

Cardiovascular remodeling

Edited by

Yan Yang, Yi Zhang, Ming Lei and Gui-Rong Li

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Cardiovascular remodeling

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Editorial: Cardiovascular remodeling

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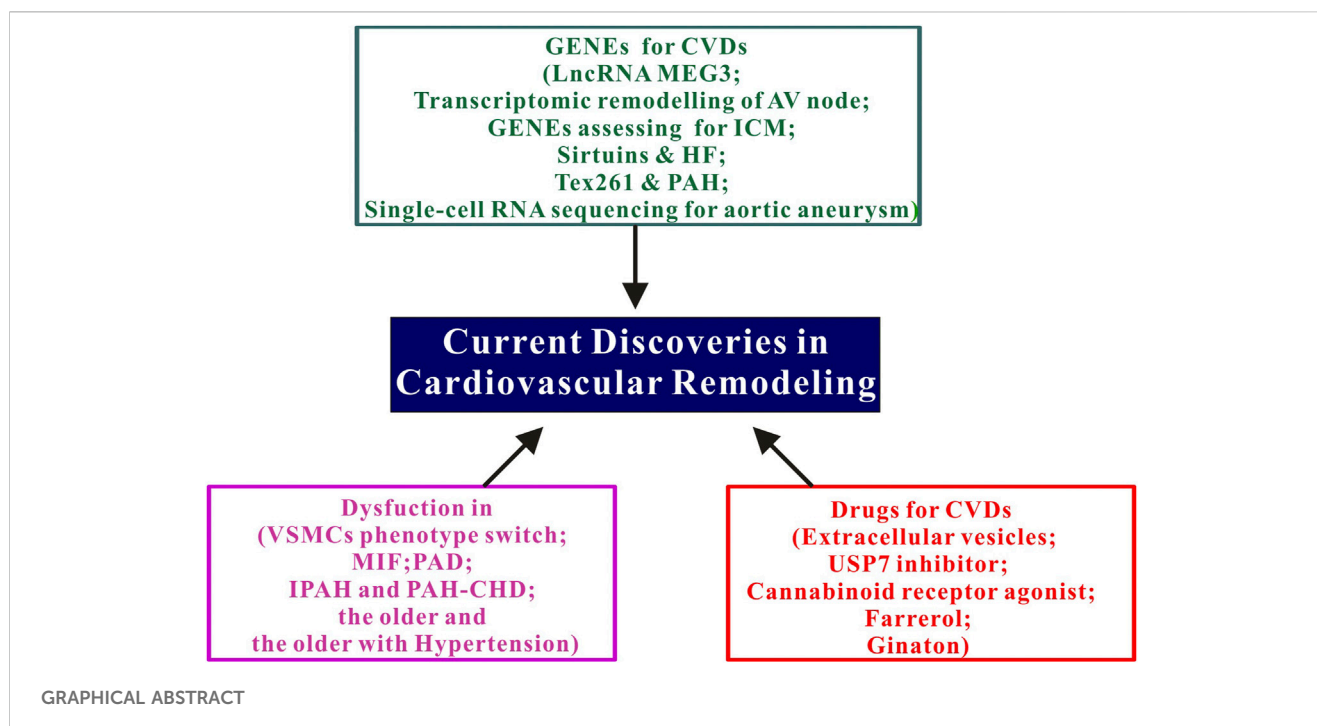
Editorial on the Research Topic Cardiovascular remodeling

1 Introduction

Cardiovascular diseases (CVDs) remain as the leading cause of death worldwide, highlighting the need for new therapeutic approaches. A significant pathogenic factor contributing to the development of CVDs is cardiovascular remodeling. As such, this Research Topic aims to explore and evaluate recent progress in molecular mechanisms, drug therapy, and innovative technologies, with the ultimate goal of enriching our understanding and management of cardiovascular remodeling. The Research Topic contains three integrated sections, as summarized in the Graphical Abstract and the subsequent parts.

2 Genetic studies in relation to cardiovascular disease

The significance of the maternally expressed gene 3 (MEG 3) is discussed in a review article [Li et al.](#) MEG3 is involved in the processes of various human diseases. P53 and PI3K/Akt are important pathways for MEG3 to participate in the regulation of apoptosis. MEG3 binds to miRNA directly or competitively and is involved in apoptosis, inflammation, oxidative stress, endoplasmic reticulum stress and epithelial-mesenchyme transition. LncRNA MEG3 is mainly involved in malignant tumors, metabolic diseases, immune system diseases, cardiovascular and cerebrovascular diseases. LncRNA MEG3 has a variety of pathological effects in cardiomyocytes, fibroblasts and endothelial cells, and has a great potential for clinical application in the prevention and treatment of atherosclerosis, myocardial ischemia-reperfusion injury, hypertension and heart failure (HF). A study conducted by [Wilson et al.](#) aimed to determine the underlying cause of atrioventricular (AV) node dysfunction in HF by analyzing changes in the transcriptome of the entire AV node. The research utilized the mouse transverse aortic constriction model, which induces HF through pressure overload. Functional changes were assessed through electrocardiography and echocardiography, while the transcriptome of the AV node was quantified using RNAseq. The findings indicate that AV node dysfunction in HF is attributed



to inflammation and a pervasive transcriptional remodeling within the AV node. Another crucial point to consider is the significance of the SIRT1 pathway in the pathophysiology of myocardial remodeling and HF, as demonstrated by the Wang et al. Sirt1, a nicotinamide adenine dinucleotide + -dependent deacetylase, emerges as a fundamental player in the pathophysiology of myocardial remodeling and HF. Its involvement in transcriptional regulation, energy metabolism, cell survival, DNA repair, inflammation, and circadian regulation positions it as a pivotal molecule for investigation. The existing literature indicates substantial advancements in understanding the role of the SIRT1 pathway in the pathophysiological mechanisms underlying myocardial remodeling and HF. The original research articles by Lu et al. primarily focused on the discovery of several newly identified therapeutic targets. They developed a risk model based on the progression of necrosis in order to identify the genes associated with this process. The objective of their study was to identify potential therapeutic agents and evaluate the progression and prognosis of ischemic cardiomyopathy (ICM) by screening for necroptosis-related genes and constructing a risk score. The findings of their study provide important insights into the significance of six necroptosis-related signature genes (STAT4, TNFSF10, CHMP5, CHMP18, JAK1, and CFLAR) in assessing the progression and prognosis of ICM. These signature genes have significant clinical value and could potentially serve as targets for enhancing cardiovascular remodeling. Tex261 is a protein-coding gene whose functional enrichment node includes the transporter activity of COP II. Chen et al. conducted a study on the correlation between Text6 and pulmonary hypertension (PAH) on the basis of multi-omics sequencing to screen target proteins. Tex261 has been found to be involved in COPII and apoptosis regulation. Hypoxia inhibits Tex261 expression by

activating HIF-1 α . Downregulation of Tex261 promoted Ndr1 and inhibited AKT activity, which in turn inhibited Sec23 activity, leading to cell proliferation and vascular remodeling. The increase of Tex261 has certain preventive and therapeutic effects on PAH in rats. These results provide a basis for exploring whether Tex261 can be used as a target for prevention and treatment of PAH, and provide a new possibility for clinical diagnosis and treatment. These positive effects are mediated through the NF- κ B signaling pathway. Most worth mentioning in this Research Topic is "Comparative analysis of thoracic and abdominal aortic aneurysm across segment and species" (Wu et al.). Single-cell RNA sequencing analysis was used to compare the differences in the cell populations of mouse and human aneurysms in the abdominal aorta and thoracic aorta, revealing the similarities and differences in the changes in the cell type components of human and mouse, and identifying the well-preserved SMCs and macrophage subpopulations. In addition, the analysis results indicated that the biological functions of thoracic and abdominal aortic aneurysms are different, and the underlying pathogenic genes are also different. This study defines the pattern of aortic aneurysms, and lays the foundation for future research into the potential of subgroups or marker genes as therapeutic targets.

3 Dysfunction and novel mechanisms in cardiovascular diseases

A bibliometric analysis (Han et al.) on the phenotype switch of vascular smooth muscle cells (VSMCPS) has garnered significant interest. To create a visual representation of the knowledge landscape in VSMCPS research, a bibliometric analysis was conducted. The Web of Science Core Research Topic Database

(SCI-EXPANDED) was utilized to search for literature on VSMCPS from 1999 to 2021. Bibliometric tools such as VOSviewer and CiteSpace were employed. The study identified the most productive researchers, journals, institutions, and countries in this field. Furthermore, trends, hot topics, and knowledge networks were analyzed and visualized, demonstrating an increasing number of annual publications in the field of VSMCPS. The primary research topics include “vascular remodeling,” “atherosclerosis,” “intimal neogenesis,” “hypertension,” and “inflammation.” The article “*Treatment of myocardial interstitial fibrosis in pathological myocardial hypertrophy*” (Zhu et al.) provides a comprehensive overview of the current understanding of the mechanisms and negative consequences associated with myocardial interstitial fibrosis (MIF) in pathological cardiac hypertrophy. Pathological myocardial hypertrophy, which can arise from various diseases, often coincides with the occurrence of MIF. This condition involves the formation of diffuse, patchy lesions comprising interstitial microscars and the deposition of collagen fibers around blood vessels. This article also explores the potential use of circulating and cardiovascular magnetic resonance (CMR) imaging biomarkers to identify MIF lesions, while also discussing existing and potential future therapeutic strategies. In a study conducted by Zhu et al., researchers investigated the primary function of the right ventricle (RV) and its subsequent response to targeted therapies in patients diagnosed with idiopathic pulmonary arterial hypertension (IPAH) and pulmonary arterial hypertension associated with congenital heart disease (PAH-CHD). The findings revealed that patients with IPAH had lower baseline RV function, a less favorable prognosis, and a limited response to targeted treatment compared to those with PAH-CHD. Multiple risk factors, such as smoking, dyslipidemia, diabetes, and hypertension, have been identified as contributors to the development of peripheral vascular diseases (PAD). The article titled “*Peripheral vascular remodeling during ischemia*” (Lin et al.) discussed in depth the functional changes observed in peripheral arterial cells during ischemic conditions and explores the underlying mechanisms responsible for the pathogenesis of PAD. Additionally, the article provides an overview of the current advancements in clinical treatments and potential therapeutic approaches for PAD, while shedding light on future perspectives in this field. The thromboxane A₂ (TXA₂) receptor (TP) is a member of the G protein-coupled receptors (GPCRs) family. The activation of TP receptor ultimately depends on the availability of a specific G protein. Zhang et al. discovered that the TXA₂-TP prostaglandin signaling pathway is impaired in elderly rats. Their research demonstrated that the contraction of mesenteric arterioles induced by thromboxane was diminished in the older and the older hypertensive rats. This suggests a weakened TXA₂-TP prostaglandin signaling pathway in the older. These findings complement the understanding of the mechanisms underlying changes in vascular TXA₂-TP signaling during vascular remodeling associated with aging.

3 Enhancement of cardiovascular health through drug therapy

One article (Cheng et al.) delves into the topic of extracellular vesicles (EVs)—bilayer lipid nanoscale vesicles—that are released into the extracellular space by eukaryotic cells. These vesicles act as transmitters of biological information and play a vital role in

intercellular communication. This article expands upon the aforementioned concept and discusses the advancements achieved in pharmacology and engineering for the purpose of targeting EVs to facilitate cardiac repair following a myocardial infarction (MI). Furthermore, this article delves into the dual functionality of the small molecule transmitters contained within EVs, with regards to their influence on the process of post-infarction cardiac remodeling. Ubiquitin specific protease 7 (USP7) plays a crucial role in various biological processes. In Gu et al.’s study, it was found that the expression of USP7 was elevated in both mice and patients with HF induced by Ang II. Additionally, the researchers observed that the inhibitor p22077 was able to mitigate cardiac hypertrophy, cardiac fibrosis, inflammation, and oxidative stress by inhibiting the AKT/ERK, TGFβ/SMAD2, NFκB/NLRP3, and NOX2/NOX4 signaling pathways. These findings indicate that USP7 could be a potential therapeutic target for managing hypertrophy remodeling and HF. It was previously hypothesized that cannabinoid receptors (CBR) possess cardioprotective properties. CB13, a compound that activates both cannabinoid receptors, has been found to stimulate AMPK signaling in ventricular cardiomyocytes. The effects of CB13 on atrial myocyte expansion and mitochondrial function in neonatal atrial rat cardiomyocytes (NRAM) stimulated by angiotensin II (AngII) were investigated in a study conducted by Altieri et al. Their findings provide further evidence that activation of CBR promotes AMPK activation in the atria, ultimately preventing myocyte enlargement, mitochondrial depolarization, and destabilization of Cx43. It has been speculated that Farrerol may have a significant role in reducing heart hypertrophy and remodeling. He et al. conducted a study to assess the role of Farrerol in cardiac remodeling. Firstly, they established a model of myocardial remodeling, and subsequently administered Farrerol through intraperitoneal injection to evaluate its effects. The results revealed that Farrerol effectively inhibited the Ang II-induced hypertrophy of cardiomyocytes, oxidative stress levels, as well as the proliferation and migration of fibroblasts. These findings suggest that Farrerol holds promise as a potential candidate for the treatment of myocardial remodeling. In addition, in traditional Chinese medicine related studies, Wang et al. demonstrated that the use of Ginaton, a natural extract derived from Ginkgo biloba, effectively inhibits the activation of M1 phenotype macrophages induced by Ang II. This treatment also prevents macrophage adhesion and attenuates the inflammatory response, thereby leading to the alleviation of hypertension and cardiac remodeling.

In summary, this topic gives us a glimpse into the ongoing research progress on cardiovascular remodeling. It is expected that these efforts will aid in future research endeavors aimed at comprehending the fundamental mechanisms of cardiovascular disease and pave the way for effective therapeutic strategies.

Author contributions

YY served as a Topic editor for this Research Topic and authored the Editorial text. YZ, G-RL, and ML also contributed

as Guest Editors, providing valuable feedback on the Editorial. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Treatment of myocardial interstitial fibrosis in pathological myocardial hypertrophy

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Pathological myocardial hypertrophy can be caused by a variety of diseases, mainly accompanied by myocardial interstitial fibrosis (MIF), which is a diffuse and patchy process, appearing as a combination of interstitial micro-scars and perivascular collagen fiber deposition. Different stimuli may trigger MIF without cell death by activating a variety of fibrotic signaling pathways in mesenchymal cells. This manuscript summarizes the current knowledge about the mechanism and harmful outcomes of MIF in pathological myocardial hypertrophy, discusses the circulating and imaging biomarkers that can be used to identify this lesion, and reviews the currently available and potential future treatments that allow the individualized management of patients with pathological myocardial hypertrophy.

KEYWORDS

pathological myocardial hypertrophy, myocardial interstitial fibrosis, myofibroblasts, anti-fibrosis, biomarkers

1 Introduction

Myocardial hypertrophy can be broadly defined as the increase of heart mass (Bernardo et al., 2010). The main function of the heart is to maintain the perfusion of peripheral organs to meet the needs of normal and stress conditions. However, the growth of postnatal heart is affected by its functional load. In order to accomplish this task with increased preload or afterload, the size of cardiomyocytes typically increases, which is called hypertrophy. Hypertrophy can be divided into physiological hypertrophy and pathological hypertrophy. Both types of hypertrophy involve the enlargement of single cardiomyocytes, which were initially developed as an adaptive response to cardiac stress, while they remarkably differ in potential molecular mechanisms, cardiac phenotypes, and prognosis (Nakamura and Sadoshima, 2018). Physiological hypertrophy is mainly caused by normal postpartum growth, pregnancy, and exercise (Perrino et al., 2006). Pathological hypertrophy is caused by chronic hypertension, aortic stenosis, mitral or aortic regurgitation, myocardial infarction, storage diseases (e.g., lipid, glycogen and misfolded protein storage diseases), and hereditary cardiomyopathy (e.g., hypertrophic cardiomyopathy) caused by gene mutations encoding sarcomere protein (Devereux et al., 2000; Turbey et al., 2010). Over time, physiological hypertrophy can maintain cardiac

function, while pathological hypertrophy is highly accompanied by cardiomyocyte death, leading to interstitial and perivascular fibrosis and adverse cardiovascular events, including heart failure (HF), arrhythmia, and death.

At the cellular level, pathological hypertrophy is caused by cardiomyocyte hypertrophy, expansion of surrounding stroma, and loss of extracellular homogeneity (Rodrigues et al., 2016). As the key component of pathological hypertrophy, interstitial expansion is the result of the joint action of extracellular edema and interstitial fibrosis, which is mainly secondary to the aggregation of mature cross-linked collagen fibers (Halliday and Prasad, 2019). The diffuse and unbalanced accumulation of collagen in myocardial stroma is called myocardial interstitial fibrosis (MIF) (González et al., 2018). MIF is a diffuse and patchy process. It is a histological marker of several heart diseases that change myocardial structure and function, and can be associated with the progression of HF. The main manifestations of MIF were interstitial micro-scar formation, perivascular collagen fiber deposition, and increased thickness of tendon collagen chain (Díez et al., 2020). Although a large number of research evidences have explained the mechanism of MIF in pathological myocardial hypertrophy, it has not yet fully realized the transformation of these knowledge into a practical and effective treatment of pathological myocardial hypertrophy. This review will focus on the currently available and potential future treatment methods of MIF in pathological myocardial hypertrophy caused by hypertensive heart disease (HHD), aortic stenosis, diabetic cardiomyopathy, hypertrophic cardiomyopathy and so on.

1.1 Classification and characteristics of myocardial interstitial fibrosis

Myocardial fibrosis is mainly divided into replacement fibrosis and interstitial fibrosis. Replacement fibrosis occurs in myocardial injury (i.e., myocardial infarction), which is characterized by excessive collagen deposition and micro-scar formation. The reason is that after myocardial cells are damaged, they are replaced by activated fibroblasts to form the main scar containing type I collagen (de Jong et al., 2011). MIF can be further divided into reactive MIF and invasive MIF (Herum et al., 2017). Reactive MIF is the accumulation of collagen in the extracellular space due to the imbalance of collagen metabolism without cell death (Liu et al., 2021). It may be manifested as a thick fibrotic sheath located around the intramural coronary artery and arterioles, or around the cardiac muscle bundle (i.e., perimuscle) and a single cardiomyocyte (i.e., intima) (Halliday and Prasad, 2019). Invasive MIF occurs in patients with Fabry disease which is a rare genetic disease characterized by dysfunction of sphingolipid function and catabolism (Disertori et al., 2017). The potential reason why MIF does not lead to cardiomyocyte death may be

that MIF is often secondary to some harmful stimuli such as pressure overload or myocardial inflammation, which do not lead to cardiomyocyte death. Replacement fibrosis and reactive interstitial fibrosis coexist in the majority of patients. It is essential to clarify whether these two histological types of fibrosis represent different entities or whether they represent different stages of development of the same disease (Halliday et al., 2017). For instance, the latest research on patients with hypertrophic cardiomyopathy showed that fibrosis is initially characterized by perivascular, perisomatic and intramuscular deposits, while as the disease progresses, fibrosis is mainly characterized by alternative scars (Galati et al., 2016).

MIF is a scar event in myocardium, which is associated with myocardial hypertrophy. It occurs in almost all types of heart disease and has different manifestations (Weber et al., 2013; Segura et al., 2014). For example, MIF in HHD is composed of fine connective tissue and mainly manifested as interfiber fibrosis, while the MIF of hypertrophic cardiomyopathy is composed of coarse collagen fibers and mainly manifested as plexiform fibrosis (Sugihara et al., 1988). Collagen deposition is the leading feature of MIF, especially type I and type III collagen, as well as (Liu et al., 2021) type I and type III collagen fibers are the main structural proteins forming fibrous tissue (Whittaker, 1995; Fomovsky et al., 2012). Although MIF is patchy, the area of fibrosis increases from the outer to the inner third of the ventricular free wall in some patients with heart diseases, such as HHD, aortic stenosis, and hypertrophic cardiomyopathy (Treibel et al., 2018). This may be related to transmural pressure, wall stress, and changes of coronary microcirculation, leading to relative endocardial ischemia (Díez et al., 2020). An evidence showed that MIF is not only related to the scope of fiber deposition, but also related to the collagen composition and physicochemical properties of fibers. For instance, in pathological myocardial hypertrophy caused by hypertension or aortic stenosis, the content of type I collagen is higher than that of type III collagen due to the excessive accumulation of type I collagen fibers (López et al., 2014; Echegaray et al., 2017). However, in diabetic cardiomyopathy, the content of type III collagen is higher than that of type I collagen (Shimizu et al., 1993). In hypertrophic cardiomyopathy, there is no difference between the content of the two types of collagen (Boerrigter et al., 1998). Collagen fibers have physical and chemical properties such as insolubility, degradation resistance and hardness. The insolubility, degradation resistance and stiffness of collagen fibers depend on the degree of intermolecular covalent bonding (i.e., cross-linking) between their constituent fibers (Díez et al., 2020). Type I collagen fibers are mainly related to the coarse fibers that give tensile strength, while type III collagen fibers usually form fine fibers and maintain the elasticity of the matrix network (Kong et al., 2014). Due to type I collagen fibers are heavily cross-linked, the stiffness of type I collagen fibers in human myocardium is higher than that of type III collagen fibers. Some studies have

proved through Young's modulus evaluation that there is a direct correlation between the ratio of collagen type I to collagen type III and myocardial stiffness in HF patients caused by severe aortic stenosis (López et al., 2021). However, it is still unclear how the ratio of type I collagen to type III collagen affects the specific pathological phenotype of MIF. Another feature of MIF is that collagen with cross-linking density matures and then forms fibrotic scars. This feature may increase the tensile strength of the scar, affect the degree of cardiac relaxation and contractility, and limit cardiac function (La et al., 2017).

2 Mechanism of myocardial interstitial fibrosis

Myocardial fibrosis is secondary to various stimuli, including ischemia, poisoning, metabolism, infection, genetics, and hemodynamics. These stimuli directly activate the fibrotic pathway or lead to cardiomyocyte dysfunction (González et al., 2018). In reactive MIF, without cell death, a variety of fibrotic signaling pathways in mesenchymal cells (e.g., fibroblasts) may be activated by different stimuli to cause fibrosis, including the following stimuli: mechanical stress associated with pressure overload of HHD and aortic stenosis, defects caused by various causal mutations in sarcomere structure and function of hypertrophic cardiomyopathy, metabolic damage associated with hyperglycemia in diabetic cardiomyopathy, or coronary microvascular endothelial damage in HF with preserved ejection fraction (HFpEF) (Diez et al., 2020). Fibroblasts undergo a response known as "activation", which is characterized by a transition of quiescent cells into myofibroblast-like phenotype, while some other cells, including macrophages, cardiomyocytes, and endothelial cells, indirectly participate in the fibrotic response by secreting fibrogenic mediators (Kong et al., 2014). Fibroblasts can sense damage-related molecular patterns (DAMP), which lead to activate pro-inflammatory responses. Molecular patterns associated with injury include proinflammatory cytokines (i.e., interleukin-1 α), intracellular molecules released by dead cells (i.e., heat shock proteins), extracellular matrix (ECM) molecules up-regulated by injury (i.e., fibronectin), or molecules modified by a pathological environment (i.e., advanced glycation end products (AGEs)). Fibroblasts also provide membrane receptors for many growth factors, cytokines, and neurohumoral factors that regulate signaling pathways, including platelet-derived growth factor (PDGF), angiotensin-II (especially type 1 receptor), connective tissue growth factor (CTGF), and transforming growth factor- β (TGF- β). In injured myocardium caused by a variety of physical and chemical factors, cardiomyocytes, macrophages, and endothelial cells can release cytokines, chemokines, and growth factors (e.g., transforming growth factor- β (TGF- β)). These factors work together with mechanical stress and

neurohormone activation to make fibroblasts proliferate and differentiate into myofibroblasts through a variety of signal pathways. These signaling pathways include DAMP-dependent pathways, inflammatory signalling cascades (triggered by cytokines and chemokines), mechanosensitive mechanisms (mediated by integrins and ion channels), and neurohumoral pathways (such as renin-angiotensin-aldosterone system). Among them, TGF- β is the main regulator of fibrogenesis through SMAD-dependent pathway and none-canonical signal cascade. Ang II also plays an important role in cardiac fibrosis, which activates profibrotic cascades, such as TGF- β /SMAD, ERK1/2 (extracellular-signal-regulated kinase1/2), AKT (protein kinase B) and MAPK (mitogen-activated protein kinase) signal transduction (Subbaiah et al., 2022). The pathophysiological role of Ang II can reconnect the transcriptome of fibroblasts to promote the activation of fibroblasts with enhanced their ability to migrate, proliferate, secrete proinflammatory mediators, and produce ECM proteins (Subbaiah et al., 2022) (Figure 1). Additionally, myofibroblasts can also be transformed from circulating fibroblast precursor cells, epicardial epithelial cells, and endothelial cells (Gyöngyösi et al., 2017).

Myofibroblasts are the main mediating cells in MIF, possessing the characteristics of proliferation and secretion. They regulate cytokine secretion and collagen synthesis, promote the formation of non-functional scar, and contribute to the renewal of extracellular matrix and collagen deposition (Liu et al., 2021). Myofibroblasts possess the characteristics of synthetic active fibroblasts because they have the combined ultrastructural and phenotypic features of smooth muscle cells obtained through the formation of contractile stress fibers and *de novo* expression of α -smooth muscle actin, as well as extensive endoplasmic reticulum (Weber et al., 2013). The fiber secretory group of myofibroblasts is composed of molecules required to change the collagen turnover of extracellular fibrils and promote MIF, as well as autocrine and paracrine factors that further simulate their proliferative and metabolic activities, so as to perpetuate the fiber formation in the damaged myocardium, thereby maintaining fiber formation (Bomb et al., 2016). The most important proteins secreted by myofibroblasts are type I and type III procollagen precursors and enzymes that directly interfere with the extracellular synthesis, deposition, and degradation of type I and type III collagen fibers, such as type I procollagen amino terminal protease (disintegrin and matrix metalloproteinase (MMP)) and type I procollagen carboxyl terminal protease, or procollagen C-proteinase (PCP, also known as bone morphogenetic protein-1) (Ricard-Blum, 2011). These enzymes convert terminal propeptides of the procollagen precursors secreted by myofibroblasts into mature collagen fibroin molecules by lysing them. Subsequently, lysyl oxidase (LOX) family plays a catalytic role in formation of the final collagen fibers that may deposit in the myocardium through covalent bonds (i.e., cross-linking) between adjacent fiber

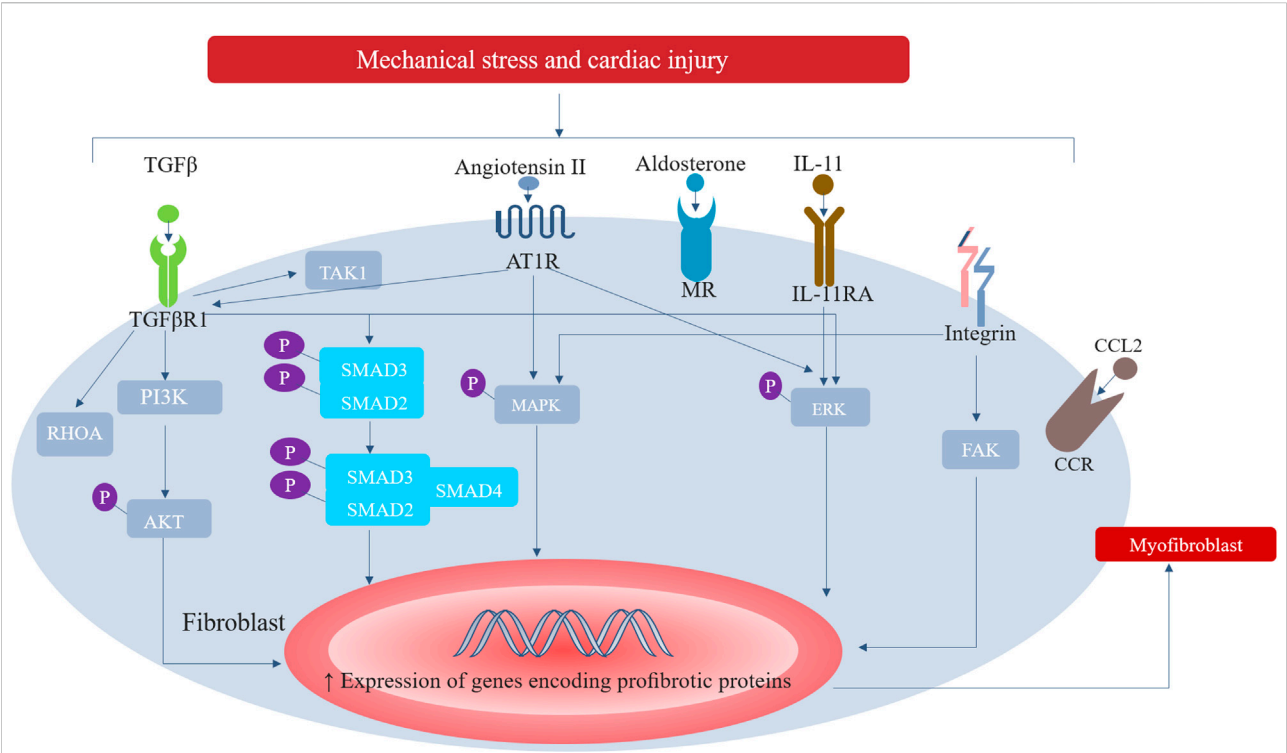


FIGURE 1
Major signalling pathways involved in the activation of cardiac fibroblasts. In response to increased mechanical stress and to cardiac injury, inflammatory signalling cascades (triggered by cytokines and chemokines), mechanosensitive mechanisms (mediated by integrins and ion channels), and neurohumoral pathways (such as renin-angiotensin-aldosterone system) can activate fibroblasts into extracellular matrix-synthesizing myofibroblasts. Among them, TGF-β is the main regulator of fibrogenesis through SMAD-dependent pathway and non-canonical signal cascade. Ang II also plays an important role in cardiac fibrosis, which activates profibrotic cascades, such as TGF-β/SMAD, ERK1/2, AKT and MAPK. AT1R, type 1 angiotensin II receptor; CCL2, CC- chemokine ligand 2; CCR, CC- chemokine receptor; ERK, extracellular-signal- regulated kinase; FAK, focal adhesion kinase; IL-11RA, IL-11 receptor subunit- α; MAPK, mitogen-activated protein kinase; MR, mineralocorticoid receptor; PI3K, phosphoinositide 3-kinase; RHOA, transforming protein RHOA; TAK1, TGFβ-activated kinase 1; TGFβR1, TGFβ receptor type 1.

