mean pressure of 90 mmHg found in the systemic arteries (Olufsen et al., 2012). This disparity is reflective of the lower resistance characteristic of the pulmonary circulation, as opposed to the higher resistance observed in the systemic circulation. Such a difference in resistance directly stems from their structural distinctions, playing a crucial role in meeting the specific functional demands of each system (Costanzo, 2009). Additionally, the pulmonary vasculature uniquely specialises in metabolising substances such as serotonin and prostaglandins, highlighting roles that extend beyond mere gas exchange (Yeazell and Littlewood, 2011) (Table 1-1).

The lower level of musculature in the pulmonary system results in lower resistance to blood flow, as compared to the systemic vasculature. The pulmonary vessels are more flexible, facilitating greater levels of distension and compression than the systemic arteries (Townsley, 2012). Combined, these factors result in substantially lower levels of pressure within the pulmonary vessels than the systemic vessels. One of the key features which distinguish pulmonary and systemic circulatory systems is their response to hypoxia. In the pulmonary system, hypoxia results in vasoconstriction, whereas it induces vasodilation in the systemic circulation (Table 1-1).

The contrasting responses to hypoxia in the pulmonary and systemic circulations are thought to have developed as a means to enhance the efficiency of oxygen distribution to various tissues under different physiological circumstances. In the systemic circulation, hypoxia prompts vasodilation, thereby enhancing the blood flow to tissues that are lacking in oxygen. However, in the pulmonary circulation, the response to hypoxia is quite the opposite: vasoconstriction takes place. This mechanism serves to redirect the blood flow from areas with poor oxygenation to those with better oxygen levels within the lungs. This adjustment ensures the optimization of gas exchange, as it improves the coordination between ventilation (air flow) and perfusion (blood flow) in the lungs (Waypa and Schumacker, 2010). The precise mechanisms underpinning these phenomena remain not fully elucidated, although several mechanisms have been proposed (reviewed recently in more detail in Böger and Hannemann (2020) and Hannemann and Böger (2022))

In response to hypoxia, the pulmonary and systemic circulations exhibit distinct physiological reactions due to varying cellular and molecular mechanisms. Pulmonary arteries constrict under hypoxic conditions, a process termed Hypoxic Pulmonary Vasoconstriction (HPV). This response is thought to involve mitochondria acting as oxygen sensors, triggering redox signalling pathways that lead to vasoconstriction. Such pathways include the inhibition of potassium channels in pulmonary arterial smooth muscle cells, leading to cell depolarization and calcium influx (Waypa and Schumacker, 2010). In contrast, systemic arteries dilate in response to hypoxia, a mechanism that involves the accumulation of metabolites like adenosine, activation of hypoxia-inducible factors (HIFs), and upregulation of vasodilators such as nitric oxide (NO) (Böger and Hannemann, 2020, Hannemann and Böger, 2022). These contrasting responses optimise oxygen delivery in the body: pulmonary vasoconstriction redirects blood flow away from hypoxic lung areas, improving gas exchange, while systemic vasodilation increases blood flow to oxygen-deprived tissues.

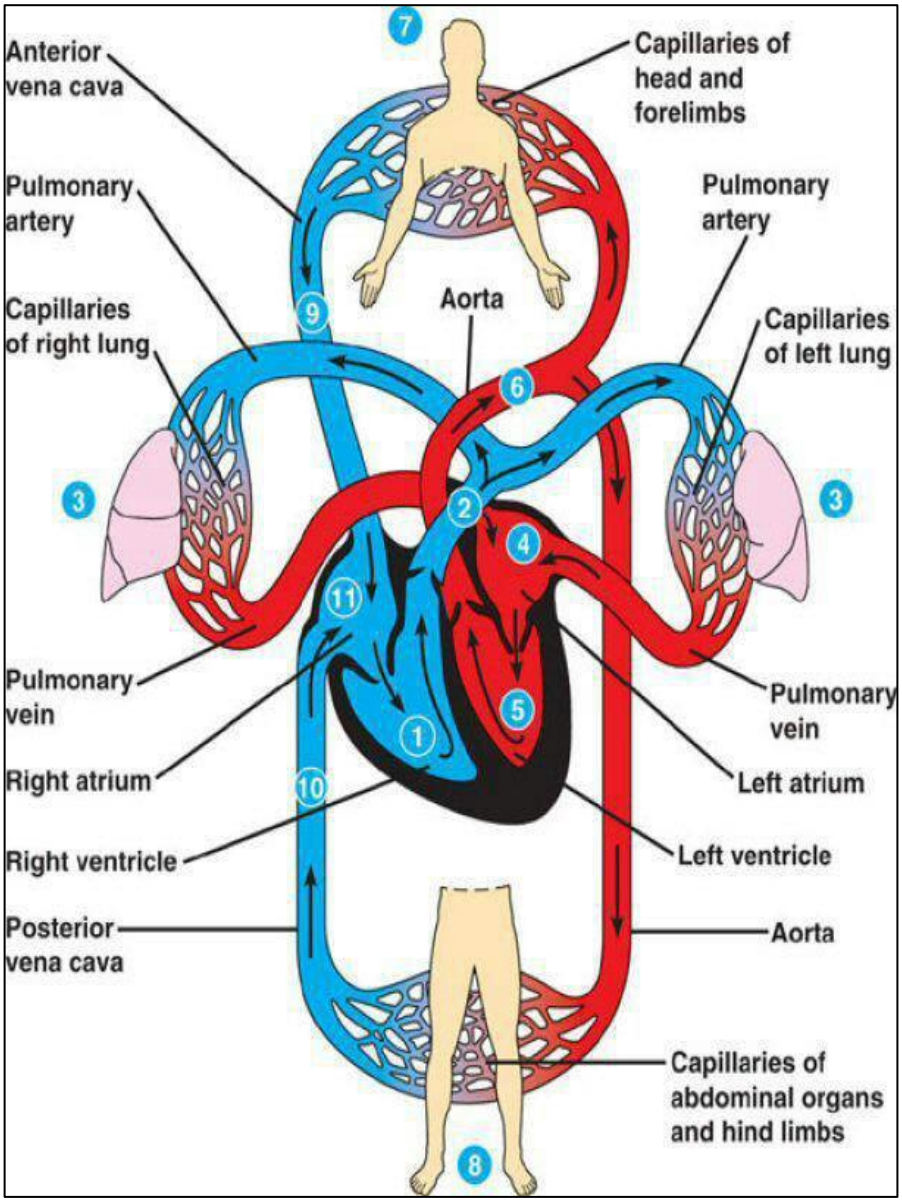


Figure 1-1. Illustration of the cardiovascular system and the route of blood circulation. Starting at the right ventricle (1), deoxygenated blood is sent to the lungs (3) via the pulmonary artery (2). After oxygenation, it returns to the left atrium (4), moves into the left ventricle (5), and is then distributed throughout the body by the aorta (6). Oxygen and nutrients are delivered to body tissues (7, 8), and the deoxygenated blood returns to the heart through the vena cava (9, 10), completing the cycle at the right atrium (11), adapted from Erlinger (2017).

A Comparison of the Pulmonary and Systemic Circulations			
Category	**Pulmonary circulation**	**Systemic circulation**	**References**
Anatomy	Thin vessels	Thick vessels	(Hughes and Morrell, 2001)
Blood volume	~500ml, in a 70kg person	~4500ml, in a 70kg person	(Burton, 2000)
Reservoir function	~10% of the total blood volume	~90% of the total blood volume	(Schönberger et al., 1995)
Blood flow	= cardiac output (~ 5L/min)	= cardiac output (~ 5L/min)	(Olufsen et al., 2012)
Blood pressure	Normal PA systolic pressure= 18-25mmHg Normal PA diastolic pressure= 8-15mmHg Normal mean pulmonary arterial pressure= 9-16mmHg	Normal aortic systolic pressure= 120mmHg Normal aortic diastolic pressure= 80mmHg Normal mean systemic pressure= 90mmHg	(Olufsen et al., 2012)
Circulatory resistance	Low resistance (100-200 dynes.sec.cm^{-5})	High resistance (900-1200 dynes.sec.cm^{-5})	Townsley, 2012)
Response to hypoxia	Vasoconstriction	Vasodilation	(Brij and Peacock, 1998)
Metabolic functions	Metabolism of -hydroxytryptamine, prostaglandins and substrates for angiotensin-converting enzyme (bradykinin and angiotensin I)	Delivery of metabolic substrates to organ systems, and removal of metabolic wastes	(Suresh and Shimoda, 2016)

Table 1-1. A comparison between the pulmonary and systemic circulations in their structure and functions. PA represents pulmonary artery. Adapted from Alex Yartsev (2021).

1.2 Pulmonary Hypertension (PH)

Pulmonary hypertension (PH) is defined by a mean pulmonary artery pressure ≥ 20 mmHg at rest with abnormal pulmonary vascular resistance of ≥ 3 Wood units ($mmHg \cdot L^{-1} \cdot min \cdot m^2$) measured during right heart catheterization (Hoeper and Humbert, 2019). The right ventricle, which pumps the blood to the lungs, experiences significant strain due to the increased pressure in the pulmonary arteries. The effect of this excess pressure on the right ventricle over time causes it to undergo structural adaptations and become weaker and less efficient at pumping blood. Ultimately, this can result in right-sided heart failure, which has the potential to be fatal. While PH is a relatively rare condition, its incidence is significant and poses a considerable health challenge. Recent studies estimate that the incidence of PH varies globally, but generally ranges from 1 to 2 cases per million people per year, emphasising its rare yet impactful nature (Ruta et al., 2023). Symptoms of pulmonary hypertension most commonly present as shortness of breath with exertion and fatigue and if untreated, the severity of the symptoms may increase (Matura et al., 2012). This in turn can have a significant effect upon the individual's physical, psychological and social well-being and ability to function (Guillevin et al., 2013). This debilitation may account for the elevated levels of depression experienced by patients with PH (McCollister et al., 2010).

1.2.1 Classification of Pulmonary Hypertension

According to Simonneau et al. (2019), PH is clinically categorised into five different classifications: 1) pulmonary arterial hypertension (PAH); 2) PH due to left heart disease; 3) PH due to lung diseases and/or hypoxia; 4) PH due to pulmonary artery obstructions; 5) PH with unclear and/or multifactorial mechanisms. It is important to note that the key difference between PAH and other PH categories is that PAH is a standalone condition with elevated pulmonary pressures, whereas other PH categories result from another underlying medical condition (Table 1-2). These categories are widely accepted and applied by pulmonary hypertension experts, the European Agency for Drug Evaluation and the US Food and Drug Administration (Simonneau et al., 2019).

PAH is a complex condition with multiple causes, including idiopathic, hereditary, and associations with other medical conditions (Table 1-2). Despite its rarity, PAH presents significant challenges and is a critical focus of research and clinical interest. Understanding its underlying mechanisms and genetics is essential for enhancing diagnosis, prognosis, and treatment.

1. **Pulmonary arterial hypertension (PAH)**
    - Idiopathic PAH (iPAH)
    - Heritable PAH (hPAH)
    - Drug and toxin-induced PAH
    - PAH associated with:
        - Connective tissue disease
        - Human immunodeficiency virus (HIV) infection
        - Portal hypertension
        - Congenital heart diseases
        - Schistosomiasis
    - PAH long-term responders to calcium channel blockers
    - PAH with overt features of venous/capillaries (pulmonary veno-occlusive disease (PVOD)/ pulmonary capillary haemangiomatosis (PCH) involvement
    - Persistent PH of the newborn syndrome

2. **Pulmonary hypertension (PH) due to left heart disease**
    - PH due to heart failure with preserved left ventricular ejection fraction (LVEF)
    - PH due to heart failure with reduced LVEF
    - Valvular heart disease
    - Congenital/acquired cardiovascular conditions leading to post-capillary PH

3. **PH due to lung diseases and/or hypoxia**
    - Obstructive pulmonary disease
    - Restrictive lung disease
    - Other lung disease with mixed restrictive/obstructive pattern
    - Hypoxia without lung disease
    - Developmental lung disorders

4. **PH due to pulmonary artery obstructions**
    - Chronic thromboembolic PH
    - Other pulmonary artery obstructions

5. **PH with unclear and/or multifactorial mechanisms**
    - Hematologic disorders
    - Systemic and metabolic disorders
    - Others
    - Complex congenital heart disease

Table 1-2. Updated clinical classification of pulmonary hypertension, adapted from (Simonneau et al., 2019).

1.2.2 Pathophysiology of Pulmonary Hypertension

PH is characterised by both pulmonary vascular remodelling (PVR) and abnormal vasoconstriction that leads to increased pulmonary vascular resistance and eventually to right-sided heart failure and death. PVR is primarily characterised by muscularisation of peripheral arteries, hypertrophy of the medial layer, loss of small precapillary arteries and neointima formation leading to occlusive changes in the pulmonary arteries and plexiform lesions and ultimately the lumen of the vessel becomes occluded resulting in loss of distal pulmonary arteries (reviewed in Rabinovitch (2012), Tuder (2017). PVR is a complicated process and many mechanisms may be involved such as: reduced K^+ channel expression and elevated Ca^{2+} levels, bone morphogenetic protein receptor type II (BMPR-II) mutation (Austin and Loyd, 2014); which will be discussed in more detail in the section on 'Genetics of Pulmonary Hypertension'. Moreover, the possible role of connexins, particularly Cx43, in the PVR process has recently been explored and discussed in more detail in (Htet et al., 2021, Boengler et al., 2021).

1.2.3 Pulmonary Vascular Remodelling in Pulmonary Hypertension

In pulmonary hypertension, the key structural changes, termed vascular remodelling, occur in all three layers of the blood vessel: the intima, an endothelial-lined component; the media, comprising of smooth muscle cells; and the adventitia, a fibroblast populated area. PVR is initially characterized by the presence of a double elastic lamina, which signifies the muscularisation of peripheral arteries. Additionally, there is an observed hypertrophy of the medial layer and disappearance of small precapillary arteries, accompanied by neointima formation. This progression leads to occlusive alterations in the pulmonary arteries and plexiform lesions, and eventually, the occlusion of the vessel's lumen, resulting in the loss of distal pulmonary arteries (Rabinovitch, 2012, Oshima, 2016, Price et al., 2019) (Figure 1-2 and Figure 1-3).

1.2.3.1 Intima remodelling

The intima represents an endothelial layer that separates the flowing blood and the media. The degree to which the intima is affected in PH remains unclear. Assessed by cross-section, the normal intima contributes to approximately 10% of the total thickness of pulmonary arteries (Stacher et al., 2012); however, in

patients with serious PH, their arteries show increased thickness of the intima to around 30% of total thickness. Critically, this increased intima generates an increase in resistance of the pulmonary arteries of around 40 times, compared to healthy patients (Stacher et al., 2012). This thickening of the intima layer in pulmonary arteries can be attributed to several factors such as excessive proliferation of cells, especially smooth muscle cells (SMCs). These cells can migrate from the media layer into the intima and start to multiply (Meyrick, 2001). This process is often stimulated by growth factors released in response to injury or inflammation or certain disease such as PH (Newby and Zaltsman, 2000). The proliferating cells within the intima also concurrently generate and release extracellular matrix proteins, including collagen and elastin. The accumulation of these proteins in the intima contributes to an increase in its thickness (Rabinovitch, 2012). Furthermore, endothelial cells, which form the lining of the intima, are critical for preserving its health and preventing its thickening. However, when these cells malfunction, they can trigger inflammatory responses and provoke the release of growth factors, leading to cell proliferation and accumulation of the extracellular matrix (Thenappan et al., 2018). Another factor that could contribute to the thickening of the intima layer in pulmonary arteries is an increase in cell size. This enlargement of cells can contribute to intima thickening, though it is generally less common and less impactful than cell proliferation and extracellular matrix deposition (Shimoda and Laurie, 2013).

1.2.3.2 Media remodelling

The SMC-dominant media has been studied previously from a pathology and pathophysiology perspective with respect to PH. The study of PH has had a major focus on the media SMCs, likely as a consequence of its association with mediating hypoxic pulmonary vasoconstriction (HPV) and the extent of medial remodelling that occurs in response to chronic hypoxia. In patients with pulmonary arterial hypertension (PAH), the medial fractional thickness is around 20% greater than in the controls (Stacher et al., 2012). The thickening of the medial layer is primarily driven by an increase in the number of SMCs and their hypertrophy. No single characteristic can fully account for the complexity of the contribution of SMCs to pulmonary vascular remodelling. As previously discussed, SMCs can proliferate and migrate to the intima in response to factors such as hypoxia or inflammation. This migration and proliferation result in the thickening of the medial layer and the

narrowing of the arterial lumen, which contributes to increased pulmonary vascular resistance- a key feature of PH (Stenmark et al., 2018). Additionally, SMCs produce and release extracellular matrix proteins, including collagen and elastin. The accumulation of these proteins can also lead to further arterial thickening and stiffness (Stenmark et al., 2018).

1.2.3.3 Adventitia remodelling

The adventitia has no clearly defined boundary, and consequently it is difficult to accurately define its limits, especially in the human lung; this introduces substantial analytical issues. However, one study showed a 2- to 4-fold increase in the thickness of the pulmonary artery adventitia in iPAH patients compared to control patients (Chazova et al., 1995). As a connective tissue sheath, the adventitia encases the airways and pulmonary arteries and within the adventitia lies the lymphatic vessels. The lymphatic system enables the transfer of mediators of inflammation, which may be directed towards the airways, as observed in both asthma and bronchiolitis, or the pulmonary arteries, as observed in PH. Irrespective of the obstacles in measuring the level of adventitia remodelling in cases of PH, Pugliese et al. (2015) and Stenmark et al. (2015) show that the adventitia is pivotal in control of the inflammatory response. The adventitia operates as a hub for inflammatory signalling by facilitating the interactions between the resident fibroblasts and the incoming macrophages. Furthermore, Majka et al. (2008) proposed that the adventitia may operate as a holding bay for bone-marrow derived progenitor cells that may induce pulmonary vascular remodelling, either directly or indirectly.

1.2.3.3.1 Role of fibroblasts in PAH

In the context of PAH, fibroblasts exhibit a variety of functional and structural responses to hypoxia, including: proliferation, differentiation, upregulation of extracellular matrix proteins, and release of factors that directly affect smooth muscle cell tone and growth (Welsh and Peacock, 2013). Hypoxia has been shown to increase the proliferation of pulmonary arterial fibroblasts (PAFs) in bovine, rat and mouse (Welsh et al., 1996, Welsh and Peacock, 2013). It has also been demonstrated that PAFs release mitogens that promote proliferation and migration of pulmonary artery smooth muscle cells (PASMCs) (Zhang et al., 2017, Carlin et al., 2012, Wilson et al., 2020). Numerous experiments have been

conducted or proposed, involving both humans and animal models, to illustrate that PAFs release mitogens. These experiments include in vitro cell culture studies where PAFs are isolated from lung tissues. Techniques such as enzyme-linked immunosorbent assay (ELISA) or Western blot are utilized to measure the production and release of known mitogens, such as p38 mitogen-activated protein kinase (MAPK) (Welsh et al., 2001, McNair et al., 2020). Another approach involves co-culturing PAFs with other cell types, such as SMCs or ECs, to demonstrate the mitogenic activity of PAFs (Welsh and Peacock, 2013). Furthermore, gene expression analysis using quantitative PCR (qPCR) or RNA sequencing techniques can be employed to determine whether PAFs express the necessary genes for mitogen production. High levels of expression of these genes would suggest that PAFs are likely producing and releasing these mitogens (Correll et al., 2018). The proinflammatory, hyperproliferative, and apoptosis-resistant phenotype of PAFs has been demonstrated in clinical and experimental studies of patients with pulmonary hypertension. The hypothesis has been proposed that these alterations might result from genetic modifications and/or "maladaptive" epigenetic processes. These were reviewed in more detail in Gamen et al. (2016).

1.2.3.3.2 Role of myofibroblasts in PAH

Beyond the scope of PAFs, the role of myofibroblasts in PAH represents a critical aspect of vascular remodelling. Myofibroblasts, which are differentiated from fibroblasts under pathological conditions, are marked by the expression of alpha-smooth muscle actin (α-SMA) and exhibit enhanced synthetic and contractile properties (Wilson et al., 2015). In the pathophysiology of PAH, myofibroblasts are instrumental in exacerbating vascular stiffness and narrowing due to their augmented production of extracellular matrix components (Thenappan et al., 2018). This shift towards a myofibroblast phenotype is often stimulated by various cytokines and growth factors, notably transforming growth factor-beta (TGF-ß) and platelet-derived growth factor (PDGF) (Nogueira-Ferreira et al., 2014). These cells, therefore, contribute significantly to the occlusive remodelling characteristic of PAH. Research has indicated that the transition of fibroblasts to myofibroblasts in PAH involves complex signalling pathways and is influenced by the surrounding microenvironment, which includes hypoxic conditions and inflammatory mediators (Hu et al., 2020).

Understanding the characteristics of myofibroblast behaviour, their origin, and the regulation of their activity is pivotal in unravelling the complex pathogenesis of PAH and could offer novel therapeutic targets for managing this debilitating disease.

Mononuclear fibrocytes are cells that bear features of both fibroblasts and leukocytes (Frid et al., 2006). Much of the remodelling of the pulmonary vasculature is attributed to mononuclear fibrocytes. As the adventitia expands, mononuclear fibrocytes migrate through the angiomata into the vessel wall (Davie et al., 2004). According to Yeager et al. (2012), some of the cells present in neointimal lesions might have originated as invading fibrocytes. The levels of circulating fibrocytes in adults and children with pulmonary hypertension are commonly high (Yeager et al., 2012). Furthermore, the incidence of PAH in children, although relatively rare compared to adults, presents unique challenges and characteristics. Paediatric PAH has an estimated incidence of 2-6 cases per million children annually, underscoring its rarity yet significant impact (Berger et al., 2012). The disease often manifests differently in children than in adults, with congenital heart disease being a predominant associated factor. Additionally, children with PAH tend to have a more rapid disease progression and a poorer prognosis compared to adults, making early diagnosis and treatment crucial (Hansmann, 2017). Genetic factors, including heritable mutations, play a more pronounced role in paediatric PAH, further complicating its management (Soubrier et al., 2013). The elevated levels of circulating fibrocytes in children with PAH could potentially serve as biomarkers for disease severity and progression, offering insights into the underlying mechanisms specific to paediatric populations (Colvin et al., 2014).

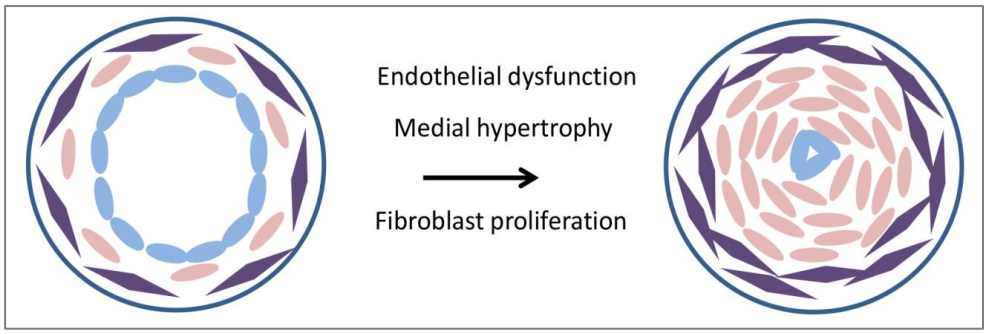


Figure 1-2. The effects of pulmonary vascular remodelling on the distal pulmonary artery. Endothelial dysfunction, represented by light blue cells in the diagram, is characterized by markers such as decreased expression of endothelial nitric oxide synthase (eNOS) and increased levels of endothelin-1 (ET-1). Hyper-proliferation in the underlying smooth muscle cells, depicted as pink cells, can be identified by increased expression of markers such as alpha-smooth muscle actin (α-SMA) and proliferating cell nuclear antigen (PCNA). Fibroblasts, shown as purple cells, exhibit markers of activation such as fibroblast-specific protein 1 (FSP1) and collagen deposition. These cellular changes contribute to the narrowing of the lumen, adapted from Dempsie et al. (2015).

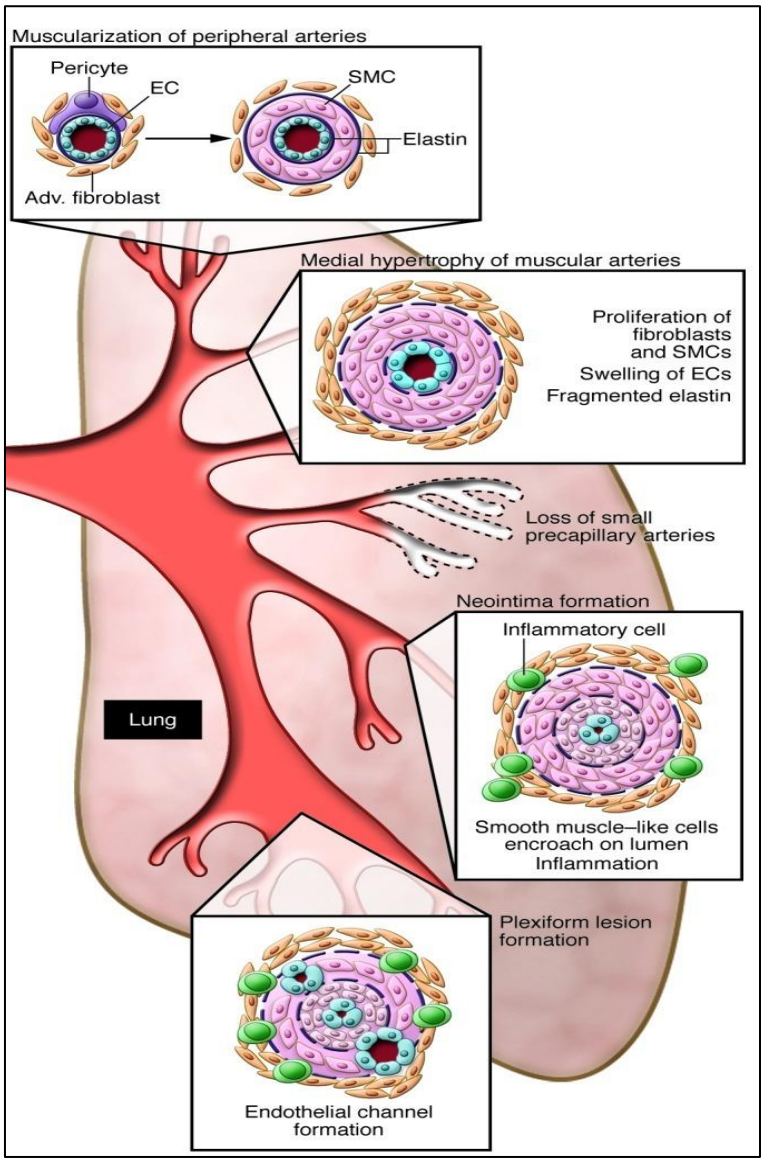


Figure 1-3. Vascular malformations within the pulmonary vasculature during PAH. They are characterised by a double elastic lamina indicating muscularisation of peripheral arteries, hypertrophy of the medial layer. These changes, along with the loss of small precapillary arteries and neointima formation, leading to occlusive changes in the pulmonary arteries and plexiform lesions. Ultimately, the lumen of the vessel becomes occluded, resulting in loss of distal pulmonary arteries. This is adapted from Rabinovitch (2012).

1.2.4 Genetics of Pulmonary Arterial Hypertension

The genetics of pulmonary hypertension are complex due to incomplete penetrance and genetic heterogeneity. Incomplete penetrance indicates that even if a person carries a gene linked to pulmonary hypertension, it doesn't necessarily mean they will develop the condition. Genetic heterogeneity, on the other hand, suggests that a variety of distinct genes could potentially contribute to the onset of the disease (Girerd et al., 2017, Ma and Chung, 2014). Interestingly, BMPR-II mutations are associated with 70% of heritable PAH (hPAH) and 10% to 40% of idiopathic PAH (iPAH) (Aldred et al., 2006, Thomson et al., 2000). PAH is also less commonly associated with mutations in other genes such as endoglin (ENG), activin-like kinase 1 (ALK1), smad1, smad4, smad5 and smad8 (Girerd et al., 2017, Austin and Loyd, 2014, Ma and Chung, 2014).

1.2.4.1 Bone morphogenetic protein receptor type II (BMPR-II)

The inclusion of the BMPR-II section is essential due to the pivotal role this receptor plays in PAH pathogenesis. BMPR-II, a member of the transforming growth factor (TGF)-ß superfamily of receptors which is essential for embryogenesis, development, and adult tissue homeostasis. Similar to the other TGF-ß family of receptors, signal transduction by BMPR-II occurs via the formation of surface heterodimers with a type I BMP receptor (BMPR-IA and BMPR-IB). In the presence of ligand, a signal transduction cascade is initiated via the serine-threonine kinase activity of the type I receptor. This cascade involves the phosphorylation of a group of signalling proteins called Smads (Miyazono, 2000). Receptor-mediated Smads (R-Smads), 1, 5 and 8 are complexed with Smad 4, the common partner molecule, prior to translocation to the nucleus. Due to the inhibitory effect of bone morphogenetic protein (BMP) on proliferation of vascular smooth muscle cells, it has been suggested that a disruption of BMP signalling pathways via downregulation of BMPR-II may cause pulmonary vascular remodelling due to the failure of antiproliferative effects in the pulmonary circulation, resulting in conditions such as PAH (Takahashi et al., 2006).

BMPR-II represents a significant anti-inflammatory mediator in the pulmonary endothelium. Burton et al. (2011) demonstrated how a loss of BMPR-II in the pulmonary vasculature results in individuals who have greater susceptibility to inflammatory tissue damage. Furthermore, BMPR-II participates in regulating the barrier role of pulmonary arterial endothelial cells (PAECs), as shown in both *in vivo* and *in vitro* studies (Burton et al., 2011), where reduced BMPR-II expression results in increased permeability of the monolayer and increased recruitment of leukocytes. Additionally, these researchers also hypothesise a regulatory role for BMPR-II in controlling secretion of inflammation-promoting cytokines, and that the endothelium may be subjected to an unnecessary release of inflammatory cytokines where BMRP-II expression is downregulated. Briefly, BMPR-II contributes to vascular integrity and reduces inflammatory signalling within the vasculature, and the loss of BMPR-II may induce in the pulmonary vasculature an excessive inflammatory response when subjected to inflammatory stimuli.

In the two animal models of PAH: chronic hypoxia (CH) and monocrotaline (MCT) models (a substance used to induce pulmonary hypertension in animal model), BMPR-II was found to be downregulated (Morty et al., 2007, Takahashi et al., 2006). However, the mechanism by which BMPR-II dysfunction is associated with the onset of pulmonary vascular disease has yet to be established, though research suggests that BMPR-II levels are reduced and aberrant BMP/TGF signalling occur in the hypoxia and MCT models. Also, the MCT model in particular, exhibits upregulation of TGF-β (Long et al., 2009, Stenmark et al., 2006). In the research conducted by Reynolds et al. (2007), the expression of pulmonary BMPR-II protein was reduced in both model systems and BMPR-II gene delivery treatment inhibited this reduction. Furthermore, treatment with BMPR-II significantly reduced the hypoxia-induced increase in vascular muscularization and also decreased the elevated levels of TGF-β in the MCT model. Thus, the discussion of BMPR-II is not only relevant but crucial for a comprehensive understanding of the genetic and molecular underpinnings of PAH. It bridges the gap between genetic predisposition and the resultant pathophysiological changes, offering insights that are critical for both the understanding and potential treatment of this complex disease.

1.2.5 Sex Differences in Pulmonary Arterial Hypertension

It is important to note that females are more likely to develop PAH compared to males (Frump and Shimoda, 2021). This is reflected in a higher female-to-male ratio found in various registries, ranging from 1.4:1 in the UK/Ireland registry, 1.6:1 in the 'European Comparative, Prospective Registry of Newly Initiated Therapies for Pulmonary Hypertension (COMPERA)' registry, to as high as 4.1:1 in the 'Registry to Evaluate Early and Long-term Disease Management in PAH (REVEAL)' registry. For more details, see recent reviews (Cheron et al., 2021, Rodriguez-Arias and García-Álvarez, 2021). However, until recently, the reasons for these sex differences were unclear. Interestingly, oestrogen has well-known cardiovascular protective effects and experimental pulmonary hypertension can be prevented and rescued by oestrogen and its metabolites (Tofovic et al., 2006, Iorga et al., 2017, Sun et al., 2021). However, an interesting counterpoint is the observation that procedures reducing oestrogen levels, such as ovariectomy, or treatments like an aromatase inhibitor anastrozole that decrease oestrogen production, can also prevent or rescue the development of pulmonary hypertension in experimental models (White et al., 2011a, Chen et al., 2017). This suggests a complex role for oestrogen in the development and progression of pulmonary hypertension that requires further investigation. There is evidence that oestrogen has both pro- and anti-proliferative effects, which will be discussed in more detail in the following sections.

The sex difference in PAH might be due to upregulation of the serotonin signalling system in females mediated via 17β-oestradiol or oestrogen (E_2). Dempsie and MacLean (2013) explained that while serotonin impacted on the pulmonary vasculature directly through contractile and proliferative effects, it also dysregulates oestrogen metabolism in favour of pro-proliferative metabolites by upregulating the enzyme cytochrome P450 1B1 (CYP1B) that metabolises oestrogen. Both human and murine research support a role for CYP1B1 in the development of PAH, with dysregulation of CYP1B1 gene expression in cultured B-lymphocytes of female PAH patients who possess a mutation in the BMPR-II (West et al., 2008a). While in the murine model, either genetic or drug-induced inhibition of CYP1B1 is protective against the induction of PAH (White et al., 2011b).

CYP1B1 catalyses the production of a number of hydroxyoestrogen metabolites from 17β-oestradiol (Badawi et al., 2001, Hanna et al., 2000), including the 2-, 4- and 16α forms. The 16α-hydroxyoestrone metabolite influences proliferation of human pulmonary artery smooth muscle cells (hPASMCs), a process which is enhanced in cells harvested from PAH patients. To evidence this, some researchers have demonstrated that dosing murine models with 16α-hydroxyoestrone induces the development of PAH (White et al., 2011b). The formation of 2-hydroxyoesterogens may occur via CYP1B1; however, the major pathway for this metabolite is via the CYP1A1/2 enzyme pathway (Hanna et al., 2000, White et al., 2011b, Badawi et al., 2001). This 2-hydroxyestradiol intermediate is then converted via catechol *O*-methyltransferase (COMT) to 2-methoxyestradiol, a substance which protects rodent models from PAH development and exhibits anti-proliferative control over cells (Tofovic et al., 2006).

Consequently, it is hypothesised that an imbalance in oestrogen metabolites, favouring the pro-proliferative metabolites, may contribute to the development of PAH in association with CYP1B1 activity. Significantly, in hPASMCs, the upregulation of CYP1B1 expression was exhibited via a serotonin-mediated mechanism, and researchers have demonstrated that CYP1B1 can be be activated by the anorectic drug (dexfenfluramine) which is a serotonin transporter substrate and an indirect serotonergic agonist (Dempsie and MacLean, 2013). Therefore, these findings indicate a potential role for increased serotonin signalling in activating CYP1B1, which could subsequently cause an imbalance of oestrogen metabolites, via a shift towards the proliferation-inducing types. However, it is important to note that serotonin is not likely the only factor affecting CYP1B1, and dysregulation in the oestrogen pathway may independently lead to PH, irrespective of alterations in serotonin signalling (White et al., 2012, Morris et al., 2021).

Although female patients are more vulnerable to developing PAH, they survive longer than male patients and this phenomenon is called the ‘oestrogen paradox’ (Austin et al., 2013). Moreover, Gabler et al. (2012) proved that the response to treatment with endothelin-1 (ET-1) antagonists ambrisentan, bosentan and sitaxsentan were better in females, and they also have greater 6-minute walking distance than male patients. The difference in the treatment response could be

because of better right ventricular adaptation in female than in male patients during PAH as they have a greater right ventricular ejection fraction (Kawut et al., 2009). Furthermore, Ventetuolo et al. (2011) found that oestrogen levels were correlated with the right ventricular ejection fraction, whereas androgen levels were associated with higher right ventricular mass and volume in both males and females.

In testing mutant BMPR-II mice, which spontaneously develop PH (as previously described by West et al. (2008b)), two oestrogen inhibitors, fulvestrant and anastrozole, were found to both prevent and reverse PAH (Chen et al., 2017, Kawut et al., 2017). Fulvestrant binds to and accelerates the degradation of oestrogen receptors, while anastrozole is an aromatase enzyme inhibitor and therefore inhibits oestrogen synthesis. The effects of these drugs included a reduction in number of metabolic defects associated with PAH, such as oxidised lipid formation, insulin resistance and rescue of peroxisome proliferator-activated receptor-γ (PPAR-γ) and CD36. These murine model data indicate that trialling drugs or studying novel mechanisms to inhibit oestrogen in human PAH are likely to be valuable, and the reduction in metabolic-related issues may contribute to a reduction in pulmonary vascular disease. Chen et al. (2017) proposed that while tamoxifen, which selectively inhibits oestrogen binding to its receptor, was less effective in these murine models, it is a favourable alternative for premenopausal females, as it confers a lower risk of menopause induction than either fulvestrant or anastrozole. Chen et al. (2017) proposed that, for the treatment of PAH, the more effective drugs, anastrozole and fulvestrant, are ideal for use in postmenopausal women and men. This hypothesis is supported by a recent phase II clinical trial (clinicaltrials.gov identifier NCT01545336) in which anastrozole was used to modulate oestrogen levels in PAH patients. This trial specifically targeted postmenopausal women and men with PAH, reflecting the potential sex and age-specific responses to oestrogen modulation in this disease. The results demonstrated that the drug was safe, well tolerated and potentially efficacious (Kawut et al., 2017). However, none of these drugs have yet been introduced for routine clinical use for PH and they are still undergoing clinical trials.

1.2.6 Treatment of Pulmonary Arterial Hypertension

The advancement in the understanding of the pathophysiology of PAH over the past two decades has significantly improved survival and led to the discovery and development of different treatments. It was well established from both clinical and non-clinical studies that ET-1 (Chen et al., 1994, Miyauchi et al., 1993, Stewart et al., 1991), NO (Xu et al., 2004, Giaid and Saleh, 1995), and PGI_2 (Hoshikawa et al., 2001, Tuder et al., 1999) signalling pathways were dysregulated during PAH.

Currently, the FDA has approved treatments for PAH patients which target the NO, PGI_2 and ET-1 signalling pathways. For example, sildenafil and tadalafil which inhibit phosphodiesterase 5 (PDE5) and riociguat which stimulates guanylyl cyclase (GC) all result in increasing cGMP levels and ultimately vasodilatation (Provencher and Granton, 2015). Interestingly, more recent study has shown that tadalafil attenuates vascular remodelling in iPAH by inhibiting cell proliferation, enhancing apoptosis, and reducing PDE5 expression in IPAH-PASMCs, thereby alleviating the severity of disease (Yamamura et al., 2017). Prostacyclin analogues such as epoprostenol and treprostinil, and the prostacyclin IP receptor agonist selexipag, are the two main groups of drugs which target the PGI_2 pathway and they exert their effects by promoting direct arterial vasodilation, inhibiting platelet aggregation and demonstrating anti-proliferative properties (Lau et al., 2017, Clapp et al., 2020). Endothelin receptor antagonists such as macitentan and bosentan which are ET_A/ET_B receptor antagonists, and ambrisentan which is a selective ET_A receptor antagonist are also currently approved treatment options for PAH patients. These ET antagonist drugs prevent phospholipase C-mediated vasoconstriction and protein kinase C-mediated cell proliferation (Ataya et al., 2016).