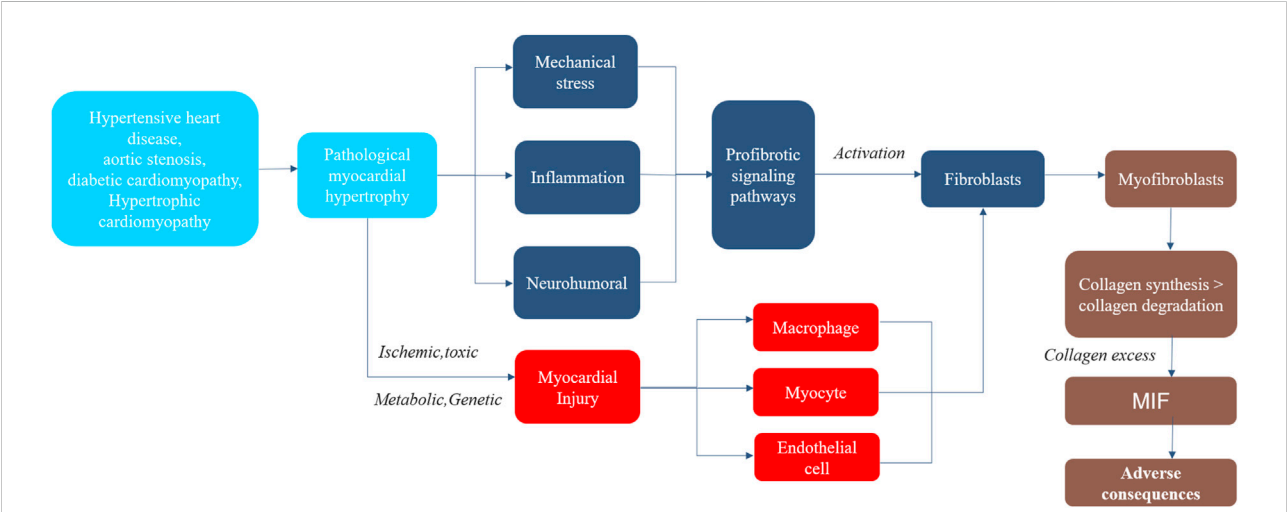
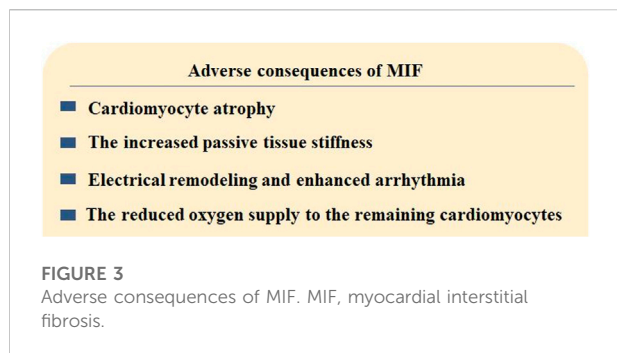


FIGURE 2
The Process of Myocardial Interstitial Fibrosis in pathological myocardial hypertrophy. Steps in the process of myocardial interstitial fibrosis, including major mechanisms and consequences. MIF, myocardial interstitial fibrosis.



polypeptide chains (Díez et al., 2020). The increase of mature fibers destroys the balance between fiber production and degradation and extracellular expansion, that is, fiber formation and deposition are dominant, rather than its degradation and removal (Bishop and Laurent, 1995). The diffuse and unbalanced aggregation of final mature fibers in myocardial stroma leads to MIF (Figure 2).

3 Adverse consequences of myocardial interstitial fibrosis

MIF is associated with the adverse effects of myocardial function and can lead to a variety of adverse consequences, including cardiomyocyte atrophy, increased passive tissue stiffness, electrical remodeling and enhanced arrhythmia, and reduced oxygen supply to the remaining cardiomyocytes (Weber et al., 2013). These adverse consequences may lead to the left ventricular (LV) dysfunction, arrhythmia, and other disorders, affecting the prognosis of cardiac function in patients with pathological myocardial hypertrophy, which indicates uncontrolled and progressive accumulation of fibrotic tissue (Figure 3).

3.1 Cardiomyocyte atrophy

Fibrosis was reported as a “key determinant” of myocardial heterogeneity in heart diseases (Weber et al., 2013). Although the size of cardiomyocytes in the myocardium is mainly variable, (Campbell et al., 1989), this heterogeneity is more prominent in the diseased heart, because fibrous collagen tendrils produced by scar tissue trap adjacent cardiomyocytes, reducing the workload of cardiomyocytes, thereby inducing cardiomyocyte atrophy (Fidziańska et al., 2010). For instance, atrophic, while viable cardiomyocytes trapped by fibrillary collagen at scar and perivascular fibrotic sites are anatomically separated from cardiomyocytes with smaller remodeling sites found in pressure-overload

hypertrophic hearts. Re-expression of the expressed β -myosin heavy chain (β -MHC) gene is a well-documented marker of pathological cardiac hypertrophy (Pandya et al., 2006; López et al., 2011).

3.2 The increased passive tissue stiffness

The increase of collagen cross-linking may lead to the hardening of fibrous tissue, and the stiffness of type I collagen fiber is higher than that of type III collagen fiber. Therefore, the passive tissue stiffness increased with the increase of the deposition of rigid and highly cross-linked type I collagen fibers (López et al., 2012). At the same time, hardened fibrous tissue can also further promotes fibrosis *via* mechanosensitive pathways and *via* liberation of latent TGF- β . With the increase of myocardial stiffness caused by collagen accumulation, diastolic dysfunction may occur (Ohsato et al., 1992; Díez et al., 2002). A previous study showed that MIF is associated with the LV stiffness and diastolic dysfunction in patients with HHD, aortic stenosis, hypertrophic cardiomyopathy, and HFpEF through histological evaluation or CMR imaging (Maragiannis et al., 2018).

3.3 Electrical remodeling and enhanced arrhythmia

The electrical remodeling and enhanced arrhythmia caused by MIF are due to the accumulation of matrix protein and the existence of myofibroblasts. The pathophysiological mechanism is mediated by intracellular Ca^{2+} -overload and oxidative stress (Hothi et al., 2008). A previous study demonstrated that MIF impairs myocardial electrophysiology by slowing down propagation of action potential, initiating reentry, promoting posterior depolarization, and increasing ectopic autonomy, which together lead to an increased risk of ventricular arrhythmia (Nguyen et al., 2017). For instance, in patients with hypertrophic cardiomyopathy, MIF has a certain influence on the electrophysiological changes that mediate ventricular arrhythmia (Kawara et al., 2001). Ventricular arrhythmia is affected by the degree of MIF, and is not correlated with other confounding factors, including the LV dysfunction. Moreover, it has been reported that the relationship between MIF and ventricular arrhythmia has no dependency on LV function in patients with HHD (McLenachan and Dargie, 1990). MIF has been recognized as a risk factor for sudden cardiac death in patients with HHD, hypertrophic cardiomyopathy, and non-ischemic dilated cardiomyopathy (Bockstall and Link, 2012; Gulati et al., 2013; Shenasa and Shenasa, 2017).

3.4 The reduced oxygen supply to the remaining cardiomyocytes

The heart is a specialized aerobic organ. Its long-term performance requires a stable oxygen supply to maintain the energy demand. The coronary vasodilatory reserve is mainly used to ensure the delivery of sufficient oxygen to meet the oxygen demand of cardiomyocytes. Therefore, the myocardium can adapt to significant changes in oxygen demand beyond its aerobic threshold and before beginning anaerobic metabolism (Weber et al., 1980). Perivascular fibrosis of intramural coronary arteries and arterioles, as well as the presence of myofibroblasts, can lead to coronary lumen stenosis and threaten the survival of cardiomyocytes (Warnes et al., 1984; Olivotto et al., 2011). In patients with HHD, hypertrophic cardiomyopathy or diabetes cardiomyopathy, the severity of MIF is related to the severity of coronary microvascular disease, including the anatomical abnormality of vascular wall and the rarefaction of capillaries (Mohammed et al., 2015).

As for whether MIF is a direct harmful cause of cardiac function, studies have evaluated collagen deposition and/or collagen cross-linking through histological analysis, proving that there is a direct correlation between MIF severity and diastolic dysfunction in patients with HF, which supports the direct involvement of MIF in LV diastolic dysfunction (Izawa et al., 2005). However, there are differences in this regard, because several articles have proved that fibrosis itself does not cause electrical problems or arrhythmias.

4 Diagnosis of myocardial interstitial fibrosis

Because MIF affects the clinical outcome of patients with pathological myocardial hypertrophy, MIF may need to be used as an evaluation indicator of clinical treatment for these patients. Endomyocardial biopsy has higher accuracy in characterizing interstitial changes and can specify their components, while it cannot be used routinely and continuously because of its limitations, such as invasiveness, complications, and sampling errors (Halliday and Prasad, 2019). Therefore, a routine practice requires alternative non-invasive methods, such as cardiovascular magnetic resonance (CMR) imaging and circulating biomarkers.

CMR imaging-derived parameters are commonly used to evaluate myocardial fibrosis, including late gadolinium enhancement (LGE) and T1 mapping, which can be divided into natural T1, post contrast T1, and extracellular volume fraction (ECV). LGE can be utilized to identify focal collagen deposition in scars after large-area infarction, while T1 mapping can be used to identify diffuse collagen deposition in MIF (Díez et al., 2020). Clinical studies have demonstrated that ECV and LGE can evaluate the degree of diffuse collagen deposition by

collagen volume fraction, and ECV is superior to LGE (Diao et al., 2016). On the other hand, neither LGE nor ECV can qualitatively judge the composition and molecular organization of collagen fibers in MIF (Messroghli et al., 2017). Therefore, due to this limitation of CMR-derived biomarkers, another method based on circulating biomarkers may be needed to quantitatively and qualitatively judge MIF.

Of the several proposed MIF biomarkers, only a limited number of them are associated with histologically confirmed MIF, such as serum carboxy terminal propeptide of type I procollagen (PICP), serum amino terminal propeptide of type III procollagen (PIIINP), and the ratio of serum type I collagen terminal peptide to serum matrix metalloproteinase 1 (López et al., 2015). PICP is formed during the extracellular conversion of type I procollagen to type I collagen by PCP enzyme. It was found that the PICP level was closely associated with the deposition of type I collagen in myocardium of patients with HHD and HF (López et al., 2012). PIIINP is produced during the extracellular transformation of type III procollagen into type III collagen by procollagen amino terminal protease. It was also found that its level is highly correlated with the degree of myocardial type III collagen deposition in patients with ischemic heart disease or idiopathic dilated cardiomyopathy and HF (Klappacher et al., 1995). Similarly, there are some limitations in the detection of MIF biomarkers. For instance, they do not have heart specificity, and the change of their concentration may represent the comprehensive abnormality of cardiovascular collagen or the influence of complications affecting collagen metabolism.

5 Treatment of myocardial interstitial fibrosis

Existing clinical evidence shows that MIF may affect the response of patients with pathological myocardial hypertrophy to treatment, and there is no routine use of specific anti fibrosis drugs in clinical practice. Therefore, for patients with pathological myocardial hypertrophy, anti-fibrosis and cardiac protection strategies need to be developed, which are mainly introduced from the following three aspects. But cardiac fibrosis is an important part of the pathophysiological response to injury. Sometimes appropriate fibrosis has a protective effect on the heart, so different pathological backgrounds of fibrosis need different treatments.

5.1 Drugs with some exhibited clinical benefit

The clinical studies have shown that MIF is a target of drugs interfering with the renin-angiotensin- aldosterone axis. A large number of drugs were found to play an anti-MIF role by acting

on this axis. For instance, in the treatment of patients with HHD, the use of angiotensin receptor blocker losartan or angiotensin-converting enzyme inhibitor lisinopril can reduce the degree of fibrosis deposition and LV stiffness, and improve LV diastolic dysfunction accordingly (Tacke and Trautwein, 2015). Similarly, mineralocorticoid receptor antagonist spironolactone can also reduce collagen deposition and LV stiffness in patients with HF and improve diastolic dysfunction (Izawa et al., 2005). These drugs have been reported to have anti-MIF efficacy and safety, but there is still a residual burden of fibrosis. Therefore, new treatment methods are urgently required for more effective anti-fibrosis treatment. For instance, torasemide, a new loop diuretic, may directly target MIF. Studies have demonstrated that the use of torasemide in patients with HHD can affect the degree of collagen deposition, reduce PCP activation and LOX expression (López et al., 2009). However, this was obtained in the study of a small number of patients receiving short-term treatment. Rigorous and large-scale clinical studies are still needed to prove whether torasemide targets MIF in a clinically effective manner (Díez et al., 2020).

5.2 Drugs that have shown promise but only in pre-clinical models

On the other hand, data obtained from experimental studies revealed that some drugs with proven safety that have been used in clinical practice may treat MIF in pathological myocardial hypertrophy through new mechanisms (Johnson et al., 2017). For instance, in HF mice with cardiac pressure overload and diabetes, the combined use of enkephalinase inhibitors and angiotensin receptor blockers, sacubitril/valsartan (a new class of drugs called angiotensin receptor neprilysin inhibitors) relieved MIF and improved LV function (Burke et al., 2019). The antifibrotic effect of sacubitril/valsartan may be due to the specific inhibition of neutral lysozyme beyond the effect of angiotensin receptor blockers. The mineralocorticoid receptor antagonist eplerenone can prevent increased collagen deposition and left ventricular systolic dysfunction in pressure overloaded mice (Franco et al., 2006). In addition, riociguat, the soluble guanylate cyclase stimulator, can reduce collagen deposition in HF mice caused by transverse aortic coarctation (Pradhan et al., 2016).

Studies have shown that sodium glucose cotransporter 2 (SGLT2) inhibitors, used as hypoglycemic drugs in patients with diabetes, also have anti MIF effects and can reduce the risk of cardiovascular death in patients with pathological myocardial hypertrophy. Such as, empagliflozin can attenuate MIF and improve diastolic dysfunction in diabetic mice (Habibi et al., 2017). Some studies have also shown that in pressure overloaded mice caused by transverse aortic coarctation, dapagliflozin treatment can reduce collagen deposition and improve left ventricular function (Shi et al., 2019). However,

because SGLT2 is not expressed in the heart and its antifibrosis effect is not related to metabolic and/or hemodynamic changes, some people believe that it may reflect the direct pleiotropic effect of drugs on myocardium (Packer et al., 2017). Therefore, it remains to be tested whether SGLT2 inhibitors can effectively reduce MIF.

Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, has been shown in a study to reduce pathological cardiac hypertrophy and fibrosis (Zhao et al., 2021). A number of evidences show that COX-2 is related to cardiac hypertrophy and fibrosis. Specific overexpression of COX2 promotes cardiac hypertrophy, while COX-2 inhibition can improve the hypertrophy and fibrosis induced by angiotensin and aldosterone (Wang et al., 2010). This study used cryoinjury (CI) to establish a mouse model of cardiac hypertrophy and fibrosis. It was found that celecoxib inhibited the production of proinflammatory cytokines and the expression of adhesion molecule genes, increased the recruitment of M1 like macrophages, and reduced cardiac hypertrophy and fibrosis (Zhao et al., 2021). However, the regulatory mechanism of COX-2 in immune-mediated cardiac hypertrophy and fibrosis is not completely clear.

Platycodon D (PD) is a triterpenoid saponin isolated from the widely used traditional Chinese medicine extracted from the root of *Platycodon grandiflorum* (PG), which has anticancer and antiangiogenic effects (Lee et al., 2015). Studies have shown that PD can reduce the incidence rate and mortality of cardiovascular disease by inhibiting cardiac fibrosis and hypertrophy (Lin et al., 2018). PD can reduce the accumulation of collagen in cardiac hypertrophy rats caused by hypertension, possibly by reducing the expression of MMP2, MMP9 and TGF- β 1 to inhibit cardiac hypertrophy and fibrosis. In addition, in preclinical animal models, PD treatment did not show related mortality at up to 2000 mg/kg, indicating that PD has great potential for clinical use because it has no obvious toxicological signs (Lee et al., 2011).

It has also been reported that the liver X receptor (LXR) agonist AZ876 can prevent pathological cardiac hypertrophy and fibrosis in a study (Cannon et al., 2015). This study has shown that AZ876 treatment can inhibit the induction of profibrotic gene expression and reduce MIF in a mouse model of pathological cardiac hypertrophy caused by chronic pressure overload. Its potential mechanism may be related to the weakening of TGF- β - Smad2/3 signal transduction. Two major cell types in the cardiac, cardiomyocytes and fibroblasts, express LXR. These two kinds of cells are the direct targets of AZ876 mediated cell protection from hypertrophy and fibrosis (Cannon et al., 2015). AZ876 activation can prevent TGF- β and Ang II induced collagen synthesis and myofibroblast differentiation. AZ876 has dual effects of anti-myocardial hypertrophy and anti-fibrosis, and can also reduce atherosclerosis without affecting the liver or plasma triglyceride levels (van der Hoorn et al., 2011). However, the

cardiac protective potential of AZ876 remains to be further explored.

Finally, pirfenidone and tranilast, two inhibitors of TGF- β signaling, have also been shown to have anti-fibrotic effects in several MIF models (Edgley et al., 2012). Because the long-term use of either of these two drugs may produce hepatotoxicity and lead to liver failure, further experiments are needed to alleviate MIF *via* studies that support safely and effectively targeting of TGF- β signaling (Fang et al., 2017).

5.3 Other potential treatments

The clinical principle of anti-MIF strategies mainly aims to reduce the excessive deposition of fibrous tissue, which may be achieved through the inactivation of inflammatory pathways and the establishment of anti-inflammatory microenvironment, or the inactivation and elimination of myofibroblasts, or the excessive synthesis and deposition of collagen (Díez et al., 2020). Based on this principle, we can find new anti-MIF markers in pathological myocardial hypertrophy, such as non-coding RNAs (ncRNAs), epigenetic modifiers, Endoplasmic reticulum (ER), mitochondrion and so on, which can be used as new molecular drug targets (Park et al., 2019). However, these new potential anti MIF strategies are still not really used in clinical treatment, and more research is needed to clarify.

NcRNAs play a key role in DNA replication, transcription and post transcriptional gene expression, chromatin processing, and maintaining mRNA stability and genomic integrity (Yang et al., 2016). In terms of pathological cardiac hypertrophy and MIF, microRNA (miRNA or miR), long-chain noncoding RNA (lncRNA) and circular RNA (circRNA) are currently the most studied ncRNAs with high therapeutic potential (He et al., 2020). It has been found that miR-21 can increase the activity of ERK-MAP kinase in fibroblasts, affect its structure and function, and control the degree of MIF (Thum et al., 2008). It has been reported that in the animal model of ischemia/reperfusion injury, inhibition of miR-21 can lead to the reduction of MIF and cardiac hypertrophy, and the overall improvement of cardiac function (Hinkel et al., 2020). In addition, in pressure overload induced cardiac hypertrophy rats, miR-1 can reduce cardiac fibrosis and reverse cardiac hypertrophy by targeting Fibulin-2 (Fbln2), a protein involved in extracellular matrix remodeling. The cardioprotective effect of miR-1 is the result of extracellular matrix remodeling, the improvement of calcium treatment and the inactivation of mitogen activated protein kinase (MAPK) (Karakikes et al., 2013). It has also been proved that inhibition of miR-221 and miR-222 can lead to increased fibrosis and left ventricular dilatation in a pressure overload induced hypertrophic mouse model. MiR-221/222 family inhibits myocardial fibrosis and plays a cardioprotective role by down-regulating JUN N-terminal kinases 1 (JNK1), a kind of regulator of TGF- β signal pathway (Verjans et al., 2018). Although ncRNA targeting therapy for MIF in pathological myocardial hypertrophy

shows good therapeutic prospects, there are still many limitations in its real use in clinical practice. For example, ncRNA can target different signal pathways in different organs at the same time, which is easy to produce adverse off target effects.

Epigenetic modification refers to dynamic and reversible changes in chromatin accessibility and post-translational modification of histone tails without changing nucleotide sequences (Prinjha et al., 2012). Histone acetylation is the earliest and most deeply studied epigenetic modification, which plays an important role in chromatin remodeling and transcriptional regulation (Scher et al., 2007). Histone acetylation regulated gene transcription relies on “epigenetic readers”, that is, acetyl-binding proteins that recognize acetylated lysine in histones and recruit transcription regulatory complexes to chromatin (Wang et al., 2021). Almost all “epigenetic readers” contain bromodomains - about 110 amino acid modules that exist in many chromatin related proteins (Scher et al., 2007). The bromodomain and extra terminal domain (BET) family is a subfamily of bromodomain containing proteins (BRDs), which is composed of four proteins (BRD2, BRD3, BRD4 and BRDT) (Gyuris et al., 2009). Therefore, epigenetic modifications and protein translation can be regulated by affecting the BET family, and protein translation regulation may inhibit the transdifferentiation of fibroblasts into myofibroblasts through transcriptional elongation or inhibition of SMADs signaling. Studies have shown that in the mouse model of cardiac hypertrophy induced by aortic coarctation, BRD4 down-regulation can reduce collagen accumulation and inhibit myocardial fibrosis by reducing the production of reactive oxygen species (ROS) (Zhu et al., 2020, 4). In cardiac fibroblasts, TGF- β 1 leads to the production of ROS that regulates the transdifferentiation of myofibroblasts. The significant role of ROS in fibrosis can be initiated by TGF- β 1/SMADs signaling (Rhyu et al., 2005). BRD4 inhibition can reduce the expression of TGF- β 1 and p-SMAD2/3 triggered by Ang II, thereby reducing ROS production to inhibit cardiac fibrosis and relieve cardiac hypertrophy (Zhu et al., 2020, 4). It has also been reported that BRD2 knockout can reverse cardiac hypertrophy, cardiac fibrosis and cardiac dysfunction in rats with pathological cardiac hypertrophy induced by isoprenaline (Lin et al., 2022). The mechanism may be related to BRD2 regulating the expression of citrate cycle gene, but the specific process is not clear. Therefore, BET family may be a new therapeutic target against MIF in pathological myocardial hypertrophy.

ER is an organelle involved in protein folding, lipid biosynthesis and calcium homeostasis (Kaufman, 1999). ER stress refers to changes in the intracellular environment that interrupt protein processing, lead to the accumulation of unfolded proteins in the endoplasmic reticulum and activate the unfolded protein response (UPR) (Ron and Walter, 2007). Although the causes of cardiac hypertrophy and fibrosis are diverse, they may all be expressed in the form of ER stress response in a common cellular mechanism (Luo et al., 2015a). At

present, the mechanism of ER stress regulating myocardial hypertrophy and interstitial fibrosis is not clear, but some people have proposed that it may be related to involvement of CaN-MEF2c signaling pathways in cardiomyocytes and TGF β 1-SMAD2/3 signaling pathways in cardiac fibroblasts (Zhang et al., 2010; Luo et al., 2015b). Studies have shown that 4-phenylbutyric acid (4-PBA), an inhibitor of ER stress, can prevent myocardial hypertrophy and interstitial fibrosis caused by pressure overload by reducing ER stress (Luo et al., 2015a). 4-PBA inhibits ER stress by directly targeting mutant and/or misfolded proteins in cells and assisting appropriate protein transport to eliminate intracellular UPR (Gonzales et al., 2015). In addition, stimulator of interferon genes (STING), which mainly exists in ER, has also been shown to affect myocardial hypertrophy and fibrosis. In aortic coarctation induced cardiac hypertrophy mice, STING knockout in cardiomyocytes inhibited ER stress and the release of inflammatory factors, thereby inhibiting the fibrosis of cardiac fibroblasts (Zhang et al., 2020). All these indicate that inhibiting ER stress may be an effective treatment strategy to improve MIF in pathological cardiac hypertrophy.

Recently, the fundamental role of mitochondrial dysfunction in fibrosis has noticeably attracted clinicians' attention. Cardiomyocytes are obligatory aerobic cells, and their survival depends on oxidative metabolism and stable demand for energy, which is provided by their mitochondria. Mitochondrial dysfunction is mainly manifested in mitochondrial structure and DNA damage, as well as changes in cellular oxidative protein activity (Li et al., 2020). Mitochondria are the main source of reactive oxygen species (ROS), a byproduct of oxygen metabolism. When mitochondria are dysfunctional, Ca²⁺ overload in mitochondria will lead to the induction of oxidative stress and the loss of ATP synthesis, resulting in an imbalance between biogenesis and the elimination of reactive oxygen species. When the production rate of ROS exceeds the detoxification rate of endogenous antioxidant defense, the permeability transition pore of mitochondrial inner membrane opens, which makes solute enter passively and leads to mitochondrial osmotic swelling (Weber et al., 2013). Eventually, these basic organelles degenerate and cardiomyocytes undergo necrosis, followed by fibrosis. This mechanism of cardiomyocyte necrosis "mitochondriocentric signal-transducer-effector" pathway. One strategy to reduce myocardial fibrosis is an intervention targeting mitochondria to prevent cardiomyocyte necrosis. The aim of this mitochondrial targeted cardiac protection strategy is to block the necrosis caused by the mitochondrial central signal transduction pathway by preventing mitochondrial Ca²⁺-overload under the sarcolemma. This includes the use of targeted antioxidants or inhibition of the opening of mitochondrial intimal permeability transition pores (Dai et al., 2011; Asemu et al., 2013; Segura et al., 2014). Such strategies include nutrients (flavonoids), drugs (cyclosporin A or third-generation β -adrenoreceptor antagonists), inhaled hydrogen or expressed microRNAs (Bomb et al., 2016). For instance, some experiments have shown that in mice with ischemia-

reperfusion injury, miRNA-214 regulates cardiomyocyte Ca²⁺ by inhibiting Na²⁺/Ca²⁺ exchange, preventing cardiomyocyte necrosis caused by cytosol and mitochondrial Ca²⁺ overload, so as to play a cardiac protective role (Aurora et al., 2012). In addition, it has also been proposed that miR-574 regulates the expression of family with sequence similarity 210 member A (FAM210A) and antagonizes cardiac fibrosis in mice with cardiac hypertrophy caused by aortic constriction. FAM210A acts as a novel regulator of the expression of mitochondrial encoded proteins, while miR-574 restricts the expression of FAM210A and may act as a molecular brake to maintain mitochondrial homeostasis and normal cardiac function (Wu et al., 2021).

6 Conclusion

MIF can be accompanied by pathological myocardial hypertrophy caused by various heart diseases, which can be induced by different stimuli in different heart diseases. In patients with pathological myocardial hypertrophy, due to different causes, the specific manifestations and characteristics of MIF are also different. And in patients with pathological myocardial hypertrophy, MIF affects their outcome and prognosis, so the evaluation of the etiology and degree of MIF should be paid attention to and are worthy of further investigation. Accordingly, the diagnosis and treatment of MIF in pathological myocardial hypertrophy should also be based on disease specificity and case-specific analysis, so as to provide individualized management for patients with pathological myocardial hypertrophy. Moreover, CMR imaging and circulating biomarkers should be reasonably used to ensure the accuracy and precision. Finally, although several anti-MIF strategies have been proposed, most of these strategies are idealized and difficult to implement in clinical practice, thus, the transformation from theory to practice still needs to be developed.

Author contributions

FZ and PL drafted the original manuscript. PL and YS revised the manuscript. YS provided the funding.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Thromboxane-induced contractile response of mesenteric arterioles is diminished in the older rats and the older hypertensive rats

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Nearly all physiological processes are controlled at some level by G-protein-coupled receptor (GPCR) signaling activity. The thromboxane A₂ (TXA₂) receptor (TP) is a member of the GPCR family. The ultimate effect of TP receptor activation depends on the availability of specific G proteins, which in turn depend on the cell type, tissue, and disease state. However, the roles of the TXA₂-TP signaling pathway executed under disease states are poorly defined. In this study, 16-week-spontaneously hypertensive rats (SHR), the 18-month-SHR (OldSHR), and the age-matched Wistar-Kyoto (WKY) rats were used to study the vasoconstriction of mesenteric resistance artery induced by TP-specific agonist, U-46619. Vasoconstriction induced by U-46619 was significantly attenuated in OldWKY and OldSHR rats, and mesenteric arteries with impaired response to U-46619 responded strongly to the adrenergic receptor agonist, phenylephrine. Similar vascular responses to U-46619 were obtained in endothelium-denuded mesenteric arteries. Accordingly, the expression of TP membrane proteins in mesenteric vessels was decreased, and the endogenous TP competitor, 8, 9-EET, in serum was increased, which was partly responsible for the decreased vascular reactivity of U-46619. Decreased TP membrane expression was associated with TP endocytosis, which involved actin cytoskeletal remodeling, including increased ratio of F-actin/G-actin in OldWKY and OldSHR rats. Hence, we studied the effects of TXA₂ and its receptors on blood vessels and found that the TXA₂-TP prostaglandin signaling pathway was impaired in older adults, which would facilitate the creation of “precision therapeutics” that possess selective efficacy in diseases.

KEYWORDS

aging-related vascular remodeling, mesenteric artery, U-46619, vasoconstriction, TXA₂-TP prostaglandin signaling pathway

Introduction

Prostaglandin signaling controls a wide range of biological processes, including blood pressure homeostasis, inflammation, pain, and cell survival. Thromboxane A₂ (TXA₂), a member of the prostaglandin family, is an arachidonic acid metabolite that is biosynthesized in cells by phospholipase A₂, cyclooxygenase (COX-1 or COX-2), and TXA₂ synthase (TXAS). TXA₂ is a lipid mediator with potent vasoconstrictive, mitogenetic, and platelet aggregative properties (Hamberg et al., 1975; Hanasaki et al., 1990). The effects of TXA₂ are mediated by specific TP and subjected to regulatory control. TP is a member of the GPCR superfamily. TP is widely expressed in various tissues and cells, including platelets, vascular systems (smooth muscle cells and endothelial cells), lung, kidney, heart, thymus, spleen, and other systems. In smooth muscle cells, activation of G protein-coupled TP induces vascular smooth muscle contraction through the initial activation of phospholipase-C (PLC), with production of 1,4,5-inositol phosphate, Ca²⁺ release from intracellular calcium stores, and phosphorylation of myosin light chains (Nakahata, 2008).

The GPCRs are known to undergo receptor desensitization following agonist stimulation (Hausdorff et al., 1990). The process involves uncoupling of G-proteins from a receptor, sequestration of receptor from cell surface, and downregulation of total receptor number (Hertel et al., 1983). Receptor phosphorylation induced by agonist stimulation is understood to contribute to each of these processes (Hausdorff et al., 1990). TP has been reported to undergo homologous desensitization after stimulation by agonists—such as U-46619 and I-BOP (Liel et al., 1988; Murray and FitzGerald, 1989; Okwu et al., 1992)—which is an agonist-dependent adaptive process in biological systems that modulates responsiveness of the cell to repeatedly stimulate over time.