1.2.6.1 Adverse Effects of the Treatment of Pulmonary Arterial Hypertension

All of the classes of drugs discussed above have several limitations and adverse side effects. Sildenafil, for instance, has quite a short half-life (4–6 h) and must be taken three times per day. On the other hand, tadalafil has a different structure to sildenafil which means that it has different pharmacokinetic properties and a longer half-life. For this reason, it only needs to be take once a

day (Galie et al., 2009). Both sildenafil and tadalafil can have side effects, such as headache, GI disturbance and vision problems. Riociguat can have side effects such as dizziness, fainting and nausea. Pregnant women should not take riociguat or any other GC stimulators because studies have shown that riociguat has teratogenic and embryotoxic effects, meaning it can cause birth defects and harm the developing embryo or fetus (Garnock-Jones, 2014, Hambly and Granton, 2015). Haemoptysis is a less common side effect of riociguat (Ghofrani et al., 2013).

The first type of prostacyclin analogue approved for PAH was intravenous epoprostenol. It is still the only therapy that has been found to increase survival rates at 12 weeks for those diagnosed with severe PAH (Sitbon and Noordegraaf, 2017). The only way to administer this drug is in the form of a continuous infusion through a central line based on its pharmacokinetic properties and a short half-life of just 2-3 minutes. Intravenous epoprostenol treatment is a complex process and, in addition to specialist nursing and medical skills, patient education and training are very important (Archer-Chicko, 2011). The half-life of treprostinil is longer and the drug has better chemical stability than epoprostenol. Moreover, treprostinil can be administered intravenously, subcutaneously, orally, or through inhalation. Epoprostenol, treprostinil, iloprost and selexipag all have possible side effects, such as nausea, headaches, skin flushing, cough, low blood pressure, jaw pain, diarrhoea, leg cramps, chest pains, IV site infections and breathlessness (Sitbon and Noordegraaf, 2017).

A common side effect of endothelin receptor antagonists is an abnormal liver function, which is experienced by 10% of PAH patients taking bosentan. Liver function must thus be monitored every month for such patients (Humbert et al., 2007). It is possible to completely reverse bosentan-induced hepatic dysfunction once the administration of the drug is stopped. The drug ambrisentan has a longer half-life than bosentan, meaning that the latter only has to be administered once a day. When treated with macitentan, a modified version of bosentan, the liver function abnormalities and fluid retention rates in PAH patients were found to be similar to those administered a placebo. However, anaemia was found to be more prevalent in patients treated with macitentan (Pulido et al., 2013). Ambrisentan has not been found to cause liver function test abnormalities when compared to the placebo (Galiè et al., 2005).

Regardless of ambritsentan's selectivity for ETA, data from non-comparative monotherapy trials failed to identify any differences in efficacy due to receptor selectivity (Humbert et al., 2014). All endothelin receptor antagonists have teratogenic adverse effects and therefore, pregnant women should not take these drugs (Zamanian et al., 2014).

Despite the multiple adverse effects of these drugs, the results from a clinical meta-analysis which was conducted over 18 years (from January 1990 to October 2008) on 23 randomised single-agent clinical trials recruiting 3199 PAH patients who were treated with these different drugs revealed a significant reduction in mortality by 43% (reviewed in detail in Galie et al. (2009). There is significant evidence to suggest that combination therapy can lower the chances of future morbidity and slow down the progression of the disease. Current research has shown that outcomes may be more favourable if combination therapy is initiated promptly after diagnosis (Boucly et al., 2021, Zelt et al., 2022). However, to date, despite the improvement in the survival rates among PAH patients, there is still no cure for PAH.

1.3 Connexins

Connexins are membrane channel forming proteins that are involved in different aspects of vascular physiology and pathology. In this part of the introduction, connexin structure, expression, functions and pharmacological and peptide modulators of the connexin channel will be discussed. In addition, as this thesis focusses on Cx43 and since Cx43 is one of the most researched connexin protein, largely owing to the fact that it is expressed in a diverse range of tissue types (Zhang et al., 2018, Söhl and Willecke, 2004), the life cycle and phosphorylation of connexin 43 will also be discussed.

1.3.1 Introduction

Connexins are transmembrane proteins in which six connexins in the plasma membrane assemble into hemichannels (HCs) (also known as connexons). Two HCs from adjacent cells dock to form a gap junction for intercellular communication (Austin and Loyd, 2014, Söhl and Willecke, 2004). The existence of gap junctions (GJs) was recognised over fifty years ago (Revel and Karnovsky, 1967, Brightman and Reese, 1969). Their component proteins, i.e. connexins, were identified several years later in the mid-1970s (Goodenough, 1974). Specific areas within the cell membrane, composed of tens to thousands of HCs, come together to form gap junctions, where direct transfer and exchange of ions such as calcium (Ca^{2+}), small metabolites such as glucose, microRNAs (miRNAs), second messengers such as 3',5'-cyclic adenosine monophosphate (cAMP), adenosine triphosphate (ATP) and inositol 1,4,5-triphosphate (IP3), between the cytoplasms of adjacent cells can occur. This is commonly referred to as gap junctional intercellular communication (GJIC) (Aucher et al., 2013, Neijssen et al., 2005). Under typical circumstances, the GJs (formed from two HCs) are open whereas the unpaired HCs are usually closed. However, in certain disease states, GJ transmission may become limited and lead to enhanced opening of the HCs. These changes can adversely affect the normal intercellular communication equilibrium.

1.3.2 Connexin Gene

In the human genome, 21 different connexin genes have been identified. The most widely studied among these is connexin 43 (Cx43). On the other hand, in mice, 20 connexin genes have been identified. These genes encode proteins with a

molecular weight (MW) ranging from 26 to 60 kiloDaltons (kDa) (Söhl and Willecke, 2004). They are divided into five subfamilies according to the homologies in their sequence and the length of cytoplasmic loops (alpha (α), beta (ß), gamma (γ), delta (δ), zeta (ζ) (Abascal and Zardoya, 2013). Furthermore, connexins are named according to their approximate MW, for example, Cx43, which is encoded by gap junction α 1 (GJA1) gene, is ~43 kDa in size. The majority of connexins have a universal gene composition, i.e. two exons with an interlinking intron sequence which is sited within the 5'-untranslated region (5'-UTR) (Valiunas et al., 2005). The connexin coding sequence and the 3'-untranslated region (3'-UTR) is found within the second exon (Willecke et al., 2002).

1.3.3 Connexin Protein

Most connexin proteins share a common structure consisting of four α helical transmembrane domain proteins (TM1-TM4), two extracellular loops (EL1 and EL2), an intracellular loop (IL), cytoplasmic amino (NH_2) and carboxy (COOH) terminals. The extracellular loops have three highly conserved cysteine residues connected by disulphide bridges which stabilize the loops during the docking of two HCs. The length and composition of all regions give rise to significant differences among connexins (Martin and Evans, 2004) (Figure 1-4). It is important to note that more than one type of connexin can be found in different tissues resulting in HCs formed by either identical connexin subunits (homomeric) or made up of various connexins (heteromeric). These HCs can form gap junctions which are either homotypic (docked with the same type of HC) and heterotypic (docked with a different type of HC) (Figure 1-5) (Račkauskas et al., 2010).

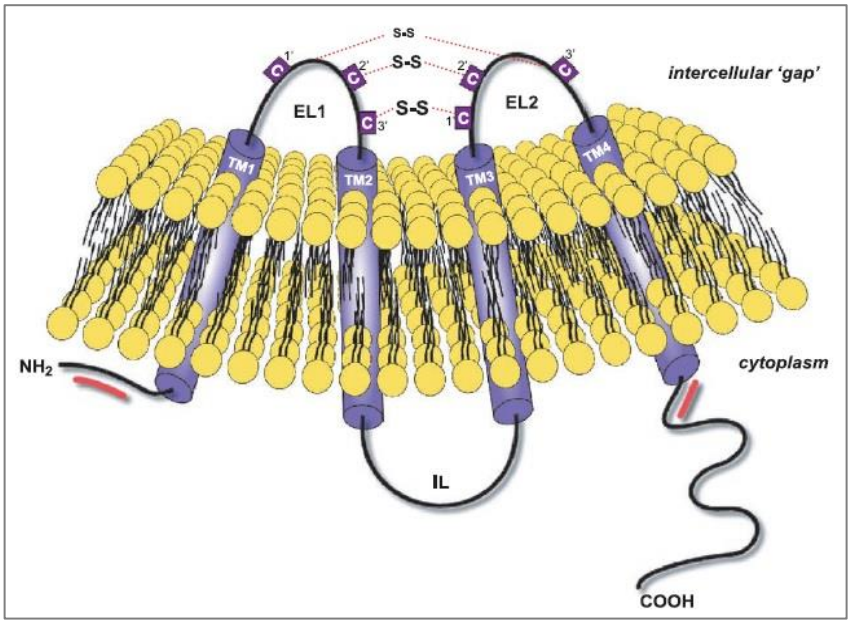


Figure 1-4. Structure of connexin proteins. They are comprised of four α helical transmembrane domain proteins (TM1-TM4), two extracellular loops (EL1 and EL2) with three highly conserved cysteine residues linked by disulphide bridges, one intracellular loop (IL), and cytoplasmic amino (NH_2) and carboxy (COOH) terminals facing the cytoplasm, (adapted from Martin and Evans (2004).

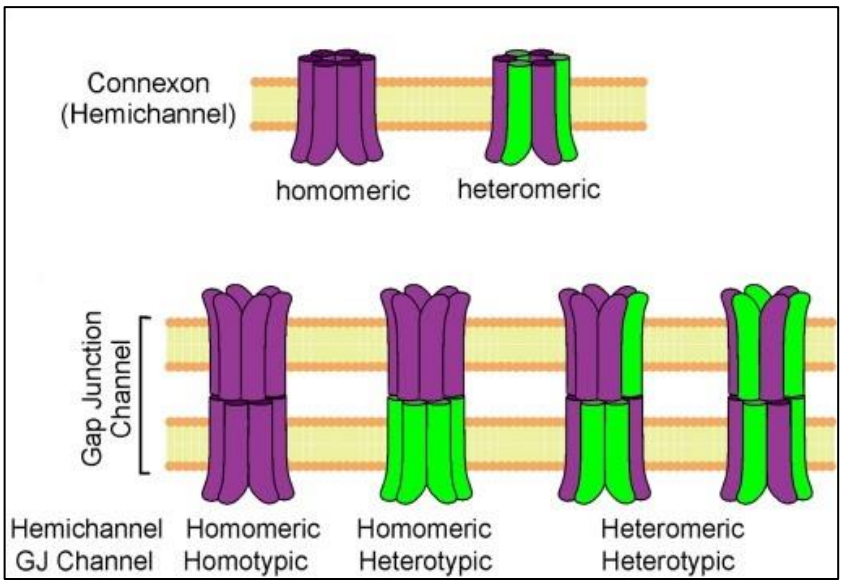


Figure 1-5. Nomenclature of connexons and GJ channels. Two types of connexons are illustrated. On the left a homomeric connexon is made up of six duplicate oligomerized connexons. On the right a heteromeric connexon is composed of several dissimilar connexins. A homomeric homotypic GJ channel is comprised of two matching homomeric connexons linked head-to-head. A homomeric heterotypic GJ channel is formed by two nonidentical homomeric connexons. A heteromeric heterotypic GJ channel is comprised of two matching heteromeric connexons, (adapted from Bai et al. (2018).

1.3.4 Connexin Expression

Connexins are expressed distinctively in almost all vertebrate cell types excluding erythrocytes, mature sperm cells, and differentiated skeletal muscle cells (Račkauskas et al., 2010). The distribution of connexins varies. Some are found predominantly in one tissue type, e.g. Cx50 which is characteristic of lens cells, whereas other connexins such as Cx43 are found in a variety of cells, and the majority of tissue types exhibit numerous connexins.

The major, most prominently expressed connexins within the vasculature are connexin 37 (Cx37), connexin 40 (Cx40), connexin 43 (Cx43) and connexin 45 (Cx45), with Cx43 being the most abundant isoform (Johnstone et al., 2009a). Cx37 and Cx40 are commonly expressed within the endothelium, while Cx43 is commonly expressed in both endothelium and smooth muscle cells (Figueroa and Duling, 2009). Cx45 expression, however, is limited to the smooth muscle cells (Li and Simard, 2001). Considerable diversity has been seen in the distribution of connexins. It has been observed that there are differences between vascular beds of one species as well as between the same vascular beds of different species (Severs et al., 2001, Oyamada et al., 2013).

1.3.5 Life Cycle of Connexins

The connexin life cycle involves several key steps: 1) trafficking of connexin proteins and formation of HCs, 2) assembly of GJs, 3) formation of GJ plaques, and finally 4) disassembly of GJs through processes of internalization and degradation (Figure 1-6). In the following paragraphs, an overview of each step is provided.

1.3.5.1 Connexin trafficking and formation of hemichannels

Connexins are transported to the plasma membrane by forward trafficking via the Golgi apparatus after being produced in the endoplasmic reticulum (ER). Hexameric connexin assemblies known as HCs are created via oligomerization, which commences in the ER and progresses through the Golgi apparatus. Hexameric connexin assemblies achieve increased structural and functional stability during their progression from the endoplasmic reticulum through the Golgi apparatus to the trans-Golgi network (Laird, 2006).

1.3.5.2 Gap junction channel assembly

The next stage in the connexin lifecycle involves forming a GJ channel through the head-to-head docking of two HCs from neighbouring cells. It has been suggested by Schubert et al. (2002) that HCs move on lipid rafts until they come across another HC with which they can dock.

1.3.5.3 Formation of gap junction plaques

The GJ channels arrange into structures known as plaques. These can significantly vary in size, ranging from barely visible by confocal microscopy to several micrometres in length. Such plaques comprise hundreds to thousands of GJ channels, with an approximate centre-to-centre separation distance of around 10nm (Klaunig and Shi, 2009). However, it is unclear how GJ channels interact to create the tightly-packed, well-organised formations that make up a GJ plaque. It's noteworthy that while HCs can aggregate in the membrane, they typically do not form plaque-like structures as GJs do. HCs generally exist as individual entities or small clusters within the membrane, serving functions distinct from those of GJs (Račkauskas et al., 2010).

1.3.5.4 Disassembling the gap junction: internalization and degradation

Early studies conducted by Lane and Swales (1980) found that junctional particles were dispersed in the plasma membrane and away from the junction. At present, the optimal characterized uptake scenario for HCs involves the internalisation of the whole GJ plaque or portions of it, which can cause an annular junction to appear in one of the cells (reviewed in Jordan et al. (2001), Falk et al. (2009).

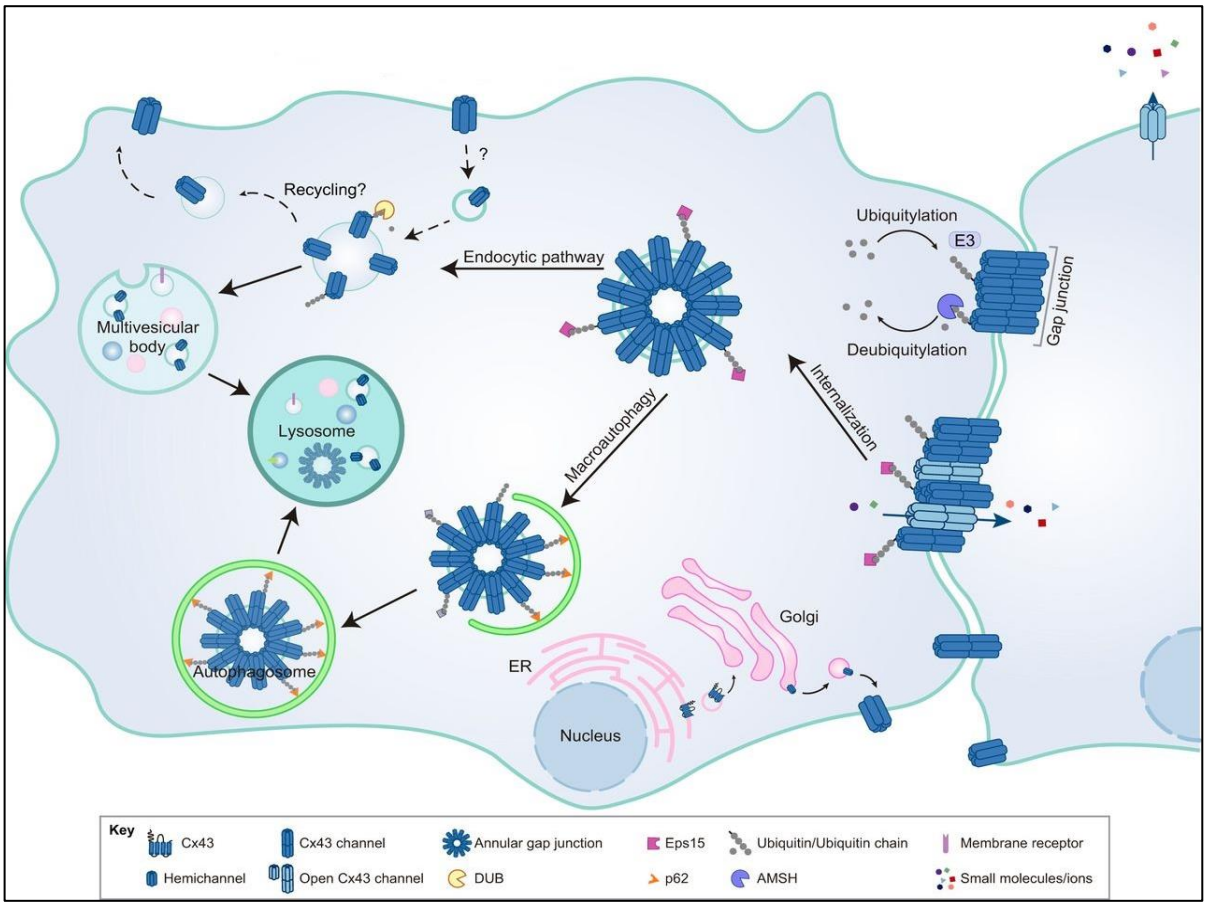


Figure 1-6. Schematic illustration of the life cycle of Cx43. Cx43 is initially synthesized in the endoplasmic reticulum (ER), where it folds into its proper conformation. It then oligomerizes, forming connexons, in the Golgi apparatus before being transported to the plasma membrane. At the plasma membrane, Cx43 functions as a hemichannel, facilitating the exchange of small molecules and ions with the extracellular environment. These hemichannels can also dock with adjacent hemichannels on neighbouring cells to form gap junctions, allowing for intercellular communication. Furthermore, the diagram depicts various post-translational modifications of Cx43, including ubiquitination, which tags the protein for degradation, and deubiquitination, which can rescue it from being broken down. The Cx43 lifecycle is subject to regulation by endocytic pathways, as shown by the figure's representation of Cx43 internalisation and possible recycling. Additionally, the figure illustrates how Cx43 can be targeted for degradation through several routes: the proteasome pathway, lysosomal degradation following endocytosis, or autophagy, where the autophagosome (highlighted for clarity in the figure) engulfs the protein for degradation in the lysosome. The various stages of the Cx43 lifecycle are marked by interactions with specific cellular components and molecular pathways, as indicated by the detailed annotations and symbols in the figure key. The portrayal of Cx43 within different cellular compartments underlines its dynamic regulation and multifaceted roles in the cell, (adapted from Ribeiro-Rodrigues et al. (2017).

1.3.6 Connexin Functions

Connexins have a variety of purposes, including channel-independent functions that are related to the connexin themselves, together with activities associated with the GJs and regulation of HC opening. The physiological functions of the GJs have been well established. In vascular cells, GJs facilitate intercellular conduction of differences in electrotonic membrane potential, acting as electrical synapses between endothelial cells (ECs), smooth muscle cells (SMCs), or between ECs and SMCs through the myoendothelial junction (MEJ). Connexins not only facilitate intercellular communication, but also fulfil the key function as transducers of retrograde vascular signalling counter to the vascular flow direction; as proven within the systemic vasculature (Figueroa and Duling, 2009, de Wit et al., 2000). GJs may also have pathologic effects, e.g. participating in the mechanism of bystander cell death (reviewed in detail in Decrock et al. (2009), Decrock et al. (2017). The physiological role of HCs remains unknown, but these structures have been associated with a variety of pathological phenomena. The GJ is approximately twice the length of the HC. Since the maximum atomic mass unit for material transport in both channels is ~1.5 kDa, GJs therefore have only half the conductance of the HCs. The HCs permit unrestricted movement of sodium (Na^+) and potassium (K^+) ions which may cause cell swelling and expedite the entry of calcium (Ca^{2+}) ions (Schalper et al., 2010, Fiori et al., 2012).

Connexins have been demonstrated to play vital roles in the normal growth and differentiation of a diverse range of tissue types, including the heart, lungs, kidneys and liver (Kar et al., 2012). Throughout the course of cell differentiation, connexin levels and their phosphorylation status is altered (Talhouk et al., 2008). Several organs require connexins in order to work effectively; one example being the coordinated function of cardiac muscle (Beauchamp et al., 2006). In addition, connexins contribute to fundamental physiological pathways involved in inflammation and cellular repair (Chanson et al., 2005). A range of pathologies such as systemic hypertension (Brisset et al., 2009), atherosclerosis (Meens et al., 2012), and a number of cancers (Aasen et al., 2019) are associated with a diminished number of connexins or with genetic mutations that disrupt their typical roles.

The first interpretation of Cx43's function was as a protein necessary for intercellular communication via the GJs; however, emerging research has significantly changed the perception of its role. Innovative studies have shown that Cx43 is able to regulate communication between cells with the use of tunnelling nanotubes (TNTs) and extracellular vesicles (Soares et al., 2015, Wang et al., 2010). TNTs are able to maintain communication between cells that are in physical proximity, whereas extracellular vesicles facilitate exchange of material between non-adjacent cells and over increased spatial areas. Recent research is offering increasing evidence for the theory that connexins have further functions in addition to their contribution to intercellular information exchange. These newly proposed functions include the regulation of gene transcription, engagement with cell growth and cell death modulators, and potential mechanical tasks (Esseltine and Laird, 2016, Leo-Macias et al., 2016, Martins-Marques et al., 2015). Connexins not only contribute to channel structure; their role in intercellular communications can also be regulated by extremely well controlled and coordinated post-translational modifications (PTMs). These modifications will be discussed in the following section. Such PTMs may have a direct impact on connexins and influence channel gating or they may offer a more indirect influence, e.g. modifying the Cx43 channel folding process, trafficking, accumulation, docking and breakdown (Axelsen et al., 2013, D'hondt et al., 2013, Pogoda et al., 2016). In particular, Cx43 phosphorylation, which is the most described PTM, controls all the phases within the life cycle of Cx43; it does not just influence channel function (Lampe and Lau, 2004, Solan and Lampe, 2014).

1.3.7 Connexin Posttranslational Modifications

Various connexin posttranslational modifications have been identified, including phosphorylation, ubiquitination, S-nitrosylation, hydroxylation, sumoylation, palmitoylation, methylation, acetylation and γ-carboxyglutamation (Laird et al., 2015). Nonetheless, it is important to note that research evidence pertaining to their incidence and biological importance varies significantly. Evidence that can indicate the importance of posttranslational changes includes the identification of the change using different analytical approaches, the discovery of the modification in cells and tissues, and the identification of any biological impacts of the posttranslational alteration. For instance, conclusive proof that a posttranslational modification has occurred could be the direct incorporation of a

radioactive element that constitutes a posttranslational modification, identification of a change using mass spectrometry, and/or antibody identification of changes to connexin purified from cells. Some connexin posttranslational modifications can be summarised as follows:

1. Phosphorylation: this is the most common connexin posttranslational modification identified in relevant research. It has been found that connexin phosphorylation can impact various processes, including protein transportation, GJ size, connexin half-life, protein turnover, incorporation into a GJ, and channel gating (recent studies include those of (Pogoda et al., 2016, Solan and Lampe, 2016). Moreover, other tissue injuries such as wounding and ischaemia can alter connexin phosphorylation. For instance, knock-in mice expressing Cx43 with mutated phosphorylation sites for mitogen-activated protein kinase (MAPK) displayed less proliferation upon arteriole injury and decreased neointima development in research performed by Johnstone et al. (2012). Additionally, after ischaemia-reperfusion injury, Cx43 knock-in mice who had a serine (Ser) to alanine (Ala) mutation at a protein kinase C (PKC) site were found to be insensitive to the cardioprotective effects of sphingosine-1-phosphate (Morel et al., 2016). These findings demonstrate that connexin phosphorylation plays a critical role in facilitating the physiological response to injury.

2. Ubiquitination: The ubiquitin protein bonds with a substrate protein during this enzymatic process. By designating substrates for degradation by the proteasome and through ubiquitin-dependent protein-protein interactions, protein ubiquitination regulates a variety of cellular functions. The range of cellular systems being studied may be the cause of the many contradictions in the literature and the complexity of ubiquitin signalling, which makes it difficult to comprehend its function in connexin biology (reviewed in details in Totland et al. (2020).

3. S-nitrosylation: The process of covalently incorporating the nitrosyl moiety of NO into another molecule is known as nitrosylation. S-nitrosylation is the process that occurs when nitrosylation occurs at the thiol group of cysteine. It is another significant posttranslational modification that occurs in both healthy and diseased states (Hess and Stamler, 2012, Hess et al., 2005). It is well known that nitric oxide (NO) affects gap junction function in the vasculature's endothelial and

smooth muscle cells (Figueroa et al., 2013, McKinnon et al., 2009, Straub et al., 2011). However, there is very little proof that the blood vessel wall's S-nitrosylation regulates the gating of connexin HCs. The mechanisms through which NO modulates connexin HC function have been examined in the central nervous system (primarily in the astrocytes) (Sáez et al., 2005, Muñoz et al., 2015). The enzyme NO synthase (NOS) is expressed by both astrocytes and endothelial cells. Therefore, there may be commonalities in the posttranslational modification systems that control connexin HC activity in these two different types of cells. Moreover, it has been suggested that S-nitrosylation may regulate the Cx43 HCs in astrocytes and cardiomyocytes. Additionally, metabolic inhibition and oxygen deprivation may increase HC activity which has been revealed by pharmacological and *in vitro* studies (Kondo et al., 2000, Retamal et al., 2006). Several studies have linked the regulation of connexin HCs with the production of NO. The functional control of vasodilator responses has been connected to the potential modification of connexin HCs by NO-mediated events. In HeLa cells, Cx43, Cx40 and Cx37 may serve as a conduit for NO transmission and may be more inclined to open when NO donors are applied (Figueroa et al., 2013). The latter research also revealed that inhibition of connexin-based channels could prevent acetylcholine (ACh)-induced vasodilation and the diffusion of NO to smooth muscle cells from endothelial cells in the mesenteric arteries. Thus, further research is required to determine the extent to which NO-activated connexin HCs influence vascular function.

1.3.7.1 Phosphorylation of Cx43

Phosphorylation is the best documented connexin PTM. Studies have described its impact on the half-life of the peptide, its trafficking, integration into a GJ, the mass of the GJ, channel gating and protein turnover (Pogoda et al., 2016, Solan and Lampe, 2016). The phosphoconnexin Cx43 is the most researched and well described. There is a whole system of kinases that are involved in the phosphorylation of Cx43. Kinase substrates include a minimum of 19/26 Ser and 4/6 Tyr in the CT region of Cx43 although there is variation in the degree of scientific documentation regarding these sites and their associated enzymes. Some more validated sites are those where MAPK family of proteins are responsible for the phosphorylation; these encompass protein kinase B, PKC and creatine kinase. Western blot and immunofluorescence studies using phosphospecific

antibodies for phosphorylation sites demonstrate the anticipated reaction in Cx43 in response to MAPK activation or inhibition. The exact location of the sites and the component measurements are not yet known. However, *in vitro* experiments have shown that if Cx43 is phosphorylated by casein kinase 1 (CK1), phosphorylation occurs at Ser-325, Ser-328, and Ser-330. This reaction is enhanced by CK1 activation and diminished by its inhibition. Three sets of double Ser, i.e. Ser-364/Ser-365, Ser-368/Ser-369, Ser-372/Ser-373, incorporated in the final 19 amino acids of Cx43, have all been proposed as phosphoacceptors (Yogo et al., 2002). *In vitro*, Cx43 is phosphorylated primarily at Ser-373 by Akt, but also at Ser-369 (Park et al., 2007). If intracellular Akt is inhibited, there is a reduction in the degree of binding of a phosphospecific antibody for phosphorylated Ser-373 (Dunn and Lampe, 2014).

In PAH, the role of Cx43's post-translational modifications (PTMs) extends beyond mere cellular response to the disease's pathological stimuli. The aberrant forces and hypoxia characteristic of PAH alter the phosphorylation and S-nitrosylation patterns of Cx43. Such modifications influence channel gating, potentially leading to endothelial dysfunction and disrupted intercellular communication (Freund-Michel et al., 2016). Stress-related ubiquitination can trigger accelerated Cx43 degradation, eroding gap junction integrity, which is essential for cellular signalling. This degradation process contributes to the vascular remodelling and inflammation seen in PAH (Wang et al., 2017, Solan and Lampe, 2014). Moreover, altered hemichannel activity due to PTMs affects calcium signalling and fibrotic responses, driving the pathophysiological changes observed in the disease (Palmer, 2008). Consequently, these PTMs do not simply occur in parallel with PAH but are implicated in its progression. Clarifying the specific PTMs of Cx43 during PAH and their impact on vascular homeostasis and response to injury is essential, as it may reveal novel therapeutic avenues to restore normal Cx43 functionality and preserve vascular communication, thereby providing new directions for PAH treatment.

1.3.8 Pharmacological Modulation of Connexin Channels

Connexin channels have complicated pharmacological properties. Unlike the availability of specific inhibitors for blocking particular types of ion channels such as Na^+, Ca^{2+}, and K^+ channels, connexin channels lack specific inhibitors that can

selectively block their activity. Over the past two decades, numerous studies have postulated that peptide-based molecules could offer higher specificity and minimal off-target effects (Leybaert et al., 2017). A further challenge is to distinguish between the properties of GJs and HCs since many agents inhibit both types of connexin-based channels. Finally, the effects of practically all inhibitors are not evident within nanomolar concentrations; they are seen in the micromolar range. Modifying the function of both channel types with the use of chemical agents can be accomplished in numerous ways; the drugs modify various levels of channel structure and function.

1.3.9 Peptide Modulators of Connexin Channels

Efforts have been made to optimise specificity by utilising peptides that directly or indirectly engage with connexins, thus regulating, inhibiting or enhancing channels' functions. The majority of peptides employed match a connexin protein sequence, e.g. Gap 26 and Gap 27, whilst some, e.g. anti-arrhythmic peptide 10 (AAP10), are quite different.

AAP10 originates from an intrinsic substance that has been extracted from bovine atria. It improves the coordination of spontaneously beating foetal chicken cardiomyocytes in cultivated clusters. AAP10 is not designed to mimic the sequences in connexin proteins. Instead, it enhances the function of gap junctions. While the exact mechanism of AAP10 remains unclear, it is postulated to increase the conductance of gap junction channels in cardiac cells, thereby potentially preventing or reducing heart arrhythmias (De Vuyst et al., 2011).

Connexin mimetic peptides that have sequences matching the extracellular loop (EL) domains were noted to inhibit the coordination of chicken myoball contractions, thus providing an indirect method to estimate GJ coupling (Warner et al., 1995). This research was based on a theory that the administration of extrinsic peptides that mimicked critical EL domains would therefore engage with the ELs, thus impeding the docking of competitive HCs. Several conserved motifs within the ELs, introduced as synthetic peptides, were noted to inhibit GJS when studied in the myoball model (this model was detailed in (Clapham et al., 1980). A half maximal inhibitory concentration (IC_{50}) of 20–30μM is typical of Gap26 and Gap27 GJ inhibition (Chaytor et al., 1997). The concentration of the two peptides

necessary to attain maximal impact is 200–300μM. These parameters were not acquired through assessment of GJ coupling directly but by indirect assessment of phenylephrine-induced rhythmic activity of endothelium-denuded rabbit superior mesenteric artery rings. GJ inhibition by Gap26/27 is never absolute and it is estimated that for Gap26, inhibition is approximately 95%. To date, the majority of research suggests that Gap26/27 peptides engage with HCs that are less associated with GJs (Leybaert et al., 2003). It is interesting to note that these peptides have a much more rapid inhibitory effect on HCs than on GJs (Desplantez et al., 2012).

Although Gap26/27 peptides demonstrate a degree of selectivity for various isoforms of connexin, they have also been observed to inhibit Pannexin 1 (Panx1) channels, which belong to the same super-family as connexins and although they can form membrane-associated channels, they do not form gap junctions and therefore do not allow direct exchange of small signalling molecules between cells (Wang et al., 2007, Dahl, 2007, Sosinsky et al., 2011).

This is not entirely surprising because of the concentrated solutions of ≥200μM required to block connexin channels which make effects on additional protein structures such as the Panx1 channels more likely to occur. However, this is a weak effect compared with their action on the connexin channels. The higher the peptide concentration, the more likely additional non-selective influences are to occur and so it is advised to work with concentrations of less than 200μM. The highest degree of off-target effects occurs at 1mM, where inhibition seen with active and disordered peptide sequences can no longer be differentiated (Wang et al., 2012).

1.4 Connexin Signalling in Large Conduit Systemic Arteries

In the context of systemic circulation, large arteries like the aorta and carotid arteries play crucial roles. The cellular behaviour within these vessels is regulated through intercellular communication, with connexin signalling playing a significant part in this process. Studies conducted using transmission and scanning electron microscopy, along with immunohistochemistry, have indicated that endothelial cells (ECs) in these large arteries effectively communicate through gap junctions. Generally speaking, both the elastic (as seen in the aorta) and muscular (as seen in coronary) arteries of many species are known to abundantly express specific connexins Cx37, Cx40, and Cx43 (Gabriels and Paul, 1998, Yeh et al., 1997, van Kempen and Jongsma, 1999). In terms of functionality, it has been demonstrated that these large-vessel ECs allow the transfer of certain dyes such as lucifer yellow and carboxyfluorescein between them, providing evidence of open gap-junction channels (Simon and McWhorter, 2003). When investigating different flow patterns across the ECs, some investigations have identified differences in connexin expression or changes in dye-transfer-mediated intercellular communication between ECs (Ebong et al., 2006). Nevertheless, it is important to note that the full functional significance of these findings remains to be fully elucidated.

In large systemic arteries, gap-junctions among smooth muscle cells (SMCs) facilitate the coordination of intracellular calcium-mediated contractions along the length of these vessels. This concept has been amply reported in the relevant literature (Fanchaouy et al., 2005). According to Reidy (1990), gap junctions in these large arteries may play additional roles as well as coordinating vascular constriction. In their studies, they observed that inserting a catheter at the anterior end of the aorta increased the SMC's uptake of thymidine (an indicator of cellular mitosis) along the whole of the thoracic and abdominal aorta (Reidy, 1990). This indicates that mitosis regulation involves long-distance communication between SMCs in the larger arteries of the systemic circulation. It is important to note that while the evidence around connexin signalling in large conduit pulmonary arteries is not extensive, the available studies suggest that connexins, particularly Cx43, may play a role in regulating vascular function in these vessels (Sedovy et al., 2022).

Further research is needed to elucidate the specific mechanisms underlying these effects and to determine their relevance to different physiological and pathological conditions.

1.4.1 Connexin 43 in Large Conduit Systemic Arteries

Cx43 is variably found in both ECs and SMCs of large systemic arteries (Bruzzone et al., 1993, van Kempen and Jongsma, 1999). In later research, it was shown that Cx43 expression is region-specific and that it is only present in the branch areas of the rat thoracic and ascending aorta, which are under high levels of shear stress (Gabriels and Paul, 1998, Hill et al., 2002). Additionally, it has been shown that Cx43 has variable expression in the ECs of bovine coronary arteries and porcine coronary arteries (van Kempen and Jongsma, 1999). Nonetheless, many investigations failed to detect Cx43 in rat or human coronary ECs (Yeh et al., 1997, van Kempen and Jongsma, 1999). On the other hand, Gabriels and Paul (1998) established Cx43 in the EC stress response when they discovered that generating vascular stress could increase Cx43 expression in rat aorta EC. These findings imply that in order to keep the large artery EC functioning properly, Cx43 expression is dynamic and controlled by several variables including sheer stress.

Cx43 is also expressed in large quantities in the vascular smooth muscle cells (VSMCs) of the large arteries of many animals. This includes rabbit, rats, mice, bovine and humans (Johnstone et al., 2012, Hill et al., 2002, Johnstone et al., 2009b). Cx43 emerges in the form of small punctates in the VSMC membrane in the carotid arteries of mice, which indicates that a gap junction is developing. Moreover, Cx43 expression varies based on vessel location as well. In rat models, the expression of Cx43 in the VSMC of the ascending aorta and arch is higher than in the descending aorta (Ko et al., 2001). Research indicates that in smooth muscle Cx43 gene knockout mice (SM Cx43 KO), there is a significant increase in smooth muscle cell proliferation in the carotid arteries (Liao et al., 2007). In contrast, a study involving a novel knock-in mouse containing Cx43-MK4A mutation, which characterized by the loss or reduction of Cx43, was found to significantly reduce VSMC proliferation in the carotid arteries (Johnstone et al., 2012). The discrepancies between these studies may be attributed to a multitude of factors, one of which could be the differing methods employed for the deletion or reduction of Cx43 levels. In the SM Cx43 KO mice, Cx43 gene in SMCs was deleted,

while in Cx43-MK4A knock-in mice, mutations were created in the Cx43 gene to mimic the effects of MAPK phosphorylation. These methodological differences could lead to varying degrees of Cx43 loss, thereby influencing the observed outcomes. Hence, these findings suggest that Cx43-mediated communication amongst the SMCs in the systemic circulation may regulate cell division rates. This could further imply that under physiological conditions, VSMCs in conduit vessels may communicate through only a restricted number of Cx43 gap junctions. It is probable that this reflects the role of the VSMC in these vessels, which supports the vessel wall against cardiac output pressures instead of coordinating contractile responses (as is the case in resistance arteries).

Alterations in the expression or function of Cx43 have been linked to various diseases, particularly those affecting the conduit vessels in the systemic circulation. For instance, changes in Cx43 expression or function have been associated with the development of atherosclerosis. The expression of Cx43 in VSMC of the human coronary artery was found to increase during the early stages of atherosclerosis, in contrast to its expression levels in healthy arteries (Blackburn et al., 1995). In the late stages of human atherosclerosis, however, the expression of Cx43 in VSMCs falls below the levels observed in healthy arteries. Similar reductions in Cx43 expression, both at the mRNA and protein levels, have been also reported in atherosclerosis models involving mice and rabbits (Blackburn et al., 1995). These observations indicate a time-dependent role of Cx43 across different stages of atherosclerotic disease (Kwak et al., 2002). Moreover, in low-density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice, a widely used model for studying atherosclerosis, Cx43 heterozygous knockdown mice ($Cx43^{+/-}$) results in reduced formation of atherosclerotic lesions compared to $Cx43^{+/+}$ $LDLR^{-/-}$ mice (Kwak et al., 2003). Furthermore, it was found that exposure to the oxidized phospholipid derivative 1-palmitoyl-2-oxovaleroyl-sn-glycerol-3-phosphorylcholine, which is associated with atherosclerosis, in mouse carotids leads to a change in the VSMC phenotype. This alteration results in an increase in VSMC proliferation, which is linked to an enhancement of mitogen-activated protein kinase (MAPK) Cx43 phosphorylation at Cx43-S279/282 (Johnstone et al., 2009b).