TXA₂ promotes various physiological responses and pathophysiological implications. Plasma TXA₂ levels correlated with the prevalence of cardiovascular diseases, including hypertension (Francois et al., 2004) and atherosclerosis (Mehta et al., 1988). Furthermore, dysregulation of TP can result in agonist hypersensitivity and hyper-responsiveness, contributing to exaggerated vasoconstriction in the hypoxic pulmonary artery in neonatal persistent pulmonary hypertension (Hinton et al., 2007; Santhosh et al., 2014). Hinton et al. (2007) found that hypoxia caused sensitization of TP in neonatal pulmonary artery myocytes, but only under controlled conditions. Neonatal pulmonary artery myocytes exposed to a moderate level of hypoxia had hypersensitive and hyperresponsive peak [Ca²⁺]_i responses to the TXA₂ agonist, U-46619, despite a reduction in cell surface TP expression. Feng et al. (2011) found that cardioplegia/reperfusion decreased the contractile response of human coronary arterioles to TXA₂ soon after cardiac

operations. They suggested that thromboxane-induced contractile response of human coronary arterioles is diminished after cardioplegic arrest, and the contractile response to U-46619 was due to activation of TP and PLC. Tang and Vanhoutte (2008) reported that the gene expression of prostanoid synthases in endothelial cells and prostanoid receptors changed in vascular smooth muscle cells in aging and hypertension. They concluded that whether the gene modifications caused by aging and hypertension directly contribute to endothelium-dependent contractions or vascular aging and the vascular complications of the hypertensive process remains uncertain.

Aging-related diseases are thought to kill nearly 100,000 individuals worldwide daily (Gladyshev and Gladyshev, 2016; Bakula et al., 2018). While the aging process itself is generally not considered to be a disease (Novoselov, 2017), it is a pathophysiological state that facilitates or even induces the occurrence of diseases, such as hypertension, in several older adults (Rattan, 2014). Although aging has been reported to affect vascular reactivity by altering the sensitivity and maximum effect of stimulating contractile agonists and endogenous vasodilators (Hongo et al., 1988; Novella et al., 2013; Cupitra et al., 2020), age-related changes in cardiovascular physiology and pathophysiology of GPCR have been reported as well (Schutzer et al., 2001; Sparks et al., 2013), few studies on the role of TXA₂ and its receptor (TP) in older individuals and older individuals with hypertension have been reported. Using the specific stable TP-specific agonist, U-46619, we found that mesenteric vasoconstriction response induced by U-46619 was diminished remarkably in OldWKY and OldSHR. Therefore, we explored the mechanisms to understand the relationship between the vascular action caused by TXA₂ and its receptor to try to further elucidate the role and significance of thromboxane and its receptor under disease conditions.

Materials and methods

Animals

Twelve-week-old male SHR and their age-matched male WKY rats were bought from Beijing Vital River Laboratory Animal Technology Co. Ltd. [License No. SCXK (Jing) 2019-0011]. Animals were fed until 16 weeks (adult) and 18 months (older adults) at the Animal Care Center of Southwest Medical University [license No. SCXK (Chuan) 2018-17]. They were further divided into four groups: WKY, SHR, OldWKY, and OldSHR groups. Animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals approved by the Ethics Committee of Southwest Medical University. The diurnal rhythm of light was maintained, and the temperature was controlled at 25°C. The animals had *ad libitum* access to food and water during

feeding. Before the experiments, blood pressure of rats was measured by femoral artery cannulation to confirm normal blood pressure or hypertension. Data on rats are shown in [Supplementary Table S1](#).

Preparation of mesenteric vascular rings and isometric tension recording

The tertiary branches of endothelium-denuded mesenteric artery segments were prepared using previously described procedures (Zhou et al., 2019; Cui et al., 2022) (Online [Supplementary Methods](#)). Briefly, after the endothelium was rubbed away, the arterial ring was suspended on the experimental system. The basal tension was adjusted, and 60 mM K⁺ solution (High K⁺) was used to detect vascular reactivity 60 min later. After the vessels reached maximum contraction, 1 μ M acetylcholine (ACh) was used to detect endothelial activity. Additionally, in some experiments, to ensure that the endothelial function is completely removed, NG-nitro-L-arginine methyl ester (L-NAME, endothelial nitric oxide synthase inhibitor) was added to block the endothelial function on the basis of removing the endothelium by mechanical eraser. If the vasodilation caused by ACh was <20%, the vessels were considered to have no endothelial residue, and the vessel could be used for subsequent experiments. High K⁺ was repeated to make the blood vessels contract twice, and the difference between the two contractions was calculated. If the difference was <10%, the maximum contraction caused by High K⁺ was considered to be stable. Thereafter, the bath was washed with Tyrode's solution, and the follow-up experiment was conducted after 30 min of stability.

RNA sequencing and functional enrichment analysis

Total RNA was isolated from the intact mesenteric arteries of rats in the WKY, SHR, OldWKY, and OldSHR groups using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. Details of the protocol are available in the online [Supplementary Methods](#). Briefly, the purified RNAs were subjected to cDNA synthesis, followed by adaptor ligation and enrichment, according to the manufacturer's instructions on NEBNext[®] Ultra[™] RNA Library. After obtaining clean data and quality control, RNA sequencing and functional enrichment analysis were performed. For the differentially expressed RNAs (DEGs), the pathways were enriched based on DEGs through the KEGG pathway. The proteins of interest in the TXA₂-TP-vasoconstriction pathways, including TXA₂, TP, arrestin, actin, G protein-linked receptor kinases (Grk), and vasoconstriction-related genes, were specifically analyzed. Tools of Heatmapper

(<http://www2.heatmapper.ca/expression/>) and Venn diagrams (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>) were used. RNA sequencing data were uploaded to the Sequence Read Archive (SRA), and the BioProject accession number is PRJNA872876.

Determination of protein expression

Total proteins or membrane proteins were extracted from the intact mesenteric artery of the rats in the WKY, SHR, OldWKY, and OldSHR groups using the liquid nitrogen grinding method or the kit method for extraction of membrane proteins (Invent, SM-005). The procedure was performed as previously reported (Cui et al., 2022). Details of the procedure are available in the Online [Supplementary Methods](#).

Determination of G actin/F actin

G/F-actin was determined by the analysis kit (G/F-actin *in vivo* analysis kit, Cat. No. bk037; cytoskeleton). The intact mesenteric artery was lysed in LAS2 buffer and the following procedure was in the online [Supplementary Methods](#).

Reagents and solutions

All reagents, which were of at least an analytical grade, used in the experiments were bought from the companies and the details are available in the online [Supplementary Materials and Methods](#). Details of the solution preparation are available in the online [Supplementary Materials and Methods](#).

Statistical analysis

Results are expressed as mean $\pm$ S. D., and all data were normally distributed. Comparisons were performed using ANOVA or unpaired Student's *t* tests, when appropriate, using GraphPad Prism 8.4.2 (GraphPad Software). *p* < 0.05 was considered statistically significant. For statistical analysis of RNA sequencing enriched in TXA₂-TP related pathways, the criteria of fold change >1.5 and adjusted *p*-value < 0.05 were used.

Results

High K⁺-induced constriction of isolated mesenteric artery rings in rats

The tertiary branches of the endothelium-denuded mesenteric arteries were used in the experiment. The maximal contractile

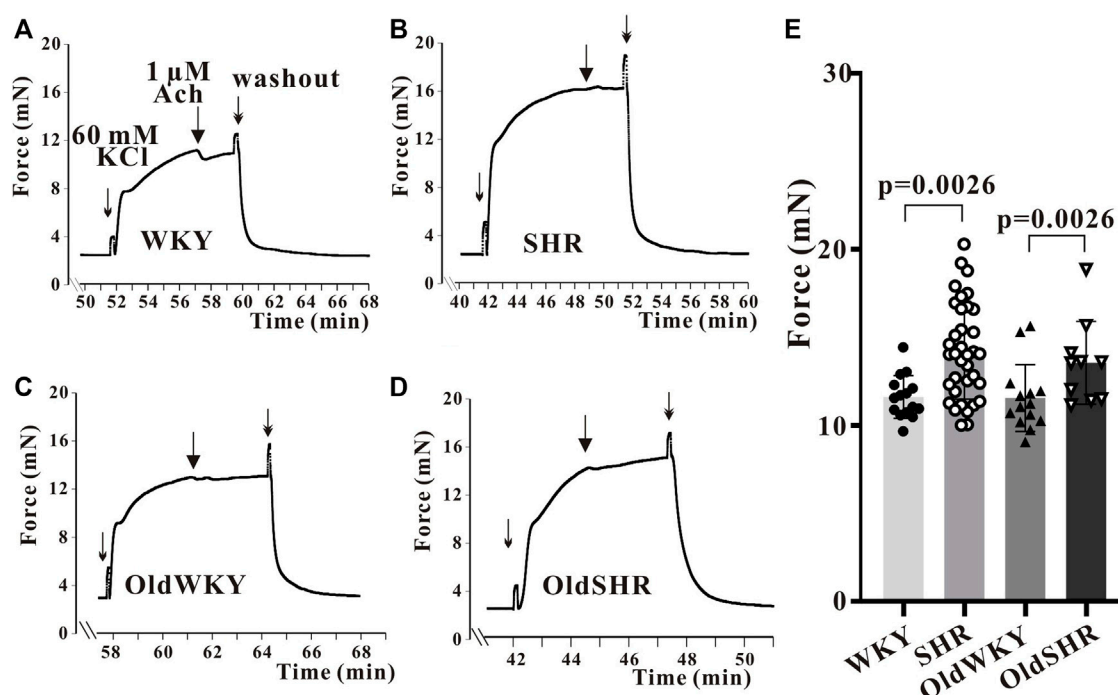


FIGURE 1

High K^+ solution induced the maximum contraction of endothelium-denuded mesenteric artery rings. (A–D) Typical recordings showing vessel response to 60 mM K^+ solution in the four groups. The X-coordinate time was recorded from the time when the vascular ring passed the activity test and was stabilized for another 30 min. (E) Summary data showed that mesenteric arteries are more sensitive to High K^+ in hypertension. The mesenteric arteries of rats were obtained from 10 WKY rats, 16 SHR, 8 OldWKY rats, and 5 OldSHR, respectively.

response (E_{max}) induced by High K^+ in the mesenteric artery was recorded and analyzed. As shown in Figure 1, E_{max} was significantly increased in SHR compared with WKY rats ($p < 0.01$); E_{max} was significantly increased in OldSHR compared with OldWKY rats ($p < 0.01$). This experiment showed that the mesenteric vessels from hypertension are more sensitive to High K^+ in hypertension, which is consistent with other reports (Wei et al., 2021; Cui et al., 2022).

Mesenteric vasoconstriction response induced by U-46619 was diminished remarkably in older rats and older hypertensive rats

First, the endothelium-denuded mesenteric artery rings from WKY rats were used to determine the optimal concentration of U-46619-induced vasoconstriction. U-46619 (10^{-10} – 10^{-5} M) induced a concentration-dependent contraction of the mesenteric artery. A dose-response curve was obtained, and the concentration of U-46619 causing 80% of E_{max} was 1 μM. Therefore, U-46619 at 1 μM was used in subsequent experiments. The mesenteric vessels were constricted with 1 μM U-46619 until E_{max} was achieved. The percentage of E_{max} caused by U-46619 relative to E_{max} caused by high K^+ solution (% E_{max}) was calculated. The vasoconstriction

induced by U-46619 was different from those of vascular responses induced by high K^+ or PE, as previously reported (Wei et al., 2021). The response of mesenteric vessels to U-46619 was significantly increased in hypertension. However, surprisingly and contrary to expectations, response to U-46619 in OldWKY and OldSHR rats was minimal. As shown in Figure 2, the E_{max} of mesenteric artery induced by 1 μM U-46619 was significantly increased in SHR compared with WKY rats ($p < 0.001$). In OldWKY rats, E_{max} was, however, significantly decreased ($p < 0.001$). No significant difference in mesenteric artery constriction induced by U-46619 was found between OldWKY and OldSHR rats ($p > 0.05$). These data clearly showed that the mesenteric vasoconstriction induced by U-46619 was obstructed in older rats and older hypertensive rats.

Vessels with impaired response to U-46619 in OldWKY and OldSHR rats had a strong contractile response to phenylephrine

To prove that the blood vessels used in the study had not been damaged by dissection or prior treatment, 5 μM phenylephrine (PE), an α_1 -adrenergic receptor (α_1 -AR)

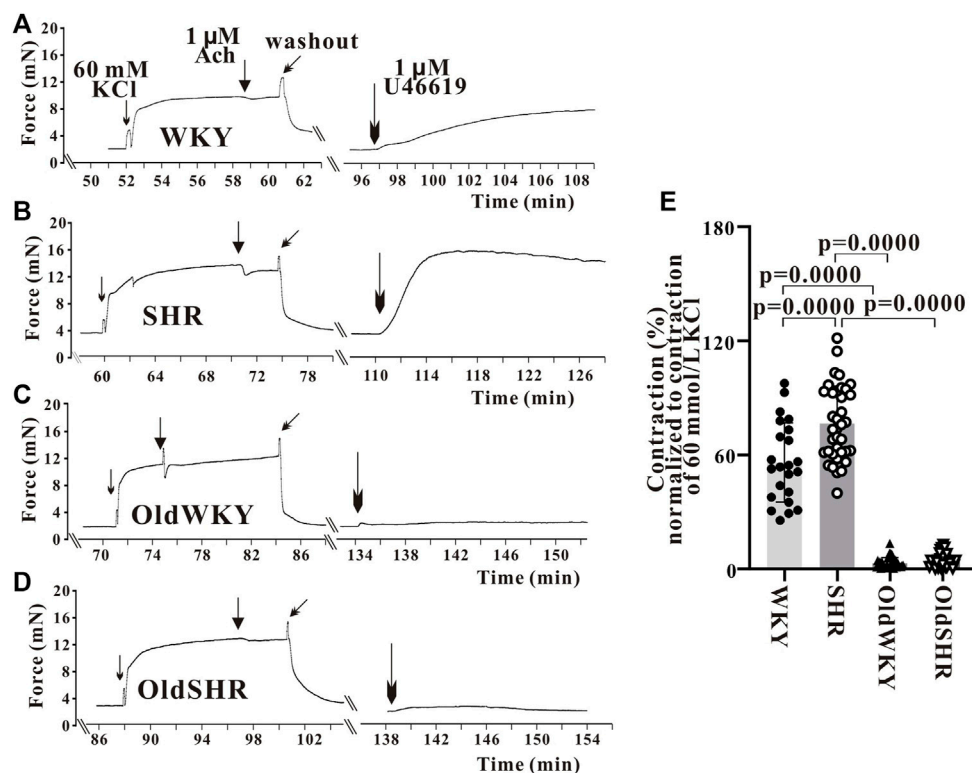


FIGURE 2

U-46619 induced the contraction of endothelium-denuded mesenteric artery rings. (A–D) Typical recordings showing vessel response to 1 μ M U-46619 in the four groups. The X-coordinate time was recorded from the time when the vascular ring passed the activity test and was stabilized for another 30 min. (E) Summary data showing that mesenteric artery response to U-46619 was diminished remarkably in OldWKY and OldSHR groups. Mesenteric vessels were obtained from 16 WKY rats, 9 SHR rats, 17 OldWKY rats, and 18 OldSHR rats.

activator, was used as stimulant to observe the vascular reactivity (Figures 3A,B). As shown in Figure 3B, the % Emax of mesenteric vasoconstriction induced by U-46619, compared with that induced by High K⁺, were 4.75 ± 5.04 ($n = 8$) and 2.31 ± 4.29 ($n = 13$) in OldWKY and OldSHR rats, respectively. The relative contractions (%Emax) induced by PE were 91.17 ± 18.41 ($n = 8$) and 95.20 ± 26.16 ($n = 13$), respectively. These results suggested that in older rats and older hypertensive rats, U-46619 hardly elicited vasoconstriction, but the vessels had a strong contractile response to PE. Additionally, a similar set of experiments was conducted to explore whether PE might sensitize the receptor TP. In this study, the vascular responsiveness was observed by preconditioning with 5 μ M PE and 1 μ M U-46619. The results showed that vessels that normally responded to PE in OldWKY or OldSHR rats did not respond to U-46619, as similarly observed in the use of U-46619 prior to PE (Figure 3C). The results indicated that PE did not sensitize TP. All these results suggested that the prostaglandin signaling pathway associated with contraction in vascular smooth muscle was abnormally weakened, but the adrenergic α receptor pathway was not.

To rule out the possibility that the reduction in vasoconstriction induced by U-46619 was due to incomplete endothelial removal, the vessels were treated with 100 nM L-NAME in addition to vascular ring friction. The vasoreactivity was measured with 1 μ M ACh, and the vessels with <20% vasodilation caused by ACh were used for the subsequent experiment. Thereafter, vascular reactivity was assessed by administering 1 μ M U-46619 and/or 5 μ M pE. Poor vascular response to U-46619 and strong response to PE in the vessels of OldWKY and OldSHR rats were recorded. This result suggested that the reduction in vasoconstriction response induced by U-46619 in OldWKY and OldSHR rats was not due to incomplete endothelial removal (typical Figure 3D).

Differentially expressed genes in the TXA2-TP signaling pathway

The study was intended to provide information for molecular biology validation experiments in the TXA2-TP-vasoconstriction signaling pathway. Similar number of DEGs

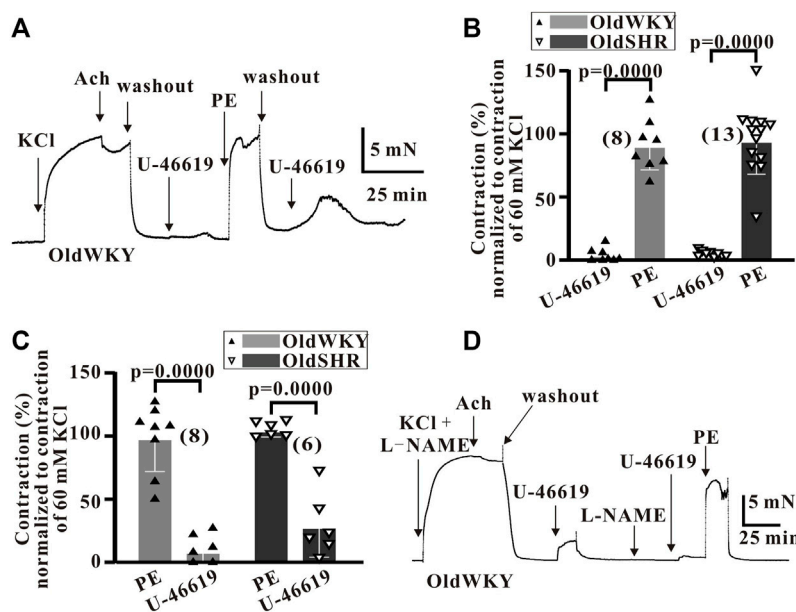


FIGURE 3

U-46619 and PE induced the contraction of endothelium-denuded mesenteric artery rings. (A) Typical recordings showing vessel response to 1 μ M U-46619 and 5 μ M in OldWKY rats, showing that the artery with impaired response to U-46619 had normal response to phenylephrine. (B,C) Summary data showing that vessels with reduced response to U-46619 in OldWKY and OldSHR rats had normal response to PE, showing similar results regardless of whether U-46619 or PE was used first. (D) Typical recordings showing vessel response to 1 μ M U-46619 and 5 μ M in OldWKY rats after the preconditioning of L-NAME, indicating that the artery with impaired response to U-46619 was not due to incomplete endothelial removal. Numbers inserted in the graphs represented the number of arteries used in the experiment. Arteries were obtained from 4–6 rats in each group.

caused by aging and hypertension (16 genes in aging and 17 genes in hypertension) were identified. The changes caused by hypertension were mainly upregulated gene expression, whereas the changes caused by aging were mainly downregulated gene expression (Figures 4A,B). The altered genes were different, and the common genes were *Myrip*, *Htr1b*, *Gja5*, *Cx3cl1*, and *Adm* (Supplementary Figure S1A), mainly reflecting in the common alteration in the myosin-interacting protein, hydroxytryptamine receptor, gap junction protein, C-X3-C motif chemokine ligand, and adrenomedullin. Changes in DEGs caused by aging factors under hypertensive conditions were more than those caused by hypertension factors under aging conditions (Figures 4C,D). Additionally, the related genes in the TXA2-TP signaling pathway of concern in this study did not change significantly. However, the common DEGs caused by aging factors and aging factors under hypertensive conditions mainly included *Ptgir* (prostaglandin I2 receptor, IP) and *Ptger3* (prostaglandin E receptor, EP) (Figures 4A–E, Supplementary Figure S1B), suggesting changes in the prostaglandin signaling pathway in the older adult. We enriched the pathways based on DEGs through the KEGG pathway, but the prostaglandin signaling pathway could not be enriched (Supplementary Figure S2, the 30 most

significant KEGG pathways were selected to construct a bar chart for display).

Expression of TXA2-TP signaling pathway-related proteins

Although experiments on these DEGs did not provide much information on the TXA2-TP signaling pathway, we designed this experiment to further study the related proteins in this pathway, considering the significance of the proteins involved in this signaling pathway on vasomotor regulation. Endogenous arachidonic acids (AA) are mainly produced by phospholipid release from cell membrane. AA is metabolized by COX-1 or COX-2 to further form TXA2. The binding of TXA2 to specific receptors (TP) produces the basic physiological function of TXA2, such as vasoconstriction. Therefore, we checked the expression of the TXA2 signaling pathway-related proteins in vasoconstriction using the mesenteric arteries or serum from rats from the four groups. As shown in Figure 5A, the protein expression of total TP in the mesenteric arteries of OldWKY and OldSHR rats determined by western blot analysis was significantly increased. The expression of COX-1 and COX-2 was shown

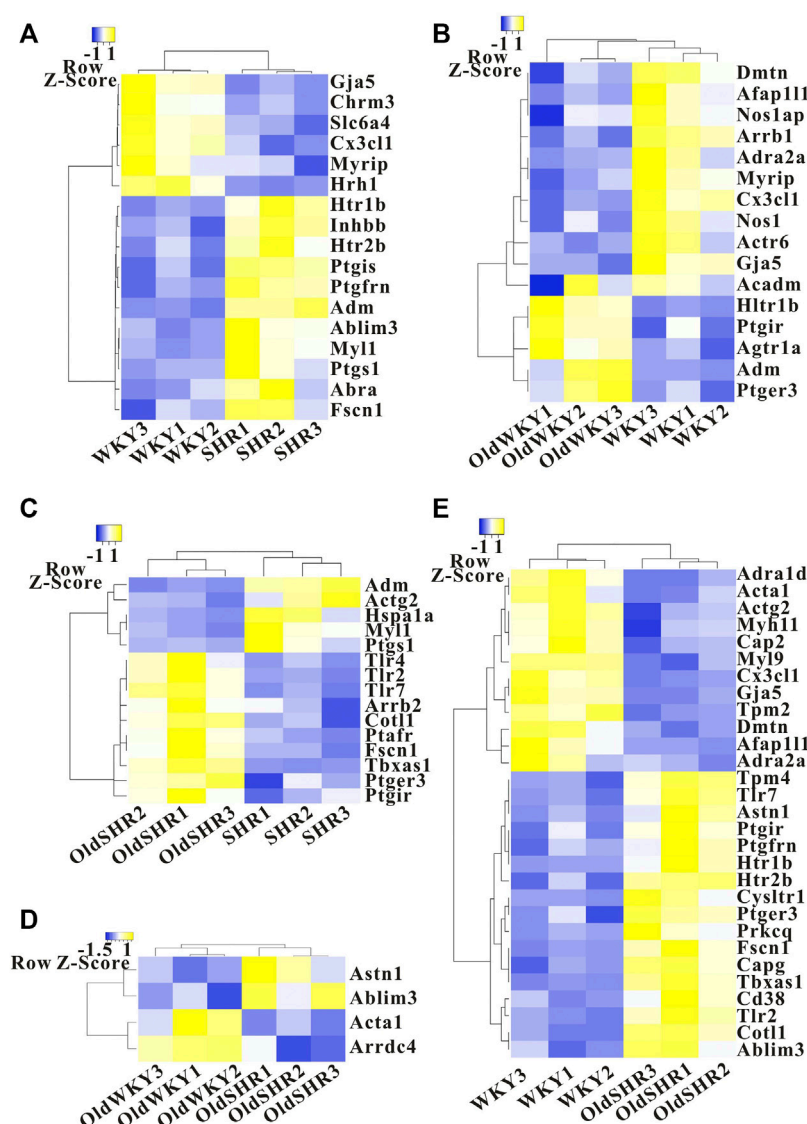


FIGURE 4

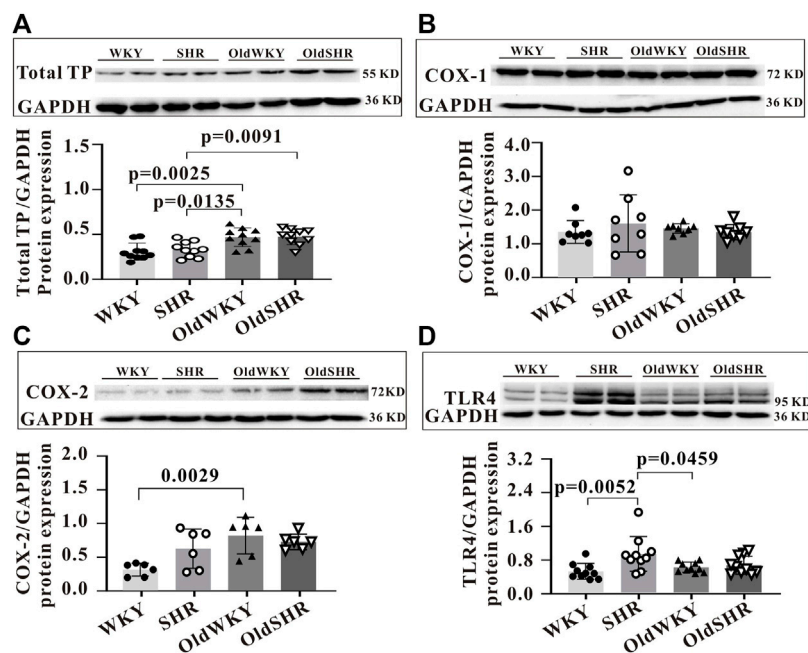
Heatmap showing differentially expressed genes in the TXA2-TP signaling pathway. (A) The SHR group was compared with the WKY group. (B) The OldWKY group was compared with the WKY group. (C) The OldSHR group was compared with the SHR group. (D) The OldSHR group was compared with the OldWKY group. (E) The OldSHR group was compared with the WKY group.

in Figures 5B,C, showing that COX-1 was not significantly altered, while COX-2 expression was significantly increased in OldWKY rats. As shown in Figure 5D, the expression of Toll-like receptor 4 (TLR4) in the mesenteric arteries of SHR was significantly increased, compared with that of WKY and OldWKY rats. Additionally, as shown in Supplementary Figure S3, findings from the ELISA showed that, compared with the WKY group, the other groups had no significant change in TXA2 expression in serum ($p > 0.05$). These results showed that the decreased vasoconstriction reactivity to U-46619 in OldWKY and OldSHR rats was inconsistent with the

expression of TXA2, total TP, and TXA2 signaling pathway-related proteins.

TP desensitization or endocytosis in OldWKY and OldSHR rats

TXA2 or thromboxane A2 analogue (U-46619) binds to its dominant receptor, TP, and mediates vasoconstriction *via* the classical pathways (Gq-PLC-IP3/DAG-Ca²⁺/PKC or G12/13-RHO-MLC). The presence of endogenous TP antagonists,

**FIGURE 5**

Expression of prostaglandin signaling pathway-related proteins. (A) Total TP protein expression, showing a significantly increased expression of TP in SHR, OldWKY, and OldSHR rats. (B,C) COX-1 and COX-2 protein expression, showing a significantly increased expression of COX-2 in SHR, OldWKY, and OldSHR rats. (D) TLR4 protein expression, showing a significantly increased expression of TLR4 in SHR and OldWKY rats. Intact mesentery arteries were collected from 5–6 rats in each group.

such as epoxyeicosatrienoic acids (EETs) (Behm et al., 2009), may cause receptor desensitization, resulting in decreased vascular response to U-46619. Therefore, the expression of total EETs in serum and mesenteric vascular tissue homogenates and the expression of EET subtypes in serum were determined (Figures 6A–D). However, no differences in the expression of total EETs in serum and mesenteric vascular tissue homogenates were observed among the four groups (data not shown). Notably, expression of some EET subtypes in serum was increased. As shown in Figure 6B, serum levels of 8,9-EET were significantly higher in OldWKY and OldSHR groups than those in the SHR group ($p < 0.01$), suggesting that attenuated vasoconstriction induced by U46619 may be associated with increased endogenous EET expression and resulting in receptor desensitization in OldWKY and OldSHR rats.

As stated, we confirmed that TP expression was significantly increased in the mesenteric arteries of SHR, OldWKY, and OldSHR rats in the above experiment. However, this finding reflected the expression of total TP protein in vascular tissues, rather than the expression of membrane surface receptors that directly interact with TXA₂. Therefore, the expression of TP in membrane extracts of mesenteric artery tissues was detected. The results clearly showed that the expression of TP on the cell surface was significantly decreased in the OldWKY and OldSHR

groups, compared with the WKY group, but significantly increased in the SHR group (Figure 6E).

Downregulation of cell surface receptors may be related to endocytosis and actin cytoskeleton during endocytosis (Laroche et al., 2005). Because our experiments showed an increase in endogenous TP stimulator EETs in older rats and older hypertensive rats, we wondered whether the actin-dependent arrestin-mediated mechanism (Laroche et al., 2005) was involved in TP endocytosis in OldWKY and OldSHR rats. Therefore, we hypothesized that the long-term increased endogenous stimulation of TP (e.g., 8, 9-EET) in older rats and older hypertensive rats, together with the remodeling of arrestins and actin cytoskeleton, led to the endocytosis of TP. We first detected the expression of β -arrestin 1 and β -arrestin 2. Unexpectedly, no significant changes of arrestins (Supplementary Figure S4) were observed in the OldWKY and OldSHR groups, which was inconsistent with the trend of vasoconstriction induced by U-46619. Thereafter, we examined the expression of F- and G-actin and found that F/G-actin ratio in the mesenteric arteries of SHR rats did not change significantly. However, the F/G-actin ratio in mesenteric arteries of OldWKY or OldSHR rats was significantly increased, compared with the ratio in SHR rats (Figure 6F, $p < 0.01$ or $p < 0.05$), suggesting that the increased F/G-actin contributed to actin-dependent TP endocytosis.

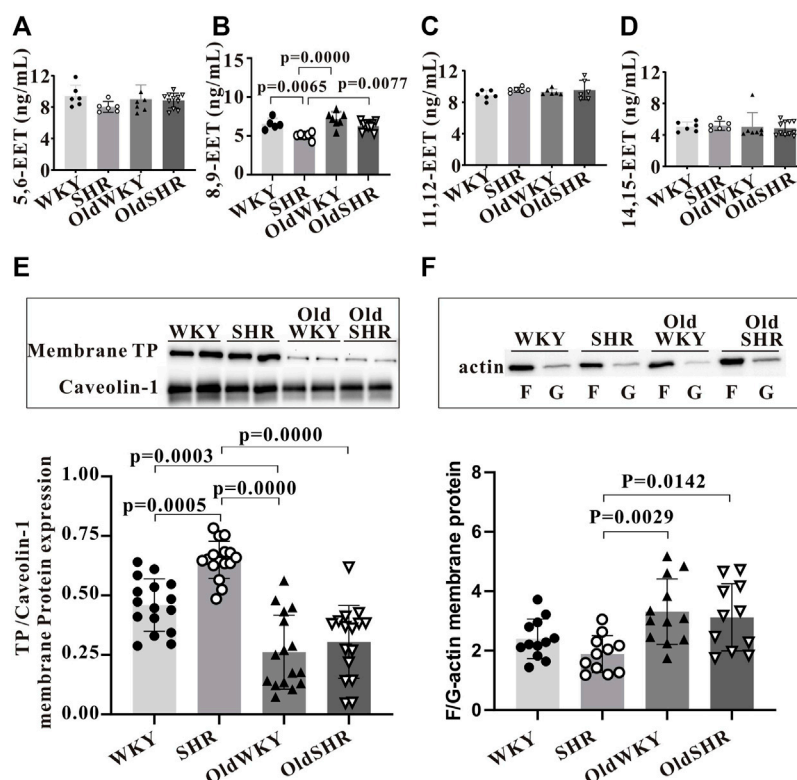


FIGURE 6

Expression of TP desensitization or endocytosis-related proteins. (A–D) Expression of EET subtypes in serum by ELISA. Summary data showing that 8, 9-EET serum levels were significantly higher in the OldWKY and OldSHR groups than in the SHR group ($p < 0.001$ and $p < 0.01$, respectively). (E) Western blot showing that expression of TP on the cell surface was significantly decreased in OldWKY or OldSHR rats ($p < 0.001$). (F) F/G-actin protein expression showing that F/G-actin ratio in mesenteric arteries of OldWKY or OldSHR rats was significantly increased, compared with SHR rats ($p < 0.01$ or $p < 0.05$). Intact mesenteric arteries were collected from 5–11 rats in each group.

Discussion

We report for the first time that the TXA₂-TP prostaglandin signaling pathway is inhibited in older rats and older hypertensive rats, as demonstrated by the TXA₂ analog, U-46619, which significantly enhanced mesenteric vasoconstriction in SHR rats. However, interestingly and contrary to expectations, U-46619 almost did not cause mesenteric vasoconstriction in OldWKY and OldSHR rats. Mesenteric arteries with impaired response to U-46619 in OldWKY and OldSHR rats had strong responses to the adrenergic receptor agonist, PE. The de-endothelialized mesenteric artery experiment confirmed that the weak vascular response of U-46619 was unrelated to the vascular endothelium. Molecular biology results showed that the decreased expression of TP cell membrane proteins in mesenteric vessels and increased endogenous TP competitor, 8, 9-EET, in serum may be partly responsible for the decreased vascular reactivity to U-46619. The decreased expression of TP on the plasma membrane suggests that the receptor protein may escape from the plasma membrane and enter the cytoplasm by

endocytosis, which may be related to actin cytoskeletal remodeling (Laroche et al., 2005; Smythe and Ayscough, 2006).

The main metabolites of endogenous AA were four types of epoxyarachidonic acid (EETs): 5, 6-; 8, 9-; 11, 12-; and 14, 15-EETs. The long-term increased endogenous stimulation of TP (such as EETs) may lead to the endocytosis or desensitization of TP. Our results showed significantly higher serum 8, 9-EET levels in OldWKY and OldSHR rats, suggesting that U46619-induced diminished vasoconstriction may be associated with increased endogenous EET expression. Moreover, AA may generate TXA₂, cycloprostaglandins (PGI₂), and prostaglandins (PGS) in the presence of COX-1 and COX-2. TXA₂ binding with its specific receptor (TP) can cause thrombosis and vasoconstriction. Our study confirmed that the total protein expression of TP in mesenteric vessels in OldWKY and OldSHR rats significantly increased (Figure 5A), which was obviously contrary to the decreased vascular response to U-46619. Additionally, upregulated or unchanged expression of proteins was associated with TXA₂-TP signaling, which is inconsistent with a decreased vascular response to U46619. The expression levels of

total TP protein were significantly increased in older rats and older hypertensive rats, reflecting the upregulation of functional requirements for this pathway.

Importantly, our membrane protein experimental results clearly showed that TP expression on OldWKY and OldSHR cell surface was significantly decreased, which may be an important mechanism of impaired vascular response to U-46619 in older rats and older hypertensive rats. Changes in U-46619-induced vasoconstriction showed that the mesenteric vessels of OldWKY and OldSHR groups were not responsive to U-46619 but to α -AR activator PE, indicating a receptor-specific change. Actin consists of monomeric globular actin (G-actin) and polymeric fibrous actin (F-actin). G- and F-actin can be converted into each other to regulate the depolymerization or polymerization of actin cytoskeleton. Previous studies demonstrated that depolymerization of actin cytoskeleton blocked agonist-induced endocytosis of TP β subtype in HEK293 cells, suggesting that TP endocytosis requires active remodeling of actin cytoskeleton (Laroche et al., 2005). Consistent with this result, our study confirmed the contribution of the increase in F/G ratio in OldWKY and OldSHR rats (Figure 6F). Taken together, our results suggested that the long-term increase in endogenous EET in older rats and older hypertensive rats, coupled with an increase in F/G-actin, resulted in endocytosis of TP.

In addition to the mechanism of TP desensitization discussed above, the DEGs were included in biological pathways for comprehensive analysis to better understand the biological process of TXA₂-TP-vasoconstriction. The degree of influence and rule of functional variation on biological pathways were analyzed for future studies. We enriched the pathways using DEGs through the KEGG pathway, but the prostaglandin signaling pathway could not be enriched (Supplementary Figure S2). We further analyzed DEGs related to prostaglandin receptors, smooth muscle contractile proteins, actin cytoskeletal remodeling, and vasoconstriction-related biological process signaling pathways (Figure 4). Similar number of DEGs caused by aging and hypertension were identified, and the common alteration mainly reflected in the myosin-interacting protein, hydroxytryptamine receptor, gap junction protein, C-X3-C motif chemokine ligand, and adrenomedullin. Importantly, the common DEGs caused by aging factors and aging factors under hypertensive conditions included Ptgir and Ptger3 (encodes prostaglandin I₂ receptor and prostaglandin E receptor 3, respectively), suggesting changes in prostaglandin signaling pathways under aging pathological conditions. Ptgir is predicted to enable prostacyclin receptor activity, which is involved in the adenylate cyclase-activating GPCR signaling pathway, negative regulation of platelet-derived growth factor receptor signaling pathway, and negative regulation of smooth muscle cell proliferation. Ptger3 enables prostaglandin E receptor activity and acts upstream of or within several processes, including GPCR signaling pathway,

bicarbonate transport, and fever generation. Combined with our findings of changes in TP receptors, F/G-actin, and DEGs, our study confirmed an age-related dysregulation of prostaglandin signaling. These changes had no significant differences between OldWKY and OldSHR rats, suggesting that the aging factor played a key role in this damaged vasoconstriction and hypertension did not exacerbate the damage.

Arrestins are adaptor proteins that function to regulate GPCR signaling and trafficking. Arrestin-mediated endocytosis of GPCRs requires the coordinated interaction between β -arrestins and clathrin, adaptor protein 2, and phosphoinositides, such as PIP₂/PIP₃ (Moore et al., 2007; Shenoy and Lefkowitz, 2011; Gurevich and Gurevich, 2015). Studies demonstrated that arrestins play essential roles not only in GPCR desensitization (Carman and Benovic, 1998) but also in receptor endocytosis (Gurevich and Gurevich, 2006). In consideration of the importance of arrestins in receptor desensitization and endocytosis, we performed special assays and found that β -arrestin 1 and β -arrestin 2 seemed not to play a major role in TP desensitization in OldWKY and OldSHR rats. In our study, DEG analysis showed that expression of the gene encoding β -Arrestin 1 was significantly downregulated in older rats (Figure 4B), whereas the expression of the gene encoding β -arrestin 2 was significantly upregulated in older hypertensive rats (Figure 4C). Protein expression analysis revealed that β -Arrestin 2 increased under hypertensive conditions (Supplementary Figure S4). The significance of these changes will be further studied in the future.

Dysregulation of TP might result in agonist hypersensitivity and hyper-responsiveness. Hinton et al. (2007) examined TP localization and kinetics following hypoxia to determine the mechanism of hypoxia-induced TXA₂ hypersensitivity. They demonstrated that TP is desensitized in neonatal pulmonary circulation, usually through PKA-mediated phosphorylation, resulting in decreased receptor-ligand affinity and reduced receptor-ligand binding to Gq. This protection is lost after hypoxic exposure. Using site-directed mutational analysis, Santhosh et al. (2014) examined the role of PKA-accessible serine residues in determining TP affinity. They found that, in hypoxic pulmonary hypertension, loss of site-specific phosphorylation of the TP receptor caused agonist hyper-responsiveness. Feng et al. (2011) reported the contractile response of human coronary microvasculature to TXA₂ before and after cardioplegia, followed by reperfusion (CP/Rep). They concluded that CP/Rep decreased the contractile response of human coronary arterioles to U-46619 soon after cardiac operations. However, they were unable to find the exact cause of the decreased vascular response to U-46619. They reported that the contractile response to TXA₂ required activation of TP and PLC but not PKC- α . They suggested that a period of intraoperative hypothermic ischemia and reperfusion may modify the functional state of TXA₂ receptor-dependent

signaling rather than the steady-state levels of their proteins. Cupitra et al. (2020) reported the influence of ageing on vascular reactivity and receptor expression in rabbit aorta. They found that ageing caused a decrease in the sensitivity of the aorta to KCl, U-46619, and isoproterenol, and an increase in the adrenergic response with aging. They indicated that the vascular reactivity to the TXA2 analogue showed a marked difference in contraction between young and old rabbits and between adult and old rabbits, suggesting that sensitivity to prostanoids remains until 6 months of development. However, the sensitivity deteriorates in old age in rabbits. Their finding indicated a significantly increased expression of $\alpha 1$ and $\beta 2$ receptors in the intima and adventitia and increased expression of TP only in the adventitia. In contrast, our previous study (Wei et al., 2021) demonstrated a significant increase in the mesenteric artery contraction induced by PE in OldSHR. The mechanism is not consistent with the downregulation of $\alpha 1$ -adrenergic receptor ($\alpha 1$ -AR) expression but related to the increased expression of the downstream signaling molecule, PKC. PKC phosphorylates PKC-potentiated phosphatase inhibitor protein, CPI-17, which inhibits the activity of myosin phosphatase target subunit 1 of MLC phosphatase. Therefore, MLC20 phosphorylation level was increased, and vascular smooth muscle contraction was enhanced.

Our results also showed that the reactivity of both OldWKY and OldSHR rats to $\alpha 1$ -AR was increased, and the reactivity to KCl was increased in the SHR and OldSHR groups. One of our previous studies (Cui et al., 2022) showed that vasodilation by NO in OldSHR rats was decreased, which significantly correlated with the reduction in the PKG-mediated effect. Our present study found that the TXA2 analog, U-46619, significantly enhanced mesenteric vasoconstriction in SHR rats but hardly caused mesenteric vasoconstriction in OldWKY and OldSHR rats. Consistent with changes in vasoconstrictor function, TP expression on the cell membrane was increased in SHR rats but decreased in the OldWKY and OldSHR groups. Our present findings indicated that the sensitivity to prostaglandins increased in hypertension, but the sensitivity deteriorated with age. This age-induced change could not be reversed even in hypertension. Additionally, findings from some reports were inconsistent with those from this study. For example, a study reported no difference in the expression of TP receptor in endothelial cells between SHR and WKY rats at 36–72 weeks (Tang and Vanhoutte, 2008). The aorta of male Fischer F344 rats at 6 and 22 months of age showed no difference in response to U-46619 stimulation (Shams et al., 1990), and the cerebral arterioles of rats showed no difference in response to the same agonist (Mayhan et al., 1990). The differences in the results from different laboratories could be due to the different animals and blood vessels used.

Agonist-induced desensitization is usually found to be associated with receptor phosphorylation by various protein kinases. The current theory is that the phosphorylation

prevents proper docking of G proteins to the receptor, thus preventing or reducing signal transmission (Biringier, 2021). Wang et al. (1998) suggested a potential source for the initial phosphorylation. They reported that a cGMP-dependent G kinase (PKG) phosphorylated the TP receptor, which in turn inhibited IP3 release. Additionally, they showed that cAMP-dependent A kinase (PKA) was not involved. GPCR desensitization is thought to involve either cross-talk/heterologous desensitization among different signaling systems or agonist-induced/homologous desensitization terminating or modulating the response to the receptor. A study by Kelley-Hickie et al. (2007) on the mechanism of desensitization of signaling by TP α , suggested a mechanism whereby U-46619-induced diacylglycerol-regulated protein kinase C (PKC) phosphorylation of ¹⁴⁵Ser partially and transiently impaired TP α signaling, whereas PKG- and PKC-phosphorylation at both ³³¹Ser and ³³⁷Thr, respectively, effectively terminated its signaling. However, unlike in TP β , G protein linked receptor kinases (GRK)/ β -arrestins had no role in TP α homologous desensitization.

Based on the results of the expression of TP membrane receptors and F/G-actin in our study, we consider the changes insufficient to explain the phenomenon that the vessels of OldWKY and OldSHR rats barely respond to U-46619. Therefore, we speculated that the loss of vascular reactivity of U-46619 in older rats and older hypertensive rats might be related to TP phosphorylation. Unfortunately, no suitable TP phosphorylation-specific antibodies are available, and the reported cell-level TP phosphorylation experiments are not applicable to animals. Therefore, TP phosphorylation was not examined in our study. We previously reported (Wei et al., 2021) that the mRNA and protein expression of PKC in rat mesenteric artery obtained from SHR, OldWKY, and OldSHR rats increased significantly. The expressions of PKC γ and PKC ζ were significantly increased in OldSHR rats, resulting in great changes in vascular function. Additionally, we reported that mRNA and protein expression of PKG was significantly elevated in SHR and OldSHR rats (Cui et al., 2022). Although we have not been able to determine PKA, our results showed that Adcy3, the main encoding gene of adenylyl cyclase, was upregulated during aging and downregulated in SHR and OldSHR rats (Cui et al., 2022). However, all these changes in PKC, PKG, or perhaps PKA that may lead to receptor phosphorylation seem to be inconsistent with the vasoconstriction changes induced by U-46619 in OldWKY and OldSHR rats. Growing evidence indicated that contraction was regulated by RhoA-Rho kinase (ROCKs). ROCK-1 expression had a large increase in hypertension, while ROCK-1 expression decreased with age. ROCK-2 expression increased significantly with age (Wei et al., 2021). However, the relationship between ROCKs and TP has not been discovered and reported, and whether ROCKs may lead to TP phosphorylation remains undetermined.

In summary, this study demonstrated for the first time the decreased vascular reactivity to U-46619 in OldWKY and OldSHR rats, indicating that the TXA₂-TP prostaglandin signaling pathway was diminished in older adults. The evidence presented here complements the mechanisms underlying changes in vascular TXA₂-TP signaling during aging-related vascular remodeling. The findings may provide a scientific rationale for developing novel therapeutic strategies for the treatment of older patients.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA872876>.

Ethics statement

The animal study was reviewed and approved by Ethics Committee of Southwest Medical University.

Author contributions

MZ, CL, CH, YC, YL, and YM conducted the experiments. JC, JW, and PL designed the experiments and analyzed the data. YY designed and arranged the experiments and wrote the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Administration of USP7 inhibitor P22077 inhibited cardiac hypertrophy and remodeling in Ang II-induced hypertensive mice

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Hypertension is one of the common causes of pathological cardiac hypertrophy and a major risk for morbidity and mortality of cardiovascular diseases worldwide. Ubiquitin-Specific Protease 7 (USP7), the first identified deubiquitinating enzymes, participated in a variety of biological processes, such as cell proliferation, DNA damage response, tumorigenesis, and apoptosis. However, its role and mechanism in cardiac remodeling remain unclear. Here, our data indicated that USP7 expression was increased during Ang II-induced cardiac hypertrophy and remodeling in mice and humans with heart failure, while the administration of its inhibitor p22077 attenuated cardiac hypertrophy, cardiac fibrosis, inflammation, and oxidative stress. Mechanistically, the administration of p22077 inhibited the multiple signaling pathways, including AKT/ERK, TGF- β /SMAD2/Collagen I/Collagen III, NF- κ B/NLRP3, and NADPH oxidases (NOX2 and NOX4). Taken together, these findings demonstrate that USP7 may be a new therapeutic target for hypertrophic remodeling and HF.

KEYWORDS

cardiac remodeling, USP7 inhibitor, P22077, inflammation, oxidative stress

Introduction

Pathological cardiac hypertrophy, characterized by increasing cardiomyocyte size, impaired Ca²⁺ handling, gap junction (GJ) dysfunction, mitochondrial dysfunction, induction of fetal gene program, fibrosis, and cardiac contract dysfunction, is a major risk factor for heart failure (HF) (McMullen and Jennings, 2007; Tham et al., 2015). Compelling evidence demonstrates that Angiotensin II (Ang II) acts as an important stimulatory factor of pathological cardiac hypertrophy and plays an important role in the progression of cardiac hypertrophy and cardiac remodeling. Ang II couples to its receptors, activates Renin-Angiotensin-Aldosterone System (RAAS) and its

downstream signaling pathways, such as PI3K/AKT/mTOR, JAK/STAT, and TGF- β /Smad pathways, and subsequently promotes hypertension, cardiac hypertrophy, cardiac fibrosis, and cardiac dysfunction (Mehta and Griendling, 2007). Moreover, Ang II could also activate the inflammation and oxidative stress in cardiac remodeling, which are common pathological alterations in cardiac remodeling, *via* activation of nuclear factor- κ B (NF- κ B) signaling or NADPH oxidases (NOX2 and NOX4). In addition, overexpression and over-activation of some of these signaling pathway proteins were found in Ang II-induced cardiac hypertrophy and cardiac remodeling, suggesting that maintaining the imbalance between protein synthesis and protein degradation of some harmful proteins was an important therapeutic strategy to protect the heart from cardiac remodeling and heart failure.

The ubiquitin-proteasome system (UPS) plays an important role in maintaining cardiovascular system homeostasis, which selectively ubiquitinylates and triggers the degradation of the misfolded, oxidized, or damaged proteins involved E1s, E2s, E3s, proteasome and deubiquitinating enzymes (DUBs) to ensure the quality and quantity control of proteins in the heart. Ubiquitin (Ub)-specific proteases (UPSs), the largest subfamily of DUBs, remove Ub chains from the substrates to regulate the stability, activity and subcellular localization of the target substrates (Lee et al., 2013; Eletr and Wilkinson, 2014). Increasing evidence has demonstrated that DUBs have a crucial role in cardiovascular diseases, such as cardiac hypertrophy, myocardial infarction, atrial fibrillation, heart failure, and others (Liu et al., 2016; Bi et al., 2020a; Bi et al., 2020b; Hu et al., 2021). USP7 (also called Herpesvirus-associated ubiquitin-specific protease, HAUSP), the first identified DUBs, participated in cell proliferation, DNA damage response, tumorigenesis, inflammation, and apoptosis by regulating its substrates, such as MDM2/p53, PTEN, FOXO4, NF- κ B, and other target proteins (Khoronenkova et al., 2012; Smits and Freire, 2016; Xu et al., 2022). Recently, two studies found that the expression of USP7 was significantly increased under hypoxia in cardiomyocytes, while its inhibition or knockdown of USP7 can protect the heart from hypoxia-induced cardiomyocyte injury or myocardial ischemia/reperfusion injury (Xue et al., 2021; Xu et al., 2022). In addition, our previous study using microarrays of gene expression has reported that USP7 is upregulated in Ang II-induced hypertrophic heart in mice, especially on day 7 (Bi et al., 2020a), suggesting that USP7 may involve in cardiac hypertrophy. However, little was known about the role and the molecular mechanism of USP7 in Ang II-induced cardiac remodeling.

Here, our study showed that inhibition of USP7 activity with selective small molecules, p22077, alleviated Ang II-induced hypertrophy, fibrosis, inflammation and oxidase stress, and protected the process from adapted hypertrophy to maladapted remodeling. Mechanically, the administration of USP7 inhibitor p22077 down-regulates multiple signal

pathways, which are involved in cardiac hypertrophy and remodeling. In addition, our studies demonstrate that USP7 contributes to the pathogenesis of cardiac hypertrophy and suggest that USP7 could be a new therapeutic target for hypertrophic diseases.

Materials and methods

Study subjects

A total of 33 patients diagnosed with HF and 37 normal controls who were admitted to the First Affiliated Hospital of Dalian Medical University from March 2021 to March 2022 were recruited into the present study. The patients with HF were defined according to the recommendation of the ESC with minor modifications (Heidenreich et al., 2022): patients aged at least 18 years with symptomatic heart failure of New York Heart Association (NYHA) functional classes II to IV, BNP $\geq$ 150 pg/ml and EF < 50%. The normal controls with normal cardiac function, and no obvious abnormalities in physical examination (routine examination, clinical examination, lab reports, and B-ultrasonography reports), were also recruited. Our study excluded patients with congenital heart disease, primary pulmonary hypertension, pericardial disease, malignancies, recent coronary bypass surgery, and severe valvular heart disease, chronic obstructive pulmonary disease (COPD), hyperthyroidism, hematological system diseases, surgery, or trauma within 2 years, acute and chronic infectious diseases, and significant renal dysfunction (estimated glomerular filtration rate <30 ml/min per 1.73 m²). Experimental protocols were approved by the ethics committee of the First Affiliated Hospital of Dalian Medical University, and patients were given informed consent for this study. Participants gave their informed consent and the study was approved by the ethics committee of the First Affiliated Hospital of Dalian Medical University. The blood samples were collected from patients with HF and normal controls for ELISA.

The study contained three male patients (median age = 48, EF = 20 $\pm$ 5%, n = 3) and three age- and gender-matched controls (median age = 46) as previously described (Xie et al., 2019; Bi et al., 2020a). Samples from patients with HF were obtained from Beijing Anzhen Hospital of the Capital University of Medical Sciences at the time of cardiac transplantation. Normal hearts (non-failing hearts as controls) were obtained from donors with normal cardiac contractile function based on echocardiography of those who had died from motor vehicle accidents. The study was performed in accordance with the Declaration of Helsinki and was approved by the Institutional Ethics Committee of First Affiliated Hospital of Dalian Medical University. Patients provided written consent. Heart tissues were fixed in neutral buffered formalin solution, embedded in paraffin, and prepared for histological examinations.

Animal and treatment

All the experiments in this study were approved by the Animal Care and Use Committee of Dalian Medical University. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No.85-23, revised 1996).

Male wild-type (WT) C57BL/6 mice (8–10 week old), obtained from The Jackson Laboratory (Bar Harbor, ME, United States), were administered intraperitoneally with USP7 inhibitor p22077 (15 mg/kg/day; S7133, Selleck Chem, United States) or DMSO (control) for 1 week, and then were infused with Ang II (1,000 ng/kg/min; A107852; Aladdin, Shanghai, China) or Saline for subsequent 2 weeks using osmotic mini-pumps (Alzet model 1,002; Durect, Cupertino, CA, United States). All mice were anaesthetized with intraperitoneal injection of 2.5% tribromoethanol (0.02 ml/g; Sigma-Aldrich, St. Louis, MO, United States). The hearts were removed and prepared for further histological and molecular analysis. The heart weight (HW), body weight (BW) and tibial length (TL) were measured, and the HW/BW and HW/TL ratios were calculated.

Primary cell isolation and culture

Neonatal rat cardiomyocytes (NRCMs) were enzymatically isolated from 1- to 3-day-old Sprague-Dawley rat hearts as described previously (Bi et al., 2020a). Briefly, the ventricle tissues were cut into about 1–3 mm² pieces, digested in trypsin (25200056; Thermo Fisher, Waltham, MA, United States) at 37°C by continually stirring the tissues, and collected into DMEM/F-12 (SH30023.01B; Hyclone, South Logan, UT, United States) containing 25% FBS (16140071; Gibco, Grand Island, NY, United States) every 3–5 min for 8–10 times. The cells were collected and incubated for 90 min at 37°C. Then, the cell suspension was collected and centrifuged at 1,000 rpm for 10 min, and then cultured in DMEM/F12 with 10% FBS, 1% penicillin/streptomycin and 100 mM BrdU. After 36 h incubation, the cells were serum starved for 12 h and then treated with Ang II (100 nM) or Saline for 24 h.

Blood pressure measurement

The blood pressure of the mice was measured at every 2 days at the same time in the morning after Ang II infusion with a tail-cuff system (BP-2010, Softron, Tokyo, JPN) on a preheated plate at 37°C, as described previously (Shu et al., 2018; Bi et al., 2020b).

Echocardiographic measurement

Mice were anaesthetized with 1.5% isoflurane (Sigma-Aldrich). Cardiac function was measured by echocardiography

in all mice at day 14 after saline or Ang II infusion using a 30 MHz probe (Vevo 770 system; VisualSonics, Toronto, Ontario, Canada) (Xie et al., 2019). Left ventricular (LV) ejection fraction (EF%) and LV fractional shortening (FS%) were calculated based on LV end-diastolic diameter (LVIDd) and LV end-systolic diameter (LVIDs) obtained from M-mode ultrasound (Bi et al., 2020a).

Histological analysis

The heart tissues were fixed in 4% paraformaldehyde for 1–3 days, embedded in paraffin and then sectioned into 4 µm thick. The sections were stained with a H&E staining kit (D006; Nanjing Jiangcheng Bio Inc., Nanjing, China) and a Masson's trichrome staining kit (D026; Nanjing Jiangcheng Bio Inc.). Immunohistochemistry was performed with anti-USP7 (1:100; 26948-1-AP; Proteintech Group Inc., Wuhan, Hubei, China), anti-BNP (1:100; 13299-1-AP; Proteintech Group Inc.), anti-α-SMA (1:150; 55135-1-AP; Proteintech Group Inc.), anti-Collagen I (1:100; 14695-1-AP; Proteintech Group Inc.), anti-CD68 (1:150; 28058-1-AP; Proteintech Group Inc.), anti-NLRP3 (1:100; 19771-1-AP; Proteintech Group Inc.), or F4/80 (1:80; 27044-1-AP; Proteintech Group Inc.) antibodies. The heart sections were stained with wheat germ agglutinin (WGA, 50 µg/ml in 1 × PBS, Vector's Laboratories, Burlingame, CA) for 60 min to evaluate cross-sectional area of myocytes. Frozen sections (5 µm thick) were stained with dihydroethidium (DHE) (BMD00001; Abbkine, Wuhan, Hubei, China) at a dose of 1 mM in PBS at 37°C for 30 min. Digital images were taken at ×200 magnifications of over 10 random fields per slide (ECLIPSE Ni-U, Nikon Instruments Inc, Tokyo, JPN) and analyzed by ImageJ.

Quantitative real-time PCR analysis

Total RNA was isolated from fresh heart tissues using TRIzol (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA, United States) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA (1–2 µg) using Random primers/Oligo (dT)-primer mix RT kits (11141ES60; Yeasen, Shanghai, China). The mRNA levels of USP7, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), collagen I, collagen III, IL-1β, IL-6, NOX2, and NOX4 were determined by real-time qPCR analysis on an Applied Biosystems 7,500 Real-Time PCR System using SYBR Green (RR820A; Takara Bio Inc., Shiga, Japan) as described previously. The values were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers were purchased from Sango Biotech (Shanghai, China). Primer sequences were provided in Table 1.

TABLE 1 The primers were used in qPCR.

Gene	Forward primer	Reverse primer
USP7	CCACAAGGAAAACGACTGGG	GTAACACGTTGCTCCCTGATT
ANP	CACAGATCTGATGGATTCAAGA	CCTCATCTTCTACCGCATC
BNP	GAAGGTGCTGTCCCAGATGA	CCAGCAGCTGCATCTTGAAT
Collagen I	AGTCGATGGTGCTCCAAAA	AGCACCACCAATGTCCAGAG
Collagen III	TCCTGGTGGTCTGCTGACTG	AGGAGAACCCTGTTGCCTG
IL-1 β	TGAAAACACAGAAGTAACGTCCG	CCCAGGAGGAAATTGTAATGGGA
IL-6	TTCCATCCAGTTGCCTTCTTG	TTGGGAGTGGTATCCTCTGTGA
NOX2	CTTCTTGGGTCAGCACTGGC	GCAGCAAGATCAGCATGCAG
NOX4	CTTGGTGAATGCCCTCAACT	TTCTGGGATCCTCATTTCTGG

Immunoblotting analysis

Protein lysates were extracted from heart tissues using radio immunoprecipitation assay buffer (Solarbio Science Technology Co., Beijing, China), and the concentration was determined using a BCA (P0010; Beyotime, Shanghai, China) protein assay. Equal amounts of protein (40–60 μ g) were separated by 8%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (ISEQ00010; Millipore, Shanghai, China), which was incubated with primary and second antibodies. All blots were developed using a chemiluminescent

system, and signal intensities were analyzed using a Gel-Pro 4.5 Analyzer (Media Cybernetics, Rockville, MD, United States), and normalized to Tubulin levels.

Antibodies

The antibodies used in immunoblotting analysis are as follows: USP7 (1:500; 26948-1-AP), TGF- β 1 (1:1,000; 21898-1-AP), NLRP3 (1:1,000; 19771-1-AP), Tubulin (1:3,000; 66031-1-Ig), NOX2 (1:1,000; 19013-1-AP), NOX4 (1:1,000; 14347-1-AP), collagen I (1:

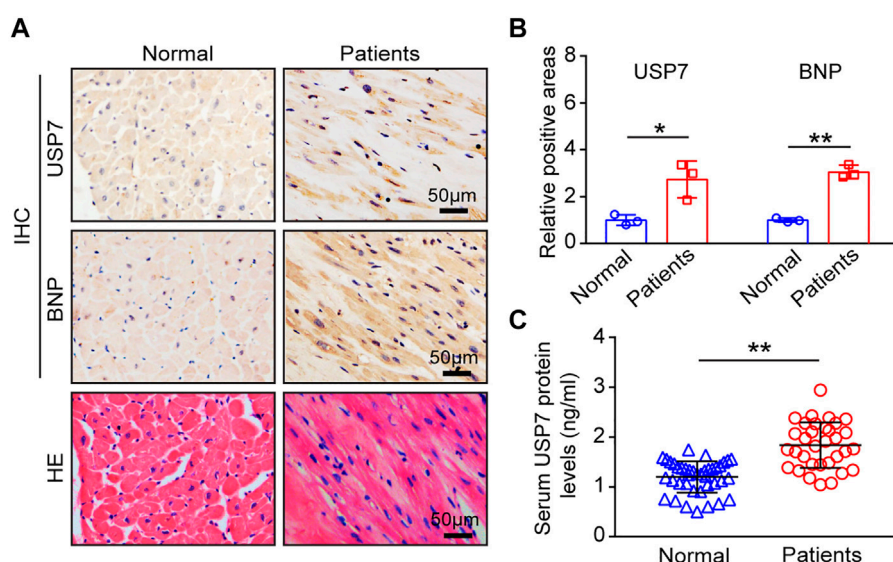


FIGURE 1

USP7 was overexpressed in patients with heart failure. (A,B) Immunohistochemical (IHC) staining of USP7 (upper) and BNP (lower) proteins in the heart tissues from normal control and HF patients (left). Scale bars, 50 μ m. Quantification of the relative USP7- and BNP-positive areas (right; $n = 3$). (C) The concentration of USP7 protein level in serum from normal controls ($n = 37$) and patients with HF ($n = 33$) was detected by ELISA assay. The data are presented as the mean $\pm$ SD, and n represents the number of animals per group. * $p < 0.05$, and ** $p < 0.01$.