These studies propose that Cx43 and gap junction signalling play a part in controlling the development of atherosclerotic plaques, and that the specific phosphorylation state of Cx43 may influence cell functions during the progression of the disease. Nonetheless, the precise role of this process in the advancement of the disease remains uncertain.

1.5 Connexin Signalling in Small Resistance Arteries

Connexin signalling plays an integral role in the functionality of the vascular system, with specific relevance to small arteries. These small arteries, crucial for peripheral resistance and blood pressure regulation, exhibit a rich expression of connexins that forms the foundation for gap junction communication. Anatomical and immunohistochemical data suggest that endothelial cells (ECs) in resistance vessels exhibit significant intercellular communication, much like those observed in the ECs of large arteries. Some earlier studies such as that performed by Little et al. (1995b) focused on addressing the functional presence of gap junctions and found that ethidium bromide, biocytin, lucifer yellow and carboxyfluorescein, could pass quickly between ECs in the arterioles present in hamster cheek pouches (Little et al., 1995b). Research indicates that Cx40 and Cx43 are both possible dye transfer mediators (Little et al., 1995a). This was investigated *in vivo* in mouse lung ECs using what are known as "caged" second-messenger compounds. These are molecules that can be activated when exposed to UV light. Upon exposure to UV light, these caged compounds liberate IP_3 or Ca^{2+}, thereby emphasising the significant role Cx43 plays in the movement of calcium within ECs. Little et al. (1995b) also observed that the release of these caged compounds induced a calcium wave among the ECs. However, mice with an EC-specific deletion of Cx43 exhibited significantly diminished calcium communication between the ECs (Parthasarathi et al., 2006).

The assumption is that gap junctional intercellular communication (GJIC) plays a significant role in coordinating arteriole constriction in SMCs. However, the specific function of gap junctions in SMCs of resistance arteries still warrants more comprehensive research to fully understand its implications. Many studies involving electrical coupling have demonstrated this (Bény, 1996). Nonetheless, studies that have examined coupling through dye transfer within resistance vessels have produced conflicting results (Little et al., 1995b, Segal and Beny, 1992).

An *in vivo* immunofluorescent punctate identification and subsequent gap-junction plaque verification can be incredibly challenging (Looft-Wilson et al., 2004).

The extent to which SMCs and ECs are integrated through direct cell contact to control vascular function is a key feature of resistance-vessel physiology. There are a number of paracrine factors such as prostaglandins and NO that facilitate this integration, which can occur through gap junctions known as MEJ. Even though some studies have examined SMC-to-EC communication through MEJ, three different model systems (two *in vivo* and one *in vitro*) have shown that increases in SMC $[Ca^{2+}]_i$ generate a resultant increase in EC $[Ca^{2+}]_i$. This process is mediated by the gap junctions at the MEJ (Isakson et al., 2007, Lamboley et al., 2005). Both of these models have shown that IP_3 passes from the SMCs to the ECs via gap junctions, resulting in changes in the EC $[Ca^{2+}]_i$ in rat mesenteric arteries (Lamboley et al., 2005) and mouse cremaster arterioles (Isakson et al., 2007). In turn, this results in the release of NO (Dora et al., 1997). Thus, this may be a key mechanism enabling these resistance vessels to manage vasoconstriction in the SMCs.

It's important to note that the role of gap junction signalling in small arteries within the pulmonary circulation is not well-studied but has recently started to receive attention. There is a downregulation of Cx37 and Cx40 expression in PAECs from patients with PAH (Kim et al., 2015). It was also found that mice lacking Cx40 are protected against hypoxic-induced PH and hypoxic pulmonary vasoconstriction (Yang et al., 2014). Therefore, dysregulation of the connexin-mediated signalling pathway may potentially contribute to the pathogenesis of certain diseases, such as pulmonary hypertension (reviewed recently in more detail in Htet et al. (2021).

1.5.1 Connexin 43 in Small Resistance Arteries

Cx43 is present in the EC of small vessel in the mice, specifically in the cremaster arterioles (Looft-Wilson et al., 2004). In addition, the rat cremaster, rat brain, and hamster cheek pouch arteriole EC all contained Cx43 (Little et al., 1995a). Cx43 was also found in the rat mesenteric artery and the mesenteric arterioles (Gustafsson et al., 2003). Additionally found in the EC, Cx43 HCs serve as purinergic release channels (Faigle et al., 2008, Begandt et al., 2017).

Purinergic ATP release regulates vascular tone by mediating the production of nitric oxide (NO)/prostacyclin, which causes vasodilation. This is also associated with immunological responses such as neutrophil activation/infiltration (Burnstock, 2017). Numerous investigations have shown that inhibiting HC activity reduces ATP release in human microvascular EC (Faigle et al., 2008, Calder et al., 2015).

There is limited research on the extent of Cx43-mediated homocellular coupling among VSMC between different species, and further investigation into specific tissue beds remains to be explored in depth. Jiang and Goodenough (1996) provided electrophysiological evidence of VSMC coupling by Cx43 and Cx40 in rat models. Recently, Borysova et al. (2018) conducted research to examine isolated rat mesenteric resistance arteries and the function of gap junctions in Ca^{2+} signalling and vasoconstriction. When the endothelium layer was removed, the non-specific gap junction inhibitor 18-glycyrrhetinic acid blocked the distribution of Ca^{2+} spikes between VSMC (Borysova et al., 2018). Additionally, Cx43 mimetic peptide and channel blocker, Gap26, successfully inhibited arginine vasopressin-induced vasoconstriction (Kwak et al., 2002, Yang et al., 2017). Furthermore, Kwak et al. (2002) found that Cx43 plays a direct role in facilitating VSMC -to-VSMC contraction in the mesenteric arteries of rats. However, there are conflicting reports of a direct link between VSMC. For instance, Haddock et al. (2006) found that endothelial denudation in the primary branches of basilar arteries in rats reduced Ca^{2+} synchronisation in VSMC. Limited lucifer yellow dye transfer between VSMC in comparison to EC was discovered by Little et al. (1995b) indicating changes in the channel number, composition and/or regulation between EC and VSMC.

It was found that renal hypertension correlates with increased gene expression of Cx43 in the aortic cells of spontaneous hypertensive rats (SHRs) (Haefliger et al., 1997). Furthermore, Gao et al. (2021) also found in SHRs that Cx43 not only plays a role in intercellular communication during hypertension, but it also promotes blood vessel contraction, increases vascular compliance, and ultimately leads to cerebral artery remodelling. It was also suggested that Cx43 appears to be a signalling molecule that promotes proliferation and migration of VSMCs from brain basilar arterioles and also contributes to vascular remodelling in SHRs through PKC and MAPK/extracellular signal-regulated kinase (ERK) signalling pathways (Gao et

al., 2021). For the development of novel therapeutic approaches, a thorough understanding of these communication systems is essential.

It has been reported in a number of studies that Cx43 plays a crucial role in pulmonary vasculature. For example, in pulmonary vasoconstriction responses, Billaud et al. (2011a) found that ^{37,43}Gap27, a pharmacological inhibition of Cx37 and Cx43, significantly reduced the contractile responses to 5-HT and phenylephrine in intra-lobar pulmonary arteries (IPAs) isolated from normoxic rats as well as in CH and MCT rats. Recently, the contractile response and potency of phenylephrine were significantly attenuated in IPAs from Cx43 heterozygous ($Cx43^{+/-}$) mice when compared to WT mice under normoxic conditions (Bouvard et al., 2020). Moreover, Bouvard et al. (2020) discovered that $Cx43^{+/-}$ mice showed partial protection against cell proliferation and intrapulmonary artery remodelling associated with chronic hypoxia-induced pulmonary hypertension (CH-PH). Specifically, in $Cx43^{+/+}$ mice exposed to CH-PH conditions, pulmonary artery remodelling characterized by smooth muscle and endothelial cell proliferation, and inflammation revealed by immune cell infiltration, were observed. In contrast, these processes did not appear in $Cx43^{+/-}$ mice with CH-PH, suggesting that blocking Cx43 could have a beneficial effect in CH-PH (Bouvard et al., 2020). Additionally, connexins also play a role in pulmonary vasodilator responses. For example, the dilatation responses to an endothelial-dependent vasodilator methacholine (MCh) was found to be decreased in IPAs in the presence of ^{37,43}Gap27 as well as in Cx43 heterozygous ($Cx43^{+/-}$) mice when compared to WT mice (Htet et al., 2018). Further, it is important to note that Cx43 gene and protein expression was significantly increased in IPAs from CH rats IPAs (Billaud et al., 2011a), as well as in rat PAFs (McNair et al., 2020). Moreover, it has been shown that hypoxia increases proliferation and migration of rat PAFs, and that these responses were inhibited both by ^{37,43}Gap27 and by genetically knocking down Cx43 by using siRNA (McNair et al., 2020).

Altogether, our work will build on the previous studies, which have demonstrated that Cx43 plays an important role in systemic and pulmonary circulation, by investigating both genetic knockout ($Cx43^{+/-}$) and pharmacological inhibition of Cx43 (^{37,43}Gap27) to further examine the effect they have on systemic and pulmonary vascular reactivity using both thoracic aortae and IPAs.

As mentioned previously, evidence suggests a co-dependent relationship between NO and connexin communication pathways, with the exact mechanisms of their interactions still unclear.

Therefore, to better understand the relationship between NO and gap junctions, this study will also investigate the production of NO and the possible interaction between the Cx43 and eNOS signalling pathways in thoracic aorta and the whole left lung lobe of $Cx43^{+/-}$ mice. Based on existing evidence that $Cx43^{+/-}$ mice are protected against hypoxia-induced pulmonary vascular remodelling (Bouvard et al., 2020), the research presented here hypothesises that Cx43 plays a critical regulatory role in both systemic and pulmonary vascular function. Specifically, it proposes that Cx43 influences the interplay between cellular communication and nitric oxide production, impacting vascular reactivity, endothelial integrity, and the response to hypoxic conditions. This hypothesis underpins our aim to assess the role of Cx43 in hypoxia-induced proliferation and migration of mouse pulmonary arterial fibroblasts (MPAFs) using both ^{37,43}Gap27 and $Cx43^{+/-}$ mice.

1.6 Aims and Objectives

- To investigate the role of connexin 43 in the systemic circulation using mouse thoracic aortae. This investigation will encompass the study of vascular reactivity, endothelial denudation, sex differences and the potential interaction between Cx43 and the eNOS signalling pathway (Chapter 3).

- To investigate the role of connexin 43 in the pulmonary circulation using mouse intra-lobar pulmonary arteries (IPAs) and lungs. This investigation will involve the study of vascular reactivity, sex differences and the possible interaction between Cx43 and the eNOS signalling pathway (Chapter 4).

- To investigate the role of connexin 43 in proliferation and migration of mouse pulmonary arterial fibroblasts (MPAFs), examining its potential impact on pulmonary vascular remodelling, a key process in the pathogenesis of pulmonary hypertension (Chapter 5).

Chapter 2 Materials and Methods

2.1 Ethical statement

All animal procedures were carried out in accordance with Directive 2010/63/EU of the European Parliament. Ethical approval for the $Cx43^{+/-}$ mouse studies was granted by the University of Strathclyde Animal Welfare and Ethics Committee. All experiments involving animals were performed under authority of Dr Yvonne Dempsie's project licence (number PEE7AAC2C). ??

2.2 Materials

All primers for genotyping were purchased from Integrated DNA technologies (IDT, Belgium). All antibodies used in Western blot analysis were purchased from Sigma Aldrich, Dorset, UK and Cell Signalling Technology, Danvers, USA. All tissue culture flasks and media were obtained from Gibco (Paisley, UK). All reagents for buffers were purchased from ThermoFisher, UK or Sigma Aldrich, unless otherwise stated. Acetylcholine (ACh) (A6625) and U46619 (538944) were bought from Sigma Aldrich. S-Nitroso-N-acetylpenicillamine (SNAP) (BML-CN210-0020) was obtained from Enzo, Exeter, UK. Gap27 (A14866-25) was obtained from Adooq Bioscience, Irvine, CA.

2.3 Methods

2.3.1 Connexin 43 heterozygous ($Cx43^{+/-}$) mouse model

The Cx43 heterozygous ($Cx43^{+/-}$) mice used in these studies were generated by homologous recombination in embryonic stem cells via insertion of a neomycin resistance gene (neo^r) into exon 2 of the Cx43 (GJA1) gene (detailed in Reaume et al. (1995) (Figure 2-1). In homozygous ($Cx43^{-/-}$) mice, a fatal condition occurs due to the failure in pulmonary gas exchange caused by swelling and blockage of the right ventricular outflow tract from the heart, rendering them unsuitable for further study (Reaume et al., 1995).

2.3.2 Breeding and ear notching

Wild-type (WT) and $Cx43^{+/-}$ mice were bred together. In order to prevent generation of $Cx43^{-/-}$ offspring which could not survive, $Cx43^{+/-}$ mice were not bred with each other. Breeding with siblings was avoided due to complications of inbreeding. Mice were numbered and ear notches were collected at 6 weeks old and stored at -20°C.

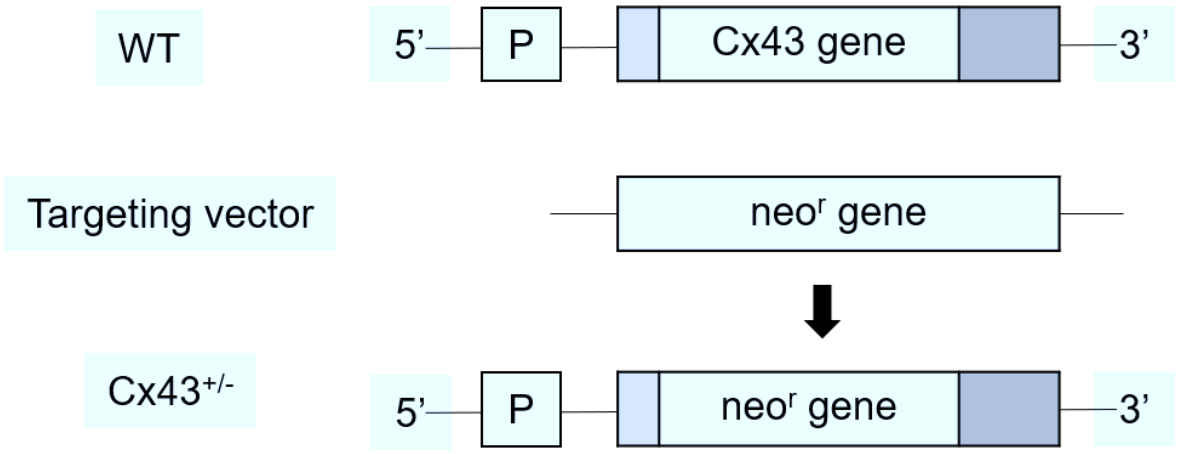


Figure 2-1. An illustration of how the Cx43 heterozygous mouse was generated by homologous recombination in embryonic stem cells. An insertion of a neomycin resistance gene (neo^r) without promoter region into exon 2 of the Cx43 gene was made.

2.3.3 Genotyping

2.3.3.1 DNA extraction

DNA was extracted from the ear notches of both male and female WT and Cx43$^{+/-}$ mice. First, 10µl of DNAreleasy (Anachem LS02, UK) was added to lyse the ear notch tissues and all samples were then incubated for 7 minutes on a thermocycler under the following conditions: initial denaturation 75°C for 5 minutes, denaturation at 96°C for 2 minutes and hold at 20°C. The DNA concentration was then diluted by adding 70µl of distilled water into 5µl DNA, and this stock DNA was stored at 4°C until used for polymerase chain reaction (PCR).

2.3.3.2 Polymerase chain reaction (PCR)

The PCR technique was used to amplify Cx43 (GJA1) and neomycin resistance (neo^r) genes. The experiment consisted of two main steps: PCR and gel electrophoresis. Each PCR tube contained a total volume of 17µl of the following: 7µl of GoTaq master mixes (Promega, UK), 7µl of Nuclease-free water, 1µl of DNA sample and 0.5µl of each of the forward and reverse primers of Cx43 and neo^r. The sequences of the primers were as follows: neo^r forward: 5' GAT CGG CCA TTG AAC AAG ATG 3', melting temperature (Tm)= 56.4°C, molecular weight (MW)= 6808.5; neo^r reverse: 5′ CCT GAT GCT CTT CGT CCA GAT 3', Tm= 57.2°C, MW= 6637.3; Cx43 forward: 5′ CAG TCT GCC TTT CGC TGT 3', Tm= 56°C, MW= 5433; Cx43 reverse: 5′ GTA GAC CGC ACT CAG GCT 3', Tm= 58°C, MW= 5485. Finally, all tubes were transferred to a thermocycler (Biometra GmbH, Analytik Jena, Germany), which was programmed to start at 95°C for three minutes to allow initial DNA denaturation. This was followed by further denaturation at 95°C, annealing at 57°C for 30 seconds and extension at 72°C for one minute. This process of denaturing, annealing and extension was repeated 40 times (Table 2-1), thereby increasing exponentially the number of copies of the Cx43 (GJA1) and neomycin resistance (neo^r) genes.

Step	Temperature	Time	Number of cycles
Initial denaturation	95°C	3 minutes	1
Denaturation	95°C	30 seconds	40
Annealing	57°C	30 seconds	
Extension	72°C	1 minute	
Final extension	72°C	15 minutes	1
Hold	4°C	-	-

Table 2-1. The PCR reaction steps in genotyping process.

2.3.3.3 Agarose gel electrophoresis and DNA detection

A 2% agarose gel was then prepared by adding 2g agarose (ThermoFisher, UK) to 100ml Tris-Acetate-EDTA (TAE) buffer (ThermoFisher, UK), and dissolved by heating in a microwave on high power for one minute. 4µl of Midori Green (Chembio, UK) was then added to the agarose solution as a nucleic acid stain, and subsequently the solution was poured into a casting tray with combs in place and allowed to set for 20 minutes at room temperature. After the gel had set, it was transferred into the electrophoresis tank and covered with TAE buffer. Fifteen microliters (15µl) of each of the samples was then loaded into separate wells and 10µl of HighRanger DNA ladder (Norgen Bitotek Corp, Canada) was loaded into a separate well. The gel was then electrophoresed at 100V for 55 minutes using a Flowgen powerpack (BIO-RAD, UK) at room temperature. Finally, the DNA bands were visualised and photographed using UV light on a transilluminator (Red™, Bio-Techne, UK) and analysed by Image Lab™ software. Both positive controls (samples from mice previously shown to be heterozygous) and negative controls (samples with no DNA, only GoTaq master mixes) were also included. PCR products of $Cx43^{+/-}$ mice yielded two distinct bands: a lower one representing the gene expression of Cx43 and an upper one representing the presence of neo^r. However, the WT mice yielded only one band which is for Cx43 (Figure 2-2).

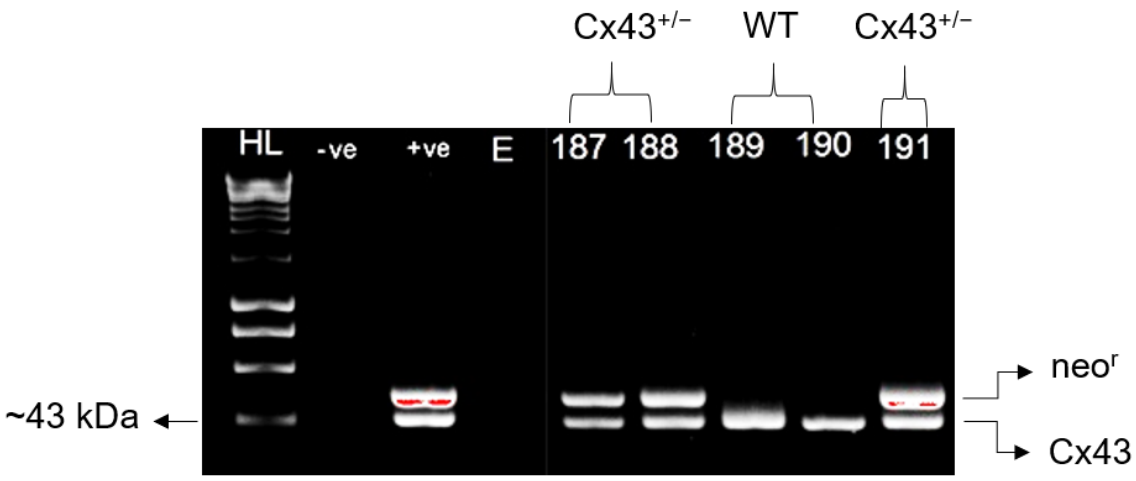


Figure 2-2. Representative result of gel electrophoresis of PCR products of Cx43 heterozygous ($Cx43^{+/-}$) and wild type (WT) mice. HL= hyperladder (1Kb); -ve= negative control; +ve= positive control; E= empty well; sample ID 187,188,191= $Cx43^{+/-}$ mice; sample ID 189 and 190= WT mice.

2.3.4 Wire myography

2.3.4.1 Animals and Euthanasia

Thoracic aortae from 4- to 6-month-old mice and intra-lobar pulmonary arteries (IPAs) from 2- to 3-month-old mice were dissected free from age-matched male and female wild-type (WT) and connexin 43 heterozygous ($Cx43^{+/-}$) littermate mice (C57BL/6 background). Prior to removal of blood vessels, mice were euthanised by intraperitoneal injection (IP) using pentobarbital sodium (Euthatal®) (60mg/kg) plus lidocaine (4mg/kg). Direct palpation of either the pulse in the carotid or femoral artery or direct cardiac palpation was then assessed to confirm the death.

2.3.4.2 Dissection and isolation of the thoracic aortae and intra-lobar pulmonary arteries (IPAs)

After euthanasia, mice were positioned in the supine position and the abdominal skin just inferior to the xiphoid process of the sternum was identified. Lateral incisions were made along the subcostal margins and the anterior part of the ribcage was removed. The chest cavity was cleaned of blood and fluid using sterile gauze. The whole heart, lungs and aortae were then isolated and transferred into a petri dish containing cold physiological salt solution (PSS) (the composition of PSS was (mM): 119.0 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 24.9 $NaHCO_3$, 1.2 KH_2PO_4, 11.1 Glucose and 2.5 $CaCl_2$). The pins were then placed at the edges of the left and right lungs and in the apex of the heart, and the thoracic aorta, which is located within the posterior mediastinal cavity, was dissected free and perivascular adipose tissue (PVAT) around it was then removed. The intra-lobar pulmonary artery (IPA), which is also known as the third order of pulmonary arteries, is a continuation of the left pulmonary artery branch inside the left lung lobe. Once isolated, the surrounding parenchyma and airway smooth muscle was carefully dissected free from the pulmonary artery.

2.3.4.3 Mounting the tissues

Following the dissection and isolation of aortae and IPAs, the vessels were cut into 2mm segments. Two wires of 2cm length and 40µm in diameter were then carefully inserted into the lumen, and the artery was mounted on a wire myograph (Multi Myograph Model 610M, Danish Myo Technology, Denmark) (Figure 2-3).

Vessels were gassed (95% O_2, 5% CO_2) and maintained at 37°C in PSS and allowed to equilibrate for 30 minutes. After equilibration of aortae, tension was added to the vessels (known as normalisation) by stretching in a stepwise manner until the required tension (9.81mN equivalent to 1g) was reached (Conklin et al., 2009). For IPAs, after equilibration, the vessels were normalised to reach a target pressure 12–15mmHg or 1.5996 – 1.9995 KPa, which is equal to 2-2.5mN. The normalisation of wire-mounted arteries is important as it sets the preparation to an internal circumference that gives a maximal response. This practice mimics *in vivo* conditions and standardises experimental conditions, ensuring consistency across studies. LabChart™ v8 Pro software (ADInstruments, Chalgrove, U.K.) was used to record vessel tension. At the beginning of each experiment, 60mM of potassium chloride (KCl) was added three times and washed out in between using PSS to check if the vessels were viable or not and to stimulate the vessels to constrict. When vessels were found to be viable, they were then pre-constricted using 30nM of the thromboxane A2 (TP) receptor agonist U46619 and relaxation responses were carried out using cumulative additions of the endothelium-dependent vasodilator ACh (0.1nM-30μM). In addition, concentration-response curves (CRCs) to U46619 (0.1nM -10μM) were constructed in vessels at baseline tension, and also to the nitric oxide donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP) (0.1nM–30μM), in vessels precontracted with 30nM of U46619. To check the effect of pharmacological inhibition of Cx43 on function of thoracic aortae and IPAs, 100μM of ^{37,43}Gap27, which is an inhibitor of Cx37 and Cx43, was added and left for 30 minutes before adding relaxant and contractile drugs.

Endothelium-free vessels were prepared by gently rubbing the lumen with a small wire of 2cm length and 40μm in diameter. To verify the removal of the endothelium, vessels were tested with the endothelium-dependent vasodilator acetylcholine (ACh) (1μM) after precontraction with 30nM of U46619. Vessels that relaxed more than 50% of U46619-induced contraction were considered as intact-endothelium, and any rings with less than 20% relaxation were counted as a denuded vessel.

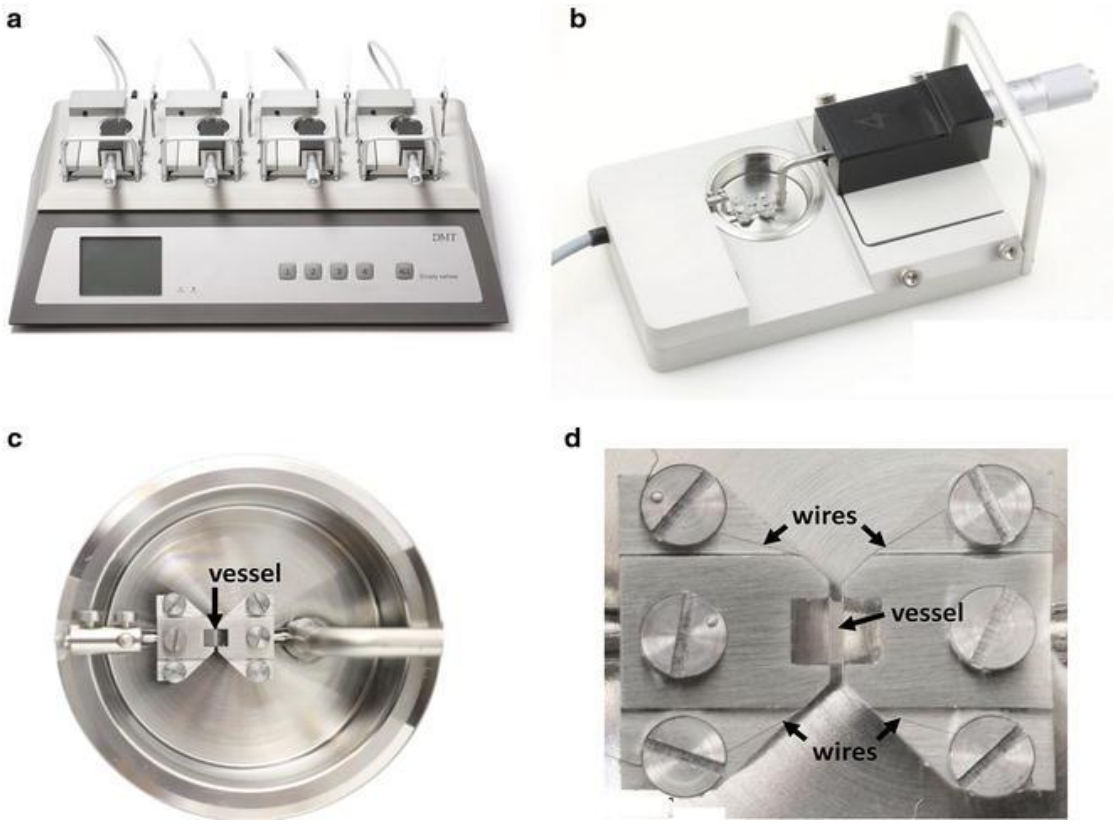


Figure 2-3. The wire myograph components. (a) DMT multi-chamber 620M Wire Myograph, (b) Myograph unit which has a force transducer (left) and a micrometer (right), (c) Organ bath unit with the jaws and a mounted vessel segment. (d) The jaws, screws, wires, and a mounted vessel segment, adapted from www.dmt.dk.

2.3.5 Cell culture

2.3.5.1 Primary culture of mouse pulmonary arterial fibroblasts (MPAFs)

Main and branch pulmonary arteries were dissected free from the lung of female mouse, cut longitudinally and opened into a flat sheet. A sterile razor blade was used to gently abrade and remove muscular tissue and the endothelial layer. To harvest enough cells, two pulmonary arteries were prepared and then both were cut into 5mm^2 pieces and distributed over the base of a 25cm^2 culture flask containing 2ml of Dulbecco's modification of Eagle's medium (DMEM) supplemented with 20% foetal bovine serum (FBS) plus 100µg/ml primocin and 2mM L-glutamine. The explants were then incubated in a humidified atmosphere of 5% CO_2 at 37°C for three days until they approached confluence before media was refreshed and tissue fragments were removed by aspiration (Figure 2-4).

2.3.5.2 Routine cell maintenance

Cells were grown in 75cm^2 culture flasks containing 10ml of DMEM supplemented with 20% FBS, 100µg/ml primocin and 2mM L-glutamine. Cells were sub-cultured or passaged into fresh growth medium once they reached ~80-90% confluency and this medium was changed every other day.

When cells were to be passaged, DMEM was aspirated off and 2ml trypsin (Life technologies, UK) was added (0.05% trypsin containing 0.02% EDTA). Trypsin is an enzyme which cleaves the molecules which are responsible for cell adhesion. The trypsinisation step was repeated two times and after trypsin was removed, flasks were then returned to a humidified incubator atmosphere of 5% CO_2 at 37°C for 5 minutes. Cells were then visualised under a light microscope (Olympus CK2) after tapping the flask to confirm that the cells were floating and detached from the flask. Cells were then resuspended in 10ml of DMEM with 20% FBS to deactivate trypsin and stop the enzyme activity. Cells were then aliquoted in 3ml portions and either transferred into 75cm^2 culture flask containing 7ml of DMEM with 20% FBS for passaging or freezing processes, or into 6 or 24- well plates after mixing with 47ml of DMEM with 20% FBS for Western blotting, migration and proliferation experiments. Experiments were started when cells reached passage 3-6.

If cells were not required immediately for use, they could be frozen for later use. In that case, cells were trypsinised twice and checked under the microscope as previously described and resuspended in 10ml of DMEM with 20% FBS. They were then transferred into a 15ml falcon tube before being centrifuged at 10,000 rpm for 10 minutes at 4°C. The medium was then removed and the pellet was washed with 1ml of this mixture: 1ml dimethyl sulfoxide (DMSO)+ 9 ml of 20% DMEM. This mixture was then transferred into a 1.2ml cryogenic vial (ThermoFisher, UK) and stored overnight at -80°C for short term storage in a Nalgene™ Cryo 1°C freezing container (ThermoFisher, UK) before they were stored at -80°C for later use.

To remove frozen cells from storage for use, the cells were defrosted by placing the vials in a water bath at 37°C for 10 minutes before transferring the contents into a 75cm^2 culture flask containing 10ml of 20% DMEM. Cells were then left overnight to adhere and attach to the flask, and the medium was then replaced with a fresh 10ml of DMEM with 20% FBS the next day.

2.3.5.3 Proliferation assay

An automated cell counter (Countless II, ThermoFisher, UK) was used to assess cell viability and proliferation. This device employs 0.4% Trypan blue stain (ThermoFisher, UK), which stains live cells green and dead cells red, providing a clear differentiation for accurate counting. This method provides a rapid and precise assessment of the proportion of viable cells in the sample. 3ml of cell suspension was diluted with 47ml of DMEM with 20% FBS and mixed by pipetting up and down. 1ml of diluted cell suspension was then added to each well a 24-well plate. After cells had grown to approximately 60% confluency, they were quiesced for 24 hours using serum-free media (SFM) to stop the cell cycle progression. SFM was used as a negative control while 1% serum was used as a positive control. Cells were then incubated in normoxic or hypoxic (5% O_2, 5% CO_2 and balance N_2) conditions for a further 24 hours. In addition, in some experiments, cells in SFM and 1% serum were both treated with 300μM of [37,43]Gap27. After 24 hours, media was then removed and cells were washed twice with 500μl of sterile phosphate buffered saline (PBS). 100μl of trypsin was then added to each well before the plates were placed in a humidified incubator atmosphere of 5% CO_2 at 37°C for 3 minutes. 500μl of DMEM with 20% FBS was then added to each well and gently mixed and then transferred into 1.5ml centrifuge

tubes and spun at 2600xg for 5 minutes at 4°C. The cell pellet was then resuspended in 10µl SFM and 10µl of 0.4% Trypan blue stain (ThermoFisher, UK), which was used to assess cell viability, was added and a drip was loaded onto a glass slide and inserted into the Countless II cell counter to determine cell concentration per millilitre (Figure 2-5). The viable cell concentration was measured in a minimum of triplicate samples. Percentage change in cell proliferation was calculated compared to normoxic SFM cells.

2.3.5.4 Migration assay

A scratch was made using a sterile surgical blade on the bottom of each 6-well plate as a reference point before cells were cultured and left to grow until 100% confluency was reached. Cells were then quiesced for 24 hours in SFM and an initial scratch was made in line with the reference scratch using a pipette tip before it was photographed using Evos (ThermoFisher, UK). SFM was used as a negative control while 0.1% serum was used as a positive control. In addition, in some experiments, cells maintained overnight in SFM and 0.1% serum were both treated with 300µM of ^{37,43}Gap27. It is important to note that 0.1% serum was high enough to induce migration but not proliferation. Cells were then incubated in normoxic or hypoxic conditions for a further 24 hours. After 24 hours, cells were photographed again for a comparison with the initial photograph after the scratch was made. Scratch or migration assay experiments were performed in a minimum of three replicate plates and using cells isolated from three different animals by measuring the percentage narrowing of the width of the scratch made in the cell layer in four different locations (Figure 2-6).

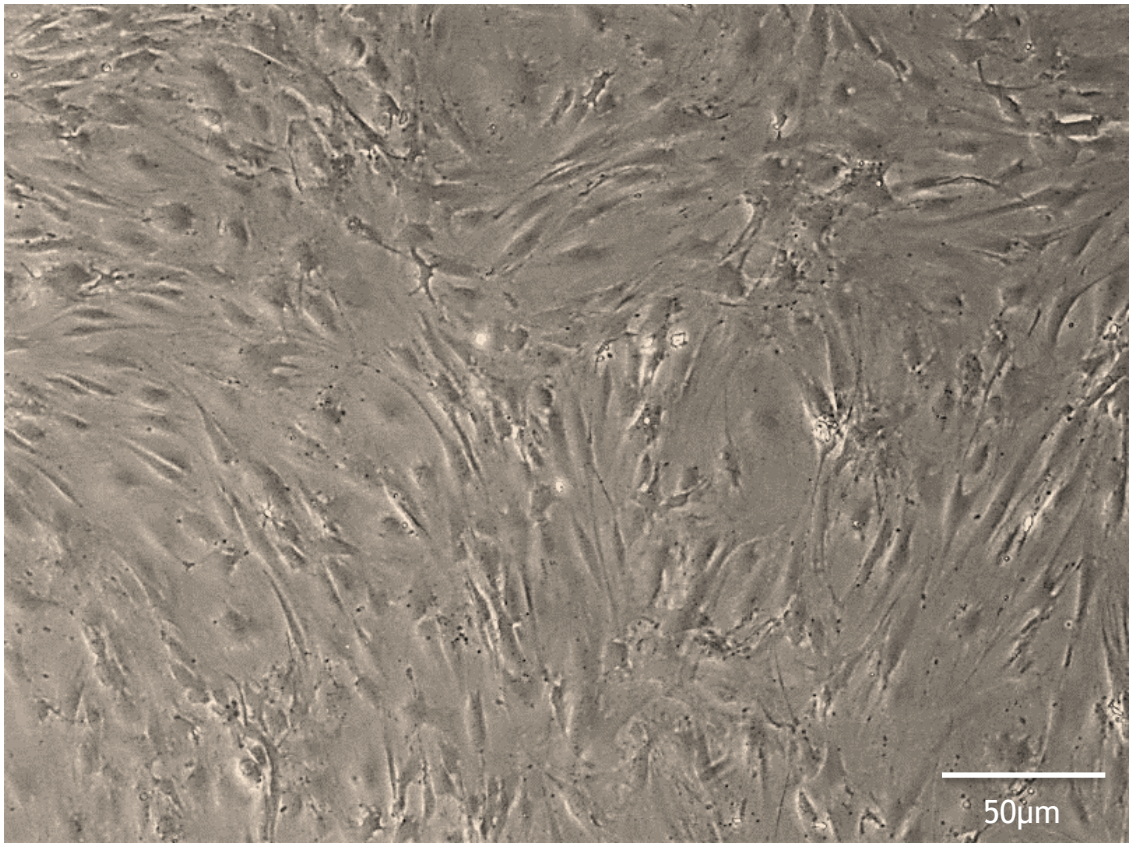


Figure 2-4. Primary mouse pulmonary arterial fibroblasts grown from explants of main and branch pulmonary arteries - photographed under 10x magnification.

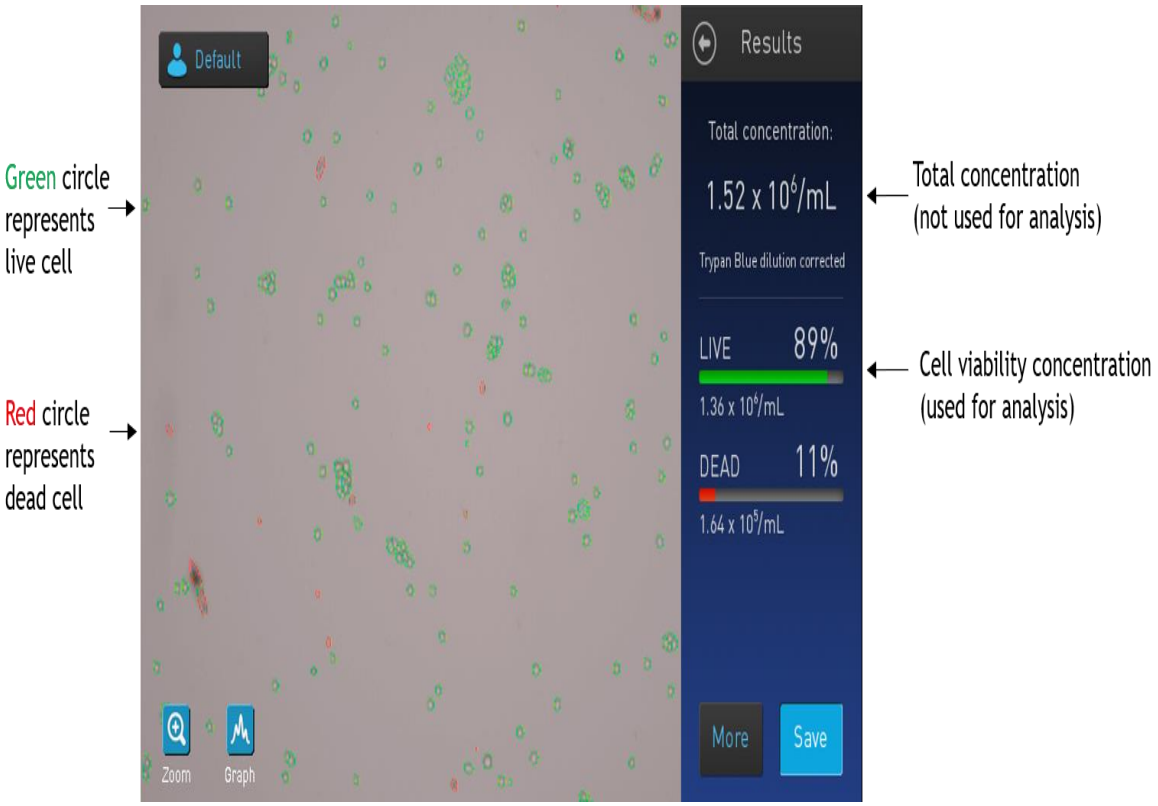


Figure 2-5. An automated cell counter display screen. The screen shows a live image where live cells are encircled in green and dead cells are encircled in red. The cell counter differentiates live cells from dead by utilizing a proprietary viability stain; live cells exclude the stain and remain unstained, while dead cells take up the stain and are marked accordingly. The right panel displays the total concentration of cells, the percentage of live and dead cells, and the absolute counts of each. This image illustrates the cell counter's output, providing a visual representation of cell viability as assessed in the study.

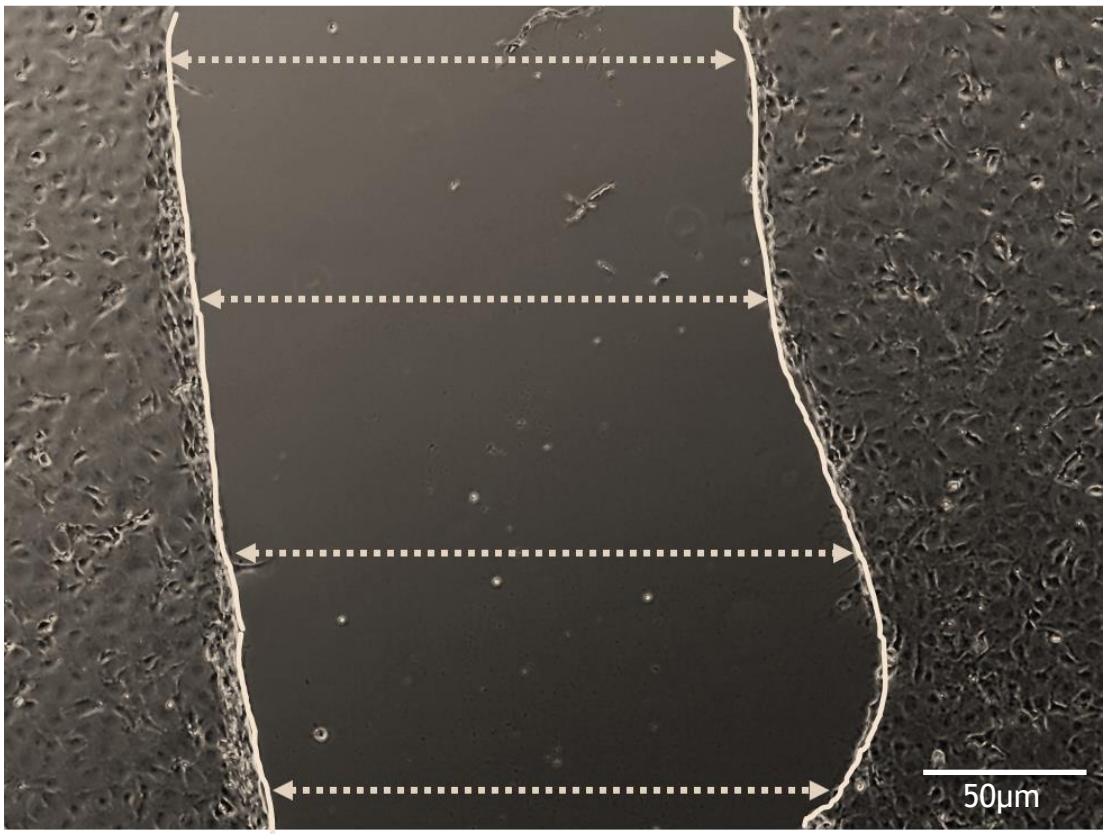


Figure 2-6. Analysis of migration assay. MPAFs were photographed under 10x magnification at 0hr and 24hr and scratch width was measured and averaged at four different locations. Cell migration was assessed at 24 hours by measuring the percentage narrowing of the scratch made in the cell layer.

2.3.6 Western Blotting

2.3.6.1 Protein Extraction

To investigate the protein expression of Cx43 gene (GJA1), protein was extracted from i) thoracic aortae after pooling two or three vessels of the same genotype in one tube ii) whole left lobe lungs and iii) MPAFs. 150-200µl of Radioimmunoprecipitation buffer (RIPA buffer) (ThermoFisher, UK) was used to homogenise the thoracic aortae, while 500µl was used with half of each left lobe lung. The RIPA buffer contained: 25mM Tris-HCl (pH 7.6), 150mM sodium chloride (NaCl), 1% nonyl phenoxypolyethoxylethanol 40 (NP-40), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS). Halt™ phosphatase inhibitor (ThermoFisher, UK) was supplemented with RIPA (10:1000µl) to preserve the phosphorylation state of proteins during and after cell lysis or tissue protein extraction. Fastprep-24 classic Instrument (MP Biomedicals, Germany), which is a high-speed benchtop homogenizer, was used to homogenise the thoracic aortae and lung tissues. The setting on the homogenizer was 5.0m/s with four 30 second intervals with 30 second pauses in between. Once homogenised, the samples were left on ice for 30 minutes to solubilise. The samples were then spun twice in a centrifuge for 10 minutes at 11,200xg at 4°C. The cell suspension was then collected in an Eppendorf tube and proteins were finally stored at -80°C for further use.

MPAFs grown in 6-well plates were scraped on ice using a plastic scraper after being washed twice with 0.5ml PBS and with the addition 50µl of RIPA to each well. This RIPA/cell mixture was then transferred into a labelled Eppendorf tube and spun at 2600xg for 10 minutes at 4°C. The cell suspension was then collected in an Eppendorf tube and proteins were stored at -80°C for further use.

2.3.6.2 Protein Quantification

The dilution series of bovine serum albumin (BSA) (2mg/ml) was used as a reference protein to determine the concentration of the protein in samples prepared from each tissue. Bicinchoninic acid (BCA) protein assay (ThermoFisher, UK) was used for quantitation of total protein in a sample, and it produces a linear calibration curve which allows accurate determination of unknown protein concentration.

After adding the BSA standard samples in duplicate into wells of a 96-well plate, tissue homogenate samples were then added in duplicate. In a fresh falcon tube, 400µl of reagent B was added to 19.6ml of reagent A, and 200µl of this mixing reagent was added to all samples. Reagent A contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide, while reagent B contains 4% cupric sulfate. The plate was then incubated at 37°C for 30 minutes to allow reaction, and then read using a microplate Epoch™ spectrophotometer (BioTek®, USA) to measure the absorbance at 562nm.

The volume of homogenate containing 30µg and 150µg of proteins from thoracic aortae and lungs, respectively, was then calculated and this volume mixed with lithium dodecyl sulfate (LDS) sample buffer (4x) (ThermoFisher, UK), and reducing agent (10x) (ThermoFisher, UK), which contains 500mM dithiothreitol (DTT) in a stabilized liquid form. Distilled water was then added for a final volume of 35-45µl. The samples were then incubated at 95°C for five minutes for thoracic aortae and lungs, and at 70°C for 10 minutes for MPAFS, before loading into the gel to help denature the proteins and allow antibody detection.

2.3.6.3 SDS-PAGE

This step involves the denaturation of proteins with the detergent SDS and using an electric current to pull them through a polyacrylamide gel by electrophoresis (PAGE) to allow separation of proteins based on their molecular weights. The samples were loaded into a precast NuPAGE Bis-Tris gels (ThermoFisher, UK). The gel tank was filled using Bolt MES SDS running Buffer (20X) (ThermoFisher, UK), which is formulated for running proteins on Bolt Bis-Tris gels. 5µl of the blue prestained protein (New England Biolabs, UK) was used as a molecular weight marker. The gel was then electrophoresed at 120V for 1hr or at 200V for 32 minutes at room temperature.

2.3.6.4 Protein Transfer

After SDS-PAGE, proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane with the iBlot 2 gel transfer device (ThermoFisher, UK) using a wet transfer method.

The transfer stacks (ThermoFisher, UK) were then subjected to a continuous voltage as follows: 20V for one minute, 23V for four minutes and 25V for two minutes. The PVDF membrane was then incubated for one hour at room temperature in 5% (w/v) skimmed milk with tris buffered saline (TBS) (10x) (ThermoFisher, UK). This incubation prevented non-specific background binding of both primary and secondary antibodies.

2.3.6.5 Immunoblotting

After the incubation with skimmed milk, membranes were then washed three times at five-minute intervals on a shaker at room temperature using a mixture of TBS and a detergent called Tween 20 (TBS-T) (1x). Primary antibody was then added and incubated with the membrane overnight at 4°C. The following primary antibodies were used: total connexin 43 (t-Cx43) and total endothelial nitric oxide synthase (t-eNOS) (Table 2-2). All primary and secondary antibodies were diluted in 5% (w/v) skimmed milk or BSA with TBS-T (1x) (dilutions listed in Table 2-2). Following incubation, the primary antibodies were collected and stored at -20°C for future use. The membrane was washed three times in TBS-T at five-minute intervals on a shaker at room temperature. Secondary antibody was then added and incubated with the membrane on a shaker for one hour at room temperature. The membrane was then washed three times again at five-minute intervals on a shaker at room temperature using TBS-T (1x).

The proteins were then visualised on the LI-COR® Odyssey system (model 2800), which utilises infrared fluorescence detection, using 700nm and 800nm wavelength setting. After protein visualisation, membranes were stripped using Restore Western blot stripping buffer (ThermoFisher, UK) for 15 minutes on a shaker at room temperature before being washed three times at five-minute intervals, and then stored in 5% (w/v) skimmed milk or BSA with TBS-T for reuse. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ß-tubulin were used as housekeeping proteins to check even protein loading. Quantification of protein expression was measured by densitometry analysis using Image Studio Lite software. For each protein of interest, the density of the band was divided by the density of the GAPDH or ß-tubulin band and then multiplied by 100 to get the percentage change in expression.

Primary antibody	Dilution	Supplier	Secondary antibody	Dilution	Supplier
t-Cx43	1:1000	Sigma, C6219	anti-rabbit	1:5000	Li-COR bioscience (IRDye 800 CW)
t-eNOS	1:5000	Sigma, N2643	anti-rabbit	1:5000	Li-COR bioscience (IRDye 800 CW)
GAPDH	1:5000	Cell signalling, 97166	anti-mouse	1:5000	Li-COR bioscience (IRDye 680 RD)
β-tubulin	1:1000	Cell signalling, 15115	anti-rabbit	1:5000	Li-COR bioscience (IRDye 800 CW)

Table 2-2. List of primary and secondary antibodies used in Western blotting and their concentrations and suppliers.

2.3.7 Nitric oxide (NO) Assay

The whole thoracic aorta and whole left lobe of the lungs was collected and weighed from both female and male WT and $Cx43^{+/-}$ mice. These tissues were then incubated for 30 minutes in 1ml oxygenated physiological buffer solution at 37°C and pH 7.4. The composition of the physiological buffer solution was (in mM): 119.0 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 24.9 $NaHCO_3$, 1.2 KH_2PO_4, 11.1 Glucose and 2.5 $CaCl_2$. After 30 minutes, 100µl of the conditioned media was collected which contains substances released from the tissues into the media during the incubation period. 400µl of methanol (v/v) was then added and the mixture centrifuged at 21,910xg at 4°C for 20 minutes to precipitate unwanted proteins. The supernatant was then collected and kept at -80°C and then assayed for nitric oxide (NO) content using a Sievers 280 NO analyser. The NO analyser calculates the amount of NO produced by the tissues by calculating the amount of nitrite (NO_2^-), which was formed by the reaction between NO released from the tissues and the dissolved oxygen in the cell culture medium. To prepare the equipment, a reducing agent composed of 5ml glacial acetic acid and 50mg of sodium iodide (NaI) dissolved in 1.5ml of de-ionised water was added to the purge vessel and flushed with nitrogen (N_2) gas to purge any trace of NO_2^- from the vessel. After 30 minutes of purging, the purge vessel was then sealed and the reducing agent was refluxed under N_2 gas. Prior to each experiment, a standard curve of NO_2^- was constructed from a standard solution of 100mM sodium nitrite ($NaNO_2$), and serial dilutions of 50µM, 10µM, 1µM and 100nM were prepared and injected into the purge vessel using an Exmire microsyringe (ITO Corporation, Japan). The NO is produced in the purge vessel via the following equation: $I^- + NO_2^- + 2H^+ \rightarrow NO + ½\ I_2 + H_2O$. The NO was then detected by the NO analyser via reaction with oxygen (O_2) to produce trioxygen or ozone (O_3), which was detected by chemiluminescence. The chemiluminescence signal was converted to an electrical potential and displayed as millivolts (mV) by the NO analyser. The amount of NO produced by duplicates of each NO_2^- standard which was recorded by the analyser was used to produce a calibration curve. After calculation of the standard curve, the supernatant samples from whole thoracic aortae or whole left lung lobe were then injected into the purge vessel. Samples were injected at one-minute intervals to allow the output curve to return to the baseline.

The output in mV was then related to the amount of NO_2^- present in the sample using the NO_2^- standard curve.

2.3.8 Co-immunoprecipitation (co-IP)

This experiment was carried out to investigate the possible interaction between Cx43 and the eNOS signalling pathway using the whole thoracic aortae and whole left lung lobe from female WT and $Cx43^{+/-}$ mice. 20-25µL of SureBeads™ protein A magnetic beads (10mg beads/ml) (BIO-RAD, UK) was used for each sample and beads were then magnetized and washed three times with 1mL PBS-T. 1µl of primary anti-eNOS antibody (Cell signalling, UK) was then added to each 1mg of beads and incubated for 2-3 hours at 4°C. Beads were then washed two times with 300µL of PBS-T. 250µg of protein from cell lysate was added to the beads/antibody complex and incubated overnight at 4°C on a rotating mixer. The next day, beads were magnetized again and supernatants, also called immunodepletes, which contained unbound proteins, were collected for future use in Western blot. Beads were then washed two times with 300µL of PBS-T and eluted using 40µL of the following mixture: 400µL LDS sample buffer plus 100µL of 500mM DTT and then incubated for 10 minutes at 75°C. Samples were then centrifuged and magnetized before loading into a precast gel, and then transferred onto a PVDF membrane and immunoblotted as mentioned previously (section 2.3.5.3 to 2.3.5.5), to detect target proteins Cx43 and eNOS.

2.4 Statistical Analysis

Data were analysed using GraphPad Prism 8.0 software. For myography studies, changes in the logarithm of median effective concentration ($LogEC_{50}$), maximal contractile (E_{max}) and relaxation responses (R_{max}) between different groups were analysed using one-way or two-way ANOVA followed by Dunnett's or Bonferroni's post-hoc test as appropriate. The appropriate post-hoc test was automatically determined by Prism. For cell culture, comparisons between groups were analysed using two-way ANOVA followed by either a Tukey or Dunnett's post hoc test as appropriate. For Western blot and co-IP, changes in protein expression were analysed using the Student's unpaired *t*-test. Quantification of protein expression was performed through densitometry analysis using Image Studio™ lite version 5.2 software. Data were shown as mean ± standard error of the mean (S.E.M). *P* value of less than 0.05 was considered statistically significant and *n* symbol indicates the number of independent experiments.

Chapter 3 Investigating the Role of Connexin 43 in Female and Male Mouse Thoracic Aortae

3.1 Introduction

Evidence is accumulating on the role of gap junctions and their subunit connexins in modulating vascular tone/vasoreactivity in the systemic vasculature. For example, Chaytor et al. (1998) found that gap junctions contributed to the contractile effects of phenylephrine and to the endothelium-dependent relaxation to acetylcholine (ACh) in an isolated rabbit conduit artery preparation as both these effects were attenuated in the presence of ^{37,43}Gap27, an inhibitory gap junction peptide which targets both connexin 37 (Cx37) and connexin 43 (Cx43). In a similar study, the contractions to phenylephrine and the thromboxane A2 (TP) receptor agonist U46619 were found to be mediated through gap junctions in aortic rings from spontaneously hypertensive rats as these effects were decreased when using the non-selective gap junction inhibitor carbenoxolone (Tang and Vanhoutte, 2008). Furthermore, multiple studies have found connexins to be important in facilitating long-distance endothelial cell communication and mediating the communication between endothelial and smooth muscle cells (reviewed in de Wit and Griffith (2010), Leybaert et al. (2017).

3.1.1 Endothelial function in the systemic circulation

A large and growing body of literature has investigated the important role of endothelium/endothelial cells in the vasculature. Damage to the endothelium can predispose to different diseases such as atherosclerosis, hypertension, diabetes and cancer (Sandoo et al., 2010). Endothelial cells control vascular function by responding to different hormones, neurotransmitters and vasoactive factors which then affect vasomotion, thrombosis, platelet aggregation and inflammation (Galley and Webster, 2004). The endothelial layer modulates a balance between vasorelaxant and vasoconstrictor responses to maintain vascular tone under physiological conditions. Endothelial cells release various important vasoactive mediators such as nitric oxide (NO), prostacyclin (PGI_2), endothelin (ET) and endothelium-derived hyperpolarizing factor (EDHF) in response to humoral or mechanical stimuli, and therefore, any changes in these mediators can significantly affect the function and structure of the vasculature (Galley and Webster, 2004). It is thought that in hypertensive patients, for example, the balance between vasoconstrictors and vasodilators produced by the endothelium is disrupted. This leads to disturbances in the NO signalling pathway which leads

to predominance of vasoconstrictors like endothelin-1 (ET-1), which contribute to high blood pressure (Nadar et al., 2004). In endothelium-denuded rat aortic rings, for example, the contractile responses to U46619, phenylephrine and potassium chloride (KCl) were significantly increased when compared to endothelium-intact aortic rings (Tang and Vanhoutte, 2008). It was found that relaxation to EDHF in subcutaneous resistance arteries and in mouse aortic rings was dependent upon Cx43 and gap junctions (Lang et al., 2007, Lopez et al., 2009). Interestingly, myoendothelial gap junctions (MEJs), which are formed by coupling between endothelial and smooth muscle cells, were found to be important in mediating the effects of NO, PGI_2, ET and EDHF mediators, since all of these mediators have been shown to transfer through the MEJs (Billaud et al., 2009, Chaytor et al., 2005, Chaytor et al., 2001, Gairhe et al., 2012, Haddock et al., 2006, Lopez et al., 2009).

3.1.2 Interplay between NO signalling and gap junction proteins

As discussed above, both the NO signalling pathway and gap junction proteins are important in mediating normal vascular function. There is a large volume of published studies describing the relationship between these signalling pathways. In intact vessels *in-vivo* or in cultured endothelial cells, production of endogenous NO and addition of exogenous NO can significantly alter the function of gap junctions, however, it is uncertain which specific vascular connexin isoforms are involved and which specific gap junction locations are targeted (Kameritsch et al., 2005, Looft-Wilson et al., 2012, McKinnon et al., 2009, Straub et al., 2011). As endothelial nitric oxide synthase (eNOS) is an important component of the NO pathway and as connexins are the subunits of gap junctions, the activity and expression of eNOS and connexins might affect each other in health and diseased states (Looft-Wilson et al., 2012). One study found that the spread of vasoconstriction along mouse cremaster arterioles, which appeared to be facilitated by gap junctions between smooth muscle cells, was impaired in the presence of both endogenous and exogenous NO due to a modulation of gap junctional conductivity (Rodenwaldt et al., 2007). In addition, the same group also found that the vasoconstriction conduction was significantly enhanced in knockout eNOS ($eNOS^{-/-}$) mice, suggesting that eNOS plays a regulatory role in the modulation of vasoconstriction conduction through gap junctions, and its absence or inhibition may lead to an increased efficiency of vasoconstriction conduction in

mice. Therefore, the relationship between Cx43 and the eNOS signalling pathway will be studied using the whole thoracic aorta.

3.1.3 Sex Differences in the systemic circulation

In the majority of pre-clinical studies, the approach taken is to use animals of a single sex, usually male. However, it is noteworthy to highlight the important role of sex in both human and animal studies; although differences in responses between sexes are not fully understood. For example, the relaxation responses to the endothelium-dependent vasodilator ACh was greater in aortic rings from female rats than males (Kauser and Rubanyi, 1994). In addition, Crews and Khalil (1999) found that the contractility to α-adrenergic receptor agonists such as norepinephrine and phenylephrine was higher in aorta from male rats than in females. Additionally, the vessel hypertrophy induced in response to pressure overload using transverse aortic constriction, which simulates hypertension and aortic stenosis, was significantly greater in male mice compared to female mice (Skavdahl et al., 2005). In addition, compared to female mice, male mice have shown to develop an earlier and severe hypertension (Girouard et al., 2008, Xue et al., 2007) and increased incidence of cardiac hypertrophy and ischaemia-reperfusion injury (Murphy and Steenbergen, 2007). Even in humans, differences caused by sex are apparent. For example, post-menopausal women are at a higher risk of developing cardiovascular diseases like myocardial infarction and diastolic heart failure, with mortality rates exceeding those of men. This increased risk can be attributed to the decrease in protective effects of oestrogen that are present during the pre-menopausal phase (Czubryt et al., 2006). In addition, forearm vasoconstriction to norepinephrine was less in women than in men, however, the relaxation response to the $ß_2$-adrenergic agonist albuterol was greater in premenopausal women, than that seen in men (Kneale et al., 2000). Furthermore, Aggarwal et al. (2013) found that the severity of aortic stenosis caused by aortic valve calcification, which was measured by multidetector computed tomography, was significantly lower in female patients versus men. Therefore, considering the functional differences observed in previous findings between sexes, it is important to address the sex differences in pre-clinical studies in order to correctly prevent, diagnose, manage and treat diverse vascular diseases in humans.

3.1.4 Summary

All previous studies conducted on the role of gap junctions/connexins in the systemic circulation indicate that connexins play a role in vascular reactivity. To the best of my knowledge, there are no previous publications on vasoreactivity of mouse thoracic aorta describing the effects of both genetic knockdown and pharmacological inhibition of Cx43 on the effects of contractile or relaxant agonists. Moreover, there is a lack of studies on NO production and the possible interaction between NO and Cx43 pathways in systemic blood vessels. Research has shown a significant sex-based variation in Cx43 expression, notably more pronounced in the cardiomyocytes of females compared to males (Stauffer et al., 2011). While this disparity has been particularly observed in cardiac tissue, including the left ventricles of aging female rats (Tribulova et al., 2005), it raises the question of whether similar sex-specific differences in Cx43 expression might exist in other types of tissues. However, further research is essential to explore and understand these potential differences beyond cardiac tissue.

3.2 Aims and objectives

- To investigate the effect of both genetic knockdown and pharmacological inhibition of Cx43 on contractile responses to U46619 and on endothelium-dependent relaxation responses to ACh and endothelium-independent relaxation responses to S-Nitroso-N-acetyl-DL-penicillamine (SNAP) using mouse thoracic aortae.

- To investigate the effect of endothelial denudation on contractile responses to U46619 using mouse thoracic aortae.

- To investigate sex differences on contractile responses to U46619, and on relaxation responses to ACh and SNAP in WT and $Cx43^{+/-}$ mice.

- To investigate the production of NO and study the possible interaction between Cx43 and the eNOS signalling pathway using the whole thoracic aortae from female WT and $Cx43^{+/-}$ mice.

3.3 Methods

Both female and male WT and Cx43$^{+/-}$ littermate mice (C57BL/6 background, 4-6 month old) were used. The thoracic aorta was dissected and rings prepared from Schedule 1 euthanised animals. Several techniques were used in this chapter: wire myography, nitric oxide assay, Western blotting and co-immunoprecipitation. All protocols were previously detailed in the materials and methods (Chapter 2). Data were analysed using GraphPad Prism 8.0 software and results were shown as mean ± standard error of the mean (S.E.M). *P* value of less than 0.05 was considered statistically significant and *n* indicates the number of independent experiments.

3.4 Results

3.4.1 Protein expression of total connexin 43 (t-Cx43) in whole thoracic aortae from WT and $Cx43^{+/-}$ mice

In order to verify that the $Cx43^{+/-}$ mouse model expressed significantly less Cx43 than WT mice in the thoracic aorta, protein expression was measured in aortic lysates by Western blotting. As expected, the protein expression of total connexin 43 (t-Cx43) in thoracic aortae was significantly attenuated in $Cx43^{+/-}$ mice compared to the WT (Figure 3-1).

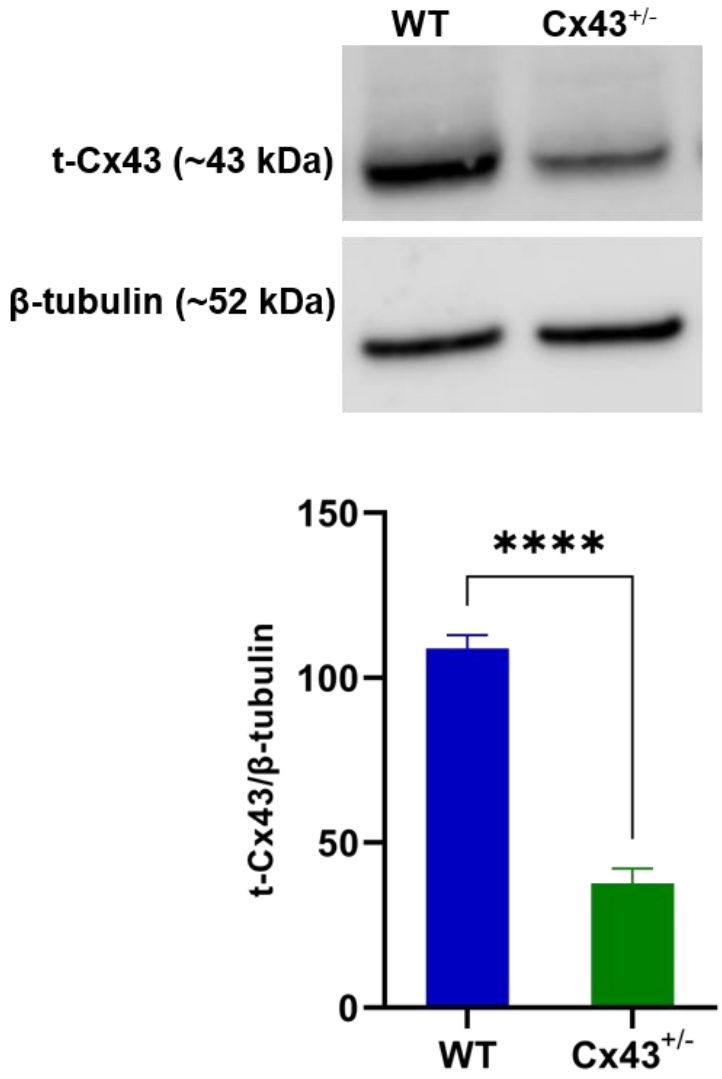


Figure 3-1. The protein expression of t-Cx43 in whole thoracic aortae normalised to a housekeeping gene- the protein β-tubulin. As expected, the protein expression of t-Cx43 was significantly attenuated in $Cx43^{+/-}$ female mice compared to female WT aortae. ****$p < 0.0001$ compared to WT within same experimental group, n=8. Data are shown as mean ± S.E.M.

3.4.2 Effect of genetic knockdown and pharmacological inhibition of Cx43 on contractile responses to U46619 in endothelium-intact and denuded female and male mouse thoracic aortae

Concentration-response curves (CRCs) were constructed to the thromboxane A2 (TP) receptor agonist U46619 (0.1nM -10μM) in thoracic aortae (both endothelium-intact and denuded) from female and male WT and $Cx43^{+/-}$ mice.

The maximal contractile response (E_{max}) and the logarithm of half-maximal effective concentration ($LogEC_{50}$) to U46619 was significantly increased ($p < 0.0001$) in endothelium-intact aortic rings from both female and male $Cx43^{+/-}$ mice compared to corresponding WT mice. Paradoxically, however, pharmacological inhibition of Cx43 using ^{37,43}Gap27 significantly reduced the contractile response to U46619 in both male and female mice ($p < 0.0001$). No significant differences between sexes were found in either $LogEC_{50}$ or E_{max} (Figure 3-2 A&B, Table 3-1).

Previous experiments with intact vessels were then repeated in endothelium-denuded vessels to investigate the role of endothelium in the observed responses. The contractile response to U46619 (E_{max} and $LogEC_{50}$) was similar in female and male $Cx43^{+/-}$ mice. However, pharmacological inhibition of Cx43 using ^{37,43}Gap27 significantly reduced the E_{max} to U46619 in denuded vessels of both female ($p < 0.0001$) and male ($p < 0.05$) WT mice. No significant differences between sexes were found in either $LogEC_{50}$ or E_{max} (Figure 3-2 C&D, Table 3-1).

A direct comparison of the intact endothelium versus denuded endothelium aortic ring data described above is shown in

Figure 3-3. Mechanical removal of the endothelial layer caused a significant increase in the contractile response to U46619 in aorta from female and male $Cx43^{+/-}$ and WT mice in both the absence and presence of ^{37,43}Gap27, when compared to endothelium intact vessels (

Figure 3-3, Table 3-1).

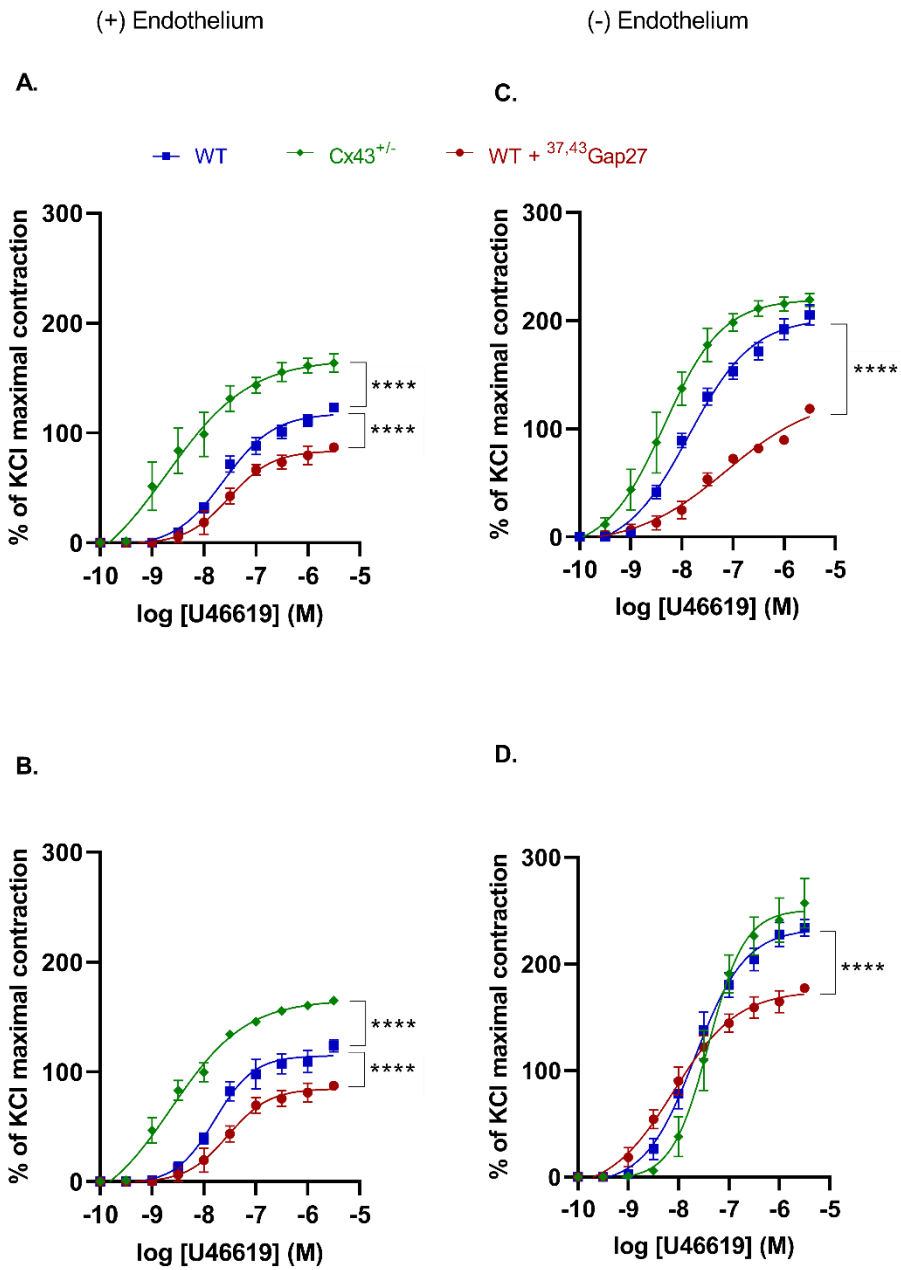


Figure 3-2. The contractile effects produced by U46619 in (A) female and (B) male endothelium-intact aortic rings, (C) female (D) male endothelium-denuded aortic rings. Contractions were significantly reduced in endothelium-intact vessels in the presence of ^{37,43}Gap27, a pharmacological inhibition of Cx37 and Cx43. However, these contractions were increased in Cx43$^{+/-}$ mice compared to WT mice (A, B). In denuded vessels, pharmacological inhibition of Cx43 using ^{37,43}Gap27 significantly reduced the contractile response to U46619 with no changes in Cx43$^{+/-}$ mice compared to WT mice (C, D). No significant differences between sexes were found in E_{max}. ****$p < 0.0001$ compared to WT controls within same experimental group, n=6 per group. Data are shown as mean ± S.E.M.

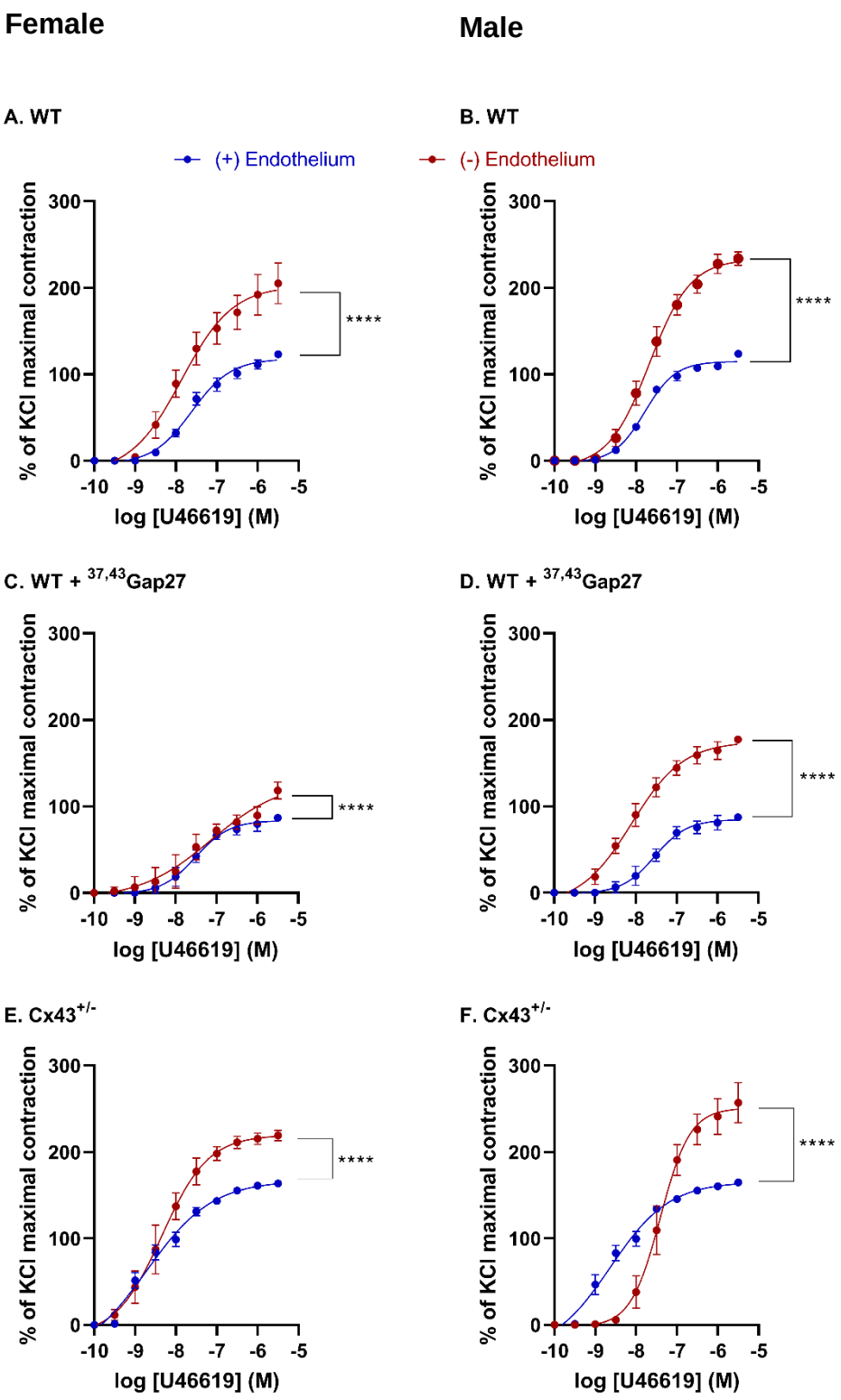


Figure 3-3. Direct comparison between denuded and endothelium-intact vessels to the contractile effects produced by U46619 in (A) female (B) male WT, (C) female (D) male WT in the presence of ^{37,43}Gap27, (E) female (F) male Cx43$^{+/-}$ mice. Compared to endothelium intact vessels, denuded vessels displayed an increased contractile response as expected. ****$p < 0.0001$, n= 6 per group. Data are shown as mean ± S.E.M.

Agonist	Groups		$LogEC_{50}$ (M)	E_{max} (%)	n
U46619	Female (Intact endothelium)	WT	-7.61 ± 0.04	123.3 ± 1.13	6
		$Cx43^{+/-}$	-8.72 ± 0.19 ****	163.6 ± 3.38 ****	
		+ ^{37,43}Gap27	-7.52 ± 0.05	86.80 ± 1.78 ****	
	Female (endothelium-denuded)	WT	-7.13 ± 0.01	205.2 ± 9.59 ‡	6
		$Cx43^{+/-}$	-7.33 ± 0.10	219.0 ± 6.13 ‡	
		+ ^{37,43}Gap27	-7.89 ± 0.09	118.5 ± 3.94 ****, ‡	
	Male (Intact endothelium)	WT	-7.79 ± 0.04	123.6 ± 2.27	6
		$Cx43^{+/-}$	-8.63 ± 0.15 ****	164.9 ± 3.87 ****	
		+ ^{37,43}Gap27	-7.54 ± 0.05	87.39 ± 1.53 ****	
	Male (endothelium-denuded)	WT	-7.67 ± 0.08	233.9 ± 7.76 ‡	6
		$Cx43^{+/-}$	-7.52 ± 0.14	257.1 ± 23.30 ‡	
		+ ^{37,43}Gap27	-8.01 ± 0.12	177.3 ± 3.47 ****, ‡	

Table 3-1. The contractile effects of U46619 in aortic rings from female and male WT and $Cx43^{+/-}$ mice. $LogEC_{50}$ indicates logarithm of median effective concentration and E_{max} represents maximal contraction effect. Changes in $LogEC_{50}$ and E_{max} between two different groups were analysed by the one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. No significant differences between female and male mice were found in either $LogEC_{50}$ or E_{max}. *$p < 0.05$, ****$p < 0.0001$ compared to WT within same experimental group, ‡ represents $p < 0.0001$ compared to endothelium intact vessels of the same sex and genotype, n= 6 per group. Data are shown as mean ± S.E.M.