1,000; 14695-1-AP), and collagen III (1:1,000; 22734-1-AP) were purchased from Proteintech Group Inc., (Wuhan, Hubei, China). p-AKT (1:1,000; #9271), AKT (1:1,000; #9272), ERK (1:1,000; #9102), Smad2 (1:1,000; #5339), p65 (1:1,000; #4764) were acquired from Cell Signaling Technologies (Danvers, Massachusetts, United States). p-p65 (1:500; 310013) was got from Zen Bioscience (Chengdu, Sichuan, China). p-Smad2 (1:1,000; ab280888) was bought from Abcam (Cambridge, MA, United States). p-ERK (1:500; WLP1512) was obtained from Wanleibio (Shenyang, Liaoning, China). Anti-mouse (1:5,000; A0216) or anti-rabbit (1:3,000; A0208) IgG secondary antibodies were purchased from Beyotime (Shanghai, China).

Enzyme-linked immunosorbent assay

Serum levels of total USP7 were determined using human USP7 ELISA kits (FS11681; Westang Bio Inc., Shanghai, China) according to manufacturer instructions.

Statistical analysis

Statistical calculations were analyzed using Graphpad Prism 8.0 and the SPSS 24.0 software package. A normality test was conducted first. If all the groups satisfied the normality criteria and variances between the groups were equal, we applied Student's *t*-test or one-way ANOVA (with the Bonferroni *post hoc* test) where appropriate; if the above conditions were not met, we used the nonparametric Mann-Whitney *U* test. Statistical differences were determined by $p < 0.05$. Results are presented as the mean $\pm$ SD.

Univariable logistic regression and multivariable logistic regression were used to investigate possible factors associated with HF. Three models were analyzed: Model 1, the crude model without covariate adjustment; Model 2, the multivariable model that adjusted for sex and age, and Model 3, the full risk-adjustment model that adjusted potential confounders that were significant on univariate analysis and those known to be associated with HF including age, sex, eGFR, and HDL. In consideration of the time lag whereby gene expression changes occur earlier than the cardiac structural functional change and the potential collinearity between the echocardiographic parameters, these echocardiographic parameters were not included in the multivariate logistic regression model.

Results

The expression of ubiquitin-specific protease 7 is upregulated in patients with heart failure

To determine the role of USP7 in human with HF, we detected the expression of USP7 both in HF patients ($n = 3$) and normal

individuals ($n = 3$) by immunohistochemistry. The expression of USP7 was significantly increased in the hearts from HF patients compared with normal controls (Figures 1A,B). Meanwhile, the expression of brain natriuretic peptide (BNP, a marker of HF) in the hearts from patients with HF was also significantly higher than those in normal controls (Figures 1A,B). Further, the serum concentration of USP7 was tested by a human USP7 Elisa kit, while the USP7 concentration was higher in HF patients compared with that in normal controls (Figure 1C; Table 2, 37 with normal controls and 33 with HF). Moreover, the basic clinical data of normal controls and patients with HF was shown in Table 2. We then evaluated the association of HF and serum USP7 by performing univariable and multivariable logistic regression analyses (Table 3). After being adjusted for the aforementioned confounding factors in model 3, we found that the elevated level of USP7 in serum was an independent predictor of HF. Specifically, the odds ratio (OR) of HF per 1 standard deviation (SD) increase in USP7 was estimated to be 6.250 [95% CI, 1.020–38.313; $p = 0.048$]. These results demonstrated that the abnormal expression of USP7 may be associated with the development of congestive HF.

The expression of ubiquitin-specific protease 7 is upregulated in angiotensin II-induced cardiac remodeling

Next, we determined the changes of USP7 in Ang II-induced hypertrophic heart tissues. Wild-type (WT) mice were treated with Ang II (1,000 ng/kg/min) or saline infusion for 14 days, and then the expression of USP7 was tested. qPCR analysis showed that the expression of USP7 was sharply increased in Ang II-induced hypertrophic heart tissues (Figure 2A). The increased expression of USP7 was also detected at the protein level by immunoblotting analysis (Figure 2B). Furthermore, immunohistochemical staining further demonstrated the increased expression of USP7 in Ang II-infused heart tissues (Figure 2C). Similarly, the protein level of USP7 was significantly augmented in Ang II-treated neonatal rat cardiomyocytes (NRCMs) (Figure 2D). Thus, the increased expression of USP7 indicated that USP7 may participate in Ang II-induced cardiac remodeling.

Administration of p22077 attenuates angiotensin II-Induced cardiac dysfunction

To determine the role of USP7 in regulating cardiac remodeling, we used P22077, a potent and selective USP7 inhibitor, which has been identified by activity-based chemical proteomics (Altun et al., 2011; Fan et al., 2013). WT mice were treated with p22077 and Ang II infusion (1,000 ng/kg/min) for 14 days (Figures 3A,B). Administration of p22077 significantly declined Ang II-

TABLE 2 Clinical characteristics.

Parameters	Normal (<i>n</i> = 37)	HF (<i>n</i> = 33)	<i>p</i> -value
Demographics			
Male sex, <i>n</i> (%)	17 (45.94)	19 (57.57)	0.607
Age, years	61.16 ± 14.92	71.06 ± 11.62	0.003**
Hemodynamic variables			
HR, beats/min	75.65 ± 10.38	81.64 ± 17.07	0.077
SBP, mmHg	121.27 ± 10.82	125.33 ± 20.39	0.294
DBP, mmHg	77.97 ± 9.27	79.45 ± 14.64	0.611
Echocardiography			
LVEF, %	59.51 ± 1.87	36.76 ± 10.49	<0.001**
IVS, mm	9.22 ± 1.03	10.24 ± 1.32	<0.001**
LVEDD, mm	45.14 ± 2.92	57.61 ± 9.66	<0.001**
LAD, mm	33.08 ± 2.81	44.91 ± 5.94	<0.001**
Blood test			
USP7, ng/ml	1.25 ± 0.27	1.51 ± 0.43	0.003*
WBC, 10 ⁹ /L	5.91 ± 1.12	6.66 ± 1.72	0.375
eGFR, ml/(min × 1.73 m ²)	91.27 ± 19.43	68.24 ± 26.23	<0.001**
Total cholesterol, mmol/L	4.66 ± 0.80	4.78 ± 1.41	0.658
LDL, mmol/L	2.67 ± 0.74	2.68 ± 0.90	0.515
HDL, mmol/L	1.25 ± 0.27	1.03 ± 0.35	0.005**
Triglycerides, mmol/L	1.48 ± 0.99	1.37 ± 1.04	0.755
BNP, pg/ml	33.25 ± 26.27	2,308.06 ± 3,873.55	<0.001**
History			
Hypertensive, <i>n</i> (%)	NA	23 (65.71)	
Diabetes, <i>n</i> (%)	NA	13 (37.14)	
coronary artery disease, <i>n</i> (%)	NA	18 (51.43)	
Myocardiopathy, <i>n</i> (%)	NA	6 (17.14)	
NYHA class, <i>n</i> (%)			
II	NA	8 (22.86)	
III	NA	15 (42.86)	
IV	NA	12 (34.28)	

p* < 0.05. *p* < 0.01 vs. normal control; HF, heart failure; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVEF, left ventricular ejection fraction; IVS, interventricular septum; LVEDD, left ventricular end diastolic diameter; LAD, left atrial diameter; USP7, Ubiquitin-Specific Protease 7; FBG, fasting blood glucose; WBC, white blood cell; SCr, serum creatinine; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BNP, B-type natriuretic peptide; NA, not applicable; NYHA, New York Heart Association. The parameters are mean (SD) or *n* (%).

induced elevation of blood pressure compared with DMSO-treated mice (Figure 3C). Echocardiography indicated that administration of p22077 sharply attenuated Ang II-induced

cardiac hypertrophy and cardiac contractile dysfunction, as reflected by delaying the decrease of left ventricular (LV) ejection fraction (EF%) and fractional shortening (FS%),

TABLE 3 Logistic regression analysis of USP7 associated with HF.

USP7, per SD increment	OR (95%CI)	p-value
Model 1	8.720 (1.873–40.604)	0.006
Model 2	9.018 (1.692–48.072)	0.010
Model 3	6.250 (1.020–38.313)	0.048

Model 1 was unadjusted, Model 2 was adjusted for sex and age, and Model 3 was additionally adjusted for all variables with p -value < 0.05 in the logistic regression analysis including age, sex, eGFR, and HDL.

compared with DMSO plus Ang II-treated mice (Figures 3D–F). Moreover, Ang II-induced cardiac hypertrophy was also indicated by an increase in the heart size, HW to BW ratio, and HW to TL ratio (Figures 3G–I). In addition, the expression of USP7 has not been affected by P22077 (Supplementary Figures S1A,B).

Administration of p22077 attenuates angiotensin II-Induced cardiac hypertrophy and fibrosis

To determine the role of p22077 in regulating cardiac remodeling, we performed WGA staining and Masson staining. As expected, the cross-sectional area of myocytes

and the area of fibrosis were significantly increased in Ang II-infusion mice, while these were markedly attenuated in p22077-treated animals (Figures 4A–D). Moreover, the Ang II-induced increase of the α -smooth muscle actin (α -SMA)-positive and collagen I-positive myofibroblasts was decreased in p22077-treated animals compared with control mice (Figures 4E–H). The expression of hypertrophic markers ANP and BNP and the expression of fibrotic markers collagen I and collagen III were markedly attenuated in p22077-treated animals (Figures 4I,J). Similarly, there was no significant difference in these parameters between the two groups after saline infusion (Figures 4A–J). These results suggest that USP7 contributes to Ang II-induced cardiac dysfunction and hypertrophic remodeling.

Administration of p22077 attenuates angiotensin II-Induced inflammation and oxidative stress

It has been reported that inflammation and oxidative stress play a critical role in regulating Ang II-induced cardiac remodeling (Crowley, 2014; de Almeida et al., 2020). We further examined the effect of p22077 on the inflammatory response and the production of reactive oxygen species (ROS) in Ang II-induced cardiac hypertrophy. Immunofluorescent assay, IHC assay and DHE staining revealed that Ang II infusion caused a marked increase in the inflammatory cells,

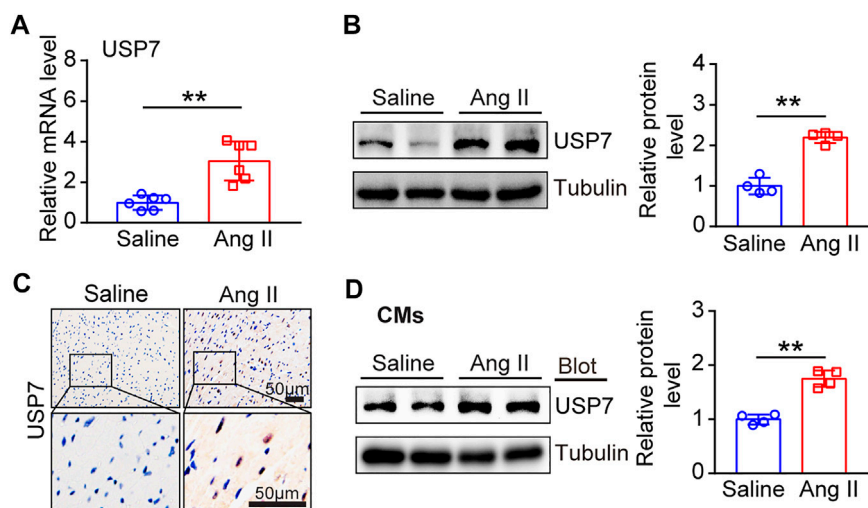


FIGURE 2

The expression of USP7 was upregulated in Ang II-induced hypertrophic hearts and myocytes. (A) qPCR analysis of USP7 mRNA expression in Ang II-infused mouse hearts ($n = 6$). (B) Representative immunoblotting analysis of USP7 protein levels in Ang II (1,000 ng/kg/min)-induced hypertrophic hearts (left). Quantification of the relative USP7 protein level (right; $n = 4$). (C) Immunohistochemical staining of USP7 in Ang II (1,000 ng/kg/min)-infused mouse hearts ($n = 6$). Scale bar: 50 μ m. (D) Representative immunoblotting analysis of USP7 protein level in NRCMs exposed to Ang II (100 nM) (left). Quantification of the relative USP7 protein level (right; $n = 3$). The data are presented as the mean $\pm$ SD, and n represents the number of animals per group. ** $p < 0.01$.

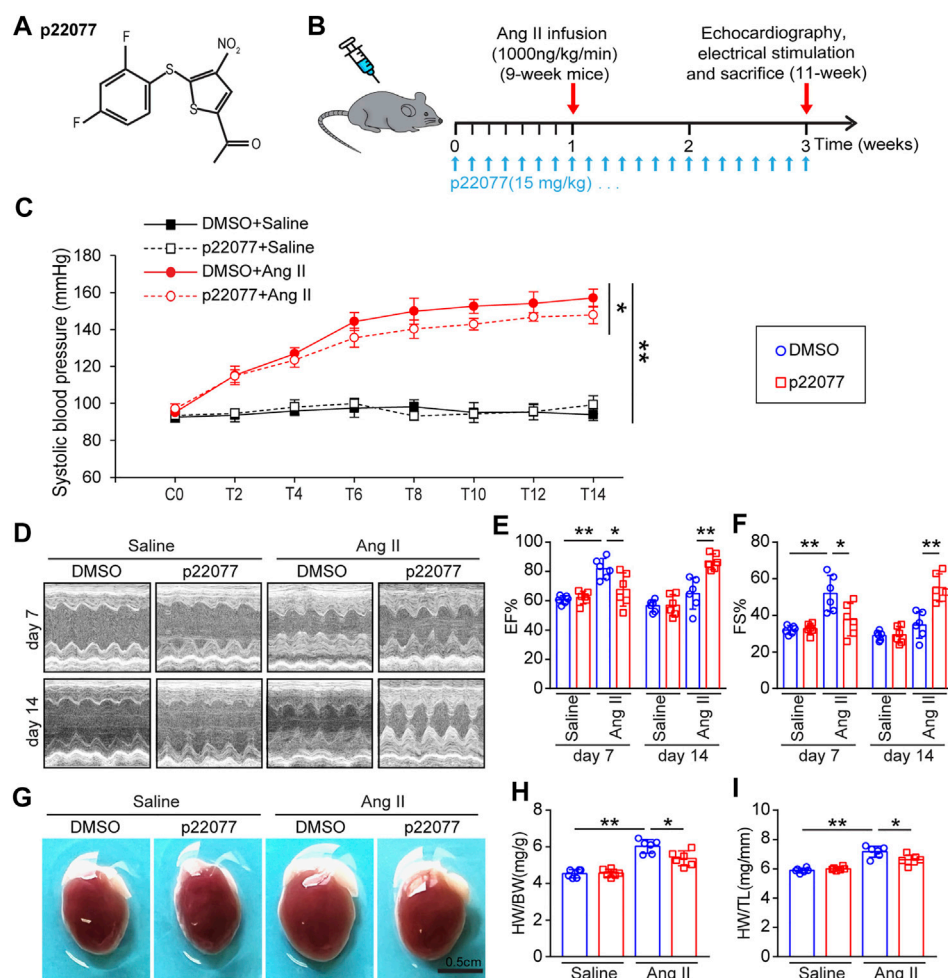


FIGURE 3

Administration of USP7 inhibitor p22077 attenuates Ang II-induced maladaptive cardiac hypertrophy. (A) The basic structure of p22077. (B) Protocol for administration of p22077 in a mouse model of cardiac hypertrophy and remodeling. WT mice were treated with p22077 for 7 days and then infused with Ang II or Saline for additional 14 days. (C) Tail-cuff method measurement of systolic blood pressure (SBP) every 3 days after Ang II infusion ($n = 6$). (D–F) Representative M-mode echocardiography of LV chamber at day 7 and day 14. Measurement of EF% and FS% ($n = 6$ mice per group). (G–I) Representative heart sizes from each group. Scale bars, 0.5 cm. The ratios of HW/BW and HW/TL ($n = 6$ per group). * $p < 0.05$, and ** $p < 0.01$.

NLRP3-inflammasome pathway and superoxide production, including that of F4/80-positive and CD68-positive macrophages, NLRP3-expression and ROS production, in the vehicle-treated mice, but this increase was attenuated in the p22077-treated mice (Figures 5A–F; Supplementary Figures S2A,B). Moreover, the Ang II-induced increase of the mRNA levels of proinflammatory (IL-1 β and IL-6, downstream targets of NLRP3-inflammasome activation) and NADPH oxidases (NOX2 and NOX4) were attenuated in p22077-treated mice (Figures 5G,H). These parameters in saline groups treated with and without p22077 have no significant difference (Figures 5A–H). These results suggest that

USP7 contributes to Ang II-induced inflammation, oxidative stress and the progression of cardiac remodeling.

Administration of p22077 reduces multiple signaling pathways

Ang II triggers multiple signaling pathways, such as AKT/mTOR/p-ERK, TGF- β /Smad2/3, NF- κ B/NLRP3, and NADPH oxidases, which are involved in hypertrophy, fibrosis formation, inflammation response and oxidative stress (Altun et al., 2011; Crowley, 2014). To determine the molecular mechanism of the

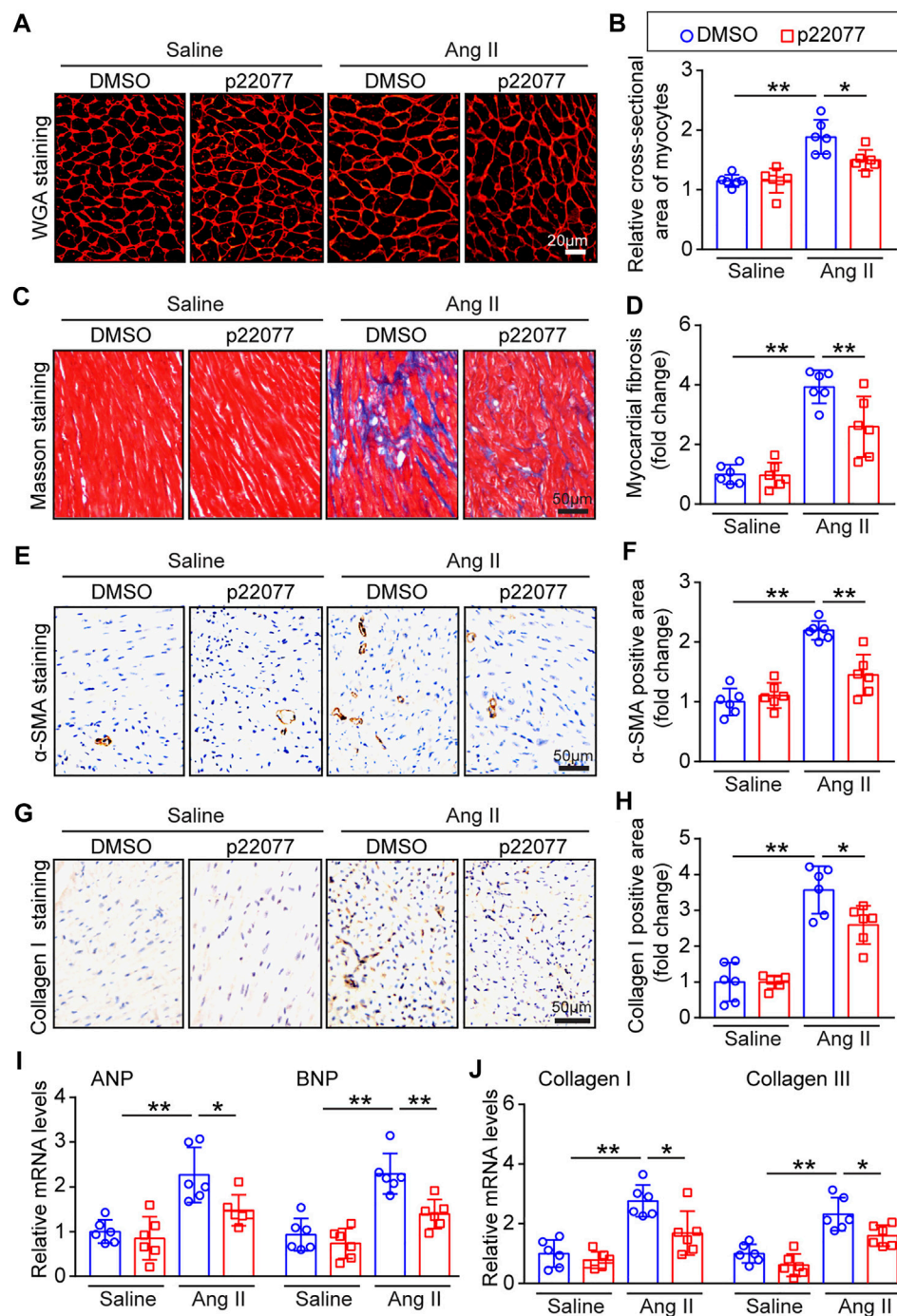


FIGURE 4

Administration of p22077 attenuates Ang II-induced cardiac hypertrophy and cardiac fibrosis. (A,B) Histological examination of cardiac hypertrophy by TRITC-WGA staining (Left) and quantification of the relative myocyte cross-sectional area (200 cells counted per heart, Right; $n = 6$). Scale bars, 20 μm. (C,D) Histological examination of myocardial fibrosis detected by Masson's trichrome staining (Left) and quantification of the relative fibrosis area (Right; $n = 6$). Scale bars, 50 μm. (E,F) Representative IHC and quantification of α-SMA-positive area ($n = 6$ mice per group). Scale bars, 50 μm. (G,H) Representative IHC and quantification of Collagen I-positive area. Scale bars, 50 μm. (I,J) qPCR analysis of ANP, BNP, collagen I and collagen III mRNA levels in the heart tissues. The data are normalized to the GAPDH expression ($n = 6$ per group). The data are presented as the mean $\pm$ SD, and n represents the number of animals per group. * $p < 0.05$, and ** $p < 0.01$.

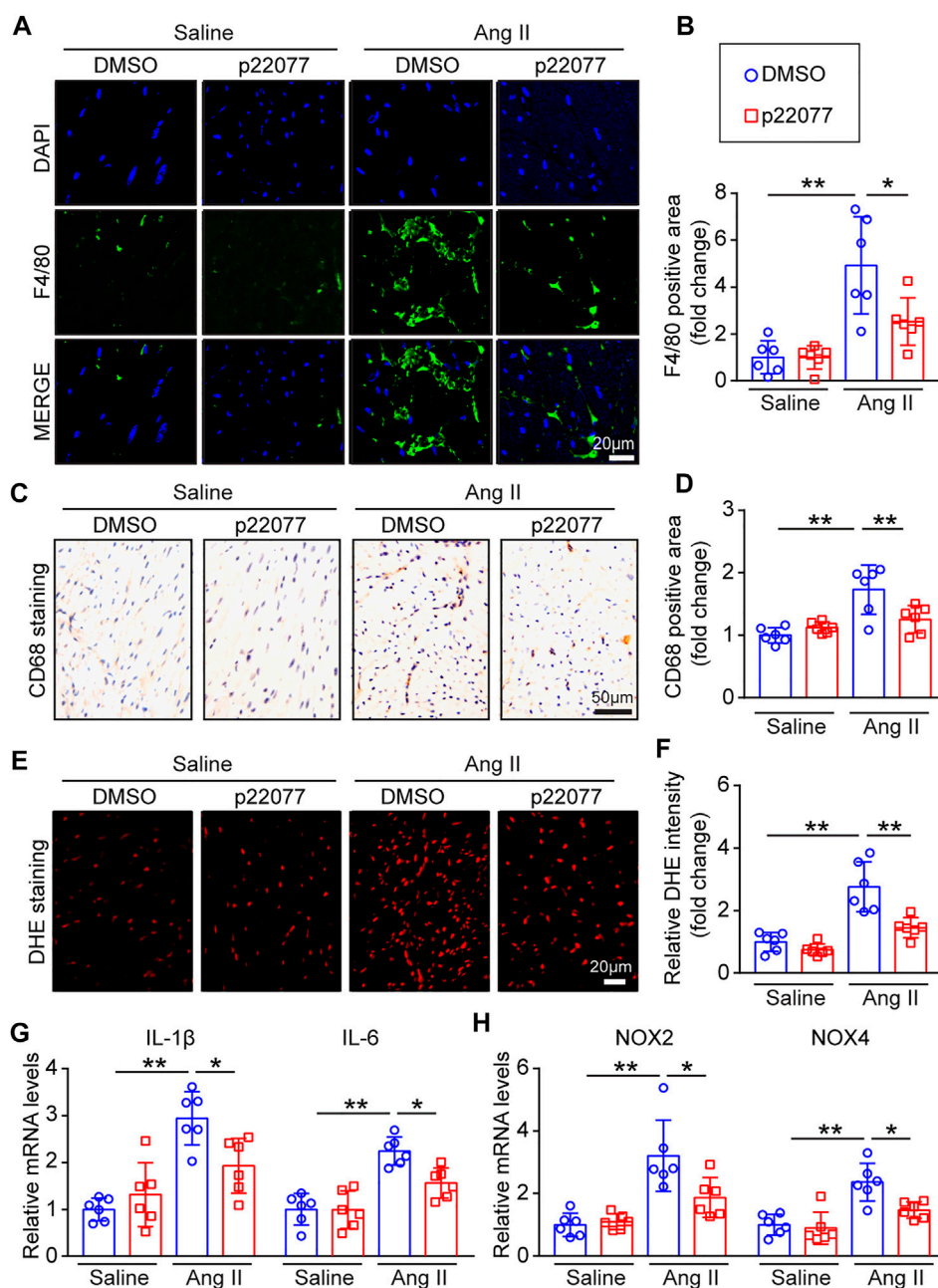


FIGURE 5

Administration of p22077 attenuates Ang II-induced inflammation and oxidase stress. (A,B) Immunofluorescent staining of heart sections for examination of F4/80-positive macrophages (green) and nuclei (DAPI, blue) ($n = 6$). Scale bar = 20 μm. Quantification of F4/80 positive area. (C,D) Representative IHC and quantification of CD68-positive area ($n = 6$ mice per group). Scale bars, 50 μm. (E,F) Dihydroethidium (DHE) staining of heart sections (left). Quantification of DHE intensity ($n = 6$). Scale bar = 20 μm. (G,H) qPCR analysis of IL-1β, IL-6, NOX2, and NOX4 in heart tissues ($n = 6$). The data are presented as the mean $\pm$ SD, and n represents the number of animals per group. * $p < 0.05$, and ** $p < 0.01$.

protective role of p22077 on Ang II-induced cardiac remodeling, we performed the immunoblotting assay to investigate several signaling pathways associated with cardiac remodeling. Compared with Ang II plus DMSO group, the administration of p22077 attenuated Ang II-induced increased expression or the

activation of p-AKT, p-ERK, TGF-β1, p-Smad2, collagen I, collagen III, p-p65, NLRP3, NOX2, and NOX4 in the Ang II plus p22077 group (Figures 6A–D). There was no significant difference in these parameters between the two groups treated with saline (Figures 6A–D).

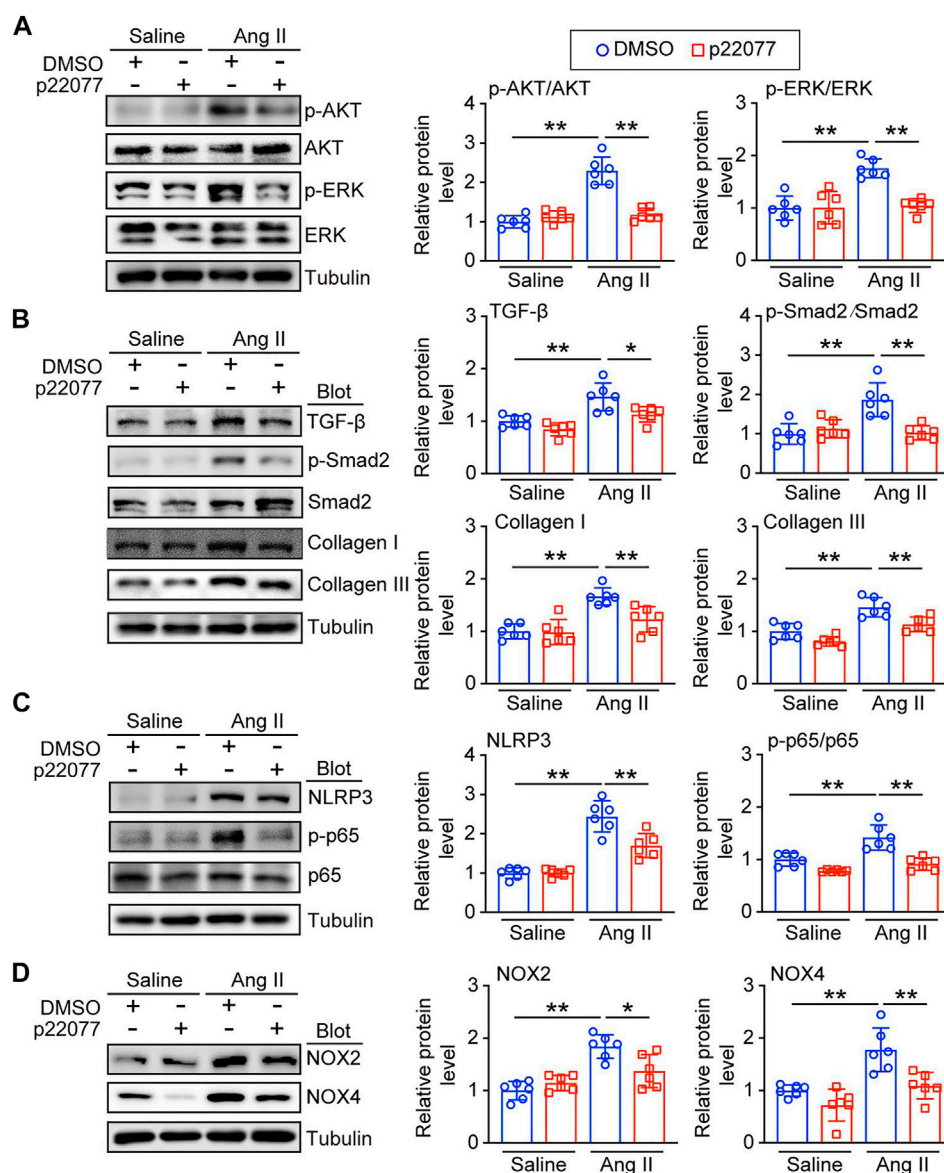


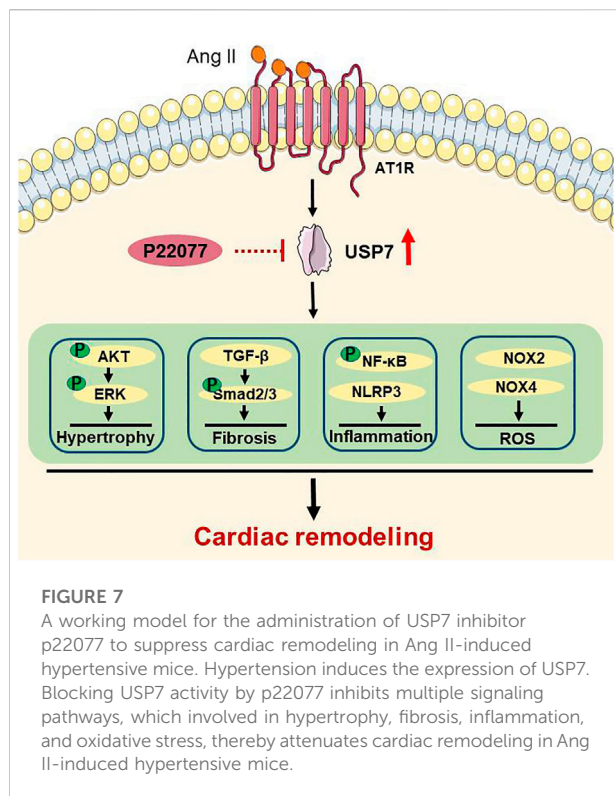
FIGURE 6

Administration of p22077 regulates multiple signaling pathways in Ang II-induced cardiac remodeling. (A) Representative immunoblotting analysis of p-AKT, AKT, p-ERK, ERK, and Tubulin in heart tissues from each group (left). Quantification of the relative protein levels (right, $n = 6$). (B) Representative immunoblotting analysis of TGF-β1, p-Smad2, Smad2, Collagen I, Collagen III, and Tubulin (left). Quantification of the relative protein levels by densitometry (right, $n = 6$). (C) Representative immunoblotting analysis of NLRP3, p65, p-p65, and Tubulin (left). Quantification of the relative protein levels (right, $n = 6$). (D) Representative immunoblotting analysis of NOX2, NOX4, and Tubulin (left). Quantification of the relative protein levels (right, $n = 6$). The data are presented as the mean $\pm$ SD, and n represents the number of animals per group. * $p < 0.05$, and ** $p < 0.01$.