3.4.3 Effect of genetic knockdown and pharmacological inhibition of Cx43 on relaxation responses to ACh in female and male mouse thoracic aortae

Having assessed the role of Cx43 in U46619-induced contraction, we then went onto assess the role of Cx43 in ACh-induced relaxation. The maximal relaxation (R_{max}) response to the endothelium-dependent vasodilator ACh was significantly decreased ($p < 0.0001$) in both female and male $Cx43^{+/-}$ mice compared to corresponding WT mice. In line with this, relaxation to ACh was also reduced ($p < 0.0001$) by pre-incubation with the gap junction blocker ^{37,43}Gap27 in both female and male WT mice. In addition, the $LogEC_{50}$ was substantially reduced ($p < 0.01$) in the presence of ^{37,43}Gap27 compared to corresponding WT mice. However, no significant differences between sexes were found in either $LogEC_{50}$ or R_{max} values (Figure 3-4, Table 3-2).

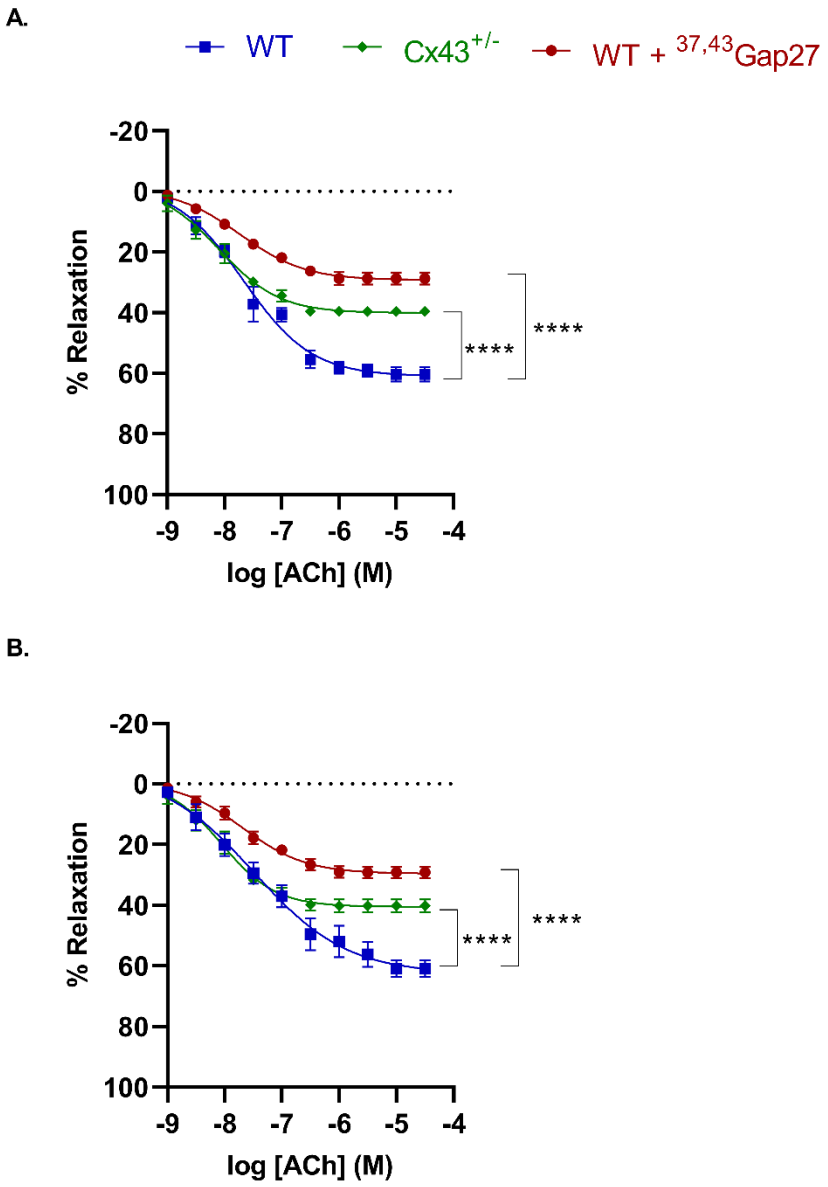


Figure 3-4. Relaxation produced by ACh in (A) female and (B) male endothelium-intact aortic rings. When compared to WT mice, the maximal relaxation (R_{max}) response to ACh was significantly reduced in $Cx43^{+/-}$ mice and in the presence of ^{37,43}Gap27. No significant differences between sexes were found in either $LogEC_{50}$ or R_{max}. ****$p < 0.0001$ compared to WT controls within same experimental group, n=6 per group. Data are shown as mean ± S.E.M.

Agonist	Groups		$LogEC_{50}$ (M)	R_{max} (%)	n
ACh	Female	WT	-7.67 ± 0.08	60.97 ± 1.61	6
		$Cx43^{+/-}$	-8.10 ± 0.09 **	40.06 ± 0.49 ****	
		+ ^{37,43}Gap27	-7.73 ± 0.09	29.21 ± 0.86 ****	
	Male	WT	-7.49 ± 0.15	62.60 ± 3.32	6
		$Cx43^{+/-}$	-8.06 ± 0.09 **	40.48 ± 1.00 ****	
		+ ^{37,43}Gap27	-7.69 ± 0.10	29.56 ± 0.93 ****	

Table 3-2. The relaxation responses to ACh in aorta of both female and male WT and $Cx43^{+/-}$ mice. $LogEC_{50}$ indicates logarithm of median effective concentration. R_{max} represents maximal relaxation effect. Changes in $LogEC_{50}$ and R_{max} between two different groups were analysed by the one-way ANOVA followed by Bonferroni's post hoc test. No significant differences between female and male were found in either $LogEC_{50}$ and R_{max}. **$p < 0.01$, ****$p < 0.0001$ compared to WT within same experimental group, n = 6 per group. Data are shown as mean ± S.E.M.

3.4.4 Effect of genetic knockdown and pharmacological inhibition of Cx43 on relaxation responses to SNAP in female and male mouse thoracic aortae

Following the reduction in relaxation observed in response to an endothelium-dependent dilator, it was of interest to assess whether the endothelium-independent vasodilator, S-Nitroso-N-acetyl-DL-penicillamine (SNAP), elicited similar responses in both female and male $Cx43^{+/-}$ mice. The maximal relaxation (R_{max}) response to SNAP was significantly decreased ($p<0.0001$) in both female and male $Cx43^{+/-}$ mice compared to corresponding WT mice. Relaxation to SNAP was also reduced ($p<0.001$) by pre-incubation with the gap junction blocker ^{37,43}Gap27 in both male and female WT mice. In addition, the $LogEC_{50}$ was significantly decreased ($p < 0.05$) in the presence of ^{37,43}Gap27 compared to corresponding WT mice. However, no significant differences between sexes were found in either $LogEC_{50}$ or R_{max} values (Figure 3-5, Table 3-3).

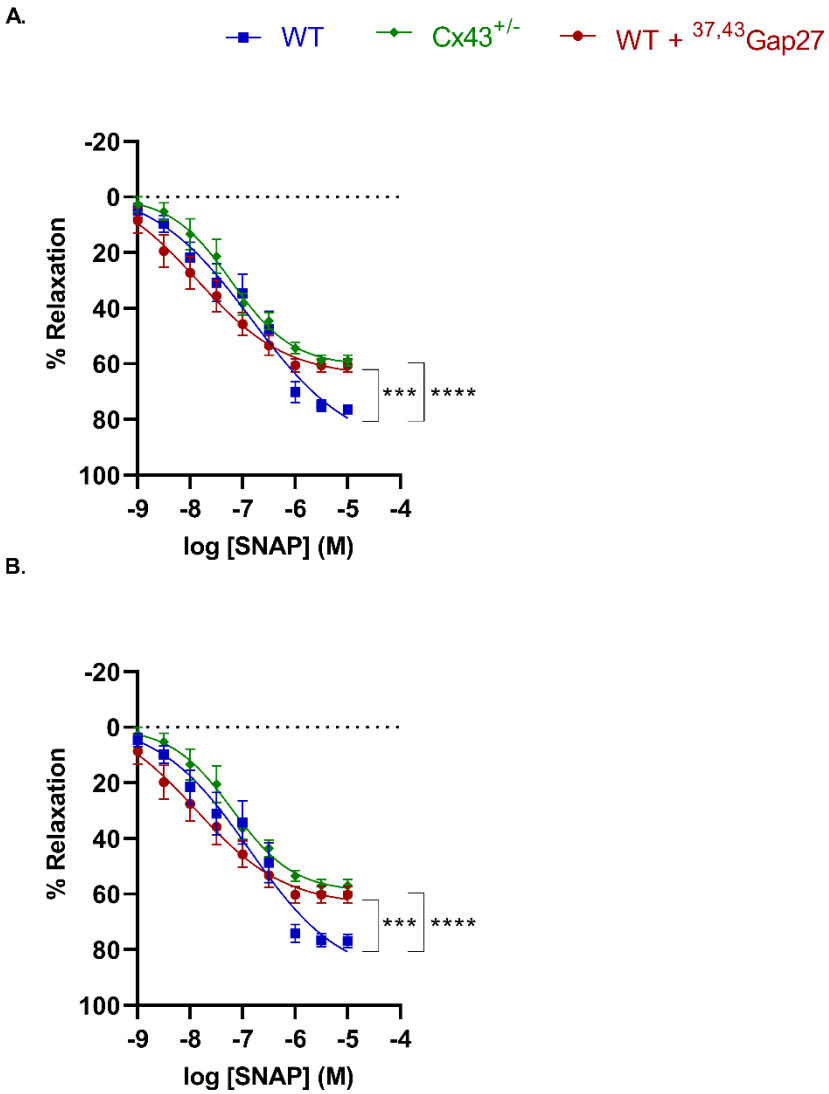


Figure 3-5. Relaxation produced by SNAP in (A) female and (B) male endothelium-intact aortic rings. When compared to WT mice, the maximal relaxation (R_{max}) response to SNAP was significantly reduced in $Cx43^{+/-}$ mice and in the presence of ^{37,43}Gap27. No significant differences between sexes were found in either $LogEC_{50}$ or R_{max}. ***p < 0.001, ****p < 0.0001 compared to WT controls within same experimental group, n=6 per group. Data are shown as mean ± S.E.M.

Agonist	Groups		$LogEC_{50}$ (M)	R_{max} (%)	n
SNAP	Female	WT	-6.83 ± 0.26	91.84 ± 12.56	6
		$Cx43^{+/-}$	-7.22 ± 0.11	60.58 ± 3.12 ****	
		+ ^{37,43}Gap27	-7.87 ± 0.20 *	64.41 ± 4.45 ***	
	Male	WT	-6.87 ± 0.24	90.57 ± 11.37	6
		$Cx43^{+/-}$	-7.20 ± 0.12	59.11 ± 3.27 ****	
		+ ^{37,43}Gap27	-7.90 ± 0.23 *	64.06 ± 4.89 ***	

Table 3-3. The relaxation responses to SNAP in aorta of both female and male WT and $Cx43^{+/-}$ mice. $LogEC_{50}$ indicates logarithm of median effective concentration. R_{max} represents maximal relaxation effect. Changes in $LogEC_{50}$ and R_{max} between two different groups were analysed by the one-way ANOVA followed by Bonferroni's post hoc test. No significant differences between female and male were found in either $LogEC_{50}$ or R_{max}. *p < 0.05, ***p < 0.001, ****p < 0.0001 compared to WT within same experimental group, n = 6 per group. Data are shown as mean ± S.E.M.

3.4.5 Investigating the production of nitric oxide (NO) in the whole thoracic aortae from female and male WT and $Cx43^{+/-}$ mice

It is known that ACh produces vasodilation through the production of NO. Therefore, based on the reduction in relaxation observed in response to ACh in female and male aortic rings from $Cx43^{+/-}$ mice and in the presence of ^{37,43}Gap27, experiments were then performed to measure NO production. Using a Sievers NO analyser, NO production was investigated in the whole thoracic aorta from female and male WT and $Cx43^{+/-}$ mice. The production of NO by the thoracic aorta was significantly decreased in the presence of ^{37,43}Gap27 as well as in the $Cx43^{+/-}$ mice compared to the WT mice. No significant differences between female and male were found in NO production by the thoracic aorta (Figure 3-6).

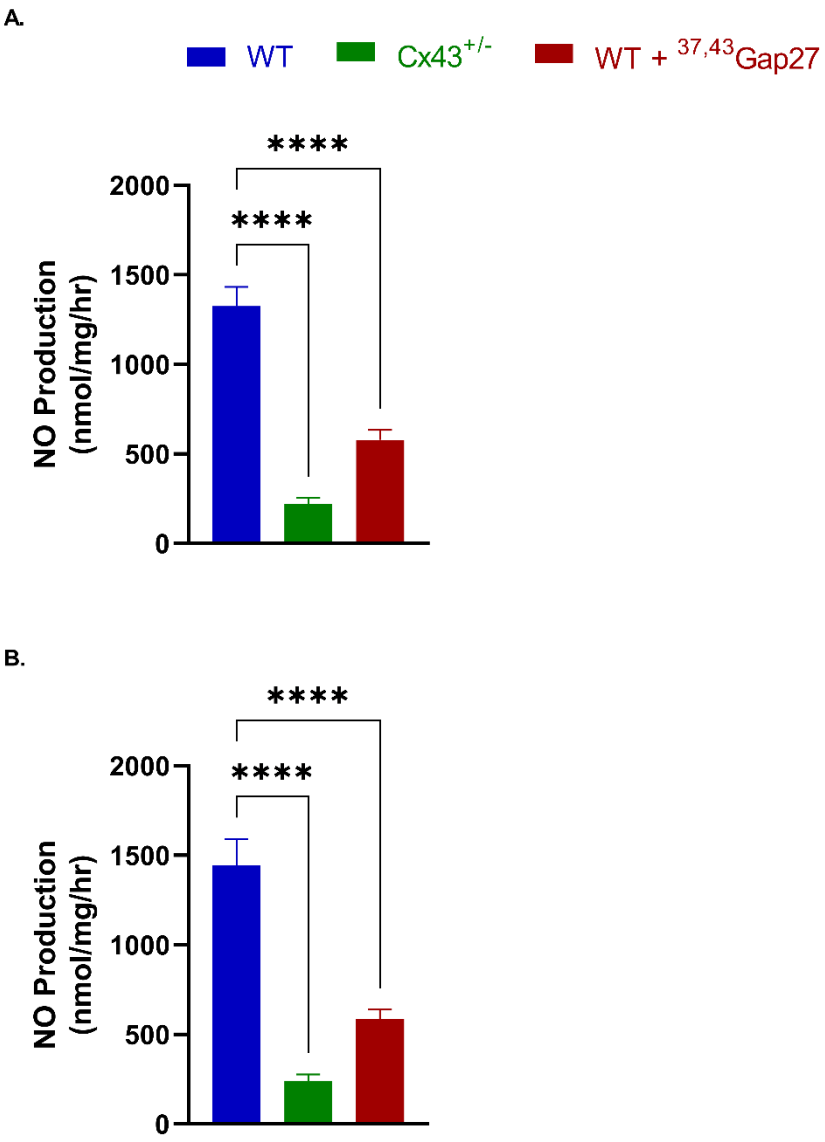


Figure 3-6. NO production by thoracic aortae in (A) female and (B) male WT and Cx43$^{+/-}$ mice. NO production was significantly reduced in thoracic aortae from Cx43$^{+/-}$ mice compared to WT mice. Cx43 blocking peptide ^{37,43}Gap27 also significantly reduced thoracic NO production when incubated with both female and male WT aortae. No significant differences between sexes were found. ****p < 0.0001 compared to WT within same experimental group, n=7 per group. Data shown represent the mean ± S.E.M.

3.4.6 Protein expression of total endothelial nitric oxide synthase (t-eNOS) in whole thoracic aortae from WT and Cx43$^{+/-}$ mice

To investigate whether the reduction in NO production caused by genetic knockdown of Cx43 is due to reduced expression of eNOS, Western blotting was used. However, the protein expression of t-eNOS was found not to differ between WT and Cx43$^{+/-}$ mice (Figure 3-7).

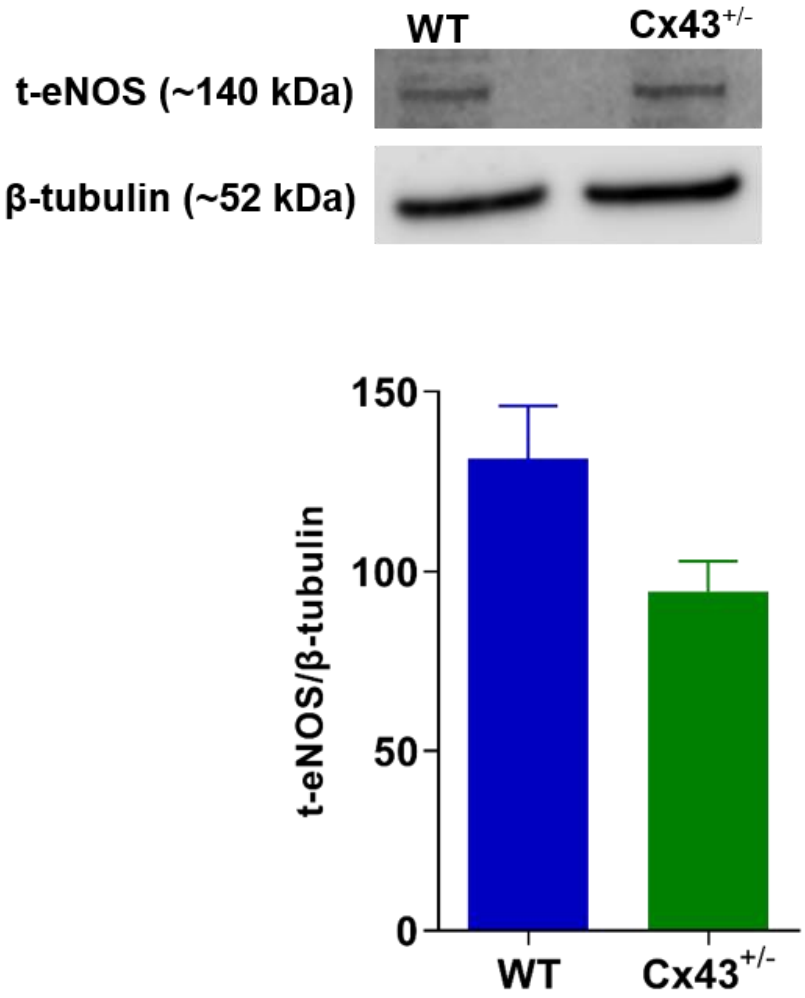


Figure 3-7. The protein expression of t-eNOS in whole thoracic aortae normalised to a housekeeping gene- the protein β-tubulin. Changes in protein expression of t-eNOS/ β-tubulin were analysed by the Student's unpaired *t*-test. No significant differences were found between WT and Cx43$^{+/-}$ mice, n=6. Data are shown as mean ± S.E.M.

3.4.7 Co-immunoprecipitation (co-IP) of eNOS and Cx43 in whole thoracic aortae from female WT and $Cx43^{+/-}$ mice

In order to investigate if Cx43 could regulate eNOS activity, co-immunoprecipitation was performed to study if the two proteins associate in aortic cells. The data demonstrate that Cx43 did associate with eNOS and existed in the same protein complexes in whole thoracic aortae from female WT and $Cx43^{+/-}$ mice. In addition, there was a trend towards a reduction, albeit not significant in the eNOS/Cx43 coupling in the $Cx43^{+/-}$ mice compared to WT (Figure 3-8).

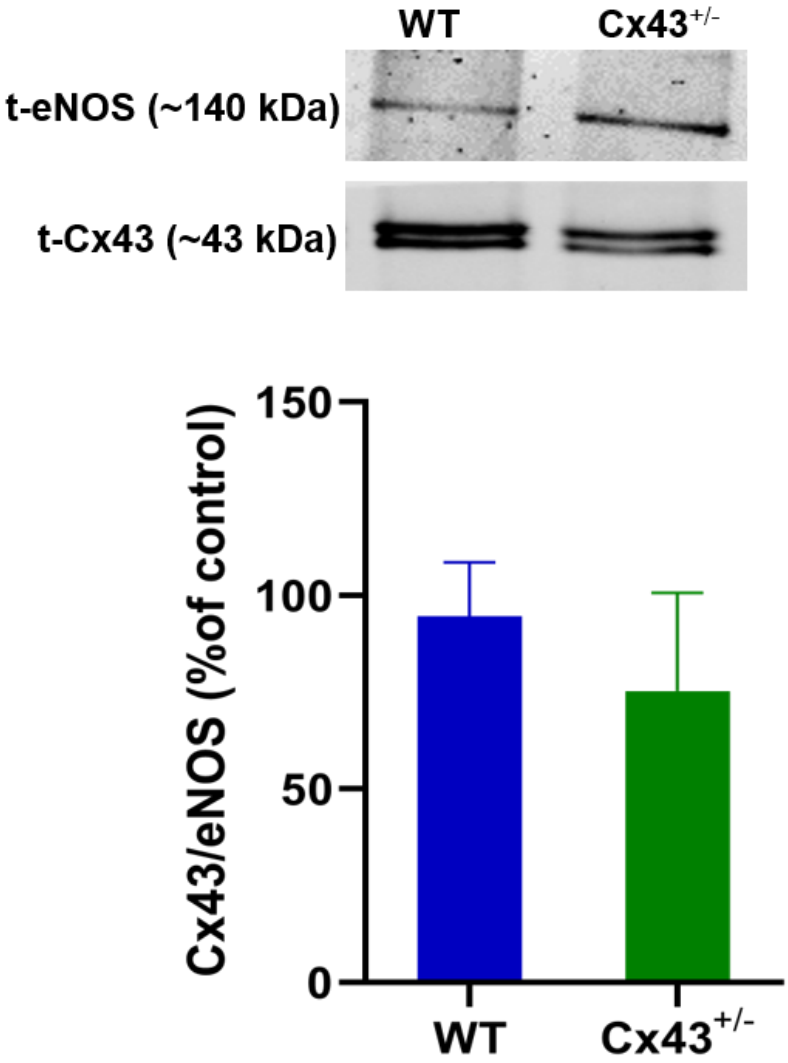


Figure 3-8. Co-immunoprecipitation of eNOS and Cx43 in whole thoracic aortae from female WT and Cx43$^{+/-}$ mice. Changes in protein expression of Cx43/eNOS were analysed by the Student's unpaired *t*-test. No significant differences were found between WT and Cx43$^{+/-}$ mice in the Cx43/eNOS ratio, n=5. The ratio between Cx43/eNOS in the WT mice was normalised and used as a control. Data are shown as mean ± S.E.M.

3.5 Discussion

This study investigated the role of both Cx43 and the endothelium on contractile responses to U46619 and on relaxation responses to ACh and SNAP, and also the role of Cx43 in the production of NO and possible interaction between Cx43 and the eNOS signalling pathway using mouse thoracic aortae. First of all, this study confirmed that as expected, the protein expression of Cx43 in the thoracic aorta of $Cx43^{+/-}$ mice was significantly attenuated compared to littermate WT mice.

Contractions to U46619 were attenuated in both sexes in the presence of ^{37,43}Gap27, however, paradoxically, the contractions to U46619 were significantly increased in both female and male $Cx43^{+/-}$ mice compared to WT mice. This discrepancy could be explained by the possibility that $Cx43^{+/-}$ mice might have compensatory changes in other connexins as shown by Htet et al. (2018), which would improve propagation of Ca^{2+} between cells and increase contraction. For example, Brisset et al. (2009) found that $Cx40^{-/-}$ mouse has decreased expression of Cx37 as well as cellular reorganisation of Cx43. Another explanation for the discrepancy in results between genetic knockdown and pharmacological inhibition is that ^{37,43}Gap27 may also have off-target effects. For example, Wang et al. (2007) found that pannexin 1 channels, which belong to the same super-family as connexins are blocked by ^{43}Gap27 and ^{32}Gap24, which targets Cx43 and Cx32, respectively. Therefore, it is possible that genetic knockdown of Cx43 and pharmacological inhibition of Cx43 using Gap27 may have different effects on other signalling pathways or proteins that are involved in smooth muscle contraction and vasoconstriction.

Given the discrepancies found in the effects of genetic knockdown of Cx43 and pharmacological inhibition of Cx43 on U46619-induced contraction, it was decided to study the effects of endothelial denudation on these responses. This approach allows to assess the potential involvement of endothelial cells in mediating the observed differences and gain insights into the interplay between endothelial and smooth muscle cells during U46619-induced contraction. In endothelial denuded aortae, contractile responses to U46619 were not affected in $Cx43^{+/-}$ mice compared to corresponding WT mice in both sexes. However in the presence of ^{37,43}Gap27, contractile responses were significantly reduced in denuded vessels from WT mice of both sexes. Moreover, and as expected in the vessels denuded of

endothelium, the contractile responses to U46619 were considerably increased in WT mice. Interestingly, even in the presence of the gap junction blocker ^{37,43}Gap27 and in the vessels from Cx43$^{+/-}$ mice, the increased contractile responses to U46619 persisted in denuded vessels compared to endothelial intact vessels. The enhanced contractile responses in denuded vessels are likely due to the removal of the opposing vasodilator effects of the endothelium caused by release of mediators such as NO, PGI_2 and EDHF. The presence of ^{37,43}Gap27 and Cx43$^{+/-}$ genotype does not appear to reverse this effect. Importantly, the denudation process may also lead to the loss of myoendothelial gap junctions (MEJs), which facilitate direct communication between endothelial and smooth muscle cells, thereby influencing vascular tone. This loss of MEJs and the subsequent disruption of cellular communication may be another contributing factor to the increased contractility observed in the denuded vessels. However, it is essential to note that our data do not show an increase in contractility in denuded vessels from Cx43$^{+/-}$ mice compared to WT mice. The lack of statistical significance indicates that additional factors may also be at play. Further investigations are needed to fully elucidate the mechanisms underlying the observed effects.

The current study also investigated the effects of both genetic knockdown and pharmacological inhibition of Cx43 on relaxation responses to the endothelium-dependent vasodilator ACh in female and male mouse thoracic aortae. The relaxation responses caused by ACh were significantly inhibited in the presence of ^{37,43}Gap27, a pharmacological inhibitor of Cx43. Similarly in Cx43 heterozygous (Cx43$^{+/-}$) mice, both females and males had a significantly reduced maximal vasodilation to ACh compared to WT mice. These findings support the previous literature which has shown that endothelium-dependent relaxations to ACh are markedly attenuated in aortic rings of the Cx43KI32 mouse model, in which the coding region of Cx43 was replaced by that of Cx32 (Lopez et al., 2009). In addition, these results also support the previous findings from Htet et al. (2018) who found that the relaxation response produced by methacholine (MCh), which is an endothelial-dependent vasodilator, was significantly reduced in intra-lobar pulmonary arteries (IPAs) of both female and male Cx43$^{+/-}$ mice when compared to WT mice.

In line with this, the current study found that the relaxation to an endothelium-independent dilator S-Nitroso-N-acetyl-DL-penicillamine (SNAP) was also substantially reduced by both genetic knockdown and pharmacological inhibition of Cx43. These data suggest that Cx43 is functional in aortic smooth muscle cells and may play a distinct role in vasoreactivity, independent of its effects in endothelial cells (Figueroa and Duling, 2009), and therefore, the role of Cx43 in smooth muscle requires further investigation. SNAP is a nitrosothiol derivative which releases NO under physiological conditions, leading to vasodilatation through various mechanisms. Theses mechanisms include the activation of stereoselective *S*-nitrosothiol recognition sites (Davisson et al., 1996), direct inhibition of Ca^{2+} channels (Marban, 1997), activation of K^{+} channels (Koh et al., 1995) and stimulation of guanylyl cyclase (GC) to produce cyclic guanosine monophosphate (cGMP), which plays an eesential role in mediating the vasodilatory effects of NO (Moro et al., 1996). Therefore, the current findings with ACh and SNAP suggest that a pathway involving both Cx43 and NO is important in mediating endothelium-dependent and -independent relaxation in the systemic circulation and requires further investigation.

To study the changes in NO-induced relaxation in more detail, nitric oxide (NO) production from the whole thoracic aorta of both female and male WT and $Cx43^{+/-}$ mice was measured. The production of NO in the thoracic aorta was significantly attenuated in the presence of ^{37,43}Gap27 as well as in the $Cx43^{+/-}$ mice compared to the WT mice. Interestingly, the link between Cx43 and NO production has been investigated in recent studies where an overall reduction in mitochondrial Cx43 expression in mouse heart was associated with a reduction in the respective mitochondrial rate of NO production (Kirca et al., 2015, Shvedova et al., 2018). However, the exact mechanism behind that effect is still unclear. We decided to investigate if the reduced NO production observed was due to decreased protein expression of eNOS in $Cx43^{+/-}$ mice. However, no significant differences in eNOS protein expression were found between WT and $Cx43^{+/-}$ mice. Suggesting that although eNOS levels were the same, there might be differences in the eNOS activity. There are various factors that can influence eNOS activity. For instance, eNOS interacts with other proteins, like caveolin-1, which can regulate eNOS activity. A plasma membrane-associated scaffolding protein caveolin-1, a key component of caveolae, acts as a negative regulator of eNOS by binding to it and

preventing it from producing NO. So, even if total eNOS levels remain constant, alterations in caveolin-1 levels or its interaction with eNOS could lead to changes in NO production (Komers et al., 2006). Given the lack of studies on eNOS levels and its activity in the aortic vessels of $Cx43^{+/-}$ mice, further work is required to investigate the activity of eNOS in both WT and $Cx43^{+/-}$ mice. This research should ideally extend to studying potential regulatory mechanisms, like the role of caveolin-1, to provide a more comprehensive understanding of the observed vascular changes.

The current study also investigated the protein interaction between Cx43 and eNOS and found that Cx43 was associated with eNOS in whole thoracic aortae from female WT and $Cx43^{+/-}$ mice. Evidence is accumulating that eNOS/NO and connexins/gap junction communication are interdependent signalling pathways, with the precise mechanisms of their interactions yet to be discovered (Looft-Wilson et al., 2012). For example, Alonso et al. (2010) found that Cx40, Cx37, and eNOS co-localise in a protein complex at intercellular junction of mouse aortic endothelial cells. In addition, they demonstrated that the proteins in this complex tightly interact with each other suggesting that the control of eNOS expression levels and function could be affected by the endothelial connexins (Looft-Wilson et al., 2012, Billaud et al., 2009). Furthermore, Pfenniger et al. (2010) showed that Cx37 interacted with eNOS in both human and mouse endothelial cells. It is important to note that there are few studies which have been done on the interrelationship of eNOS/NO and connexins/Cx43 in large arteries compared to the studies conducted on the resistance vasculature.

It is important to mention that the current study did not uncover any differences between sexes on (i) contractile responses to U46619, (ii) relaxation responses to ACh and SNAP, (iii) NO production in both WT and $Cx43^{+/-}$ mice. There is a paucity of research conducted on sex differences in vasoreactivity in the systemic circulation. It is important to address the issue of the oestrous cycle in female mice. The stages of oestrous can markedly influence vascular reactivity due to fluctuating levels of hormones such as oestrogen and progesterone (Schwertz and Penckofer, 2001). This variable was not controlled in the current study, which could potentially have influenced the results. Ultimately, exploring potential sex

disparities is vital in all pre-clinical and clinical research to ensure that everyone, male or female, receives the optimal treatment for their condition.

In conclusion, the current study has shown that Cx43 is important in mediating systemic vasoreactivity and has close relations with the NO signalling pathway in the endothelium. The next chapter will discuss the role of Cx43 in the pulmonary vasculature.

Chapter 4 Investigating the Role of Connexin 43 in Female and Male Mouse intra-lobar Pulmonary Arteries and Lungs

4.1 Introduction

As the role of gap junctions and their subunit connexins in modulating vascular tone/vasoreactivity in the systemic vasculature was discussed in the previous chapter, this chapter will focus on extending these findings to study the role of connexins in the pulmonary vasculature. There have been several studies in the literature reporting a significant role for gap junctions/connexins in the vasoreactivity of pulmonary circulation. For example, the contractile response to serotonin (5-HT) was significantly decreased by pre-incubation with the gap junction blocker ^{37,43}Gap27 in intra-lobar pulmonary arteries (IPAs) isolated from normoxic rats (Billaud et al., 2011a). Billaud and colleagues also found that contractions to phenylephrine were reduced in the presence of ^{37,43}Gap27 in rats exposed to chronic hypoxia (CH) or treated with monocrotaline (MCT), a substance used to induce pulmonary hypertension in animals. In addition, they determined that the sensitivity to contractile responses induced by high potassium solutions was increased in CH rats and this sensitivity was inhibited in the presence of ^{37,43}Gap27 (Billaud et al., 2011a). However, not all contractile responses are reduced when the function or expression of connexin is interfered with. More recently, the responses to the endogenous contractile agent endothelin-1 (ET-1), a powerful vasoconstrictor substance released by the endothelium, was actually increased in IPAs derived from Cx43 heterozygous ($Cx43^{+/-}$) mice compared to littermate wild-type mice (Bouvard et al., 2020, Htet et al., 2018). This finding aligns with the observations in the systemic circulation in the previous chapter where the contractions to U46619 were significantly increased in both female and male $Cx43^{+/-}$ mice compared to WT mice. In terms of vasodilation, gap junctions/connexins may also be involved within the pulmonary circulation. Recently, Htet et al. (2018) found that the dilatation response to methacholine (MCh), which is an endothelial-dependent vasodilator, was significantly attenuated in IPAs derived from $Cx43^{+/-}$ mice compared to WT mice and also in the presence of ^{37,43}Gap27. Therefore, as dysregulation of the connexin-mediated signalling pathway could associate with pathogenesis of pulmonary diseases such as pulmonary hypertension (reviewed in Htet et al. (2021), this study will expand the data available on how connexins influence pulmonary vascular reactivity. In the current study, the effect of both genetic knockdown and pharmacological inhibition of Cx43 on IPA contraction responses to thromboxane A2 (TP) receptor

agonist U46619, and on IPA relaxation responses to endothelium-dependent vasodilator acetylcholine (ACh) and endothelium-independent vasodilator S-Nitroso-N-acetyl-DL-penicillamine (SNAP), will be studied.

In the previous chapter, data was presented on the relationship between nitric oxide (NO) signalling pathway and gap junction proteins in the systemic vasculature, and so, in this chapter this relationship will be investigated in the pulmonary vascular beds. There is a large volume of published studies indicating that the communication between NO and gap junction pathways is co-dependent (reviewed in Looft-Wilson et al. (2012). In an *in-vitro* study for example, connexin 40 (Cx40) was found to be responsible for the NO-dependent enhancement in the coupling of gap junctions in HeLa cells (Hoffmann et al., 2003). In addition, Hoffmann et al. (2003) determined that in human umbilical vein endothelial cells (HUVECs), trafficking of Cx40 from cytoplasmic compartments to the plasma membrane was enhanced by the nitric oxide donor SNAP. Additionally, Straub et al. (2011) found that in co-cultured endothelial and smooth muscle cells isolated from mice thoracodorsal arteries, Cx43 and endothelial nitric oxide synthase (eNOS) was enriched at the MEJs, which are formed by coupling between endothelial and smooth muscle cells. They also found that the communication of MEJs was enhanced by adding NO donor. Moreover, they confirmed that Cx43 and its gap junction function can be regulated by constitutively *S*-nitrosylated at cysteine 271 by NO at the MEJs. This alters the contraction and relaxation responses of thoracodorsal arteries and regulates gap junction permeability (Straub et al., 2011). Therefore, in an attempt to understand the link between NO and gap junctions better, this study will investigate the production of NO and consider the possible interaction between Cx43 and eNOS signalling pathways using the whole left lung lobe from the $Cx43^{+/-}$ mouse model.

As discussed previously (section 1.2.5), there is a large female sex bias in patients with PH. Therefore, this chapter will also investigate the role of Cx43 in the vasoreactivity of IPAs from both female and male WT and $Cx43^{+/-}$ mice using a contractile drug U46619, and some relaxant drugs such as ACh and SNAP. In addition, the production of NO in both sexes will be studied using the whole left lung lobe from both genotypes.

4.2 Aims and objectives

- To investigate the effect of both genetic knockdown and pharmacological inhibition of Cx43 on contractile responses to U46619 and on relaxation responses to ACh and S-Nitroso-N-acetyl-DL-penicillamine (SNAP) using intra-lobar pulmonary arteries (IPAs).

- To investigate sex differences on contractile responses to U46619, and on relaxation responses to ACh and SNAP in WT and $Cx43^{+/-}$ mice.

- To investigate the production of NO and study the possible interaction between Cx43 and the eNOS signalling pathway using the whole left lung lobe from female WT and $Cx43^{+/-}$ mice.

4.3 Methods

Both female and male WT and Cx43$^{+/-}$ littermate mice (C57BL/6 background, 2-3 months old) were used. The whole left lung lobe and intra-lobar pulmonary artery (IPA), which is a continuation of the left pulmonary artery branch inside the left lung lobe, were isolated from Schedule 1 euthanised animals, and the surrounding parenchyma and airway smooth muscle was carefully dissected free from the pulmonary artery. Several techniques were used in this chapter: wire myography, nitric oxide assay, Western blotting and co-immunoprecipitation. All protocols were previously detailed in the materials and methods (Chapter 2). Data were analysed using GraphPad Prism 8.0 software and results were shown as mean ± standard error of the mean (S.E.M). *P* value of less than 0.05 was considered statistically significant and *n* indicates the number of independent experiments.

4.4 Results

4.4.1 Protein expression of total connexin 43 (t-Cx43) in whole left lung lobe from WT and $Cx43^{+/-}$ mice

In order to verify that the $Cx43^{+/-}$ mouse model expressed significantly less Cx43 than WT mice in the whole left lung lobe, protein expression of Cx43 was measured in whole left lung lobe lysates by Western blotting. As expected, the protein expression was significantly attenuated in $Cx43^{+/-}$ mice compared to the WT (Figure 4-1).

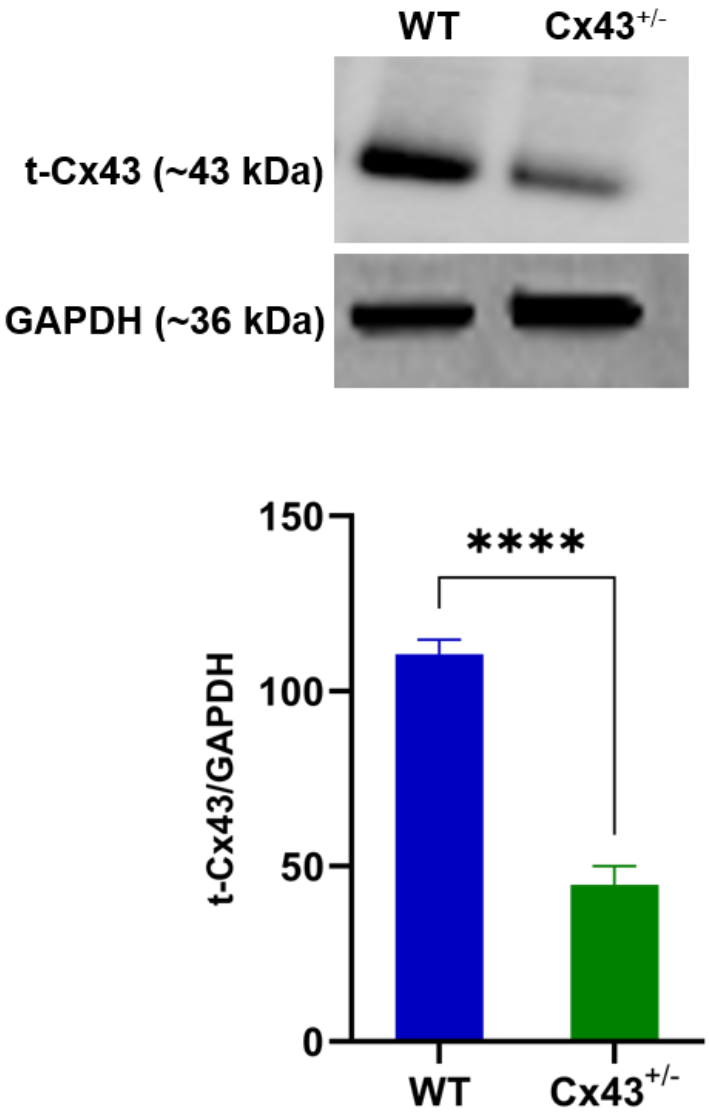


Figure 4-1. The protein expression of t-Cx43 in whole left lung lobe normalised to a housekeeping protein- the protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). As expected, the protein expression of t-Cx43 was significantly attenuated in the lungs of $Cx43^{+/-}$ female mice compared to female WT lungs. ****$p < 0.0001$ compared to WT within same experimental group, n=9. Data are shown as mean ± S.E.M.

4.4.2 Effect of genetic knockdown and pharmacological inhibition of Cx43 on contractile responses to U46619 in IPAs of female and male mice

Concentration-response curves (CRCs) were constructed to the thromboxane A2 (TP) receptor agonist U46619 (0.1nM -10μM) in both female and male WT and $Cx43^{+/-}$ mice. The maximal contractile response (E_{max}) and the logarithm of half-maximal effective concentration ($LogEC_{50}$) to U46619 were significantly decreased ($p < 0.0001$) in both female and male $Cx43^{+/-}$ mice compared to corresponding WT mice. In addition, pharmacological inhibition of Cx43 using ^{37,43}Gap27 significantly reduced the contractile response to U46619 ($p < 0.0001$) in both female and male WT mice. No significant differences between sexes were found in either $LogEC_{50}$ or E_{max} (Figure 4-2, Table 4-1).

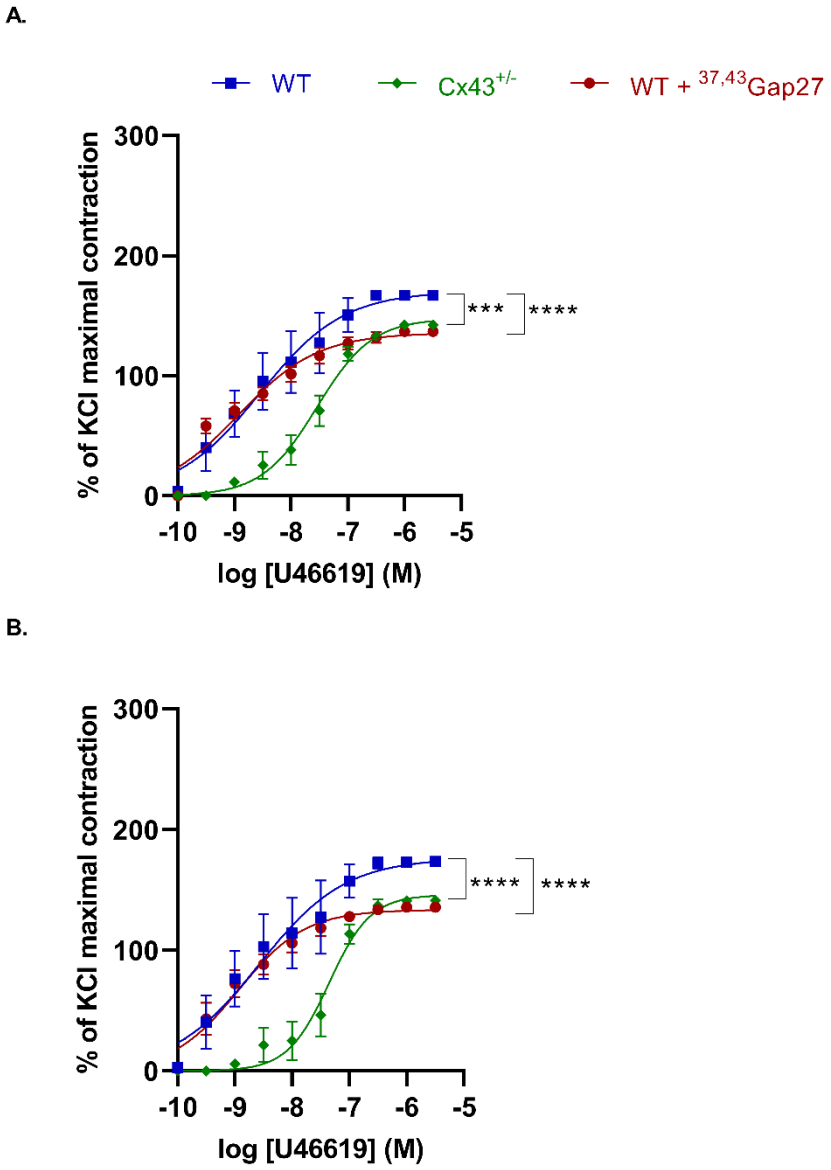


Figure 4-2. The contractile response to U46619 in IPAs of (A) female and (B) male mice. No significant differences between sexes were found in either $LogEC_{50}$ or E_{max}. ***p < 0.001, ****p < 0.0001 compared to WT controls within same experimental group, n=6 per group. Data are shown as mean ± S.E.M.

Agonist	Groups		$LogEC_{50}$ (M)	E_{max} (%)	n
U46619	Female	WT	-8.55 ± 0.21	170.4 ± 12.91	6
		$Cx43^{+/-}$	-7.55 ± 0.08 ***	147.7 ± 6.21 ***	
		+ ^{37,43}Gap27	-8.92 ± 0.09	135.7 ± 4.17 ****	
	Male	WT	-8.58 ± 0.24	176.3 ± 14.98	6
		$Cx43^{+/-}$	-7.35 ± 0.09 ***	145.9 ± 7.40 ****	
		+ ^{37,43}Gap27	-8.94 ± 0.09	133.6 ± 4.17 ****	

Table 4-1. The contractile effects of U46619 in IPAs from female and male WT and $Cx43^{+/-}$ mice. $LogEC_{50}$ indicates logarithm of median effective concentration. E_{max} represents maximal contraction effect. Changes in $LogEC_{50}$ and E_{max} between two different groups were analysed by the Student's unpaired *t*-test. No significant differences between female and male mice were found in either $LogEC_{50}$ or E_{max}. ***$p < 0.001$, ****$p < 0.0001$ compared to WT within same experimental group, n = 6 per group. Data are shown as mean ± S.E.M.

4.4.3 Effect of genetic knockdown and pharmacological inhibition of Cx43 on relaxation responses to ACh in IPAs of female and male mice

Having assessed the role of Cx43 in U46619-induced contraction, we then went onto assess the role of Cx43 in ACh-induced relaxation. The maximal relaxation (R_{max}) response induced by the endothelium-dependent dilator ACh was significantly decreased in both female ($p < 0.001$) and male ($p < 0.0001$) $Cx43^{+/-}$ mice compared to corresponding WT mice. In line with this, relaxation to ACh was also reduced by pre-incubation with the gap junction blocker ^{37,43}Gap27 in both female ($p < 0.001$) and male ($p < 0.0001$) WT mice. However, no significant differences between sexes were found in either $LogEC_{50}$ or R_{max} values (Figure 4-3,Table 4-2)

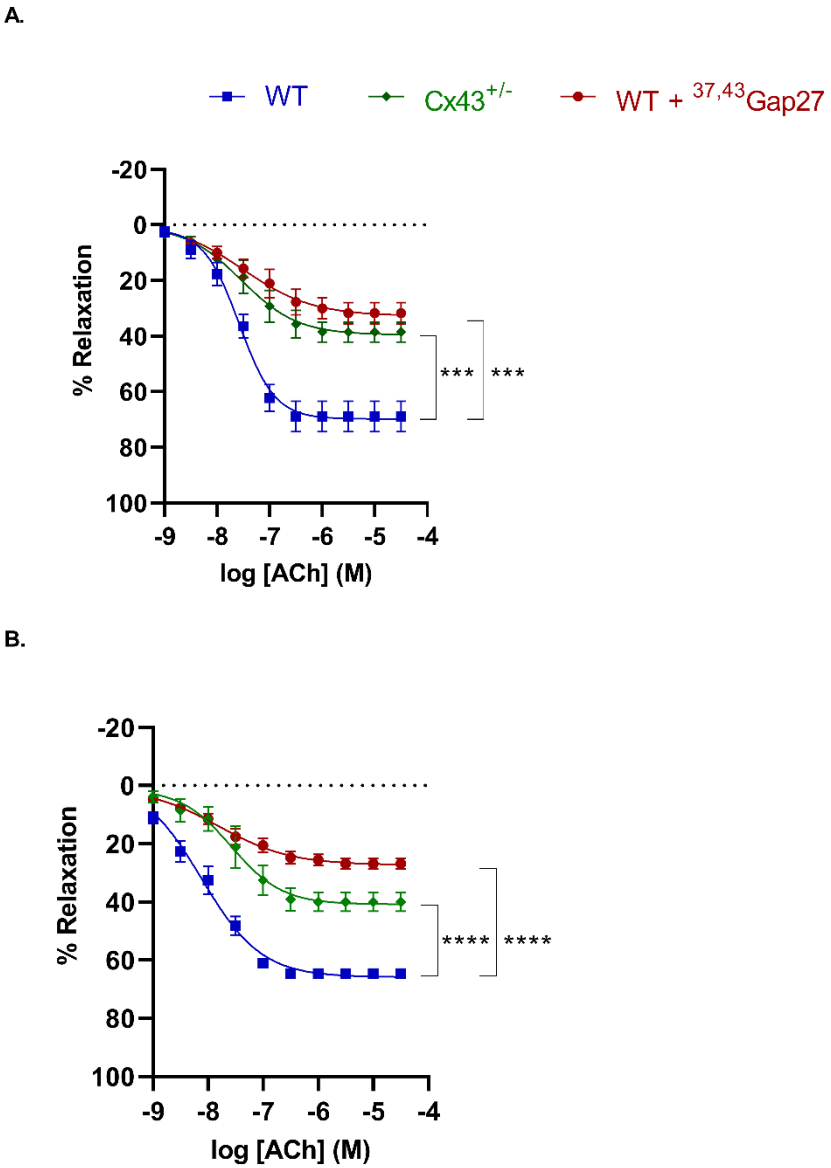


Figure 4-3. Relaxation produced by ACh in IPAs of (A) female and (B) male mice. No significant differences between sexes were found in either $LogEC_{50}$ or R_{max}. ***p < 0.001, ****p < 0.0001 compared to WT controls within same experimental group, n=6 per group. Data are shown as mean ± S.E.M.

Agonist	Groups		LogEC$_{50}$ (M)	R_{max} (%)	n
ACh	Female	WT	-7.58 ± 0.07	69.76 ± 1.97	6
		Cx43$^{+/-}$	-7.54 ± 0.17	39.47 ± 2.21 ***	
		+ ^{37,43}Gap27	-7.51 ± 0.21	32.70 ± 2.30 ***	
	Male	WT	-8.11 ± 0.07	65.82 ± 1.21	6
		Cx43$^{+/-}$	-7.57 ± 0.14	40.79 ± 1.96 ****	
		+ ^{37,43}Gap27	-7.81 ± 0.16	27.24 ± 1.13 ****	

Table 4-2. The relaxation responses to ACh in IPAs from female and male WT and Cx43$^{+/-}$ mice. LogEC$_{50}$ indicates logarithm of median effective concentration. R_{max} represents maximal relaxation effect. Changes in LogEC$_{50}$ and R_{max} between two different groups were analysed by the Student's unpaired *t*-test. No significant differences between female and male were found in either LogEC$_{50}$ or R_{max}. ***p < 0.001, ****p < 0.0001 compared to WT within same experimental group, n = 6 per group. Data are shown as mean ± S.E.M.

4.4.4 Effect of genetic knockdown and pharmacological inhibition of Cx43 on relaxation responses to SNAP in IPAs of female and male mice

Since there was a reduction in relaxation in response to ACh in the $Cx43^{+/-}$ mice, it was of interest to assess whether the endothelium-independent vasodilator, S-Nitroso-N-acetyl-DL-penicillamine (SNAP), elicited similar responses in IPAs of both female and male $Cx43^{+/-}$ mice. SNAP is a compound that induces relaxation by activating endothelial muscarinic receptors and directly releasing NO. The maximal relaxation (R_{max}) response to SNAP was significantly decreased ($p<0.0001$) compared to corresponding WT mice. Relaxation to SNAP was also reduced ($p<0.001$) by pre-incubation with the gap junction blocker ^{37,43}Gap27 in both male and female WT mice. However, no significant differences between sexes were found in either $LogEC_{50}$ or R_{max} values (Figure 4-4, Table 4-3).

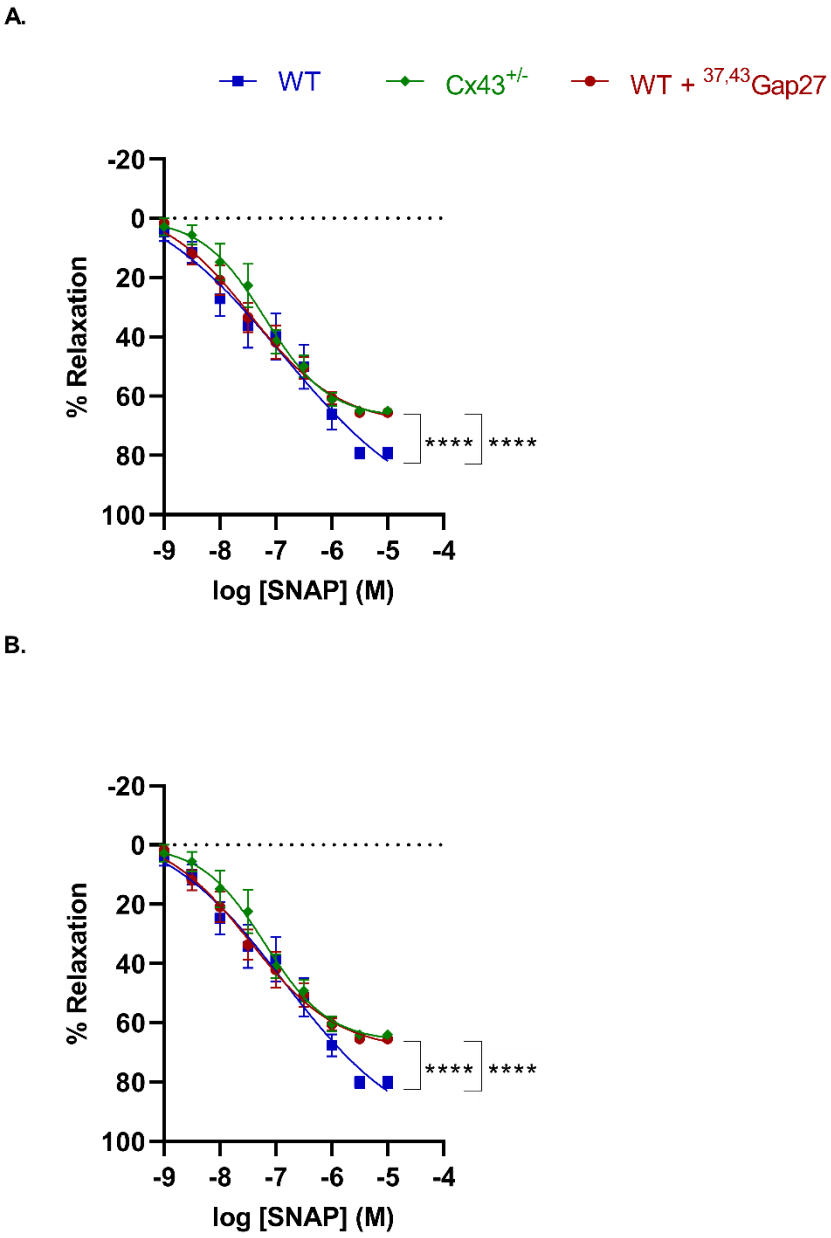


Figure 4-4. Relaxation produced by SNAP in IPAs of (A) female and (B) male mice. No significant differences between sexes were found in either $LogEC_{50}$ or R_{max}. ****$p < 0.0001$ compared to WT controls within same experimental group, n=6 per group. Data are shown as mean ± S.E.M.

Agonist	Groups		LogEC$_{50}$ (M)	R_{max} (%)	n
SNAP	Female	WT	-6.88 ± 0.46	103.6 ± 25.11	6
		Cx43$^{+/-}$	-7.19 ± 0.11	67.20 ± 3.39 ****	
		+ ^{37,43}Gap27	-7.46 ± 0.13	69.74 ± 4.21 ****	
	Male	WT	-6.85 ± 0.32	99.90 ± 16.88	6
		Cx43$^{+/-}$	-7.20 ± 0.11	66.34 ± 3.44 ****	
		+ ^{37,43}Gap27	-7.47 ± 0.14	69.44 ± 4.24 ****	

Table 4-3. The relaxation responses to SNAP in IPAs from female and male WT and Cx43$^{+/-}$ mice. LogEC$_{50}$ indicates logarithm of median effective concentration. R_{max} represents maximal relaxation effect. Changes in LogEC$_{50}$ and R_{max} between two different groups were analysed by the Student's unpaired *t*-test. No significant differences between female and male were found in either LogEC$_{50}$ or R_{max}. ****p < 0.0001 compared to WT within same experimental group, n = 6 per group. Data are shown as mean ± S.E.M.

4.4.5 Investigating the production of nitric oxide (NO) in the whole left lung lobe from female and male WT and $Cx43^{+/-}$ mice

Based on the reduction in relaxation observed in response to ACh and SNAP in both aortic rings (Chapter 3) and IPAs from $Cx43^{+/-}$ mice, we decided to measure the basal NO formation in both $Cx43^{+/-}$ mice and in the presence of ^{37,43}Gap27 in the whole left lung lobe using a Sievers NO analyser. The production of NO was significantly decreased in the presence of ^{37,43}Gap27 as well as in the $Cx43^{+/-}$ mice compared to the WT mice but no significant differences between sexes were found (Figure 4-5).

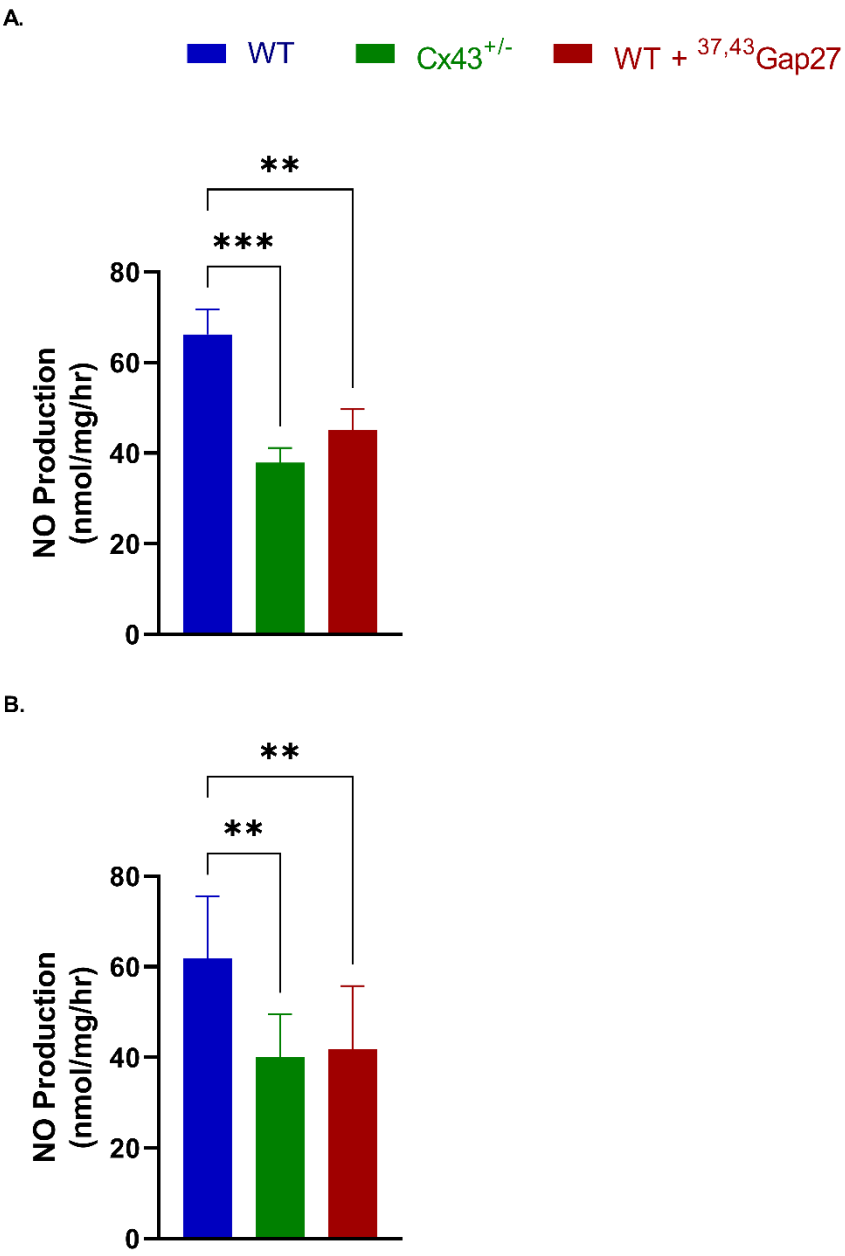


Figure 4-5. NO production by IPAs in (A) female and (B) male WT and Cx43$^{+/-}$ mice. No significant differences between female and male were found. However, there was a significant reduction in NO production between WT and Cx43$^{+/-}$ mice and the Cx43 blocking peptide significantly reduced left lung lobe NO production when incubated with both female and male WT lungs. **p < 0.01, ***p < 0.001 compared to WT within same experimental group, n=7 per group. Data shown represent the mean ± S.E.M.

4.4.6 Protein expression of total endothelial nitric oxide synthase (t-eNOS) in whole left lung lobes from WT and Cx43$^{+/-}$ mice

To investigate whether the reduction in basal NO production caused by genetic knockdown of Cx43 is due to reduced expression of eNOS, Western blotting was used. However, the protein expression of t-eNOS was found not to differ between WT and Cx43$^{+/-}$ mice (Figure 4-6).

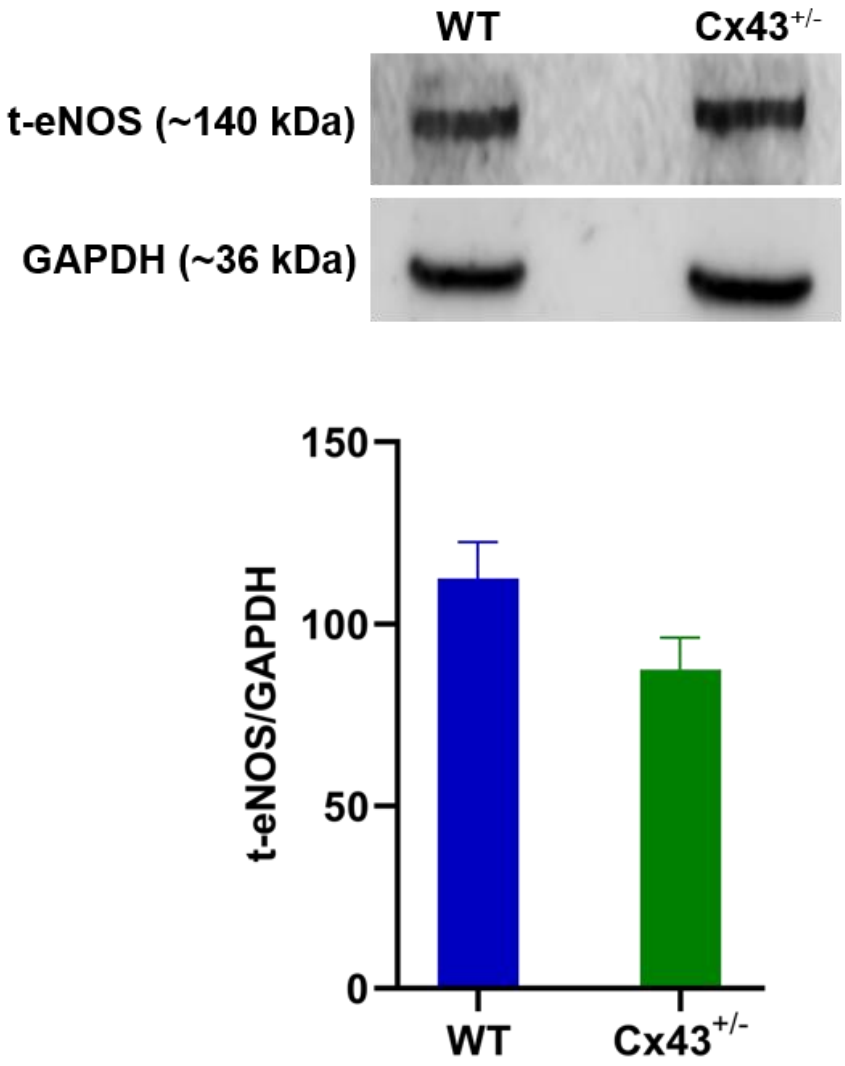


Figure 4-6. The protein expression of t-eNOS in whole left lung lobes normalised to a housekeeping protein- the protein GAPDH. No significant differences were found between WT and Cx43$^{+/-}$ mice, n=6. Data are shown as mean ± S.E.M.

4.4.7 Co-immunoprecipitation (co-IP) of eNOS and Cx43 in whole left lung lobes from female WT and Cx43$^{+/-}$ mice

In order to investigate if Cx43 could regulate eNOS activity, co-immunoprecipitation was performed to study if the two proteins associate in the lungs. The data demonstrate that Cx43 did associate with eNOS, colocalizes in the same cellular domains and exists in the same protein complexes in whole left lung lobes from female WT and Cx43$^{+/-}$ mice. In addition, there was a trend towards a reduction, albeit not significant in the eNOS/Cx43 coupling in the Cx43$^{+/-}$ mice compared to WT (Figure 4-7).

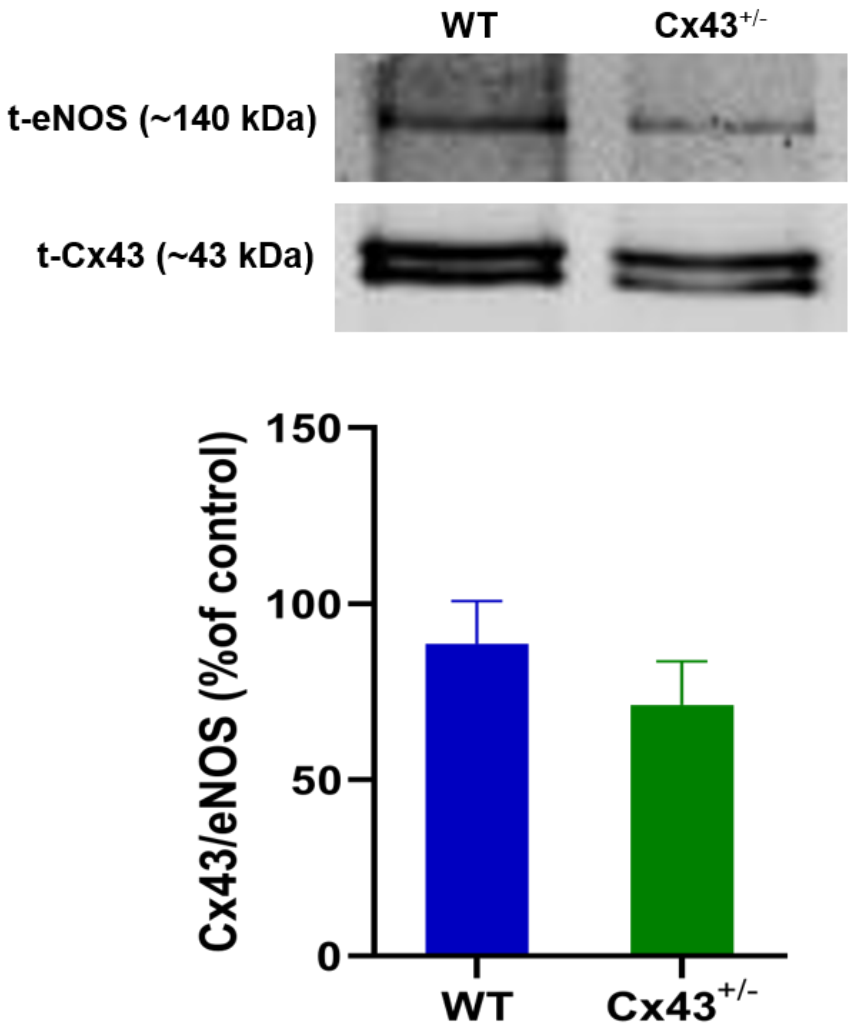


Figure 4-7. Co- immunoprecipitation of eNOS and Cx43 in whole left lung lobes from female WT and Cx43$^{+/-}$ mice. No significant differences were found between WT and Cx43$^{+/-}$ mice in the eNOS/Cx43 ratio, n=5. The ratio between t-Cx43/t-eNOS in the WT mice was normalised and used as a control. Data are shown as mean ± S.E.M.

4.5 Discussion

In this chapter, the role of Cx43 on contractile responses to U46619 and on relaxation responses to ACh and SNAP in IPAs, and also the role of Cx43 in the basal production of NO and possible interaction between Cx43 and the eNOS signalling pathway using mouse lungs were investigated. First of all, this study confirmed that as expected and as seen in mouse thoracic aortae in the previous chapter, the protein expression of Cx43 in the whole left lung lobes was significantly attenuated in $Cx43^{+/-}$ mice compared to littermate WT mice.

This study investigated the effects of both genetic knockdown and pharmacological inhibition of Cx43 on contractile responses to U46619 in IPAs of female and male mice. In both sexes, the contractile effects to U46619 were significantly reduced in the presence of ^{37,43}Gap27 as well as in the $Cx43^{+/-}$ mice compared to WT mice. These results are consistent with those obtained by Billaud et al. (2011a) who found that the contraction to serotonin (5-HT) in IPAs from normoxic rats, and the contraction to phenylephrine in IPAs from CH and MCT rats were substantially decreased after treatment with ^{37,43}Gap27. More recently, using mouse pulmonary arteries under normoxic conditions, the contractile response and potency of phenylephrine was significantly attenuated in $Cx43^{+/-}$ mice compared to WT mice (Bouvard et al., 2020). Therefore, the present study agrees with all previous literature to indicate that Cx43 is involved in the vasoconstriction of pulmonary vasculature and requires further investigation to determine how connexins might be involved. It can be speculated that gap junctions and their subunit connexins might be involved in pulmonary vasoreactivity by their relationship with calcium (Ca^{2+}). For example, calmodulin, which is the main receptor for Ca^{2+} ion in most tissues, was found to be associated with gap junctions and plays a direct role in chemical gate control of Cx32-containing gap junctions in frog oocytes (Peracchia et al., 2000). In addition, two distinct amino acid sequences have been identified in Cx32 that are capable of binding calmodulin (Torok et al., 1997). A study by Lurtz and Louis (2007) showed that corresponding physiological concentrations of $[Ca^{2+}]_i$ in HeLa cells that were transfected with Cx43 were able to modify the permeability of Cx43 via the interaction between calmodulin and a cytoplasmic region of Cx43 in response to physiological concentrations of $[Ca^{2+}]_i$.

It is well known that the concentration of intracellular calcium $[Ca^{2+}]_i$ is involved in modulating smooth muscle cell-membrane potential and gap junctions are known to play an important role in controlling the function of adjacent smooth muscle cells in terms of both membrane potential and $[Ca^{2+}]_i$ (Christ et al., 1992, Christ et al., 1996). Moreover, Srisakuldee et al. (2014) found that blocking Cx43 channels using ^{37,43}Gap27 reduces $[Ca^{2+}]_i$ in subsarcolemmal mitochondria of rat hearts. Consequently, a genetic or pharmacological reduction in Cx43 expression could potentially lead to diminished levels of calmodulin and Ca^{2+}, resulting in a possible attenuation of contractile responses to various agonists

The reduction in contraction to U46619 in pulmonary arteries of $Cx43^{+/-}$ mice, despite their lower production of NO in the whole lung, raises an important point to consider. While reduced NO production would typically lead to increased contraction, several factors might explain this discrepancy. For example, compensatory mechanisms or the involvement of other vasoactive substances in the pulmonary arteries of $Cx43^{+/-}$ mice could be contributing to the observed reduction in contraction. EDHF or PGI_2 may counterbalance the effects of decreased NO. Additionally, alterations in the expression or function of other connexins or gap junction proteins in $Cx43^{+/-}$ mice could be influencing the observed reduction in contractile responses (Htet et al., 2018). Hence, understanding these complexities will require further investigation, considering regional differences in vascular beds and specific cell types involved in the pulmonary arteries.

The differences in contractile responses to U46619 in $Cx43^{+/-}$ mice, with increased sensitivity in the thoracic aorta but decreased sensitivity in the pulmonary arteries, may be due to compensatory changes in other connexins promoting Ca^{2+} propagation in the aorta, as discussed in the previous chapter. Furthermore, the off-target effects of the pharmacological inhibitor ^{37,43}Gap27 on other connexins could also be influencing the responses, as previously discussed. Moreover, the role of calmodulin in regulating gap junctions and intracellular calcium concentration might impact contractile responses differently in the two vascular beds (Peracchia et al., 2000, Lurtz and Louis, 2007, Srisakuldee et al., 2014). Further investigation is needed to fully understand these tissue-specific mechanisms.

There is an important point to note that there was no examination of the effect of endothelial denudation on contractile responses to U46619 in IPAs in the current study compared to what was observed in the previous chapter with mouse thoracic aortae. This is because of the smaller diameter of the lumen of IPAs, which presented technical difficulties for the removal of the endothelium compared to aortic rings. Therefore, future experiments could mechanically try to remove endothelium using a smaller size of wire (<40µm) or with a strand of moose mane hair as described in (Jernigan et al., 2004). Further, endothelium can also be removed using 0.3% of a zwitterionic non-denaturing detergent 3-[(3-cholamidopropyl)-dimethylammonio] 1-propanesulfonate (CHAPS). Previous studies perfused CHAPS through the lumen of the IPAs as described in (Pourageaud et al., 2005, Robert et al., 2007). The absence of a relaxation response to 10µM carbamylcholine, which is an endothelium-dependent dilator, following precontraction induced by 0.3µM phenylephrine, confirmed the effect of CHAPS (Pourageaud et al., 2005, Robert et al., 2007).

The present study also examined the effects of both genetic knockdown and pharmacological inhibition of Cx43 on relaxation responses to the endothelium-dependent vasodilator ACh in IPAs of both sexes. The relaxation responses caused by ACh were significantly inhibited in $Cx43^{+/-}$ mice and in the presence of ^{37,43}Gap27 compared to WT mice. These results support the previous findings from Htet et al. (2018) who found that the relaxation response produced by methacholine (MCh), which is an endothelial-dependent vasodilator, was significantly reduced in IPAs of both female and male mice in response to ^{37,43}Gap27 as well as in $Cx43^{+/-}$ mice when compared to WT mice. More recently, Mondejar-Parreño et al. (2019) found that microRNA-1 (miR-1) reduced the gap junction protein alpha 1 (Gja1), which is the gene encoding expression of Cx43 at the mRNA level. In addition, they found that rat pulmonary arteries transfected with miR-1 showed a decreased relaxant response to ACh associated with Cx43 downregulation, and therefore, Cx43 could be a potential target of miR-1 (Mondejar-Parreño et al., 2019). It is also interesting since among all miRNAs, miR-1 was the most upregulated in plasma from idiopathic pulmonary hypertension patients (8-12-fold increase) (Sarrion et al., 2015).

In addition, Mondejar-Parreño et al. (2019) determined that lungs from rats with pulmonary hypertension induced by Su5416, a vascular endothelial growth factor type 2 receptor inhibitor, plus three weeks of chronic hypoxia displayed a substantial upregulation of miR-1. Hence, the present study supports the previous literature that Cx43 is involved in vasorelaxation of the pulmonary vasculature, and that blocking Cx43 channels or reducing expression of Cx43 reduced relaxation responses. This suggests that in future, targeting Cx43 could be an effective way of treating diseases such as pulmonary hypertension, which is mainly characterised by both vasoconstriction and vascular remodelling.

In the current study and in line with ACh-induced relaxation, the relaxation to an endothelium-independent dilator S-Nitroso-N-acetyl-DL-penicillamine (SNAP) was also substantially reduced by both genetic knockdown and pharmacological inhibition of Cx43. This might support the idea that Cx43 is commonly expressed in both endothelium and smooth muscle cells (Figueroa and Duling, 2009). Therefore, from the current findings with ACh and SNAP, communication between Cx43 and NO is important in mediating endothelium-dependent and -independent relaxation in the pulmonary circulation.

As reduction in relaxation responses to ACh and SNAP in IPAs of both sexes was observed, the production of basal NO was then measured in the whole left lung lobe from both female and male WT and $Cx43^{+/-}$ mice. The production of NO was significantly attenuated in the presence of ^{37,43}Gap27 as well as in the $Cx43^{+/-}$ mice compared to the WT mice. This reduction in basal NO formation in genetic knockdown or pharmacological inhibition of Cx43 could be associated with pulmonary diseases such as PAH. Zhang et al. (2015b) found that NO bioavailability was significantly reduced in PAH patients. There has been an increasing use of NO as a long-term therapeutic option for patients with pulmonary hypertension in the United States since 1999, when it was approved by the Food and Drug Administration (FDA) for the purpose of treating pulmonary hypertension patients (Hill et al., 2015). It was found that, on inhaling the gas, it quickly diffused through the alveolar-capillary membrane and pulmonary arteries' muscles.

This leads to an increase in the concentration of intracellular cyclic guanosine monophosphate (cGMP), following activation of soluble guanylate cyclase (Sim, 2010). Additionally, NO was found to significantly reduce pulmonary resistance without affecting systemic vascular resistance in patients with high pulmonary blood pressure (Frostell et al., 1991, Pepke-Zaba et al., 1991). In addition, It was observed that patients with chronic inflammatory respiratory disease that lacked pulmonary vasodilator function showed a reduced expression of eNOS and NO activity (Barberá et al., 2001). Therefore, further work is required to build on the data presented and to investigate the interaction between the eNOS/NO and Cx43 pathways.