Discussion

In this study, we for the first time demonstrated that the deubiquitinase USP7 has a crucial role in regulating Ang II-induced cardiac hypertrophy and cardiac remodeling. The major findings are as follows: 1) The expression of USP7 was upregulated both in heart tissues and serum from patients with heart failure, and in Ang II-induced hypertrophic mice

hearts. 2) The inhibition of USP7 by p22077 significantly reduced the Ang II-induced cardiac hypertrophy, fibrosis, inflammation and oxidative stress, and recovery of cardiac contractile dysfunction. 3) Mechanically, the administration of USP7 inhibitor p22077 reduced the multiple cell signals, including AKT/ERK, TGF-β/SMAD, NF-κB, NOX2, and NOX4, which are associated the cardiac remodeling. Thus, our data indicated that USP7 plays an important role in Ang



II-induced cardiac hypertrophy and remodeling and may be a novel target for the treatment of hypertrophic cardiovascular diseases. The proposed working model was shown in Figure 7.

Pathological hypertrophy and remodeling can be stimulated by prolonged and abnormal stress, such as hypertension, pressure overload, and myocardial infarction, which promote the progression from adaptive compensation to maladaptive decompensation and finally heart failure (Opie et al., 2006). Ang II, a powerful effector peptide of the RAAS and a hypertrophic agent, can induce cardiac hypertrophy and maladaptive cardiac remodeling by activating different signaling pathways, such as AKT/ERK, TGF- β /SMAD/collagen I/collagen III, NF- κ B/NLRP3, NADPH kinases (NOX2 and NOX4) (Sag et al., 2014; Wang et al., 2019). Increasing evidence demonstrated that oxidative stress plays an important role in cardiac remodeling (Wen et al., 2012). Currently, the increased expression and activity of NADPH oxidases play a central position in ROS production leading to cardiac remodeling, which causes the activation of the hypertrophic signal pathway ERK1/2, AKT/mTOR and inflammation response signaling pathway NF- κ B/NLRP3 and dysregulation of cardiac fetal contractile genes (Zhao et al., 2015). Here, our studies provided convincing evidence that the administration of p22077 decreased the Ang II-induced oxidative stress, reflected by inhibiting the ROS production and the expression of oxidative stress response

proteins (NOX2 and NOX4) (Figures 5C,D,F). Moreover, the Ang II-induced cardiac hypertrophy, fibrosis and inflammation, which can be activated by ROS, were also attenuated with the treatment of USP7 inhibitor p22077 (Figures 4, 5), suggesting that the protective effect of p22077 on the Ang II-induced cardiac hypertrophy, fibrosis and inflammation may be related to oxidative stress. In addition, the administration of p22077 extremely abolished the Ang II-induced augment of the expression of NOX2 and NOX4 as well as the activity of its target signaling pathway, including ERK1/2, AKT/mTOR and NF- κ B/NLRP3 (Figure 6A–D). Meanwhile, previous studies report that USP7 could target and deubiquitylate NOX4 (Liu et al., 2020).

USP7, as an important deubiquitinase, has been reported to participate in different biological activities by stabilizing and deubiquitylating several substrate proteins, such as MDM2, SMAD3, PTEN, NF- κ B, Keap1, NLRP3 and others (Colleran et al., 2013; Huang et al., 2021; Xu et al., 2022). A study has shown that the USP7 inhibitor p22077 blocks inflammation response and the activation of NLRP3 inflammasome (Palazon-Riquelme et al., 2018), while activation of NLRP3 inflammasome involved in cardiac remodeling, suggesting that USP7 may play an important role in cardiac remodeling. Moreover, inhibition of USP7 activated p53 and decreased TfR1, and then attenuates ferroptosis and protects the heart from myocardial I/R injury (Tang et al., 2021), suggesting that inhibition of USP7 may be a potential therapy for cardiovascular disease. Consistent with these reports, our data demonstrated that administration of USP7 inhibitor p22077 attenuated the Ang II-induced increase of the inflammation response marker (IL-1 β and IL-6) and the F4/80-positive macrophages (Figures 5A,E). Taken together, these results indicate that the USP7 inhibitor p22077 may modulate cardiac remodeling by targeting and stabilizing a series of substrate proteins, which play important roles cardiac hypertrophy, fibrosis, inflammation, oxidative stress, and the progression of heart failure (Figure 7).

There are several limitations in our study. 1) Our previous studies and present study have demonstrated that Ang II upregulated USP7 both in mRNA and protein levels in hypertrophic hearts (Figure 1) (Bi et al., 2020a). However, the upstream mechanism in Ang II-induced increasing expression of USP7 needs to be tested in the future. 2) NOX2 and NOX4 are the two NADPH oxidases in the heart, which are the major generators of ROS. Also, our studies demonstrated that p22077 could significantly down-regulate the protein level of NOX2 and NOX4 (Figure 6). Since both enzymes share sequence homology, and previous study demonstrated that USP7 could target and deubiquitylate NOX4 (Liu et al., 2020), whether USP7 could also target and deubiquitylate NOX2 and whether USP7 regulate the

location and the activation of NOX2 in the myocyte need to be studied in further.

Conclusion

The current study demonstrated that the deubiquitinase USP7 was upregulated in cardiac hypertrophy and the administration of its inhibitor p22077 attenuated Ang II-induced cardiac hypertrophy, fibrosis, inflammation, and oxidative stress, suggesting that USP7 might be a novel therapeutic target and a hypertrophy marker for cardiac hypertrophy and heart failure.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the First Affiliated Hospital of Dalian Medical University (PJ-KS-KY-2021-229, to X-HY). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Care and Use Committee of Dalian Medical University.

Author contributions

H-LB and H-HL conceived the project. Y-HG, K-WR, and YW performed the experiments and analyzed the data. S-HW, X-HY, and L-WX were responsible for human clinical studies and analyses. H-LB wrote the paper with input from all authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.1021361/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

The effect of p22077 on the expression of USP7 in Ang II-induced cardiac remodeling. (A) Representative immunoblotting analysis of USP7 and Tubulin in heart tissues from each group (left, $n = 6$). (B) Quantification of the relative protein levels (right, $n = 6$). The data are presented as the mean $\pm$ SD, and n represents the number of animals per group. ** $p < 0.01$ and NS, not significant.

SUPPLEMENTARY FIGURE S2

Administration of p22077 attenuates Ang II-induced inflammation. (A,B) Representative IHC and quantification of NLRP3-positive area ($n = 6$ mice per group). Scale bars, 50 μ m. The data are presented as the mean $\pm$ SD, and n represents the number of animals per group. ** $p < 0.01$.

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Supplementation with Tex261 provides a possible preventive treatment for hypoxic pulmonary artery hypertension

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Objectives: Pulmonary artery hypertension (PAH) is a serious disease for which there is no effective treatment. Its pathogenesis is complex and has not yet been clarified. Tex261 is a protein-coding gene whose functional enrichment nodes include the transporter activity of COP II. However, the role of Tex261 in PAH remains unknown.

Methods: Sugen5416/Hypoxic PAH models were established, and pulmonary arteries (PAs) were isolated for proteomic sequencing. The binding sites between Hif-1 α and Tex261 were verified by dual-luciferase reporter gene assay. Cell proliferation was detected by MTS and EdU assays. For determination of the preventive and therapeutic effects of Tex261, intratracheal instillation of adeno-associated virus (AAV6) with Tex261 vectors was performed.

Results: Tex261 was screened according to the proteomic sequencing data. Hif-1 α inhibited Tex261 promoter activity under hypoxia. Decreased Tex261 expression promoted PASMC proliferation. Tex261 regulated Sec23 via the Ndr1-mediated Akt pathway. Tex261 overexpression improved the pressure and vessel remodeling of PAs induced by Sugen5416/hypoxia.

Conclusion: Hypoxia suppressed Tex261 expression through Hif-1 α activation. The decreased Tex261 could promote Ndr1 and depress Akt activity and then inhibit Sec23 activity, which leads to cell proliferation and vessel remodeling. Elevated Tex261 has some preventive and therapeutic effects on rats with PAH.

KEYWORDS

pulmonary artery hypertension, smooth muscle cells, Tex261, Sec23, ndr1

1 Introduction

Pulmonary artery hypertension (PAH) is diagnosed based on a resting pulmonary artery pressure >25 mmHg. This condition is characterized by pulmonary artery hyperplasia and pulmonary artery remodeling (PAR), leading to right heart failure and even death (Mejía Chew et al., 2016; Simon and Hedderich, 2020). Abnormal proliferation of pulmonary artery smooth muscle cells (PASMCs) is the main cause of pulmonary vascular pressure increases and poor efficacy of drugs (Harding et al., 2003; Kim and George, 2019). Therefore, reversing PASMC proliferation is the key to the treatment of PAH.

Testis expressed 261 (Tex261) is a protein-coding gene whose functional enrichment nodes include coat protein II (COP II) transporter activity. Studies have reported that Tex261 may be involved in regulating excitatory toxic cell death induced by NMDA receptor activation (Miller et al., 2003). Tex261 shares homology with StAR, a steroidogenic acute regulatory protein, but may have different functions (Miller et al., 2003). Therefore, Tex261 is thought to be involved in the formation of COP II, mediating protein transport from the endoplasmic reticulum to the Golgi apparatus (Mosessova et al., 2003). Tex261, as a target gene of miR-28-5p, affects cell proliferation, survival, and apoptosis in PCa. However, the function and possible mechanisms of Tex261 in PAH are unknown.

In this study, significantly different Tex261 expression in Sugen5416/hypoxia induced PAH rats was identified by proteomic sequencing. We investigated the regulatory effect of hypoxia on Tex261 and its role in hypoxia-induced PASMC proliferation and further explored the underlying mechanisms, which will provide a new theoretical basis for the pathogenesis of PAH and identify potential therapeutic and preventive targets for PAH.

2 Materials and methods

2.1 Establishment of hypoxia models

Male SD rats were supplied by the animal center of Fujian Medical University. We used the animals under the guidelines and principles of the animal experiment committee of Fujian Medical University as well as the experimental program. For animal studies, all *in vivo* procedures were approved by the local authorities (Regierung von Unterfranken) and conformed to the guidelines of the European Parliament directive 2010/63/EU on the protection of animals used for scientific purposes. Rats (200 g) were randomly divided into the control group and hypoxia with Su5416 group (Sugen5416/Hypoxia (Su/Hx)). Su5416 (20 mg/kg, one time) was intraperitoneally injected (IP) before hypoxia. Rats were kept in a hypoxic (FiO_2 10%) or normal (FiO_2 21%) environment for 3 weeks (W). The right

ventricle pressure (PVP) was monitored through the right jugular vein cannula. The rats were killed by cervical dislocation after anesthesia (20% urethane, 5 mg/kg, IP), and then, the heart and lung tissues were collected. The left and right atria and blood vessels were cut off, and the right ventricle (RV) was separated from the left ventricle (LV) and the interventricular septum (S). These samples were dried and weighed separately, and then, the $\text{RV}/(\text{LV} + \text{VS.})$ was calculated as the right ventricular mass index (RVMI).

2.2 Tandem mass tags

The pulmonary arteries (PAs) of rats were quantitatively analyzed by TMT proteomics (LC-BIO Technologies (Hangzhou) Co., Ltd.). Based on criteria for screening differentially expressed proteins (fold-change >1.2 , $p < 0.05$), a total of 811 differentially expressed proteins were obtained: 356 were upregulated and 455 were downregulated. The detailed procedures were described in our previous study (Zhang et al., 2019). Heatmaps were obtained based on the heatmap function of R language. Moreover, the heatmaps of the top 20 upregulated and downregulated proteins with the most significant changes are shown (standardized heatmaps of hypoxia/control). In this study, the Tex261 protein ($p < 0.001$) was chosen for functional and mechanistic studies.

2.3 Hematoxylin-eosin staining and masson staining

Rats were anesthetized with 20% urethane (5 mg/kg, IP), followed by cervical dislocation. Lung tissues were collected, and the detailed procedures were described previously (Zhang et al., 2014; Zhang et al., 2021). Briefly, tissues were cut into 5 μm thick sections and fixed in 4% formaldehyde for 24 h. After the samples were embedded in paraffin and sectioned, the slides were subjected to HE staining and Masson staining according to the manuals. The morphology and collagen deposition of PAs were observed under a microscope. The vascular media thickness and collagen area were measured by Image-Pro Plus 6.0 software, and then the ratio of the wall thickness of PAs to the artery diameter, and the ratio of the collagen positive staining area (blue) to the total area were calculated. The data were used for statistical analysis.

2.4 Immunofluorescence

The sections were placed in 0.1 mol/L citrate repair solution ($\text{pH} = 6$), heated in microwave oven for 6 min at medium heat to slightly boiling, maintained at medium and low heat for 10 min, and cooled naturally for 20–30 min after stopping heating

(6 min $\times$ 4 times). The sections were blocked with serum for 30 min and then incubated with primary antibodies overnight at 4°C. The next day, the secondary antibodies were reacted for 1 h in the dark. After washing, DAPI was added for 10 min away from light. Finally, the slides were mounted with glycerol, and immediately observed under a fluorescence microscope.

2.5 *In situ* hybridization

For inactivation of endogenous enzymes, the slices were dehydrated and reacted with 3% H₂O₂ for 10 min, and then, 3% citric acid with freshly diluted pepsin was added and digested at 37°C for 30 min. After that, sections were washed and fixed with 1% paraformaldehyde, and then, 20 μ l of prehybridization solution was incubated at 38°C for 3 h. The solution was discarded, and 20 μ l of hybridization solution was added to each slice and incubated overnight at 38°C.

The targeted *TEX261* gene sequences were as follows:

5'-ACTACCTTGCAGAGCTGATTGAAGAGTACACGG
TGGCCAC-3'
5'-TCACCAACCTGGTCTACTTTGGCCTTCTCCAGA
CCTTCC-3'
5'-ACTTCACCAAAGGCAAGCGAGGCAAGCGCTTAG
GCATCCT-3'

The next day, sections were washed successively twice with 2xSSC, 0.5xSSC, and 0.2xSSC at 37°C for 15 min. The slides were reacted successively with blocking for 30 min, biotinylated mouse anti-digoxigenin for 60 min, strept avidin-biotin complex (SABC) for 20 min, and then biotinylated peroxidase for 20 min, at 37°C. After displaying color visualization with DAB, hematoxylin was counterstained before the sections were observed under a microscope.

2.6 Pulmonary artery smooth muscle cell culture

Pulmonary artery smooth muscle cells (PASMCs) were cultured by enzyme digestion (Wang et al., 2019; Zhang et al., 2021). Briefly, lung tissues were removed by thoracotomy after normal rats were anesthetized and then PAs were isolated under a microscope. The PAs were isolated and placed in the enzyme solution to digest for 30–40 min. The cell suspension was placed into a culture flask with 4 ml of DMEM containing 1% penicillin and 10% fetal bovine serum. After cells grew over 90% of the culture flask, they were passaged. Cells passaged 1–5 times were used for experiments. Before hypoxia, the culture medium was replaced with DMEM containing 5% FBS, and then the cells were placed under a 3% O₂ condition for 24–48 h.

2.7 Cell transient transfection

For silencing or overexpression of *Tex261*, PASMCs were transfected with RNAi or vectors, and the detailed processes are shown in our previous work (Cangul et al., 2002; Zhang et al., 2021).

2.7.1 Transfection of siRNA

Cells in six-well plates were replaced with DMEM before transfection. Lipofectamine RNAiMAX transfection reagent (Invitrogen) and siRNAs (IBSBIO, Shanghai) were diluted with DMEM. Five microliters of transfection reagent with 100 μ l of DMEM and 60 pmol of siRNA with 100 μ l of DMEM were mixed and placed for 20 min. Then, 200 μ l of the mixture was added to each well, mixed gently, and incubated at 37°C for 6 h. Then, the medium was changed, and the cells were cultured for another 24 h. The siRNAs and nontargeted control sequences with FAM tags were as follows:

Tex261-1:5'-GGCUGUCGUGUUAUCCAGG-3'
Tex261-2:5'-CCAGCAGAAUCAUAAAUAACA-3'
Tex261-3:5'-GCGAUGACGUGGUCUCAAUU-3'
Ndr1-1:5'-CGAAGACCACCCUACUCAAGA-3'
Ndr1-2:5'-GGUCUGUGAUAGCACGGAAAU-3'
Ndr1-3:5'-GGAUCUUGGAGUUGCUAGAGG-3'
NC control:5'-UUCUCCGAACGUGUCACGUTT-3'

2.7.2 Plasmid transfection

100 μ l strain was added to each tube and shaken overnight. Follow the instructions of the plasmid extraction kit. 10 μ l of plasmid was added with endonuclease reaction buffer, restriction nuclease and water, and placed at 37°C for 1–2 h for enzyme digestion. The digestion products were subjected to agarose gel electrophoresis to test whether the digestion was successful. The required fragment was cut down and connected with the carrier. The competent state was made according to the transformation steps, and the above linked products were tested and cultured on coated plates at 37°C for 12–16 h. The monoclones were selected and put into liquid medium containing LB and AMP, and the bacteria were shaken for 4–5 h. The products were detected by electrophoresis. 100 μ l of the correct band was extracted, added to LB, AMP + culture medium, shaken overnight, and took 1 ml solution for sequencing. The cloning vector was pcDNA3.1 (+). Cloning sites of *Tex261* was BamHI-EcoRI and *Sec23* was BamHI (GGATCC)-XhoI (CTCGAG). The plasmids and vectors were constructed by IBSBIO (Shanghai). Lipo6000 transfection reagent and plasmids were diluted with DMEM: 2.5 μ l transfection reagent with 125 μ l DMEM; 2.5 μ g vectors with 125 μ l DMEM. After remaining for 5 min, the two solutions were mixed gently and allowed to

stand for another 20 min. Then, 250 μ l of the mixture was added to each well, mixed gently and incubated at 37°C. Six hours later, the medium was changed, and the cells were cultured for 48 h.

2.8 MTS assay

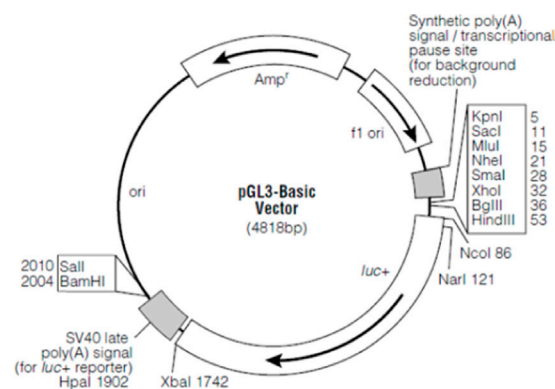
PASMCs in 96-well plates with different treatments, 100 μ l of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) mixture (20 μ l of MTS with 80 μ l of DMEM) per well were incubated for 1 h. Then, the absorbance value of the plate was read at 490 nm wavelength (Wang et al., 2021; Zhang et al., 2021).

2.9 EdU assay

PASMCs were incubated with 50 μ M 5-ethynyl-2'-deoxyuridine (EdU) for 2 h and then fixed with 4% paraformaldehyde. The cells were decolorized using 2 mg/ml glycine, and then incubated with 0.5% Triton X-100 for 20 min. After the cells were washed, they were reacted with click-iT EdU mixtures for 30 min and then incubated with Hoechst 33342 for 30 min in the dark. The images were taken with a fluorescence microscope (Zhang et al., 2018; Zhang et al., 2021). The ratio of EdU positive cells to Hoechst positive cells was calculated.

2.10 Dual-luciferase[®] reporter assay

The successfully constructed overexpression vectors were inoculated into 5 ml LB medium, and the plasmids were prepared by shaking (220 rpm) at a 37°C overnight, and the high purity plasmid extraction kit was used. Then, the reporter gene plasmids were transfected into HEK293 cells (DMEM 50 μ l, gene overexpression plasmids (OE/NC) 450 ng, promoter reporter plasmids (Pro/NC) 75 ng, pRL-TK 25 ng, HG transgene reagent 1.5 μ g), and the samples were collected. The samples were tested according to the instructions of the reporter gene detection kit (Dual-Luciferase[®] Reporter Assay System, Promega, USA). Briefly, the lysate was centrifuged at 13,000 rpm for 5 min, and the supernatant was pooled. 20 μ l samples were collected into a test tube, and then 20 μ l of firefly luciferase assay reagent was added and mixed well to measure the relative light units (RLUs). Cell lysis buffer was used as a blank control well. Then, 20 μ l of the prepared Renilla Luciferase Assay working solution was added to the tested sample, and the RLU was determined. The activation degree of the reporter gene was statistically analyzed. Pgl3-basic vector map and its vector information:



Pgl3-basic-Tex261 promoter WT: Cloning sites, KpnI (GGTACC)-XhoI (CTCGAG), Insert size, 612 bp;

Pgl3-basic-Tex261 promoter Mut: Cloning sites, KpnI (GGTACC)-XhoI (CTCGAG), Insert size, 604 bp;

pCDNA3.1-Hif1a: Cloning sites, BamHI (GGATCC)-XhoI (CTCGAG), Insert size, 2472 bp.

2.11 Real-time PCR

Total RNA from PAs and PASMCs was extracted by TRIzol, and then reverse transcribed into cDNA using a Roche Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). The fluorescence signal from SYBR Green combined with the newly synthesized double-stranded DNA was used for quantitative analysis (Roche, Germany). The primers used in this study are shown.

Tex261:sense, 5'-TTGATGAAGGAGAAGACAACCAGG ATG-3'
antisens, 5'-CCATGCAGCCAGGCGATGAC-3'
HIF-1a:sense, 5'-TGAGGCTGTCCGACTGTGAGTAC-3'
antisens, 5'-CACCGCAACTTGCCACCACTG-3'
 β -actin: sense, 5'-CCATCGGCAATGAGCGGTTCC-3'
antisense, 5'-CGTGTGGCGTAGAGGTCCTTG-3'

The specificity of the fluorescence signal was detected according to the melting curves. The data were obtained using a relative quantitative method (Zhang et al., 2018; Zhang et al., 2019).

2.12 Western blot

Total proteins were extracted from PAs of animal models and PASMCs with different treatments. RIPA (P0013B, Beyotime) contained PMSF (ST506, Beyotime) were added to lyse tissue or cells, and then centrifuged at 4°C, 14,000 rpm for 10 min. The

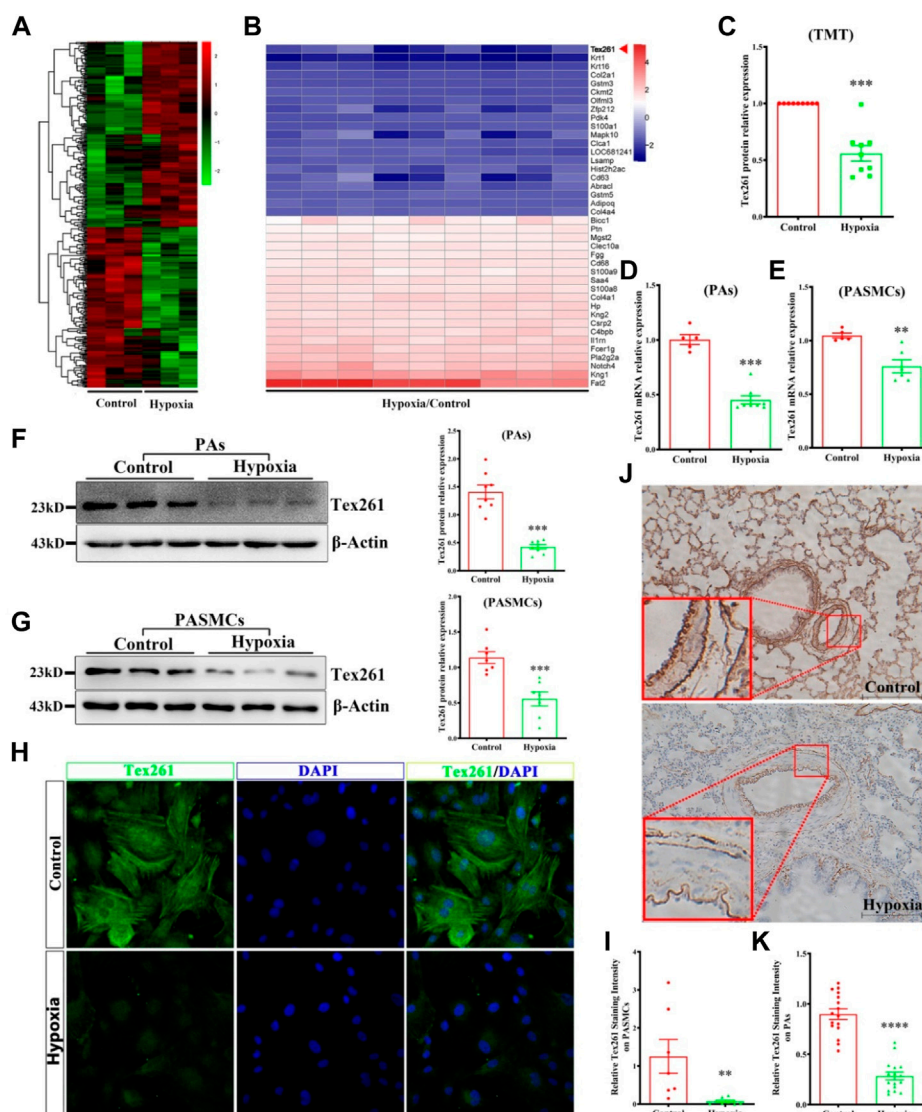


FIGURE 1

Tex261 is downregulated in hypoxic PAs and PSMCs. (A) Protein expression heatmap of hypoxia versus the control ($n = 15$): unsupervised hierarchical clustering analysis of the significantly dysregulated proteins. Red: upregulation; green: downregulation. (B) The heatmap shows the Top 20 of the significantly upregulated and downregulated proteins in the hypoxia group. (C) Tex261 protein relative expression chart in TMT data. (D,E) The mRNA levels of Tex261 in PAs and PSMCs were detected by real-time PCR ($n = 5-8$). (F,G) The protein expression of Tex261 in PAs and PSMCs was measured by Western blotting ($n = 7-8$). (H,I) The localization and expression of Tex261 in PSMCs were detected by immunofluorescence and observed under a $\times 20$ microscope. (J,K) Tex261 mRNA was measured through ISH and observed under a $\times 20$ microscope. The values are the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. the control.

supernatant was collected for Western blot experiments. 50 μ g samples were isolated with a 10% SDS polyacrylamide gel and then transferred to PVDF membranes. After blocking with 5% skim milk for 2 h, the membranes were incubated with primary antibodies against Tex261 (1:500, Novus), Hif-1a, PCNA, Ndgr1, p-Akt, Akt (1:500, Beyotime) and Sec23a (1:5000, Abcam) at 4 $^{\circ}$ C overnight. After secondary antibodies were incubated for 2 h, the blots were reacted with enhanced chemiluminescence (ECL) reagent (Thermo, United States) for 5 min. Exposure detection

was performed under a Biomolecular Imager. The relative optical density was analyzed, and β -actin was used as the control.

2.13 Intratracheal instillation of AVV6

2.13.1 Prevention models

Rats were randomly divided into four groups: control + AAV6-GFP, control + AAV6-Tex261-GFP, Sugen5416/

Hypoxia (Su/Hx)+AAV6-GFP, and Sugen5416/Hypoxia (Su/Hx)+AAV6-*Tex261*-GFP. AAV6 was intratracheally instilled first for 2 w and then subjected to hypoxia after Su5416 injection for 21 days to establish PAH models.

2.13.2 Treatment models

Rats were randomly divided into three groups: control + AAV6-GFP, Sugen5416/Hypoxia (Su/Hx)+AAV6-GFP, and Sugen5416/Hypoxia (Su/Hx)+AAV6-*Tex261*-GFP. For establishment of the PAH model, first, the rats were injected with Su5416 and exposure to hypoxia for 21 days, and then instilled with AAV6 and rose for another 2 w in a normal environment.

2.14 Data analysis

Data are expressed as the mean $\pm$ SEM, and statistical analysis and mapping of the data were performed using GraphPad Prism 5. *T* tests were used for the comparison of the mean between the two groups, and one-way ANOVA was used for the comparison of the mean among the groups. $p < 0.05$ indicated a statistically significant difference.

3 Results

3.1 *Tex261* is downregulated in hypoxic pulmonary arteries and pulmonary artery smooth muscle cells

Based on TMT data, 811 differentially expressed proteins were obtained according to the criteria (fold-change >1.2 , $p < 0.05$); 356 were upregulated and 455 were downregulated. The heatmap of the differentially expressed proteins is shown in Figure 1A. The top 20 upregulated and downregulated proteins are shown in Figure 1B. In this study, the *Tex261* protein was selected because it was significantly reduced in hypoxic PAs (Figure 1C). To verify the mRNA and protein expression of *Tex261* in both PAs and PSMCs after hypoxia, we used real-time PCR and Western blotting, respectively. As shown in Figures 1D–G, hypoxia indeed reduced the mRNA and protein expression levels of *Tex261* in both PAs and PSMCs. Moreover, cell immunofluorescence showed that the *Tex261* intensity was weakened in PSMCs after hypoxia and mainly existed in the cytoplasm, cytoskeleton and apparently stress fibers. (Figures 1H,I). *Tex261* appears to be seen in the medial layers of vessels, suggesting its expression in smooth muscle, observed by ISH (Figures 1J,K). These results indicated that *Tex261* was downregulated in both PAs and PSMCs under hypoxia.

3.2 *Tex261* is negatively related to hypoxia and regulated by Hif-1 α

To prove the relationship between *Tex261* and PAH, we established animal models with different hypoxia durations (0 W, 1 W, 2 W, 3 W). As shown in Figures 2A–C, RVSP and RVMI gradually increased with prolonged hypoxia times. We also isolated PAs to detect the mRNA levels of *Tex261*. The results showed that the *Tex261* mRNA levels decreased as the hypoxia time increased, as shown in Figure 2D. The correlations between *Tex261* mRNAs and RVSP or RVMI were analyzed. We observed that both RVSP and RVMI had a negative correlation with *Tex261* expression (Figures 2E,F). Furthermore, we found that the expression of Hif-1 α and *Tex261* had a negative correlation, as shown in Figure 2G. We examined whether *Tex261* is regulated by Hif-1 α . Overexpression of Hif-1 α with vectors and *Tex261* was detected in PSMCs. As shown in Figure 2H, elevated Hif-1 α depressed *Tex261* expression (Figure 2H). Moreover, Hif-1 α was silenced in PSMCs with siRNAs, and *Tex261* was detected in PSMCs after hypoxia. As shown in Figure 2I, Hif-1 α knockdown reversed the hypoxia-induced *Tex261* decrease. The JASPAR database (<http://jaspar.genereg.net/>) was used to predict the binding sites between Hif-1 α and *Tex261* (Figure 2J). The binding site was confirmed by luciferase reporter gene assay, as shown in Figure 2K. These results suggest that *Tex261* is involved in the development of PAH and is regulated by Hif-1 α under hypoxia.

3.3 *Tex261* regulates the proliferation of pulmonary artery smooth muscle cells

To observe the effect of *Tex261* on the proliferation of PSMCs, we knocked down *Tex261* expression, and the interference efficiency was verified, as shown in Figures 3A,B siR2-*Tex261* has the most obvious interference efficiency, so it was used in subsequent experiments. Cell viability was detected by MTS assays. As shown in Figure 3C, silencing *Tex261* enhanced the viability of PSMCs. PCNA expression was also significantly increased (Figure 3D). We also detected the cell proliferation rate after *Tex261* knockdown through EdU incorporation assays. The results showed that the proportion of proliferating cells was significantly increased after *Tex261* inhibition (Figures 3E,F). Correspondingly, cells overexpressed *Tex261* vectors, as verified in Figure 3G. Figure 3H showed that *Tex261* overexpression inhibited cell viability caused by hypoxia. Meanwhile, PCNA expression (Figure 3I) and cell proliferation (Figures 3J,K) were resumed after *Tex261* overexpression. These results suggest that *Tex261* regulates hypoxia-induced PSMC proliferation.

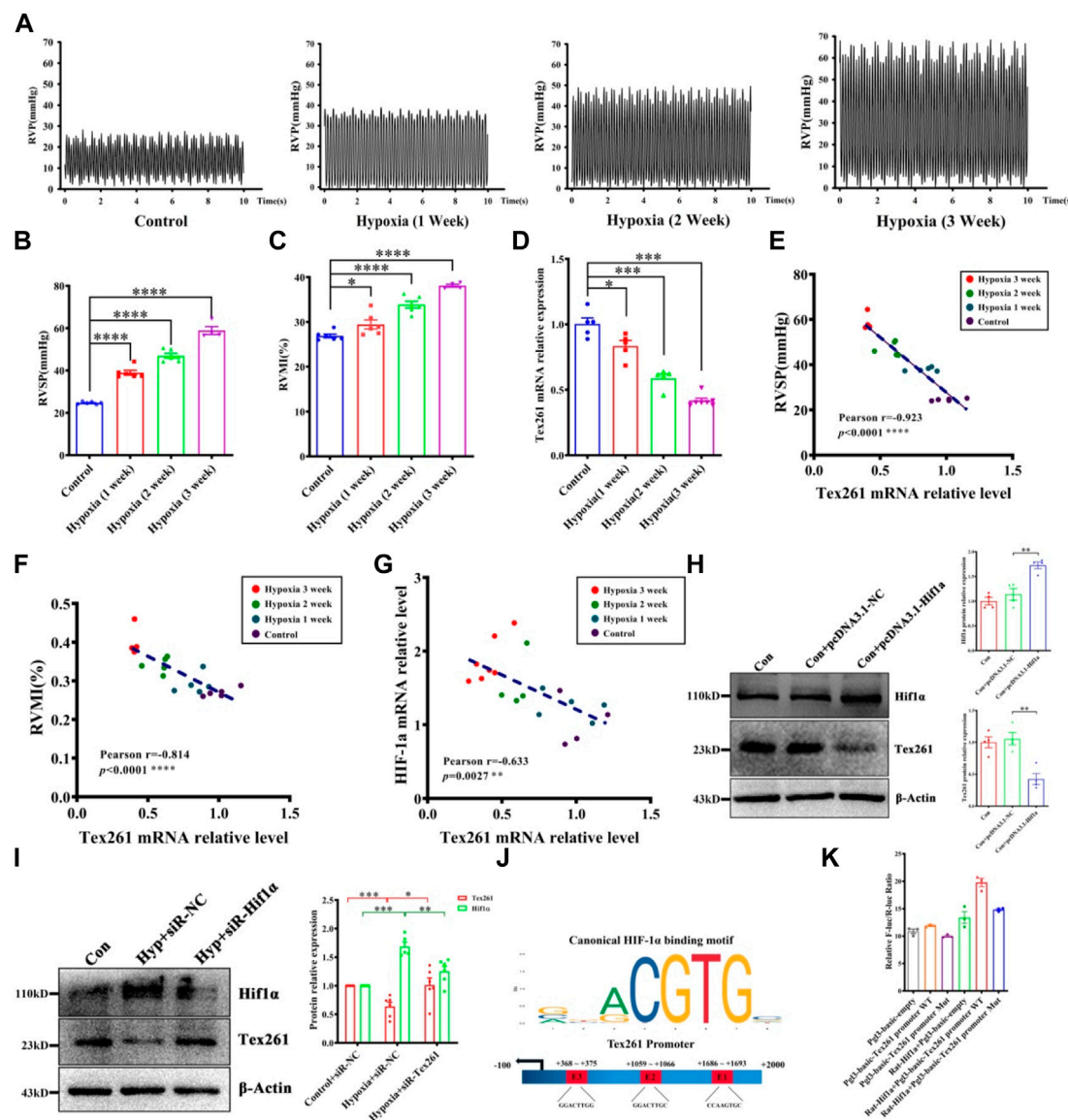


FIGURE 2

Tex261 is negatively related to hypoxia and regulated by Hif-1α. (A) Representative tracings of right ventricle pressure (PVP) recorded from different hypoxia time courses (0 W, 1 W, 2 W, 3 W). (B, C) The right ventricular systolic pressure (RVSP) and right ventricular mass index (RVMI) statistical graphs of different hypoxia time courses. ($n = 4-6$) (D) The mRNA levels of Tex261 in different hypoxia time courses ($n = 5-7$). (E, F) Correlation analysis between the mRNA levels of Tex261 and RVSP or RVMI. (G) Correlation analysis of the mRNA levels of Tex261 and Hif-1α. (H) Tex261 expression was detected after Hif-1α overexpression by Western blots ($n = 4$). (I) Tex261 expression was detected after Hif-1α interference by Western blots ($n = 6$). (J) Prediction of the binding sites between Hif-1α and Tex261. (K) Hif-1α binding with the promoter of Tex261 was identified by luciferase reporter gene assays ($n = 3$). The values are the mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ vs. the control. ** $p < 0.01$ vs. con + pcDNA3.1-NC. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. the control + siR-NC or hypoxia + siR-NC.

3.4 Sec23 is involved in Tex261-induced cell proliferation

Studies have shown that Tex261 is involved in the formation of COP II (Koyama et al., 2014). Through prediction, we found that Tex261 may interact with Sec23. Therefore, this study investigated the possible role of Sec23 in Tex261-induced cell

proliferation. As shown in Figures 4A,B, Sec23 expression was decreased in both hypoxic PAs and PSMCs. After silencing of Tex261, Sec23 was decreased, while after Tex261 overexpression, Sec23 was increased in PSMCs (Figures 4C,D). The efficiency of Sec23 plasmid transfection is shown in Figure 4E. Overexpression of Sec23 inhibited hypoxia-enhanced cell viability and proliferation (Figures 4F-H), and also PCNA

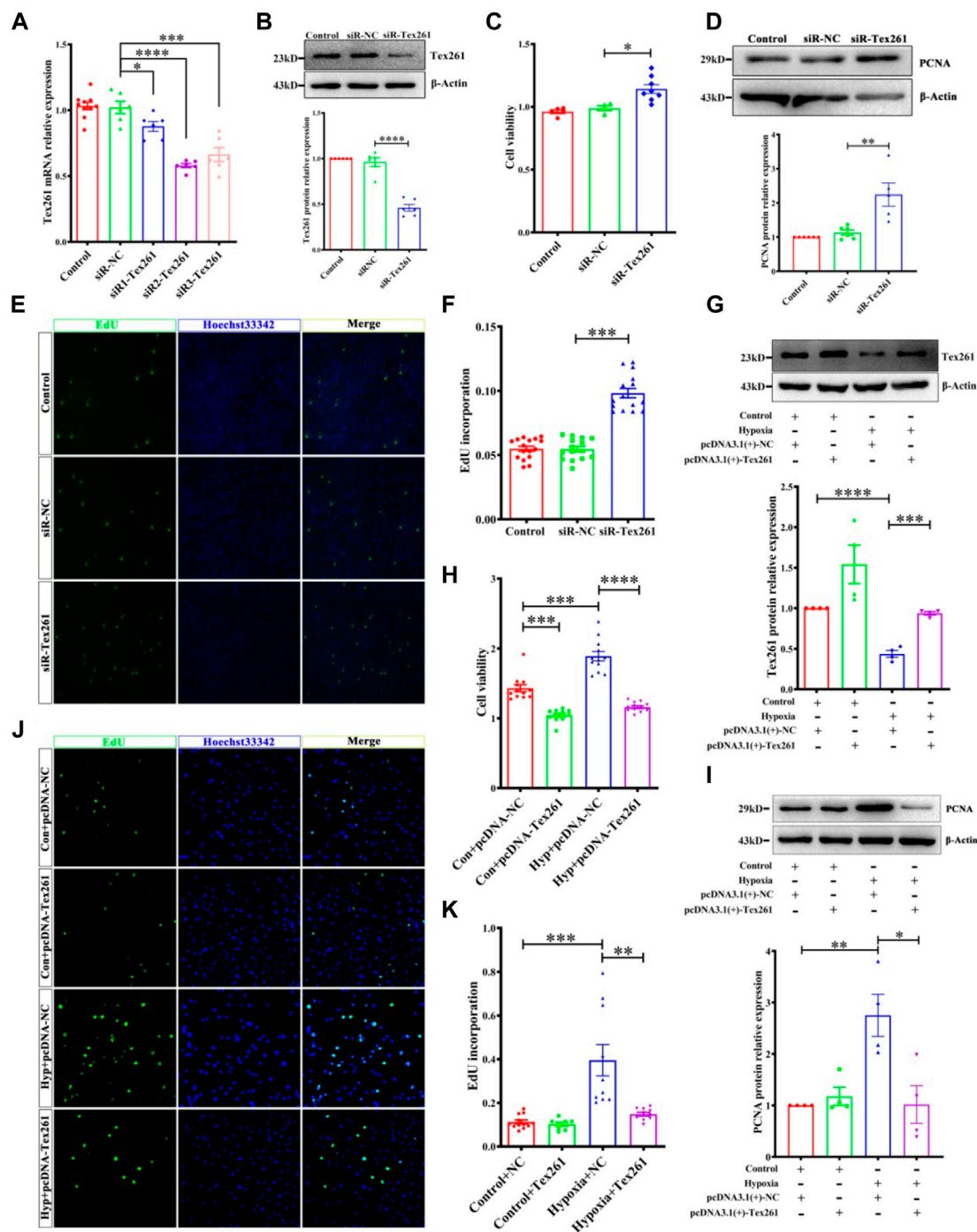


FIGURE 3

Tex261 regulates the proliferation of PSMCs. (A,B) Cell knockdown of Tex261 with siRNAs was verified in PSMCs ($n = 6$). (C) Cell viability was detected after transfection with siR-Tex261 or siR-NC in PSMCs by MTS assays ($n = 4-8$). (D) PCNA expression was measured after Tex261 silencing ($n = 6$). (E,F) The proportion of PSMC proliferation after Tex261 silencing was detected by EdU assays and observed by x20 magnification ($n = 6$). (G) Western blot results showed Tex261 overexpression by transfection with plasmids in PSMCs ($n = 4$). (H) Cell viability was detected after Tex261 overexpression by MTS. (I) PCNA expression was measured after Tex261 overexpression ($n = 4$). (J,K) The proportion of PSMCs proliferating was detected by EdU assays and observed by x20 microscope. Data are presented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. siR-NC. *** $p < 0.001$ and **** $p < 0.0001$ vs. the control + pcDNA-NC or hypoxia + pcDNA-NC.

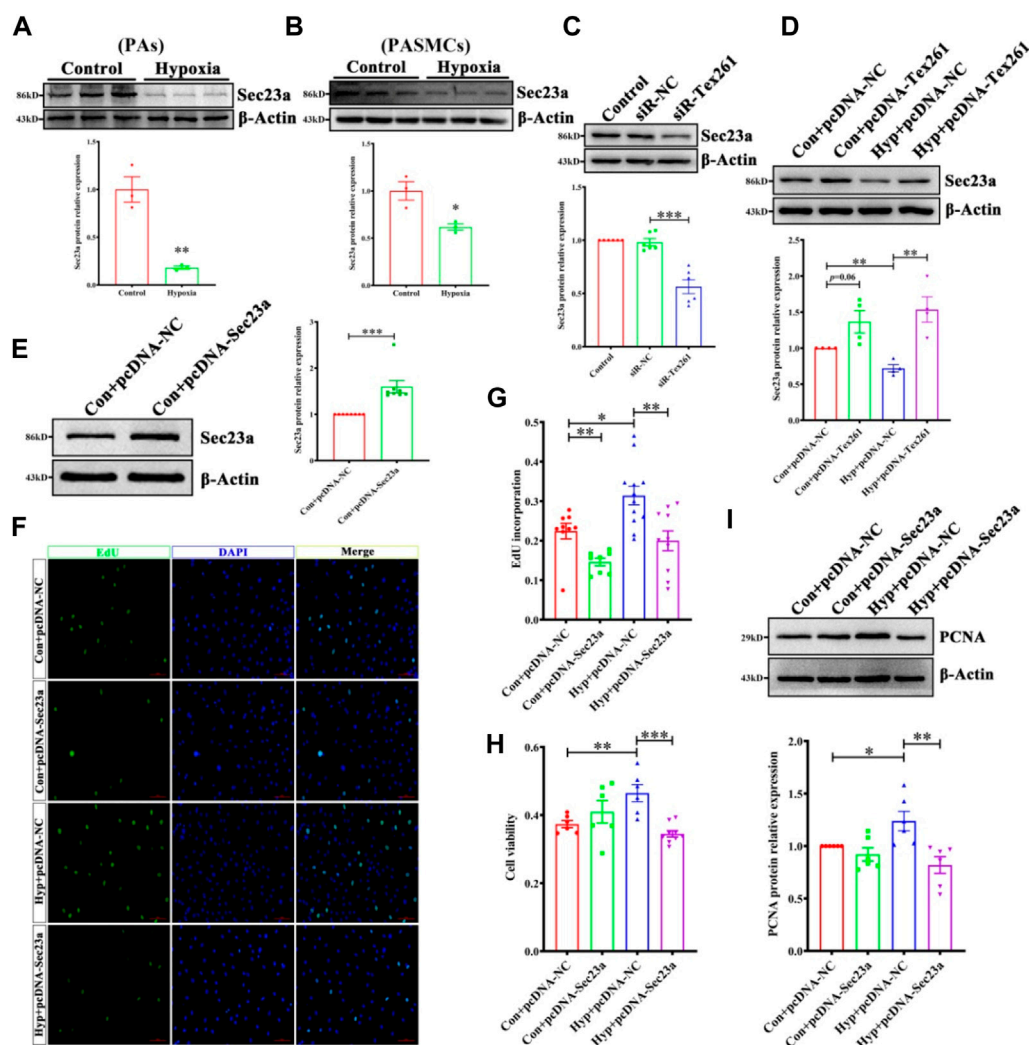


FIGURE 4

Sec23 is involved in Tex261-induced cell proliferation. (A,B) Sec23 expression was detected in hypoxic PAs and PSMCs ($n = 3$). (C) After Tex261 inhibition, Sec23 expression in PSMCs was detected by Western blots ($n = 6$). (D) After Tex261 overexpression, Sec23 was detected by Western blots ($n = 4$). (E) Western blot results showed that Sec23 increased after transfection with plasmids in PSMCs ($n = 8$). (F,G) The proportion of PSMCs proliferating was detected by EdU assays and observed by $\times 20$ magnification ($n = 6$). (H) Cell viability was detected after Sec23 overexpression by MTS assays ($n = 6$). (I) PCNA expression was measured after Sec23 overexpression ($n = 6$). Data are presented as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ vs. the control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. hypoxia + pcDNA.

expression (Figure 4I), suggesting that Sec23 was involved in Tex261-induced PSMC proliferation.

3.5 Tex261 regulates Sec23 through Ndrp1 mediated akt pathways

Ndrp1 may be a controversial factor in cancers (Bandyopadhyay et al., 2004; Wang et al., 2019). Whether Ndrp1 is regulated by Tex261 is unclear. First, we detected Ndrp1 expression after hypoxia. The data showed that hypoxia increased Ndrp1 in both PAs and PSMCs (Figures

5A,B). Moreover, Tex261 knockdown enhanced Ndrp1 expression, while Tex261 overexpression suppressed it (Figures 5C,D). When Ndrp1 was silenced, Sec23 expression increased, as shown in Figure 5E. Furthermore, Ndrp1 may regulate Akt pathway activity. In our study, hypoxia inactivated the Akt pathway (Figure 5F). Cells were pretreated with the Akt pathway agonist SC-97, and then silenced Tex261, Sec23 expression was resumed (Figure 5G). Cells pretreated with the Akt inhibitor MK2206 before Ndrp1 knockdown reversed Sec23 expression, as shown in Figure 5H. These results indicated that Ndrp1 mediated Akt is involved in Tex261 regulation of Sec23.

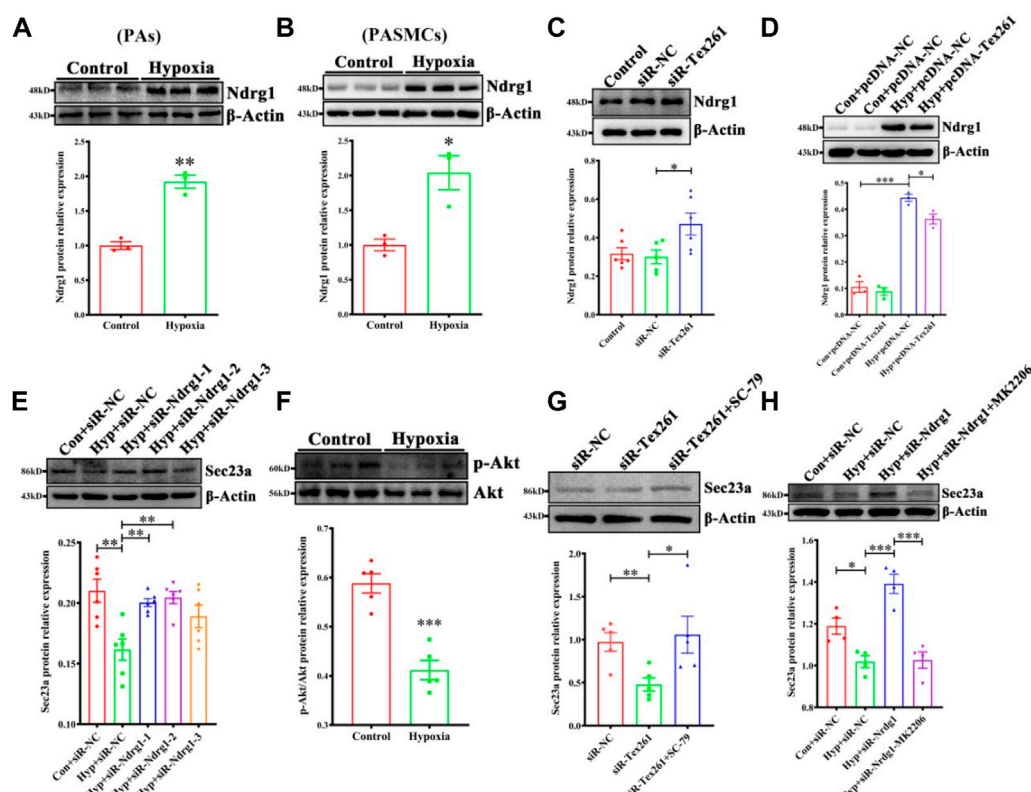


FIGURE 5

Tex261 regulates Sec23 through Ndrgr1 mediated Akt pathways. (A,B) Ndrgr1 expression was detected in hypoxic PAs and PSMCs ($n = 3$). (C) After Tex261 silencing, Ndrgr1 expression was detected in PSMCs by Western blots ($n = 6$). (D) After Tex261 overexpression, Ndrgr1 was measured ($n = 4$). (E) After Ndrgr1 knockdown, Sec23 expression was examined ($n = 6$). (F) The Akt pathway was detected after PSMCs were exposed to hypoxia ($n = 5$). (G) Cells were pretreated with the Akt agonist SC-79 before Tex261 knockdown, and Sec23 was then measured ($n = 5$). (H) Cells were pretreated with the Akt blocker MK2206 before silencing Ndrgr1, and Sec23 was then tested ($n = 4$). Data are presented as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ vs. control. * $p < 0.05$ and *** $p < 0.001$ vs. con + pcDNA3.1-NC. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. the control + siR-NC or hypoxia + siR-NC.

3.6 Preventive effect of Tex261 on rats with pulmonary artery hypertension

Tex261 was overexpressed by adeno-associated virus 6 (AAV6), and the construction mode is shown in Figure 6A. Two weeks after tracheal infusion of AAV6-Tex261-GFP or AAV6-GFP, rats were fed in a normal or hypoxic environment for another 3 weeks after Su5416 injection, to observe the preventive effect of Tex261 expression on PAH rats. The diagram of the specific animal experiment scheme is shown in Figure 6B. Lung infection with AAV6-GFP was observed through a frozen section scan. The images showed that AAV6 had been integrated into the lung tissues and expressed (Figure 6C). Then, RVSP was monitored, and RVMI was calculated. We found that the RVSP and RVMI of Sugen5416/hypoxic rats were significantly relieved after injection of the Tex261 virus (Figures 6D–F). Pulmonary vessel walls were thinned with AAV-Tex261 (Figures 6G,H). Artery fibrosis also

resumed after the Tex261 increase (Figures 6I,J). These results indicated that Tex261 could prevent PAH to some extent.

3.7 The therapeutic effect of Tex261 on rats with pulmonary artery hypertension

Rats were injected with Su5416 and then subjected to hypoxia for 3 W to establish PAH models, and then AAV6 w as injected and breasted for another 2 W, to observe the therapeutic effect of Tex261 expression on PAH rats. The diagram of the specific animal experiment scheme is shown in Figure 7A. Sugen5416/Hypoxia significantly increased RVSP and RVMI, while treatment with AAV6-Tex261 rescued them (Figures 7B–D). HE and Masson staining results showed that Tex261 overexpression alleviated PAR and fibrosis (Figures 7E,F). The results suggested that Tex261 may have a therapeutic effect on rats with PAH.

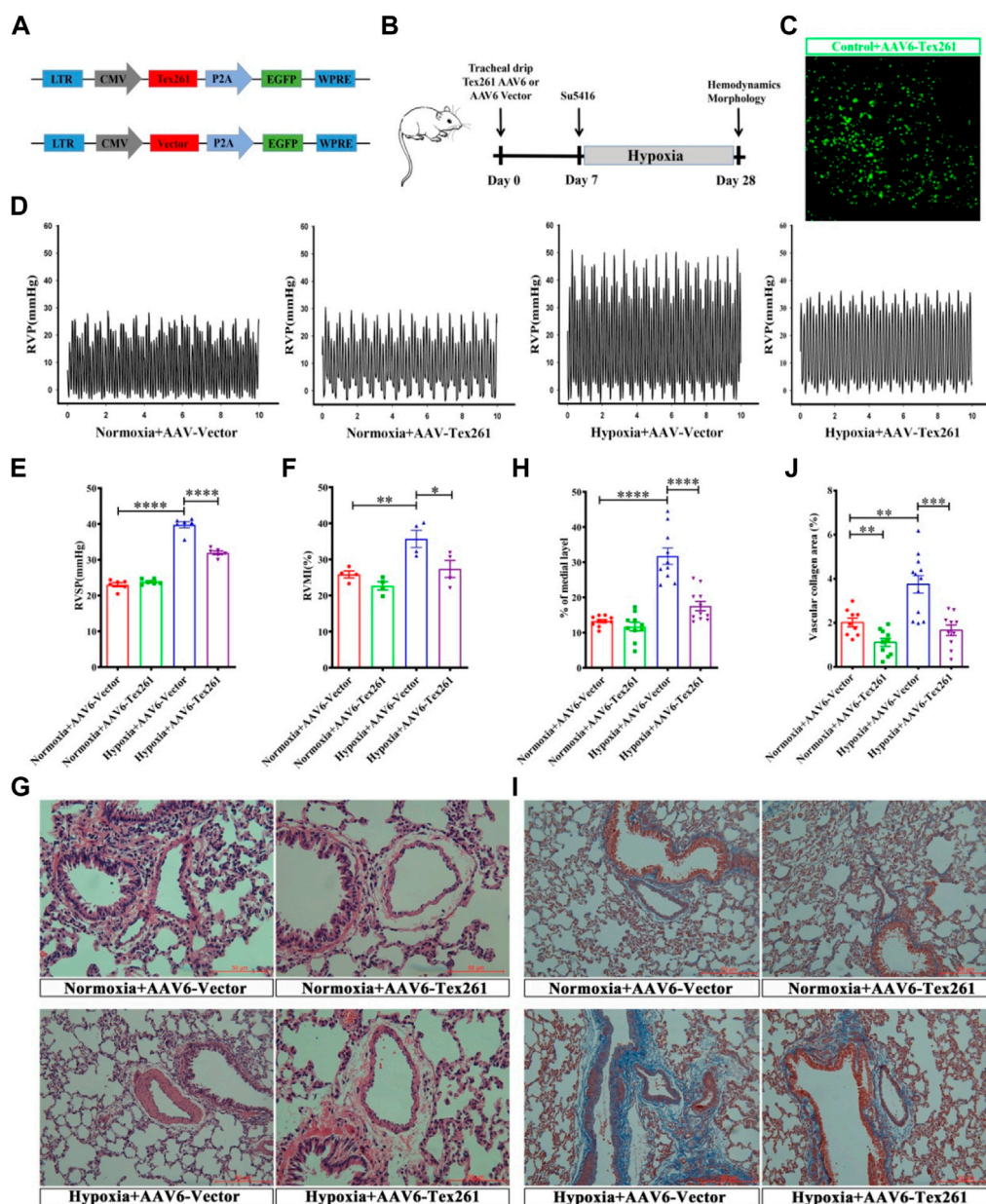


FIGURE 6

The preventative effects of Tex261 on rats with PAH. **(A)** Schematic diagram of the structure of the AAV6-Tex261-GFP shuttle. **(B)** Animal treatment protocol for the preventative effects of Tex261. After transfection of AAV6, hemodynamics and morphology were recorded after Su5416 administration at the beginning of hypoxia. **(C)** Verification of Tex261 transfection in lung tissues with immunofluorescence and observation by 10x confocal microscopy. **(D–F)** Representative tracings of RVP, RVSP and RVMI recorded from the control and hypoxic rats treated with AAV6-GFP and AAV6-Tex261-GFP, respectively. **(G,H)** PAR was observed through HE staining and observed by a x20 microscope. The PAR was calculated according to the average of the four diagonal thicknesses divided by the diameter of the vessel. **(I,J)** Vascular fibrosis was detected with Masson staining and observed by x20 microscopy. Data are presented as the mean $\pm$ SEM. $n = 6$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. hypoxia + AAV6-GFP.

4 Discussion

PAH is an extremely malignant cardiovascular disease with progressive PAR or occlusion, and eventually leads to death

(Southgate et al., 2020). Currently, the drugs for PAH treatment mainly aim to reduce the symptoms of patients and improve the quality of life of patients (Coons et al., 2019; Galiè et al., 2019). However, they cannot reverse the progression of the disease.