To investigate if reduced NO production could be due to changes in eNOS expression, protein expression of eNOS was investigated in WT and $Cx43^{+/-}$ mice by Western blotting. However, no significant differences in eNOS protein expression were found between WT and $Cx43^{+/-}$ mice. The present finding is in agreement with data published by Htet et al. (2018) who found that the gene expression of eNOS (encoded by the nitric oxide synthase 3 (NOS3) gene), was not affected by the genetic knockdown of Cx43 in pulmonary artery of female and male mice under both normoxic and hypoxic conditions. In other studies, the link between Cx43 and NO production has also been investigated and an overall reduction in mitochondrial Cx43 expression in mouse heart was associated with a reduction in the respective mitochondrial rate of NO production (Kirca et al., 2015, Shvedova et al., 2018). However, the exact mechanism behind that effect was still unclear. Therefore, the results from the current study suggest that although eNOS levels were the same in lung tissue from WT and $Cx43^{+/-}$, reduced NO release from $Cx43^{+/-}$ lung tissue suggest differences in the eNOS activity or regulation. There are a variety of physical and chemical factors that affect the expression and activity of eNOS both *in vitro* and *in vivo*. As an example, there is evidence that fluid shear stress, which is generated when blood flows over the surface of endothelial cells, plays an important role in regulating expression of eNOS mRNA and eNOS protein both in cultured endothelial cells and in intact arteries (Fleming, 2010).

It is important to note that eNOS expression is regulated by a complex set of signalling pathways, and a variety of transcription factors have been implicated in the regulation of eNOS expression, including nuclear factor-κB (NFκB), Kruppel-like factor 2 (KLF-2), forkhead box O1 (FoxO1) and specific microRNAs (reviewed in detail by Balligand et al. (2009). In addition, there is a considerable amount of regulation of the activity of eNOS at the post-translational level based on substrate availability, cofactor availability, endogenous inhibitors, lipid modification, protein-protein interactions, phosphorylation, O-linked glycosylation, and S-nitrosylation, all of which are detailed in Balligand et al. (2009). Therefore, it can be concluded from the data from the current study that reduction in Cx43 genetically or pharmacologically could in turn lead to a reduction of basal NO production in the pulmonary vasculature, potentially contributing to the worsening of conditions such as pulmonary hypertension.

The current study also considered the protein interaction between Cx43 and eNOS and found that Cx43 was associated with eNOS and colocalized in the same cellular domains and exists in the same protein complexes in whole left lung lobes from female WT and $Cx43^{+/-}$ mice. In line with the current findings, Straub et al. (2011) found that MEJs, which are formed by coupling between endothelial and smooth muscle cells, were enriched by the increased expression of Cx43 and eNOS co-localisation in mouse thoracodorsal arteries. A considerable amount of literature has been published on the relationship between eNOS/NO and connexins/gap junction communication which were found to be co-dependent signalling pathways, with the exact mechanisms of their interactions still unclear (Looft-Wilson et al., 2012). For example, in HUVECs and mouse microvascular endothelial cells, NO worked as a negative regulator to Cx37 and reduced dye transfer and electrical coupling mediated by Cx37 (Kameritsch et al., 2005, McKinnon et al., 2009). This reduction in gap junction permeability would be expected to reduce the amount of contractile signals that are transferred. Clinically, Tsang et al. (2013) found that dysfunctional gap junction communication was exhibited by blood outgrowth endothelial cells from PAH patients, whilst gap junction communication in human pulmonary arterial endothelial cells (PAECs) was suppressed in PAH patients due to the elevated expression of asymmetric dimethyl arginine (ADMA), an inhibitor of nitric oxide synthase.

They also found that excessive Cx43 expression or a modulator of connexin 43 phosphorylation, rotigaptide, which improved gap junction coupling, prevented ADMA impact (Tsang et al., 2013). Moreover, Garcia et al. (2018) have described that NO can act as a modulator in the properties of HCs and gap junction channels, however, it is not well understood how NO regulates these channels.

The findings from the current study did not uncover any differences between sexes on (i) contractile responses to U46619, (ii) relaxation responses to ACh and SNAP, (iii) NO production in both WT and $Cx43^{+/-}$ mice. Interestingly, previous studies have uncovered some differences between female and male mice, albeit with using different constrictor agents. For example, Htet et al. (2018) observed notable sex differences in the vasoreactivity of IPAs. They found that ET-1 was more potent in IPAs from male $Cx43^{+/-}$ mice compared to male WT mice with no changes in the maximal responses. On the other hand, contractile responses to ET-1 were found to be similar in female $Cx43^{+/-}$ and WT mice (Htet et al., 2018). However, both female and male $Cx43^{+/-}$ mice had similar contractile responses to 5-HT when compared to WT controls. Therefore, these differences in contractile responses between sexes could be due to agonists used which support the conclusions of Billaud et al. (2011a) who confirmed that the effects of ^{37,43}Gap27 on contractile responses to phenylephrine, 5-HT and ET-1 in IPAs varied based on agonists used. Therefore, in order to better diagnose and treat diseases which might affect both sexes differently, it is still important to address the likely difference between sexes in all pre-clinical and clinical research.

In conclusion, the current study has shown that Cx43 is important in mediating pulmonary vasoreactivity and has close relations with the NO signalling pathway. The next chapter will discuss the role of Cx43 in proliferation and migration of mouse pulmonary arterial fibroblasts (MPAFs).

Chapter 5 Investigating the Role of Connexin 43 in Proliferation and Migration of Mouse Pulmonary Arterial Fibroblasts

5.1 Introduction

Following the discussion of the role of connexin 43 (Cx43) in modulating vascular tone and vasoreactivity within the pulmonary vasculature in the previous chapter, this chapter will examine the role of Cx43 in pulmonary fibroblasts, a major cell type in the pulmonary artery. Three main types of cells constitute the pulmonary artery, as described in chapter one: fibroblasts, smooth muscle and endothelial cells. Pulmonary artery fibroblasts (PAFs) play a significant role in pulmonary vascular remodelling (Stenmark et al., 2011, Welsh and Peacock, 2013). The study of connexin expression and function in pulmonary vasculature has primarily focused on pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs), with only a few studies conducted on PAFs to date. It is important to note that one of the first connexins to be discovered in fibroblasts was Cx43 (Yamasaki and Naus, 1996), and it is the most abundant connexin found in human lung fibroblasts (Yamasaki et al., 1999). Moreover, the recent research by McNair et al. (2020) found that among all the vascular connexins in rat PAFs and PASMCs, the Cx43 gene (GJA1) was the most abundantly expressed connexin.

Cell proliferation, cell growth and development are all coordinated physiological processes regulated by the cell cycle. In response to stress or injury, fibroblasts are often the first cell type to become activated, proliferate, and differentiate (Stenmark et al., 2013). A deviation from their physiological cell cycle, resulting from homeostatic imbalance, may eventually lead to uncontrolled cell proliferation activity (Malumbres and Barbacid, 2009, Suryadinata et al., 2010). Interestingly, from cell growth to cell death, gap junctions were found to be responsible for controlling every aspect of cellular life cycle (Vinken et al., 2006). While 21 named connexin isoforms have been identified in humans, only a limited number of these isoforms have been consistently linked to cell proliferation, including Cx26, Cx30, Cx32, and Cx43 (Aasen, 2015).

Hypoxia, as discussed in chapter one, refers to a reduction in oxygen availability in either the whole organism or in a specific tissue or cell within the organism. Hypoxia causes constriction in the pulmonary vessels, but dilation in the systemic vessels (Euler and Liljestrand, 1946). Hypoxia is one of the most important factors

that affects cell proliferation and migration and exposure of the pulmonary artery to hypoxia can result in vasoconstriction and pulmonary vascular remodelling, which in turn result in the development of pulmonary hypertension (Welsh et al., 2006). For this reason, hypoxia is a common model used to study pulmonary hypertension both in *in vitro* and *in vivo*.

In response to hypoxia, a variety of functional and structural behaviours can be exhibited by fibroblasts including: proliferation, differentiation, upregulation of contractile and extracellular matrix proteins and release of factors which directly affect smooth muscle cell tone and growth (Welsh and Peacock, 2013). It has been demonstrated that hypoxia increases proliferation of bovine PAFs (Welsh et al., 1996). Additionally, it has been demonstrated that PAFs cause PASMCs proliferation and migration by releasing mitogens (Zhang et al., 2017, Carlin et al., 2012, Wilson et al., 2020). Several studies have demonstrated that PAFs derived clinically from pulmonary hypertension patients and experimentally from animal models of pulmonary hypertension possess a pro-inflammatory, hyperproliferative and apoptosis-resistant phenotype (Gamen et al., 2016). There is a great deal of diversity and controversy regarding reports related to regulation of cell proliferation by connexins. Several contradictory studies report pro- or anti-proliferative effects, which are often influenced by the type of cell or tissue (reviewed in details in Aasen (2015).

It is important to note that the gap junctions and their subunit connexins are also regulated by hypoxia. For example, Billaud et al. (2011b) found that gene and protein expression of Cx43 was significantly increased in pulmonary arteries from rats exposed to chronic hypoxia for three weeks. More recently, McNair et al. (2020) also found that protein expression of Cx43 after exposure to hypoxia for 24h was significantly upregulated in rat PAFs. However, gene expression of Cx43 was found to be decreased in pulmonary arteries from mice exposed to chronic hypoxia for 14 days (Htet et al., 2018). In addition, in rat PASMCs, protein expression of Cx43 was significantly reduced as a result of 24h acute hypoxia (Chen et al., 2014). There is a possibility that the differences between these studies may be caused by the type of tissue or cell, the length of hypoxia and/or the concentration of oxygen.

Recent research performed by McNair et al. (2020) found that proliferation and migration of rat PAFs were increased in response to hypoxia, and this response was inhibited both by genetic knockdown of Cx43 using small interfering RNA (siRNA) and by pharmacological inhibition of Cx43 ^{37,43}Gap27. This highlights the importance of Cx43 in modulating hypoxia-induced cellular behaviours. As it has been shown that hypoxic-induced pulmonary vascular remodelling was decreased in Cx43$^{+/-}$ mice (Bouvard et al., 2020), it is important to investigate the hypoxic-induced proliferation and migration response in PAFs derived from Cx43$^{+/-}$ mice.

It has been established that mitogen-activated protein kinase (MAPK) cascades govern diverse cellular processes, including proliferation, differentiation, metabolism, migration and apoptosis. Several evolutionarily-conserved kinases are involved in MAPK pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and c-Jun NH2-terminal kinase (JNK). This study will focus only on the ERK pathway since it is the most extensively researched MAPK signalling pathway and is closely related to cell proliferation and differentiation and plays a crucial role in the signalling of cells (Guo et al., 2020). In rat PAFs, it was found that the phosphorylation of both p38 MAPK and ERK1/2 is increased as a result of hypoxia, and the hypoxic-induced proliferation and migration of cells requires p38 MAPK in particular (Welsh and Peacock, 2013). Recently, in hypoxic rat PAFs, McNair et al. (2020) found that ^{37,43}Gap27, a pharmacological inhibitor of Cx37 and Cx43, inhibited phosphorylation of both p38 MAPK and ERK1/2, suggesting that connexin-mediated signalling is required for MAPK-mediated proliferation and migration of rat PAFs. Therefore, this study will investigate the role of Cx43 in MPAFs using both pharmacological inhibition and heterozygous mice.

5.2 Aims and objectives

- To investigate the protein expression of total connexin 43 (t-Cx43) in MPAFs from WT and and $Cx43^{+/-}$ mice.

- To investigate the effects of Cx43 on proliferative responses of MPAFs to serum under normoxic and hypoxic conditions in WT and $Cx43^{+/-}$ mice.

- To investigate the effects of Cx43 on migratory responses of MPAFs to serum under normoxic and hypoxic conditions in WT and $Cx43^{+/-}$ mice.

- To investigate the protein expression of p-ERK in MPAFs from WT and $Cx43^{+/-}$ mice.

5.3 Methods

Female WT and $Cx43^{+/-}$ littermate mice (C57BL/6 background, 2-4 months old) were used. Primary mouse pulmonary arterial fibroblasts (MPAFs) were obtained from main and branch pulmonary arteries and grown in Dulbecco's modification of Eagle's medium (DMEM). Cells were pre-treated with $^{37\ 43}$Gap27 (300μM; an inhibitor of Cx37 and Cx43) before being placed in normoxic or hypoxic (5% O_2) conditions for 24h. Proliferation of MPAFs was assessed using an automated cell counter, while migration of MPAFs was assessed using a scratch assay. All protocols were previously detailed in the materials and methods section (Chapter 2). Data were analysed using a two-way analysis of variance (ANOVA) followed by Tukey post hoc test using GraphPad Prism 8.0 software. Results were shown as mean ± standard error of the mean (S.E.M). *P* value of less than 0.05 was considered statistically significant and *n* indicates the number of independent experiments.

5.4 Results

5.4.1 Protein expression of total connexin 43 (t-Cx43) in MPAFs from WT and genetic knockdown of Cx43

By Western blotting, the protein expression of total connexin 43 (t-Cx43) was determined in MPAFs from female mice in order to confirm that MPAFs derived from $Cx43^{+/-}$ mice expressed significantly less Cx43 compared with those derived from WT mice. As expected, the t-Cx43 protein expression was significantly lower in $Cx43^{+/-}$ MPAFs in comparison with WT (Figure 5-1).

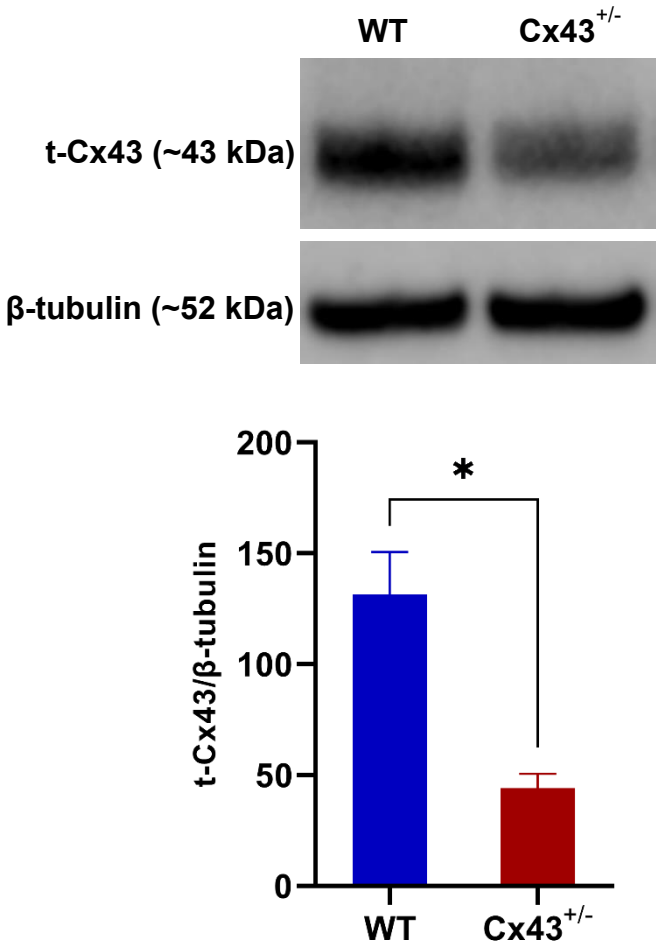
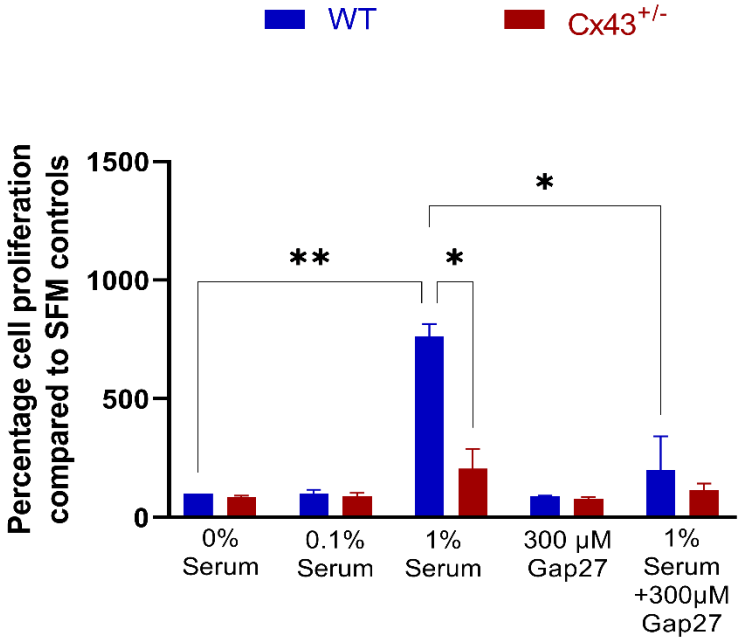


Figure 5-1. The protein expression of total connexin 43 (t-Cx43) in MPAFs normalised to a housekeeping gene- the protein β-tubulin. As expected, the protein expression of t-Cx43 was significantly attenuated in MPAFs derived from $Cx43^{+/-}$ mice compared to those derived from WT mice. *$p < 0.05$ compared to WT within same experimental group, n=6. Data are shown as mean ± S.E.M.

5.4.2 Effects of Cx43 on proliferative responses of MPAFs to 1% serum under normoxic and hypoxic conditions in WT and $Cx43^{+/-}$ mice

MPAFs derived from WT mice showed a proliferative response to 1% serum under both normoxic and hypoxic conditions. MPAFs derived from $Cx43^{+/-}$ mice had a proliferative response to 1% serum under hypoxic conditions only. This was of a similar magnitude to hypoxia-induced proliferation of WT MPAFs in the presence of 1% serum. ^{37,43}Gap27, a pharmacological inhibition of Cx37 and Cx43, reduced 1% serum-induced proliferation of WT MPAFs under both normoxic and hypoxic conditions and $Cx43^{+/-}$ MPAFs under hypoxic conditions (Figure 5-2, Figure 5-3).

A. Normoxia



B. Hypoxia

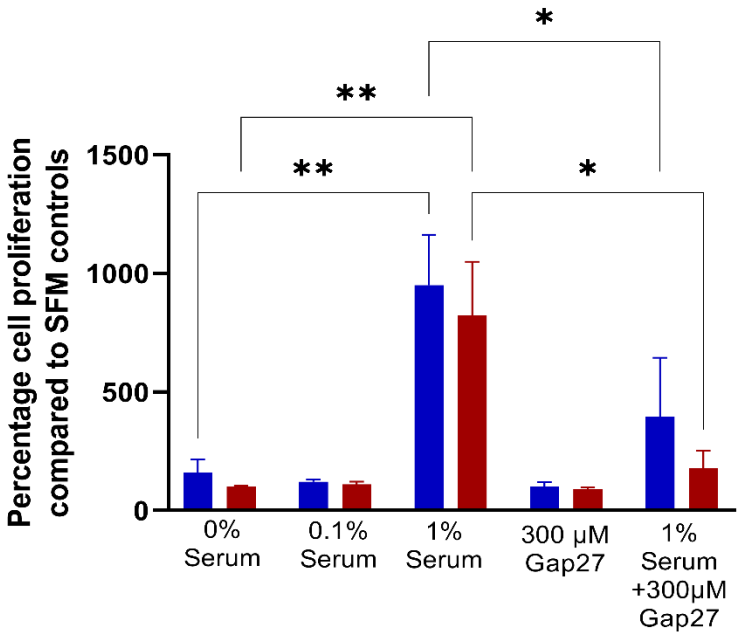
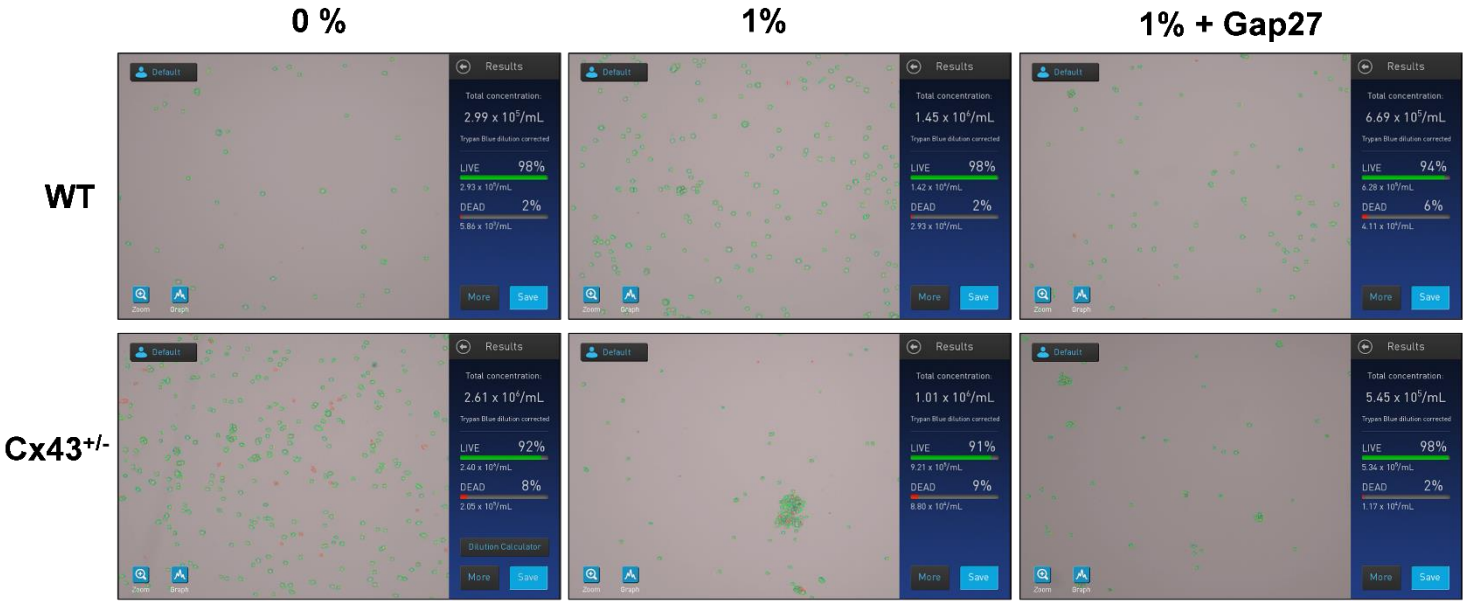


Figure 5-2. Effects of Cx43 on proliferation responses of MPAFs from WT and Cx43$^{+/-}$ mice to serum under (A) normoxic and (B) hypoxic conditions. MPAFs from Cx43$^{+/-}$ mice showed increased proliferation with 1% serum only under hypoxic conditions. However, treatment with ^{37,43}Gap27 reduced 1% serum-induced proliferation in both normoxic and hypoxic conditions in WT MPAFs and under hypoxic conditions in Cx43$^{+/-}$ MPAFs. Data are shown as mean ± S.E.M. *$p < 0.05$, **$p < 0.01$, n=3.

A. Normoxia



B. Hypoxia

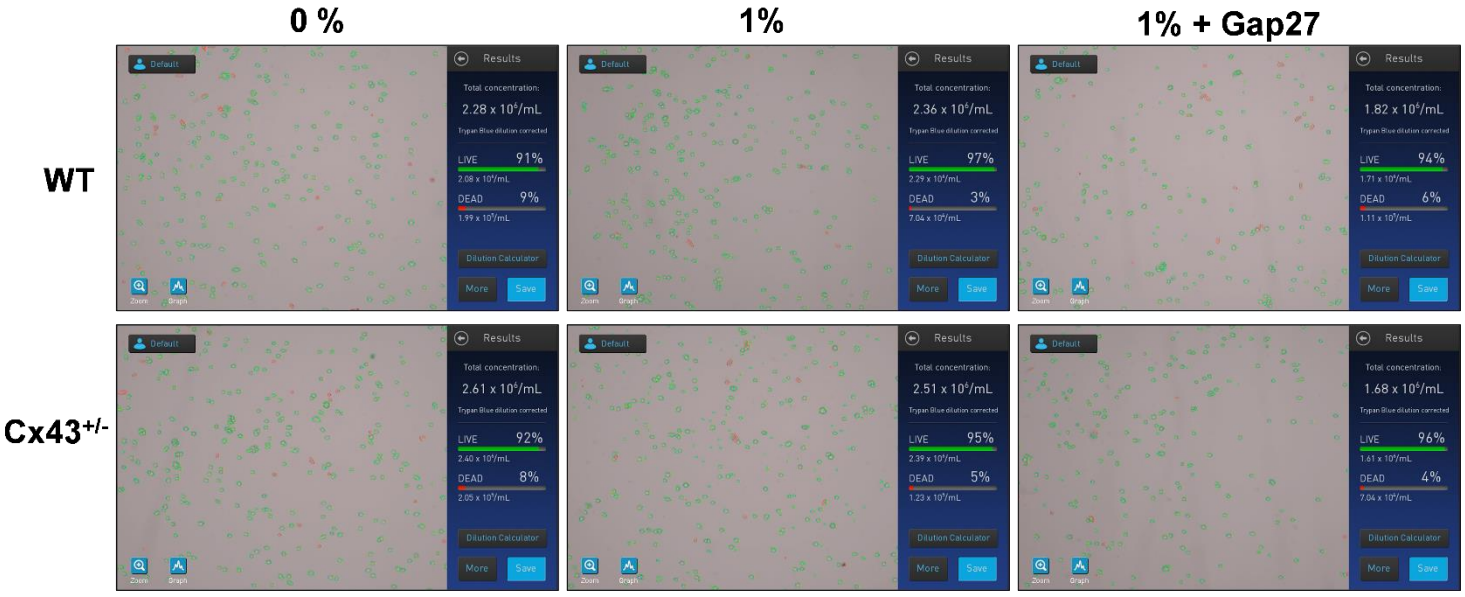
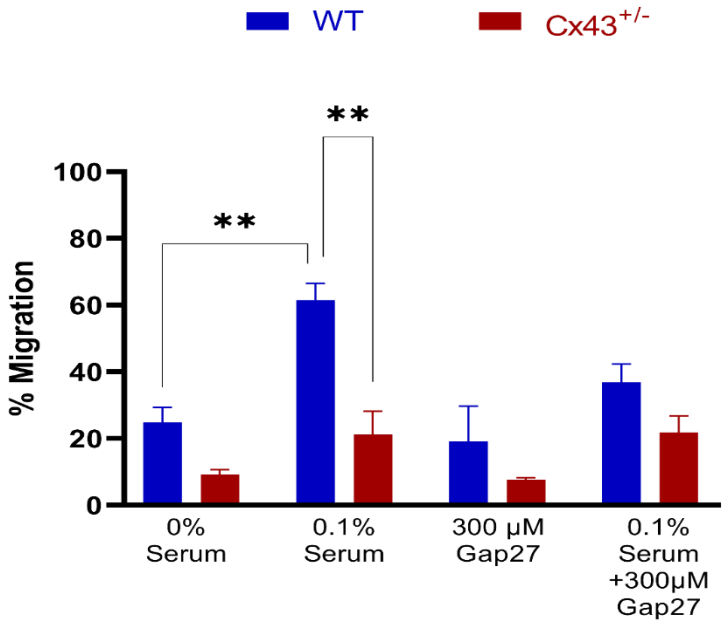


Figure 5-3. Proliferation responses of MPAFs from WT and Cx43$^{+/-}$ mice to serum exposure under (A) normoxic and (B) hypoxic conditions. The panels display cell counts indicating proliferation rates under control (0% serum), stimulated (1% serum), and inhibited conditions (1% serum + Gap27) for both WT and Cx43$^{+/-}$ MPAFs. Cell viability is visually represented by cell staining, with viable cells in green and non-viable cells in red.

5.4.3 Effects of Cx43 on migratory responses of MPAFs to 0.1% serum under normoxic and hypoxic conditions in WT and $Cx43^{+/-}$ mice

MPAFs derived from WT mice had a migratory response to 0.1% serum under normoxic and hypoxic conditions. MPAFs derived from $Cx43^{+/-}$ mice had a migratory response to 0.1% serum under hypoxic conditions only. Migration under hypoxic conditions was similar between WT and $Cx43^{+/-}$ MPAFs. ^{37,43}Gap27 reduced 0.1% serum-induced migration of both WT and $Cx43^{+/-}$ MPAFs under hypoxic conditions, although there was a trend towards a decrease under normoxic conditions, this was not statistically significant (Figure 5-4, Figure 5-5). As seen above in the proliferation responses, it is important to note that 0.1% serum was high enough to induce migration but not proliferation (Figure 5-2), (Figure 5-4).

A. Normoxia



B. Hypoxia

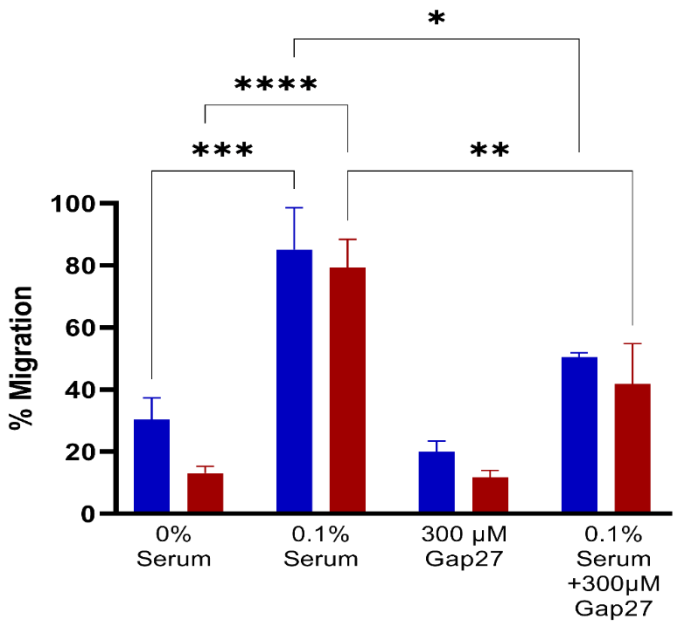
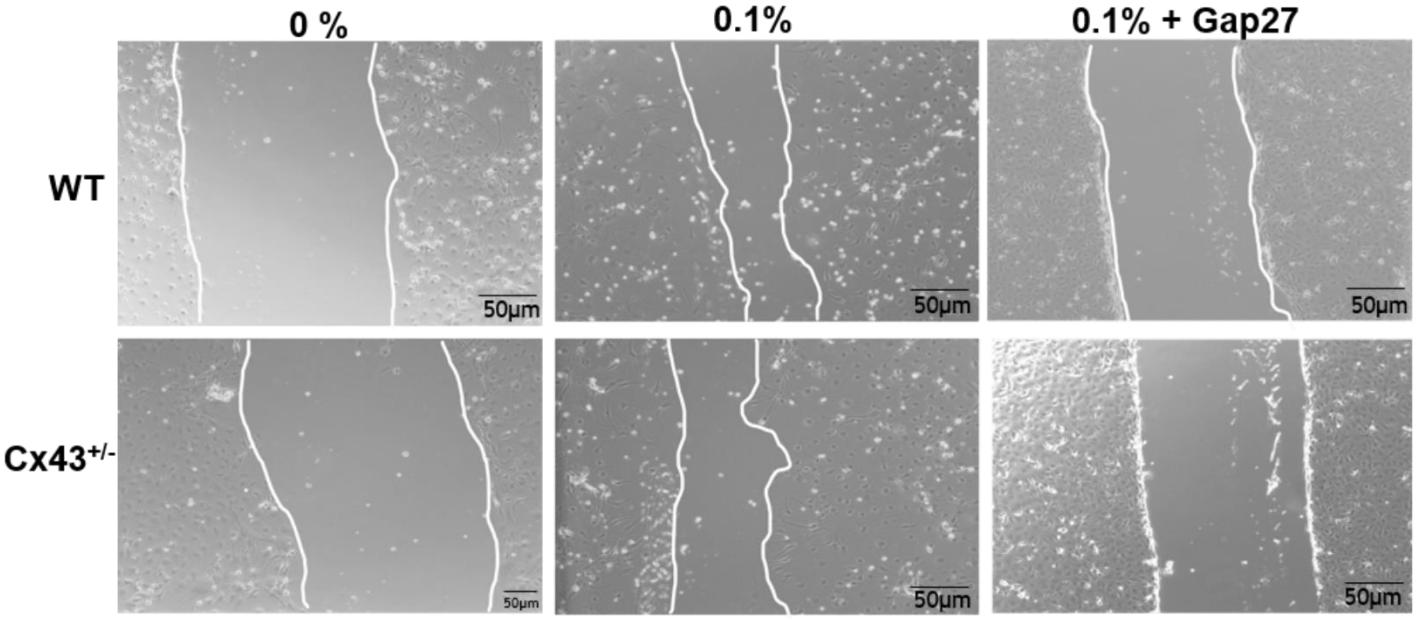


Figure 5-4. Effects of Cx43 on migration responses of MPAFs from WT and Cx43$^{+/-}$ mice to serum under (A) normoxic and (B) hypoxic conditions. MPAFs from Cx43$^{+/-}$ mice showed increased migration with 0.1% serum only under hypoxic conditions. However, treatment with ^{37,43}Gap27 reduced 0.1% serum-induced migration of both WT and Cx43$^{+/-}$ MPAFs under hypoxic conditions. Data are shown as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n=3.

A. Normoxia



B. Hypoxia

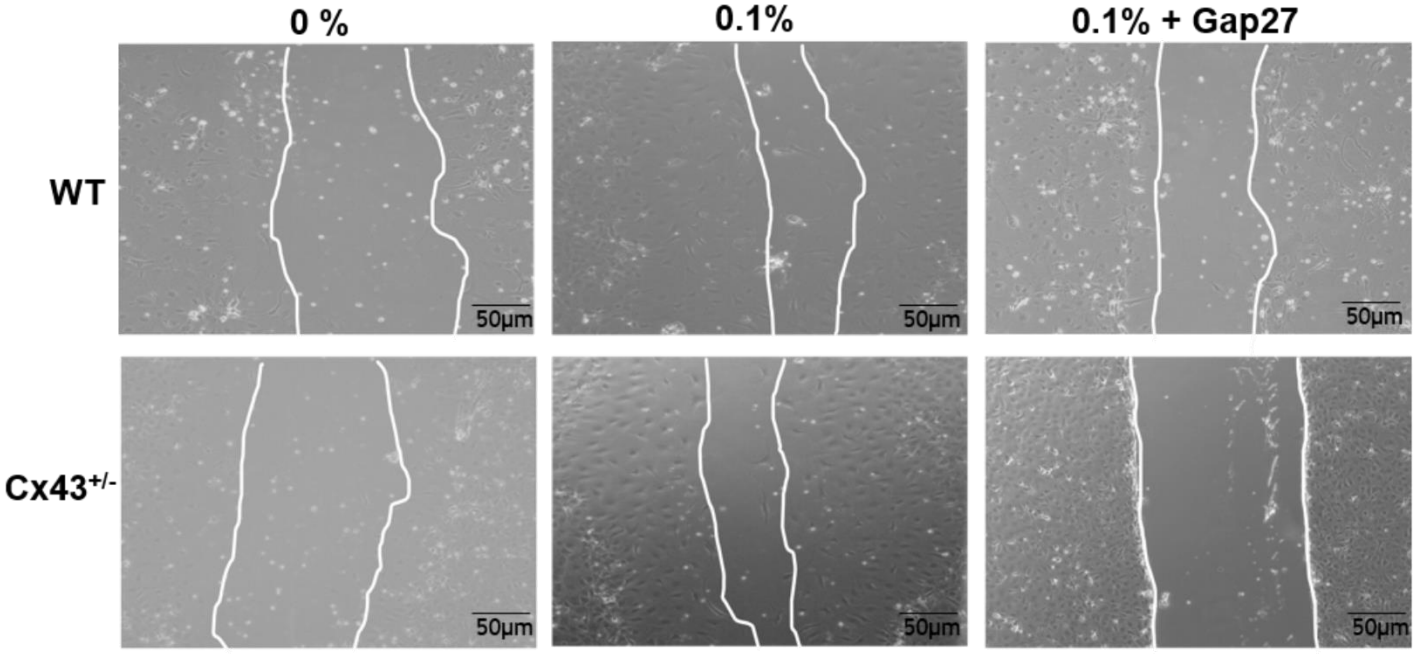
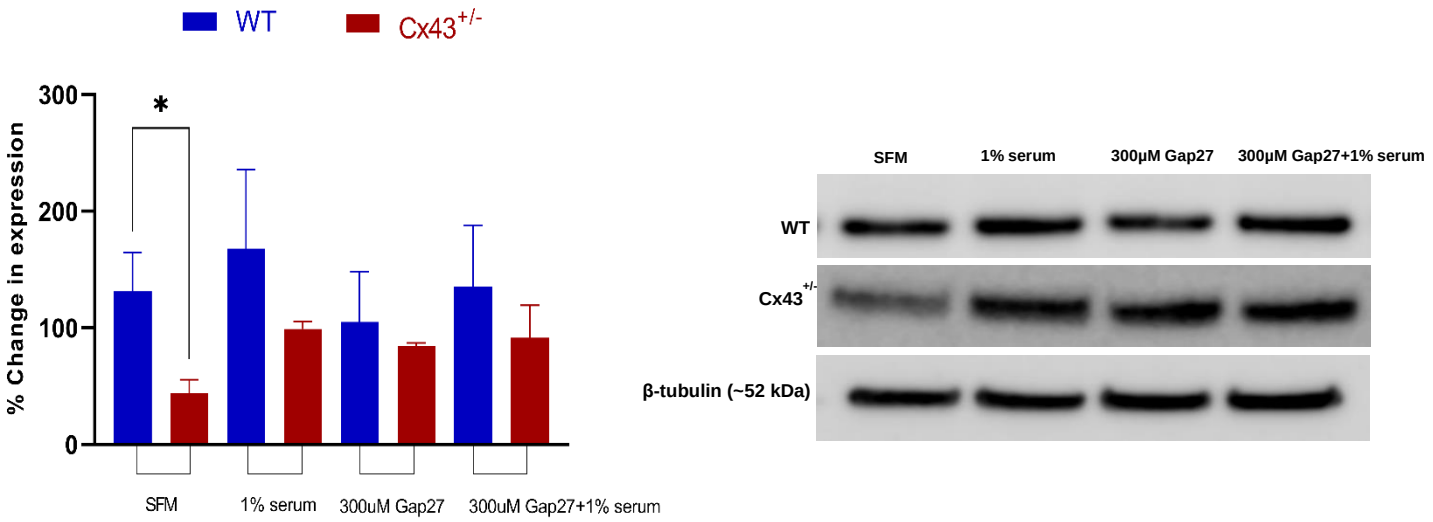


Figure 5-5. Migration responses of MPAFs from WT and Cx43$^{+/-}$ mice in response to serum exposure under (A) normoxic and (B) hypoxic conditions. The images depict the gap closure in scratch assays, indicative of cell migration, with serum concentrations of 0%, 0.1%, and 0.1% + Gap27. Migration is observed in both WT and Cx43$^{+/-}$ MPAFs, under each condition and treatment, providing insights into the role of Cx43 in cellular migration dynamics.

Protein expression of total connexin 43 (t-Cx43) in MPAFs from WT and genetic knockdown of Cx43 under normoxic and hypoxic conditions

Following the results from cell proliferation and migration, Western blotting was then conducted to investigate the protein expression of total connexin 43 (t-Cx43) in MPAFs from WT and $Cx43^{+/-}$ mice under both normoxic and hypoxic conditions. The protein expression of t-Cx43 was significantly decreased in $Cx43^{+/-}$ mice compared to WT in serum-free media (SFM) under normoxic condition but not hypoxic conditions (Figure 5-6). There was no significant effect of serum or the connexin inhibitor peptide on t-Cx43 expression.

A. Normoxia



B. Hypoxia

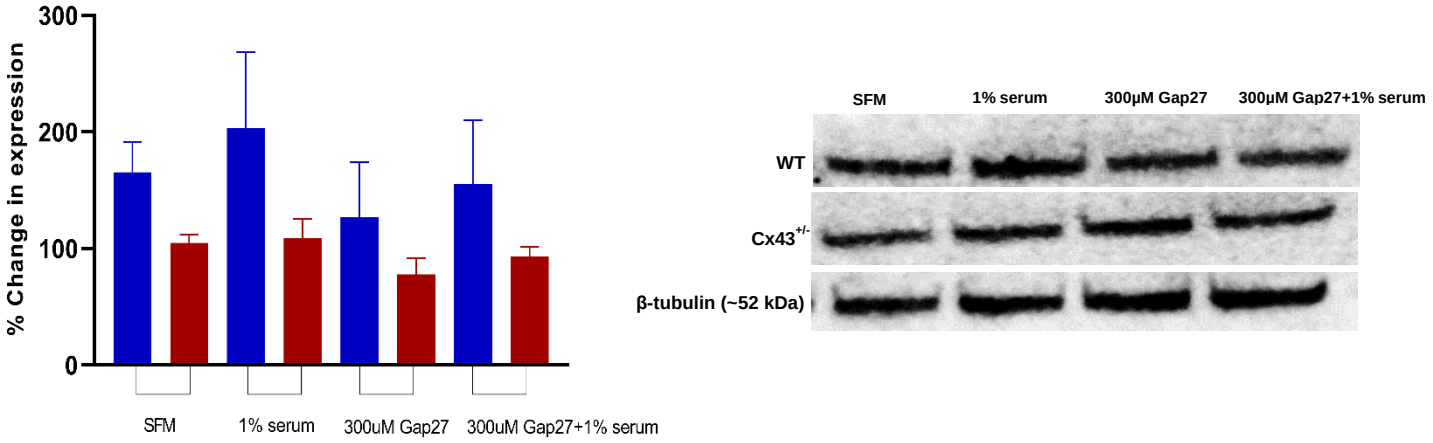
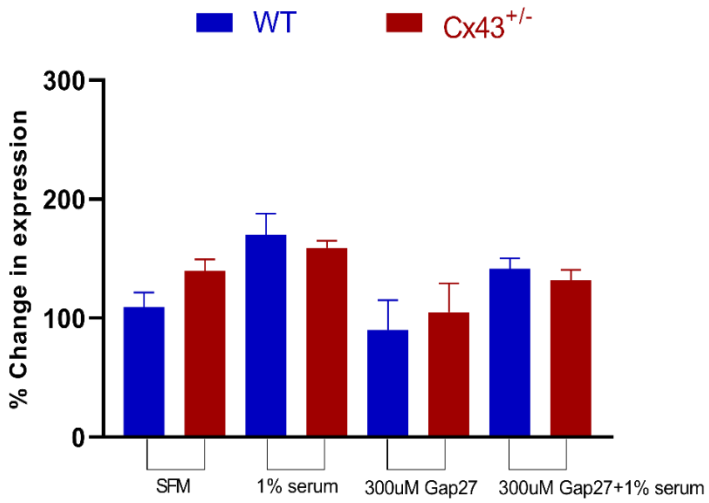


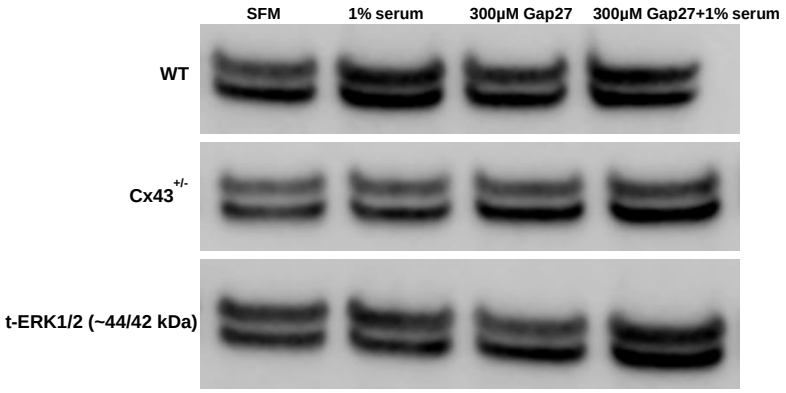
Figure 5-6. The protein expression of t-Cx43 in MPAFs normalised to a housekeeping gene- the protein β-tubulin under (A) normoxic and (B) hypoxic conditions in WT and Cx43$^{+/-}$ mice. A representative densitometry blot of t-Cx43 (~43 kDa) under normoxic and hypoxic conditions contained serum-free media (SFM) in lane 1, 1% serum in lane 2, 300μM Gap27 in lane 3 and 300μM Gap27 plus 1% serum in lane 4. Only in the absence of serum under normoxic condition was the protein expression of t-Cx43 significantly (*$p < 0.05$) decreased in Cx43$^{+/-}$ mice compared to WT, n=5. Data are shown as mean ± S.E.M.

5.4.5 Protein expression of p-ERK in MPAFs from WT and genetic knockdown of Cx43

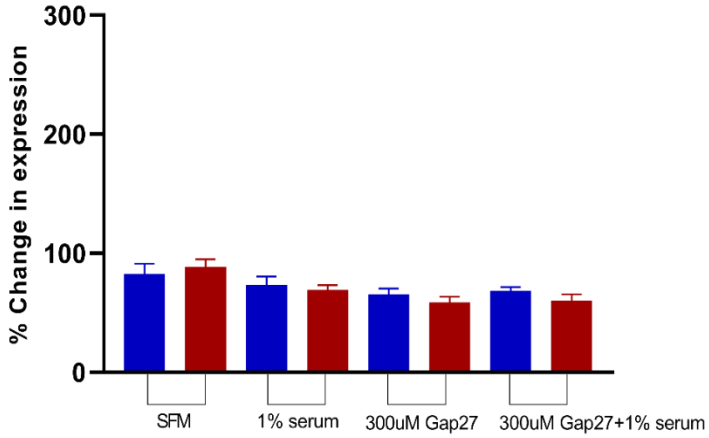
The protein expression of p-ERK 1/2 was conducted in MPAFs from both WT and $Cx43^{+/-}$ mice under varying oxygen conditions. The results indicated that there were no significant differences in the levels of activated p-ERK 1/2 between the WT and $Cx43^{+/-}$ mice, regardless of whether the cells were exposed to normoxic or hypoxic environments, or to treatments with serum and the gap junction blocker Gap27 (Figure 5-7).

A. Normoxia





B. Hypoxia



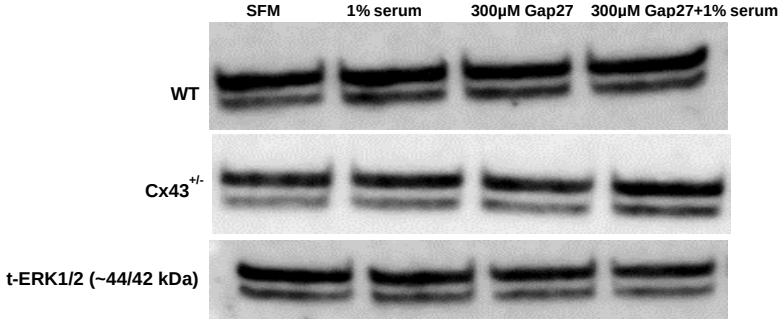


Figure 5-7. The protein expression of phospho-p44/42 MAPK (ERK1/2) (p-ERK1/2) in MPAFs normalised to the total ERK1/2 (t-ERK1/2). A representative densitometry blot of p-ERK1/2 (~44/42 kDa) under normoxic and hypoxic conditions contained serum-free media (SFM) in lane 1, 1% serum in lane 2, 300μM Gap27 in lane 3 and 300μM Gap27 plus 1% serum in lane 4. There were no significant differences were found between WT and Cx43$^{+/-}$ mice under both normoxic and hypoxic conditions, n=6. Data are shown as mean ± S.E.M.

5.5 Discussion

In this chapter, the role of Cx43 in proliferation and migration of MPAFS was investigated, as was the protein expression of t-Cx43 and p-ERK in MPAFs from WT and $Cx43^{+/-}$ mice under both normoxic and hypoxic conditions. A first observation from this study is that, as expected, and as seen previously in chapters pertaining to the thoracic aortae and lungs of mice, MPAFs from $Cx43^{+/-}$ mice displayed significantly lower protein levels of Cx43 compared to littermate WT mice.

Foetal bovine serum (FBS) is one of the most powerful additives that can be used in cell culture to enhance cell proliferation (Walenda et al., 2011, Greiner et al., 2011, Yamaguchi et al., 2002). The current study used 1% serum for proliferation experiments because research has shown that this serum concentration is the optimal concentration for enhancing the proliferation of rat PAFs (Welsh et al., 2004, Wilson et al., 2020, McNair et al., 2020) and MPAFs (Dempsie et al., 2008) in response to hypoxia. In the current study, MPAFs derived from WT mice had a proliferative response to 1% serum under both normoxic and hypoxic conditions, which was inhibited by ^{37,43}Gap27, a pharmacological inhibition of Cx37 and Cx43. In addition, MPAFs derived from $Cx43^{+/-}$ mice exhibited a proliferative response to 1% serum under hypoxic conditions only, and this response was also inhibited by ^{37,43}Gap27. These findings are in accordance with recent research performed by McNair et al. (2020) who found that 1% serum resulted in an increase in rat PAFs proliferation only when hypoxic exposure was present, and this response was inhibited both by genetic knockdown of Cx43 using small interfering RNA (siRNA) and by pharmacological inhibition of Cx43 ^{37,43}Gap27. Therefore, as a result, these studies have provided evidence that Cx43 has an important role to play in the proliferation of both rat and mouse PAFs. Although the exact mechanism by which Cx43 facilitates the proliferation of cells in MPAFs remains unclear, MAPK pathway was thought or suggested to be involved in this process. For example, Welsh et al. (2001) found that hypoxic-induced proliferation of rat PAFs is primarily mediated through p38 MAPK pathway. The previous researchers also found that hypoxia increases the phosphorylation of p38 MAPK, and hypoxia-induced cell proliferation requires p38 MAPK in particular. Furthermore, in the presence of ^{37,43}Gap27, this phosphorylation was inhibited in rat PAFs (McNair et al., 2020), suggesting that connexin signalling is essential for MAPK-mediated proliferation.

This study then investigated the effects of Cx43 on migratory responses of MPAFs to 0.1% serum under normoxic and hypoxic conditions in WT and $Cx43^{+/-}$ mice. The results of this study confirm that a low serum concentration (0.1%) was sufficient to enhance migration but not proliferation, as reported by others (Welsh et al., 2004, Wilson et al., 2020, McNair et al., 2020). According to the current study, MPAFs derived from WT mice had a migratory response to 0.1% serum under both normoxic and hypoxic conditions. In addition, MPAFs isolated from $Cx43^{+/-}$ mice had a migratory response to 0.1% serum under hypoxic conditions only, and ^{37,43}Gap27 inhibited this response. These findings are in agreement with recent research conducted by McNair et al. (2020) who found that in hypoxic conditions, 0.1% serum caused a significant increase in migration in rat PAFs, and this response was inhibited both genetically by using a siRNA approach and pharmacologically by using ^{37,43}Gap27. It has been shown that connexins, and specifically Cx43, play a role in the migration of cancer cells. For example, a study conducted by Ogawa et al. (2012) demonstrated that suppression of Cx43 expression in rat hepatocellular carcinoma cells using Cx43-siRNA reduced invasion and migration capacity *in vitro*, as well as metastatic ability *in-vivo*, which suggests that Cx43 can be used to prevent cancer metastasis in tumours that overexpress Cx43. In addition, Cx43 was shown to be related to an increased risk of prostate cancer metastasis malignancy and progression through the enhancement of cell migration and invasion (Zhang et al., 2015a). Therefore, these studies have demonstrated that Cx43 plays an important role in cell migration, and there is a need to investigate the underlying mechanism. In contrast to the current findings, the migration rates in cultured normal human epidermal keratinocytes and dermal fibroblasts was found to be increased in response to Cx43 siRNA as well as connexin mimetic peptides such as ^{37,43}Gap27 (Wright et al., 2009, Wright et al., 2012). In addition, Cogliati et al. (2015) found that wound re-epithelialization and closure were accelerated, and dermal fibroblast proliferation and activation are increased, and extracellular matrix remodelling mediators were increased in Cx43 deficiency in Cx43 heterozygous ($Cx43^{+/-}$) mice. It was noted, however, that ^{37,43}Gap27 did not affect the migration of human dermal fibroblasts, either neonatal or adult (Faniku et al., 2018). It is possible that these disparities may be caused by the variation in the species or differences in the types of cells.

The observed proliferation and migration of MPAFs under hypoxic conditions only may be attributed to several factors. For example, hypoxia triggers various signaling pathways and transcription factors, like hypoxia-inducible factors, that regulate cell survival and adaptation. It is possible that the lack of responsiveness in $Cx43^{+/-}$ MPAFs to serum alone needs the supplementary trigger of hypoxia, leading to their proliferative and migratory responses specifically under hypoxic conditions (Sedovy et al., 2022). Further research is needed to fully understand the underlying mechanisms behind the observed proliferative and migratory responses of MPAFs from $Cx43^{+/-}$ mice under hypoxic conditions. Investigating the expression levels of other connexins, signaling pathways, and interactions with neighboring cells could provide valuable insights into the complex regulatory networks that govern these cellular behaviors.

It has been shown that connexins play a crucial role in coordination of number of physiological and pathological processes, including immune responses, wound healing, cancer metastasis, osteoporosis and embryonic development during cell migration. For example, during wound-induced migration of endothelial cells, an increased expression of Cx43 and a decreased expression of Cx37 was observed at the injury site. The wound repair rate was significantly reduced when cells were transfected with pEFZ and p3243H7Et, both dominant negative connexin inhibitors (Kwak et al., 2001). pEFZ and p3243H7Et are both expression vectors which encode full-length mouse Cx43 polypeptide fused at its COOH terminus and encode 12CA5 epitope-tagged chimeric polypeptide, respectively. It has been shown that both the fusion protein and the chimeric protein exert dominant negative inhibition on the gap junction channels in relation to their activity (Paul et al., 1995, Sullivan and Lo, 1995). The extracellular matrix remodelling mediators include collagen type I and III, matrix metalloproteinase-2 (MMP-2) and transforming growth factor beta1 (TGFß-1).

This study has also investigated the protein expression of t-Cx43 and p-ERK in MPAFs from WT and $Cx43^{+/-}$ mice under normoxic and hypoxic conditions. Interestingly, our findings indicate no significant difference in the expression of any of these proteins under either normoxic or hypoxic conditions. This observation contrasts with the results reported by McNair et al. (2020), who found that hypoxia increased the protein expression of Cx37 and Cx43 in rat PAFs. They

also found that upon hypoxia, phosphorylation of both p38 MAPK and ERK1/2 was significantly upregulated and both of these responses were inhibited by ^{37,43}Gap27, as revealed by Western blotting. It is important to note that we conducted these measurements at a 24-hour time point, consistent with the protocol of McNair et al. (2020), and both studies utilized the same oxygen concentration, maintaining 5% O_2 levels throughout the experiments. Notably, the carboxyl tail of Cx43 contains MAPK phosphorylation sites, which has previously been shown to enhance the migration of cells mediated by p38 MAPK (Behrens et al., 2010). The disparities in protein expression of Cx43 and ERK1/2 between this study and McNair et al. (2020) may be attributed to species differences. Therefore, the effects of hypoxia on ERK1/2 gene expression as well as in phosphorylation of Cx43 in MPAFs from WT and $Cx43^{+/-}$ mice should be investigated in future studies to gain a deeper understanding of these variations.

In conclusion, the current study has demonstrated that Cx43 is an important factor in promoting MPAFs proliferation and migration when hypoxic conditions are present. This study, together with all previous literature, provides further evidence that Cx43 is involved in cell proliferation which results in the remodelling of the pulmonary vasculature in response to hypoxia, leading to pulmonary hypertension. Further investigation is required regarding the precise mechanism behind this phenomenon.

Chapter 6 General Discussion

6.1 Summary and Discussion of Results

This thesis provides insights into the role of Cx43 in both the systemic and pulmonary circulation.

Even though the systemic and pulmonary circulatory systems differ physiologically, connexin-mediated communication is a common mechanism for cellular coordination. As mentioned previously, a number of cardiovascular, neurodegenerative and metabolic disorders as well as lung diseases have been associated with mutations or alterations in connexins (reviewed in Yamasaki (2018). As an example, mutations of connexins in the systemic circulation can result in cardiac arrhythmias (Lambiase and Tinker, 2015), and connexin dysfunction in the pulmonary circulation may have implications in pulmonary hypertension (Htet et al., 2021), with limited evidence in patients.

This is general discussion provides a concise summary of the key findings from both chapter 3 and chapter 4. In chapter 3 and using mouse thoracic aortae, the focus was to investigate the role of both Cx43 and the endothelium on contractile responses to U46619 and on relaxation responses to ACh and SNAP, as well as on the possible interaction of Cx43 with eNOS signalling. As far as can be ascertained from published literature, this is the first study to demonstrate that Cx43 plays a role in the contraction of mouse aorta in response to U46619. In chapter 4, the focus switched to study the importance of connexins in the pulmonary vasculature using mouse lungs and IPAs. All experiments conducted in chapter 3 using thoracic aortae were repeated in this chapter using IPAs, with the exception of the endothelium removal experiments. The results of both chapters suggest a similarity in the role of connexin 43 between the systemic and pulmonary circulations, despite their physiological differences as discussed in the opening chapter. The only difference found in this study in terms of vasoreactivity was in the contractile responses to U46619 in $Cx43^{+/-}$ mice, which showed increased sensitivity in the thoracic aorta but decreased sensitivity in the IPAs, as discussed in the discussion sections in chapter 3 and 4. It has been shown that connexins can indirectly influence relaxation to ACh and SNAP by facilitating the spreading of NO signals among adjacent cells, which allows small molecules, including NO, to diffuse from cell to cell through gap junction channels formed by connexins (Looft-Wilson et al., 2012).

As a result of the diffusion of NO, smooth muscle cells can be stimulated to relax by the vasodilatory effects propagated by the diffusion of NO. This vasodilation increases the diameter of blood vessels, reduces vascular resistance, lowering blood pressure and enhances blood flow. Therefore, as a result of combining all the data from chapter 3 and chapter 4, there could be an association between a reduction in Cx43 genetically or pharmacologically with a reduction in NO production in the systemic and pulmonary vasculature, which could exacerbate some conditions, such as systemic and pulmonary hypertension.

As discussed previously in chapter 3 and 4, there were no significant differences between male and female mice in terms of: (i) contractile responses to U46619, (ii) relaxation responses to ACh and SNAP, (iii) NO production in both WT and $Cx43^{+/-}$ mice. Although sex-related differences in connexin expression and function have been observed in some contexts, it is essential to acknowledge that hormonal influences, such as those related to the oestrous cycle, can play a significant role. The specific experimental conditions and parameters investigated in the current study were not particularly sensitive to sex-specific effects. It is possible that the regulation of connexins and their functional implications exhibits a high degree of similarity between female and male mice, which may explain the absence of significant differences observed between sexes in connexin experiments in both systemic and pulmonary circulation. However, it is important to recognise the importance of sex-related effects, particularly in the context of certain diseases such as PAH. Further research into potential disparities between the sexes, considering hormonal fluctuations and their potential impact, both at the pre-clinical and clinical levels, remains essential for a comprehensive understanding of connexin-related mechanisms and their relevance to diseases affecting individuals of both sexes.

Moving forward to chapter 5, which investigates the role of Cx43 in mouse pulmonary fibroblasts (MPAFs). Recent work has shown that $CX43^{+/-}$ mice are protected against pulmonary vascular remodelling (Bouvard et al., 2020). It is well documented that PAFs are involved in hypoxic-induced remodelling of the pulmonary vasculature (Welsh et al., 1996, Welsh et al., 2006, Stenmark et al., 2011). Previous work from our group showed Cx43 to be involved in hypoxic-induced proliferation and migration of rat PAFs (McNair et al., 2020).

However, as of yet no research has been performed on the effects of Cx43 on mouse PAF (MPAF) proliferation and migration. WT MPAFs proliferated and migrated significantly more than $Cx43^{+/-}$ MPAFs when exposed to serum, whereas WT and $Cx43^{+/-}$ MPAFs responded similarly under hypoxic conditions. MPAFs derived from $Cx43^{+/-}$ mice, however, showed proliferative and migratory responses to serum only under hypoxic conditions, which were inhibited by ^{37,43}Gap27. Additionally, ^{37,43}Gap27 also inhibited WT MPAF proliferation and migration under hypoxic conditions induced by serum. These findings are in line with those of the McNair et al. (2020) and have been discussed in detail in chapter 5. Thus, the data in chapter 5, in conjunction with all previous literature, provides further evidence that Cx43 is involved in cell proliferation and migration, which may result in the remodelling of the pulmonary vasculature in response to hypoxia, resulting in pulmonary hypertension as a result.

The results obtained from this study shed light on the complex role of Cx43 in the pathophysiology of PAH. In blood vessels and in fibroblasts, it is evident that Cx43 inhibition has complex effects, both favourable and unfavourable, which could impact on the progression of PAH in humans or animals. On one hand, the observed reduction in proliferation and migration of vascular cells following Cx43 inhibition suggests a potential benefit in slowing down the vascular remodelling associated with PAH. These effects align with the current understanding that abnormal cell proliferation and migration contribute significantly to the vascular remodelling seen in PAH as discussed previously. Therefore, targeting Cx43 may hold promise as a therapeutic strategy aimed at mitigating these processes and attenuating the progression of PAH. However, the findings also reveal a paradoxical effect of Cx43 inhibition, where relaxation is impaired. This raises concerns about the potential adverse consequences of targeting Cx43 for PAH treatment, as impaired relaxation could exacerbate the already compromised haemodynamics observed in PAH patients. Therefore, while targeting Cx43 holds promise for mitigating specific PAH pathologies, a balanced therapeutic strategy is essential.

It has been previously noted that cell contact, coupling, and communication are essential to maintaining normal physiological activity. Studies have suggested that altered Cx43 expression and function could be associated with a number of diseases, including conditions related to the heart, lungs, liver, eyes, and kidneys (Leybaert et al., 2023, Cliff et al., 2022, Katturajan and Prince, 2021,

Swartzendruber et al., 2020, Htet et al., 2021). Due to this, connexin-targeted therapeutics have attracted considerable attention in recent years. As noted in chapter one, current drugs targeting specific connexins are mostly peptides, which are currently receiving growing attention in the areas of wound healing, cancer, neurological and cardiovascular diseases (Naus and Giaume, 2016). A growing body of evidence supports the notion that peptide-based molecules offer better specificity and fewer off-target effects (Leybaert et al., 2017, Acosta et al., 2021). However, both pre-clinical and translational research are impacted by the limited bioavailability of these peptides. Thus, in order to improve that, many research efforts are being conducted, such as using exosomes for the delivery of peptides (Marsh et al., 2021). The idea behind this is that the extracellular vesicle shields and protects fragile drug charges during transport within the body (Pinheiro et al., 2018, Batrakova and Kim, 2015). Another approach involves encapsulating these peptides in nanoparticles, which shields them from degradation and enhances their stability during delivery (Marsh et al., 2021). Additionally, modifying connexin-targeting peptides with cell-penetrating peptides (CPPs) improves their cellular uptake (Caufriez et al., 2020, Marsh et al., 2021). These methods aim to enhance the efficacy of connexin-targeting peptides in drug development.

A number of peptides are now being used in clinical practice to treat a variety of diseases. For example, α-connexin carboxyl terminal (ACT1) has been shown to stimulate gap junction activity in functional assays by maintaining Cx43 at gap junction sites at cell membrane borders of breast cancer cells. This leads to impaired breast cancer cell proliferation and survival. Additionally, ACT1 aids in re-establishing a normal wound repair cascade when applied to cutaneous wounds because it restores gap junctions lost in diabetic-related injured tissues (Ghatnekar et al., 2015). One of the other peptides that has entered clinical trials is rotigaptide, a peptide that increases gap junction electrical conductance used to treat cardiac arrhythmias (Macia et al., 2011). Other peptides such as Cx43 antisense oligonucleotides (ASOs), Xentry-Gap19 and Peptide5 also appear to be effective therapeutic options for chronic inflammatory diseases of the eye surface and retina (Acosta et al., 2021). Furthermore, Cx43 ASOs have also been used to treat severe ocular surface burns, and it is currently being tested in stage 3 clinical trials for ocular surface indications (Ormonde et al., 2012, Acosta et al., 2021).

6.2 Limitations

There are several noteworthy limitations that warrant attention within the scope of the current study. Although this investigation yields valuable insights into the intricate role of Cx43 in both systemic and pulmonary circulation utilising the $Cx43^{+/-}$ mouse model, it is important to acknowledge that the direct translation of these findings to clinical applications may encounter limitations. While mouse models serve as informative tools, they might not fully recapitulate the complexities of human physiology and disease. Thus, it becomes important to broaden the scope of this study, extending it to encompass investigations that incorporate human tissue samples, human cell lines, or certain animal models, such as the Sugen/hypoxia (SuHx) rat model of PAH (widely considered to be the 'gold standard/model of disease). This expansion is vital to determine the clinical relevance of the current findings within a broader and more clinically translatable context.

While centring primarily on the role of Cx43 in a specific cell type, namely fibroblasts, within the vasculature is undoubtedly insightful, it is also important to broaden our understanding by exploring the involvement of other connexins in various cell types. It is essential to acknowledge the inherent complexity of the vascular system, which comprises a dynamic interplay of various cell types. Smooth muscle cells, endothelial cells, immune cells, and perivascular cells are just a few examples of the multifaceted cellular landscape. These diverse cell types possess the potential to exert significant contributions and interactions, as reviewed in detail by Chang et al. (2020). Therefore, to gain a more comprehensive understanding of the roles played by connexins in vascular physiology and pathophysiology, we should consider studying other connexins in addition to Cx43. This approach will enable us to explore the broader cellular context and its impact on the overall outcomes and interpretation of the current findings in this thesis.

In this study, the selection of the 4-6 month age group for thoracic aorta samples, while initially based on the availability of subjects, serendipitously aligned with a crucial phase in murine vascular development. This phase is characterized by mature vascular structures that have not yet experienced significant age-related alterations (Fritze et al., 2011), thus providing a unique opportunity to study

Cx43's role in a well-developed yet still dynamically responsive vascular system. However, it is important to also acknowledge this as a limitation, as the initial choice was not driven by a pre-defined scientific rationale. While the results obtained from this age group have contributed valuable insights, future studies could be designed with a more systematic approach to age selection, allowing for a more controlled examination of age-related vascular changes. Similarly, the deliberate choice of the 2-3 month age group for pulmonary studies, aimed at exploring responses in early adult stages, underscores the need for a consistent and scientifically-driven approach in age selection across different vascular systems. This discrepancy in the rationale for age selection between thoracic aorta and pulmonary studies highlights a potential area for refinement in future research designs, ensuring that age-related variables are consistently accounted for and their impacts thoroughly understood.

Another limitation worth of note is the absence of blinding and randomization in the experimental design. While these methodologies are recognized for their ability to reduce bias and enhance the validity of results, especially in biomedical research, they were not implemented in our investigations. The lack of blinding and randomization could potentially introduce a degree of bias in the interpretation of the data and the assignment of subjects to experimental groups. Future studies could benefit from incorporating these strategies to strengthen the reliability of findings. This limitation highlights the need for cautious interpretation of our results and underscores the importance of considering these factors in experimental design.

In the present study, the decision to focus predominantly on female mice in the WB and co-IP experiments, as well as in the NO assay and in MPAFs studies in Chapter 5, was based on initial evaluations. These evaluations included comparative functional studies and NO experiments across both sexes. These preliminary investigations did not reveal significant differences in Cx43-related responses between male and female mice in the specific functional aspects we explored. Consequently, to adhere to the principles of the 3Rs (Replacement, Reduction, and Refinement) in animal research, the study focused only on female mice. This approach aimed to minimise animal use without compromising the scientific objectives and integrity of the research. While the study did encompass initial comparative analysis between sexes, the decision to focus on female mice

in certain experiments can be seen as a limitation. Thus, while the current study's findings are based on comprehensive preliminary analyses, future research could benefit from a more extensive exploration of sex differences in Cx43 expression and functionality, particularly within the areas of systemic and pulmonary vascular physiology. This extended approach could provide a deeper and more detailed understanding of Cx43's roles across sexes.

In this study, as outlined in chapter 2, we established 'normoxic' conditions at 95% O_2 and hypoxia at 5% O_2, following the standard practices in our laboratory. However, a notable limitation is the lack of validation of hypoxia using specific markers such as hypoxia-inducible factor (HIF). The inclusion of these markers would have been crucial to confirm the actual induction of hypoxia in MPAFs, providing a more robust and definitive understanding of the cellular environment. Moving forward, it's essential to incorporate such validation steps in future experiments. This will not only ensure the accuracy of the hypoxic conditions but also strengthen the overall findings by clearly outlining the cellular response to hypoxia.

Other notable limitation of this research was the lack of monitoring of Cx43 expression changes in response to pharmacological agents. Although the study involved comprehensive investigations into the roles of Cx43 in vascular reactivity, NO production, and its interaction with eNOS, it did not specifically track the dynamic changes in Cx43 levels in response to each treatment condition. Measuring any changes in Cx43 expression could have provided deeper insights into the regulation of Cx43 and whether this could underlie any of the effects seen under varying experimental treatments. Such monitoring might have revealed important information about the adaptive responses of Cx43 to different physiological and experimental contexts, which would be beneficial for a more detailed understanding of its role in vascular function and pathology.

6.3 Future Directions

Chapter 4 presented data relating to pulmonary vasoreactivity and is similar in some respects to the data presented by Htet et al. (2018) and Bouvard et al. (2020) who used ET-1, 5-HT and phenylephrine as contractile agents, and MCh and carbachol as relaxant agents. Here, U46619 and ACh were used as contractile and relaxant agents, respectively. 5-HT, ET-1, and thromboxane signalling have been well established as contributing to PAH pathogenesis (Dempsie et al., 2008, Chester and Yacoub, 2014, Alqarni, 2023), and hence, it would be interesting to study the effect of these agents in IPAs derived from Sugen/hypoxia (SuHx) rat model of PAH. This model induces PAH by Su5416, an inhibitor of vascular endothelial growth factor type 2 receptors, plus three weeks of hypoxia, as described in (Vitali et al., 2014). In addition, it would be worthwhile to examine the effect of the previous contractile and relaxant agents on IPAs from hypoxic WT and $Cx43^{+/-}$ mice to better understand the differences in signalling pathways activated by these agents and to investigate the relationship between hypoxia and Cx43 in the pulmonary vasoreactivity.

Earlier in this thesis, it was mentioned that one hypothesis is that gap junctions and their subunit connexins might play a role in pulmonary vasoreactivity through their interaction with Ca^{2+} (reviewed in Peracchia et al. (2000), Srisakuldee et al. (2014). Therefore, further investigation is needed to address the relationship between different connexins and Ca^{2+} using some techniques such as calcium imaging, calcium flux assay, electrophysiological techniques and biochemical analysis (reviewed in Decrock et al. (2011), Decrock et al. (2017).

Reflecting on the discussions presented in Chapter 1 regarding the role of Cx43 phosphorylation in modulating gap junction communication and connexin-mediated cell signalling (page 51), it is clear that future investigations should rigorously pursue the study of Cx43's phosphorylation states. This aspect was not addressed within the scope of the current study, leaving a critical gap in our understanding of how phosphorylation influences Cx43 functionality, particularly under pathological conditions such as pulmonary hypertension. Investigating the phosphorylated forms of Cx43 is crucial for elucidating the molecular mechanisms that govern vascular reactivity and the progression of disease. Such focused research will enhance our comprehension of the complex regulatory networks

involving Cx43 and potentially reveal novel therapeutic avenues aimed at correcting dysfunctional connexin signalling in vascular diseases. This direction is vital for advancing our knowledge on the multifaceted roles of Cx43 phosphorylation in health and disease, promising to yield significant insights into the development of targeted interventions.

Future experiments could also be conducted to determine how eNOS/NO interact with connexins and gap junctions. To the best of my knowledge, $Cx43^{+/-}$ mice have not been studied for the activities of eNOS in thoracic aortae, so further research is needed to investigate eNOS activity in both WT and Cx43-genetically or -pharmacologically modified mice. Various factors, including protein interactions with caveolin-1, co-factor availability and oxidant stress can influence eNOS activity and affect NO production, as discussed in chapter 3. Therefore, further investigation is needed to understand eNOS activity in aortic vessels of $Cx43^{+/-}$ mice, including potential regulatory mechanisms like caveolin-1. A growing body of evidence suggests that eNOS/NO and connexins/gap junctions are interdependent signalling pathways, though the exact mechanisms are unknown (reviewed in Looft-Wilson et al. (2012).

In endothelial, smooth muscle and fibroblast cells, as well as vascular tissue, immunofluorescence microscopy can be used to determine eNOS subcellular localization and co-localization. In addition to staining cells and tissue samples, fluorescence signals can be visualized and quantified using specific antibodies against eNOS and Cx43. Analysis of colocalization can provide information about the spatial relationship between eNOS and Cx43, suggesting the possibility that they might interact and function cooperatively. A variety of pharmacological approaches, such as the use of eNOS inhibitors or NO donors to modulate the eNOS/NO pathway and the application of connexin-specific modulators or mimetics to manipulate Cx43 function, can also be employed to manipulate eNOS/NO and Cx43 pathways independently and assess their interactions. Using techniques such as Western blotting or immunofluorescence, the subsequent impact on Cx43 expression or function can be evaluated when eNOS activity is modulated with NO donors (such as sodium nitroprusside) or inhibitors (such as L-N^G-Nitro arginine methyl ester (L-NAME). A similar approach can be used to manipulate Cx43 by using agonists (such as retinoic acid) or inhibitors (such as ^{37,43}Gap27 or carbenoxolone) and to measure the subsequent changes in eNOS/NO

signalling. Furthermore, *in vivo*, eNOS and Cx43 expression or activity can be manipulated and assessed using several techniques, including pressure myograph and telemetry to assess vascular function, blood pressure, and endothelial responses of eNOS knockout mice or $Cx43^{+/-}$ mice. Immunohistochemistry can also be used as it allows researchers to visualise eNOS and Cx43 proteins within tissues at a microscopic level using specific antibodies, revealing their distribution and abundance. Staining patterns and intensity provide insights into changes in protein expression, offering valuable information about their roles in observed physiological changes.

There is increasing evidence that perivascular adipose tissue (PVAT) surrounding aortae and connective tissues surrounding IPAs play a significant role in vascular biology and have implications for cardiovascular disease pathogenesis (reviewed in Grigoras et al. (2019), Faffe and Zin (2009), Vonk et al. (2021). In the current study, the thoracic aortae and IPAs were dissected free from their surrounding tissues and the PVAT was removed. However, it would be worthwhile to investigate whether these surrounding tissues are involved in connexins/Cx43-mediating vascular tone/vasoreactivity in the systemic and pulmonary vasculature. This could be accomplished by repeating prior experiments and then comparing the results in the presence of PVAT or connective tissues. Furthermore, imaging techniques could be conducted to visualize and quantify dynamic vascular responses, both in the presence and absence of surrounding tissues.

As compared to chapters 4 and 5, which employed both vessels (lungs and IPAs) and cells (MPAFs), chapter 3 used only the whole thoracic aorta. It may therefore be worthwhile for future studies to investigate the three distinct layers of the aorta: endothelial cells, smooth muscle cells and fibroblasts. These layers have been shown to play an important role in the normal functioning of the systemic vasculature (reviewed in Iddawela et al. (2021). Therefore, comparing the results with MPAFs, it may be advantageous to investigate the role of Cx43 in proliferation and migration of mouse thoracic aorta fibroblasts under normoxic and hypoxic conditions in WT and $Cx43^{+/-}$ mice. In the aortic wall, aortic fibroblasts actively participate in remodelling of the extracellular matrix. Various matrix components are secreted by these cells, including collagen, elastin, and proteoglycans, which contribute to the aorta's structural integrity and mechanical properties (Singh and Torzewski, 2019). Cell-cell communication and coordination in remodelling

processes are mediated by connexins. It is therefore possible to gain insight into the role of connexins in tissue remodelling and matrix synthesis by studying them in aortic fibroblasts. As a result of dysregulation of connexins and impaired intercellular communication, aortic fibroblasts are implicated in numerous cardiovascular diseases, including aortic aneurysms and atherosclerosis (Trollope and Golledge, 2011). It is possible to gain insights into the role of connexins in disease development, progression, and potential therapeutic targets by studying them in aortic fibroblasts. Thus, the study of connexins in aortic fibroblasts will allow researchers to investigate their role in intercellular communication as well as how it influences cell behaviour, tissue homeostasis, and pathological processes.

The results of this thesis indicate that NO production from lung homogenates was much smaller in comparison to the aorta as much less nitrite, which is a stable breakdown product of NO, was detected per mg of tissue. In addition, this study observed a reduction in NO release from $Cx43^{+/-}$ thoracic aortae and lung tissue, indicating potential differences in eNOS activity in these mice. Therefore, a co-immunoprecipitation technique was attempted to investigate if Cx43 could regulate eNOS activity. The data demonstrate that Cx43 and eNOS colocalize in the same cellular domains and are present in the same protein complexes in both thoracic aortae and lungs from WT and $Cx43^{+/-}$ mice. Thus, as there is a lack of studies on eNOS expression and its regulation in the aortic and pulmonary vessels and cells of $Cx43^{+/-}$ mice, further research is needed in mice with Cx43 deficiency, either genetically or pharmacologically in both systemic and resistance vessels, to examine the regulation of eNOS through transcriptional and posttranslational mechanisms, including protein-protein interactions (all these factors were reviewed in Balligand et al. (2009).

Other future plans to take the work performed in this thesis forward could include assessing whether Cx43 is involved in the mechanism of action of current PAH treatments (such as sildenafil, prostacyclin and bosentan), and current systemic hypertension treatments (such as thiazide diuretics, amlodipine, ramipril, telmisartan and atenolol). The effects of these drugs alone or in combination could be assessed on vascular reactivity of aortae and IPAs derived from both wildtype and $Cx43^{+/-}$ mice. Additionally, *in-vivo* experiments could be used to assess the role of connexin 43 in the development of PAH. While Bouvard et al.

(2020) explored the hypobaric hypoxic model, future work may consider assessing PAH in Sugen/hypoxia (SuHx) WT and $Cx43^{+/-}$ mice. This approach will allow for the examination of the effects of genetic reduction of Cx43 on the development of PAH. Heart, lung and pulmonary arterial tissue could be taken from control and SuHx/hypoxic WT and $Cx43^{+/-}$ mice and expression of various connexins and signalling molecules assessed by qPCR, Western blotting and confocal immunohistochemistry. To quantify pulmonary haemodynamics following exposure to hypoxia, pulmonary arterial pressure and flow could be measured simultaneously *in vivo*. This can be achieved through techniques such as right heart catheterization, which involves catheter insertion for direct measurements, and non-invasive methods like Doppler echocardiography. These approaches would enable a comprehensive assessment of pulmonary haemodynamics where expression of connexin 43 is reduced.

6.4 Conclusions

In conclusion, the current study has demonstrated the significance of Cx43 in modulating both systemic and pulmonary vasoreactivity, highlighting its close relationship with the NO signalling pathway. These findings suggest the potential for Cx43 as a valuable target for pharmacological interventions. Further, this study provides further evidence that Cx43 plays a role in cell proliferation which results in remodelling of the pulmonary vasculature upon hypoxia, leading to pulmonary hypertension. Therefore, the potential of targeting Cx43 in the treatment of PAH warrants further investigation, emphasizing the need for a balanced therapeutic approach that optimizes its benefits while minimizing adverse effects, ultimately advancing our understanding of PAH management.

Chapter 7 References

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