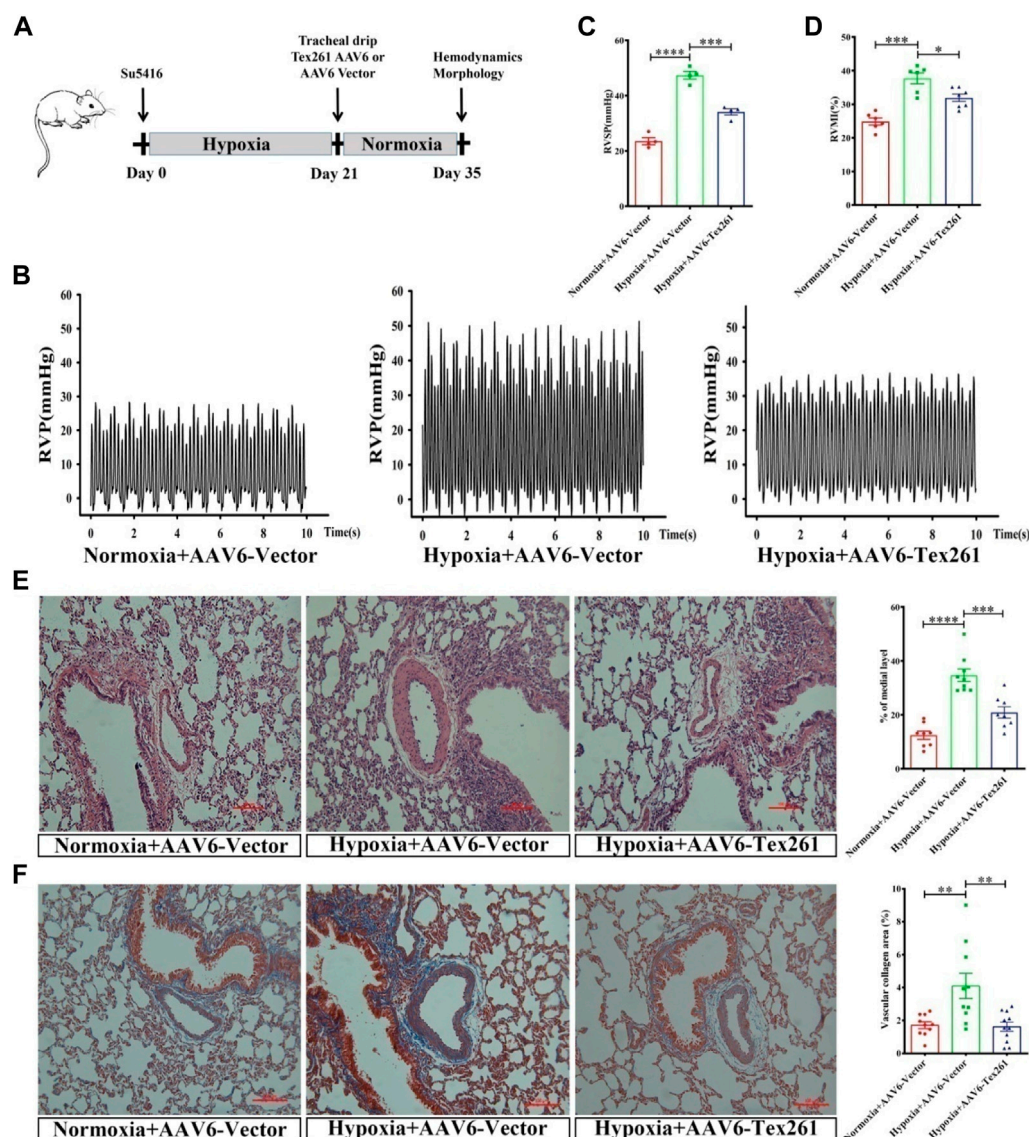


FIGURE 7

The therapeutic effect of Tex261 on rats with PAH. (A) Animal treatment protocols for the therapeutic effect of Tex261. After administration of Su5416, rats were exposed to hypoxia for 3 W to establish a PAH model, and then, AAV6 was injected and the animals were fed for 2 W. Hemodynamics and morphology were recorded. (B–D) Representative tracings of RVP, RVSP and RVMI recorded from the control + AAV6-GFP, hypoxia + AAV6-GFP, and hypoxia + AAV6-Tex261-GFP mice. (E) PAR was observed by HE staining and observed by a $\times 20$ microscopy. The PAR was calculated according to the average of the four diagonal thicknesses divided by the diameter of the vessel. (F) Vascular fibrosis was detected by Masson staining and observed by $\times 20$ microscopy. Data are presented as the mean $\pm$ SEM. $n = 4-6$, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs. hypoxia + AAV6-GFP.

Therefore, there is an urgent need to search for new targets for drugs. This study indicated that Tex261 was downregulated in both rats with PAH and PSMCs. Hif-1 α regulated Tex261 expression under hypoxia. Tex261 inhibited PSMC proliferation. Overexpression of Tex261 using AAV6 alleviated the increase in RVP and PAR caused by Sugeng5416/hypoxia, which may have certain preventive and therapeutic effects on rats with PAH.

Tex261 is mainly involved in the formation of COP II. COP II is known to mediate the transport of proteins from the endoplasmic reticulum to the Golgi apparatus (Mosessoova et al., 2003). Studies have found that Tex261 might regulate excitotoxic cell death induced by NMDA receptor activation (Miller et al., 2003). Tex261, as a target of miR-28-5p in prostate cancer, affects cell proliferation, survival and apoptosis (Rizzo et al., 2017). Moreover, the Tex261 family

contains many members, among which Tex9 is believed to cooperate with eIF3b to promote proliferation and inhibit apoptosis of esophageal squamous cells and carcinoma occurrence by activating the AKT signaling pathway (Burg et al., 2008). In this study, we found that Tex261 was significantly reduced in TMT sequencing data. To prove that there was no a concomitant change in PAH, we established different time points in hypoxic rats and detected the possible roles of Tex261 in the development of PAH. The results showed that Tex261 gradually decreased with prolonged hypoxia and increased PVP, suggesting that Tex261 may be a factor in the onset of PAH. Moreover, decreased Tex261 could regulate PASM C proliferation and participate in PVR. Proliferating smooth muscle cells could migrate to intima and participate in intima remodeling as reported. We are also checking the effects of Tex261 on the migration, phenotype and apoptosis of PASM Cs, which is a follow-up work of this study and are thinking to include those data in their next paper. Furthermore, we also found that overexpression of Tex261 alleviated fibrosis, and the possible mechanism was that Tex261 has a certain regulatory effect on adventitia as we observed it was decreased after hypoxia in Figure 1J.

Hif-1 α is closely related to PAH, and may be a driving factor upstream of PAH (Kojima et al., 2019; Luo et al., 2019). How hypoxia inhibits Tex261 expression has not yet been reported. We found that the expression of Hif-1 α and Tex261 was negatively correlated under hypoxia. The JASPAR database was used to predict the binding sequence sites of the Hif-1 α and Tex261 promoters. The results also verified that Hif-1 α regulated the promoter activity of Tex261 and suppressed Tex261 expression.

Tex261 is closely related to COP II vesicle transport, and Sec23 is one of the important components of COP II. Reports have shown that the abnormal expression and mutation of Sec23 cause various diseases (Boyadjiev et al., 2006; Lang et al., 2006; Fromme et al., 2007). Sec23 is a direct target of miR-200c, which mediated the secretion of metastasis inhibitory proteins such as IGFBP4. Sec23 down-regulation was closely related to proliferation and metastasis (Long et al., 2013). Overexpression of miR-21 inhibited Sec23 and promoted the proliferation, migration and invasion of DLD-1 cells. The same results were obtained by knocking out Sec23 in DLD-1 cells (Yang et al., 2013). In LNCaP and DU145 CaP cells, Sec23 overexpression suppressed cell growth, while Sec23 inhibition promoted cell proliferation (Jones et al., 2003). We observed that Sec23 was reduced after hypoxia and participated in the regulation of PASM C proliferation and PAR.

Ndr g1 is a component of ER stress because it is very sensitive to the redox state of cells and intracellular calcium concentration (Okuda et al., 2005). Ndr g1 is involved in the induction of ER-induced partners, which

may itself be a companion protein or a target of these partners (Kokame et al., 1998; Lan Chun Tu et al., 2007). Ndr g1 belongs to a new protein family that does not contain protein motifs with known functions (Zhou et al., 2001). Studies have shown that Ndr g1-interacting proteins contain Sec23a through LC-MS/MS³¹, but their role has not been reported in PAH and other diseases. Therefore, we tested whether Sec23 was regulated by Tex261 and mediated by Ndr g1. We found hypoxia increased Ndr g1 and was regulated by Tex261, while Ndr g1 knockdown restored Sec23 expression. The results seemed contradictory with others (Lan Chun Tu et al., 2007). Reports have shown that Ndr g1 is increased in liver cancer, kidney cancer and other solid cancers, but it is decreased in colon, nervous system and other tumors (Melotte et al., 2010). Sec23 was reduced in PASM Cs after hypoxia, so we speculated that Ndr g1 may not directly bind to Sec23 in PASM Cs. This molecular may regulate Sec23 in other ways. To explore this problem, we focused on the Akt pathways because they play an important role in regulating cell functions. Studies have reported that increased Ndr g1 suppresses angiogenesis *via* the PI3K/Akt pathways in human placental cells (Dai et al., 2020). Ndr g1 suppressed EMT through activation of the Wnt/ β -catenin signaling pathway (Chen et al., 2018). Ndr g1 was shown to modulate the Wnt- β -catenin pathway by inhibiting the nuclear translocation of β -catenin (Jin et al., 2014). Our results showed that hypoxia induced Akt inactivation, but treatment with Akt agonists or blockers reversed the change in Sec23 after Ndr g1 interference, indicating that Ndr g1 regulates Sec23 through Akt pathways. Moreover, Akt pathways were reported to play a role in PAH (de Jesus Perez et al., 2014; Li et al., 2020), and our results also showed that the pathways were involved in PASM C proliferation after hypoxia. Furthermore, we observed that Ndr g1 was enhanced and mainly expressed in the cytoplasm, while Sec23 was weakened, mainly in the nucleus after hypoxia stimulation through immunofluorescence, indicating that there were differences in expression in time and space between the two proteins. This finding may explain why there was no direct combination of Ndr g1 and Sec23 but indirect regulation of Sec23 by Ndr g1 through the Akt pathway in PASM Cs.

5 Conclusion

Hypoxia inhibits Tex261 expression through Hif-1 α , and lowered Tex261 promotes PASM C proliferation and PAH development through Ndr g1-Akt-induced Sec23 downregulation. Tex261 elevation alleviates PAH in rats. This study provides a new theoretical basis for elucidating the pathogenesis of PAH and potential treatment and prevention targets for PAH.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the animals under the guidelines and principles of the animal experiment committee of Fujian Medical University as well as the experimental program.

Author contributions

LZ: Conceptualization, writing-original draft preparation. SC: Methodology, investigation. XW: Investigation, data curation. XZ: Methodology, software. MY: Investigation. ZQ: Project administration. LC: Writing-reviewing and editing, funding acquisition. All authors were final approval of the version to be submitted.

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Conflict of interest

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Global trends and Frontier topics about vascular smooth muscle cells phenotype switch: A bibliometric analysis from 1999 to 2021

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Objective: Vascular smooth muscle cell phenotype switch (VSMCPS) plays a significant role in vascular remodeling. This study aimed to conduct a bibliometric analysis and visualize the knowledge map of research on VSMCPS.

Methods: We retrieved publications focusing on VSMCPS from the Web of Science Core Collection database (SCI-EXPANDED) from 1999 to 2021. Using bibliometric tools, VOSviewer and CiteSpace, we identified the most productive researchers, journals, institutions, and countries. At the same time, the trends, hot topics, and knowledge networks were analyzed and visualized.

Results: A total of 2213 publications were included in this analysis. The number of annual publications in the VSMCPS field exhibited an upward trend and could be roughly divided into three phases. Until 2006, the most prolific authors were from the United States. As of 2008, the number of articles published in China increased dramatically to reach 126 papers in 2020. As of 2014, China was the most productive country in this field. The United States ranked first in the number of highly-influential authors, institutions, and literature from 1999 to 2022. Owens GK, Hata, Akiko, and Wen, jin-kun were the most prolific authors. *Arteriosclerosis Thrombosis and Vascular Biology*, *Circulation Research*, and *Cardiovascular Research* were the top-ranked journals in this field. "Vascular remodeling," "atherosclerosis," "neointima," "hypertension", and "inflammation" were the main researched topics. New diseases, new mechanisms, and new phenotype (e.g., micro RNA, macrophage-like-cell, hypoxia, autophagy, long noncoding RNA, oxidative stress, endoplasmic reticulum stress, senescence, aging, abdominal aortic aneurysm, and aortic dissection) represent the trending topics in recent years.

Conclusion: This study systematically analyzed and visualized the knowledge map of VSMCPS over the past 2 decades. Our findings provide a comprehensive

overview for scholars who want to understand current trends and new research frontiers in this area.

KEYWORDS

vascular smooth muscle cells, phenotype switching, bibliometric, citespace, VOSviewer

1 Introduction

Vascular remodeling is an active process of structural alteration involving vascular wall thickening, thinning, and/or lumen stenosis as a result of endothelial injury, vascular smooth muscle cells (VSMCs) proliferation and migration, and extracellular matrix deposition (Galis and Khatri, 2002). It may result in atherosclerosis and/or stenosis, followed by myocardial infarction, stroke, etc. In healthy blood vessels, VSMC interact with the changing hemodynamics and provide structural and functional integrity to the vessel wall. When the endothelium is damaged, VSMCs are exposed to changes in growth factors (PDGF-BB, EGF, TGF, etc.), cytokines (IL-6, CSF, TNF- α , etc.), extracellular matrix (ECM) components, shear stress, oxygen free radicals, and other stimuli (Raines, 2000; Cordes et al., 2009; Vengrenyuk et al., 2015). This phenomenon induces VSMCs decreased expression of contractile proteins and myofilament density, increased expression of ECM components and ECM-remodeling enzymes, meanwhile enhancing the migration and proliferation abilities. This transition from a relatively quiescent contractile phenotype to an active synthetic phenotype is called VSMC phenotype switching (VSMCPS). Current evidence suggests that VSMCPS is involved in the initiation and progression of many cardiovascular and cerebrovascular diseases, such as atherosclerosis, in-stent stenosis, intracranial aneurysms, aortic aneurysms and aortic dissection (Alexander and Owens, 2012; Huang et al., 2020). It is widely thought that VSMCPS plays an important role during the initiation, development, and plaque formation of atherosclerosis (Ikari et al., 1999). At the early stage of atherosclerosis, VSMCs migrated from preexisting medial SMCs or SMC-like-cells derived from circulating blood in neointima tissues exhibit a proliferative and migrative state, decreased contractility and expression of smooth muscle markers, which accelerate the plaque formation (Owens et al., 2004; Shankman et al., 2016; Misra et al., 2018). During the late stages of atherosclerosis, VSMCs can switch to macrophage-like cells, foam cells, mesenchymal stem cell-like cells, extracellular-matrix-producing cells, and osteochondrogenic cells (Allahverdian et al., 2014; Jacobsen et al., 2017; Basatemur et al., 2019). Eventually, it may result in plaque calcification (Steitz et al., 2001; Naik et al., 2012). Indeed, many biological functions of VSMCPS and new therapeutic approaches targeting this process have significant value. In addition, clarifying the

current status and hot topics may help new researchers gain a quick overview of this area for future research design, given the huge volumes of data reported in the literature.

Bibliometric analysis is a big data-based scientific mapping method used to investigate the key contributors (e.g., country, institution, author, and journal) and identify the collaborative network between them in a given field. Additionally, it can identify the main Frontier research topics (Zhang et al., 2022a; Zhang et al., 2022b). Several tools, such as CiteSpace and VOSviewer, are popularly used for bibliometric analysis, primarily to explore the potential relationships between the published literatures and assess progress made in a particular field (Zhang et al., 2022a). Over the years, these two bibliometric tools have been widely used to predict hot topics and future research trends in several medical domains, such as cardiovascular diseases, ARDS, sepsis, host immune response, etc., (Chen, 2006; Avcu et al., 2015; Perianes-Rodriguez et al., 2016; Yao et al., 2020; Ma et al., 2021). To date, no bibliometric analysis of VSMCPS has been reported. Therefore, this study aims to analyze the publication trends and visualize the knowledge network of all publications regarding VSMCPS research. Importantly, this study's findings can help researchers quickly grasp current knowledge and identify emerging Frontier topics regarding VSMCPS.

2 Methods

2.1 Data sources

We searched the literature for publications related to VSMCPS from the Web of Science Core Collection database (SCI-EXPANDED). The following search terms and Boolean operators were entered for a database search: TS= (vascular-smooth-muscle-cell* OR vascular SMC* OR VSMC*) AND TS= (phenotyp* transform* OR phenotyp* switch* OR phenotyp* alter* OR phenotyp* chang* OR phenotyp* modulat* OR phenotyp* transition OR phenotyp* diversity) AND Article OR Review Article (Document Types) AND English (Languages). The time span was from 1999-01-01 to 2022-09-22.

2.2 Bibliometric analysis

After screening by titles and abstracts, we downloaded the retrieved publications as "Full Record and Cited

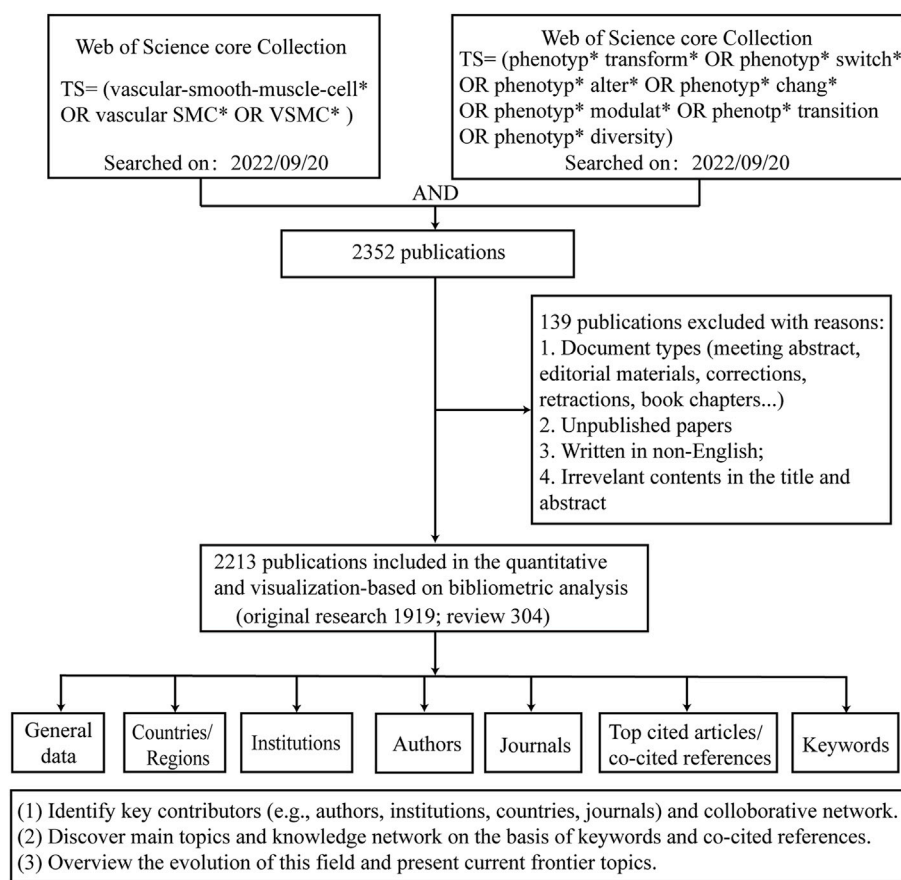


FIGURE 1

The workflow of data collection and bibliometric analysis.

References” and imported the search results into Excel 2010 to analyze the publication trend and major contributors (e.g., authors, institutions, countries, and journals). CiteSpace (Version 6.1. R1) and VOSviewer (version 1.6.11, Leiden University, Leiden, Netherlands) were used to construct the knowledge network, identify the collaborative network, the keyword co-occurrence network, and the co-citation references network. In VOSviewer and CiteSpace, the node size was positively related to the number of publications, and total link strength (TLS) was positively correlated with the cooperation strength.

3 Results

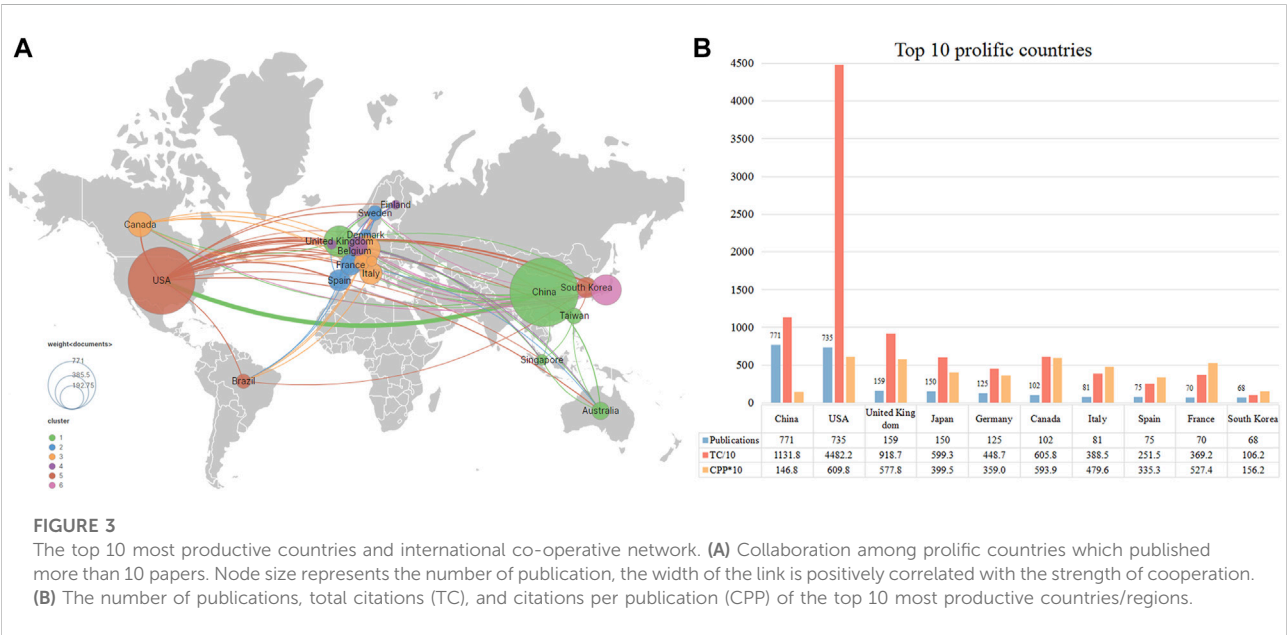
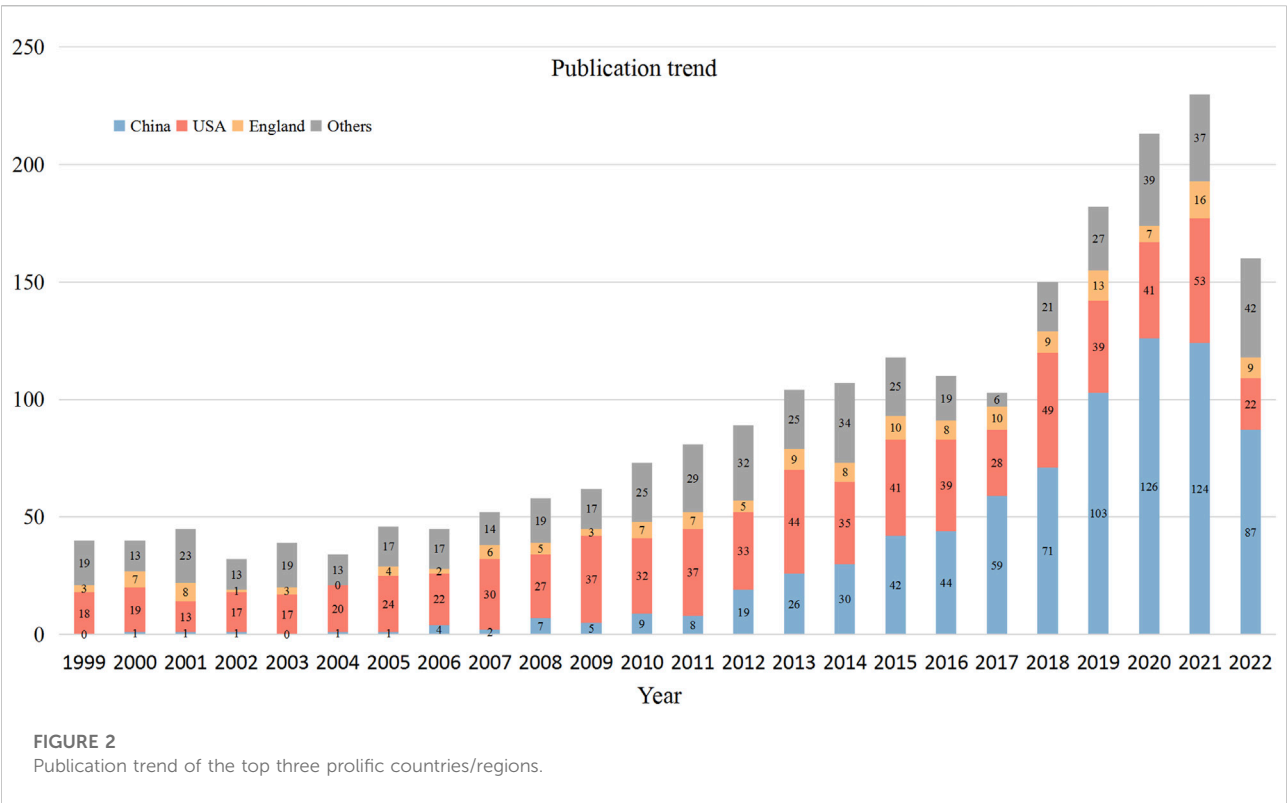
3.1 General data

Figure 1 shows the process of literature screening and bibliometric analysis. A total of 2213 publications were

retrieved and imported into bibliometric tools (VOSviewer and CiteSpace) for further analysis. The number of total citations (TC) was 82,487, and the number of citations per publication (CPP) was 37.27. Overall, a total of 65 countries/regions, 1,933 institutions, 11,912 authors, and 579 journals contributed to this field.

3.2 Publication trend

The number of annual publications in the VSMCPS field exhibited an upward trend and could be roughly divided into three phases. Phase I was from 1999 to 2006, with below 50 publications per year. Phase II was from 2007 to 2015, and the number of publications steadily increased from 50 to over 100 per year. Phase III was from 2016 to 2022, the number of publications slightly decreased in 2016 and 2017 and increased sharply after 2017 to over 200 publications per year. Figure 2 presents the annual output of the top three contributing countries.



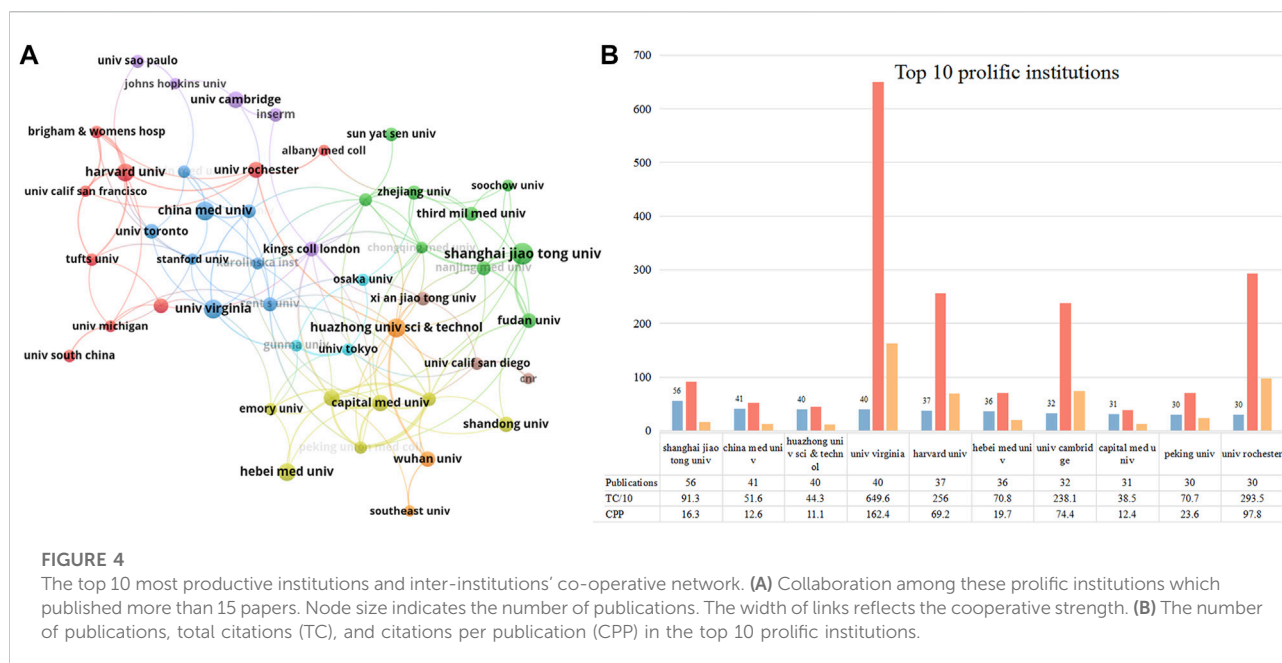


FIGURE 4

The top 10 most productive institutions and inter-institutions' co-operative network. (A) Collaboration among these prolific institutions which published more than 15 papers. Node size indicates the number of publications. The width of links reflects the cooperative strength. (B) The number of publications, total citations (TC), and citations per publication (CPP) in the top 10 prolific institutions.

3.3 Countries/regions

Figure 3A displays the international collaborative network formed by countries that published at least ten publications in this field. The United States and China were the most significant nodes with the largest number of publications. At the same time, China (TLS = 171) and the United States (348) were actively involved with other countries/regions. Figure 3B lists the top ten contributing countries in this field. China ranked first with 771 publications, followed by the United States (735 publications) and the United Kingdom (159 publications). The top three countries accounted for 75.23% of the total publications. Regarding citations, the United States hold the highest number of TC ($n = 44,822$) and CPP ($n = 60.98$). China ranked second in the number of TC ($n = 11,318$) but ranked 10th in terms of CPP ($n = 14.68$).

(Shanghai Jiaotong Univ, Capital Med Univ, and Heibei Med Univ). We used the timeline view in VOSviewer to visualize the activity of these institutions (Supplementary Figure S1). Institutions in the United States, the United Kingdom, and Japan started relatively early in this domain compared to China. Besides Shanghai Jiaotong Univ and Soochow Univ, other Chinese institutions became active after 2015. Figure 4B lists the top ten contributing institutions in this field. Shanghai Jiaotong Univ (56 publications) ranked first, followed by China Medical Univ (41 publications) and Huazhong Univ Sci&Tec (40 publications). Regarding citations, Univ Virginia scored the highest TC ($n = 6,496$) and CPP ($n = 162.4$). Univ Rochester ranked second with 2,935 TC and 97.8 CPP. Overall, of the top 10 most productive institutions, institutions from China had relatively few citations compared with those from other countries.

3.4 Institutions

Figure 4A presents the collaborative network between institutions that published more than 15 publications. Several clusters were formed, suggesting a closed cooperative relationship among them. Institutions in Cluster# red and blue were located in North America (e.g., Harvard Univ, Univ Rochester, and Univ Toronto). Institutions in Cluster# purple were located in Europe (Univ Cambridge, Kings Coll London, and Inserm). Institutions in Cluster# light blue were located in Japan (Osaka Univ, Gunma Univ, and Univ Tokyo). Institutions in Cluster# green, yellow, and brown were located in China

3.5 Authors

Figure 5A shows the collaborative network between authors who have published over six papers. The biggest cluster consisted of 46 authors, with frequent cooperation among them. Owens GK, Hata, Akiko, and Wen, jin-kun were the central nodes in the cooperative map. Similar to institutions, we used the timeline view in VOSviewer to visualize the activity of these prolific authors (Supplementary Figure S2). Most Chinese scholars were active after 2015 (e.g., Sun Yingxian, Yu Tao, and Zhang Jing). Owens GK, Zhang Wei, Fukuda N, Hata, and Akiko started exploring this field relatively early (around 2010). The top 12 prolific authors who published over ten papers in this field

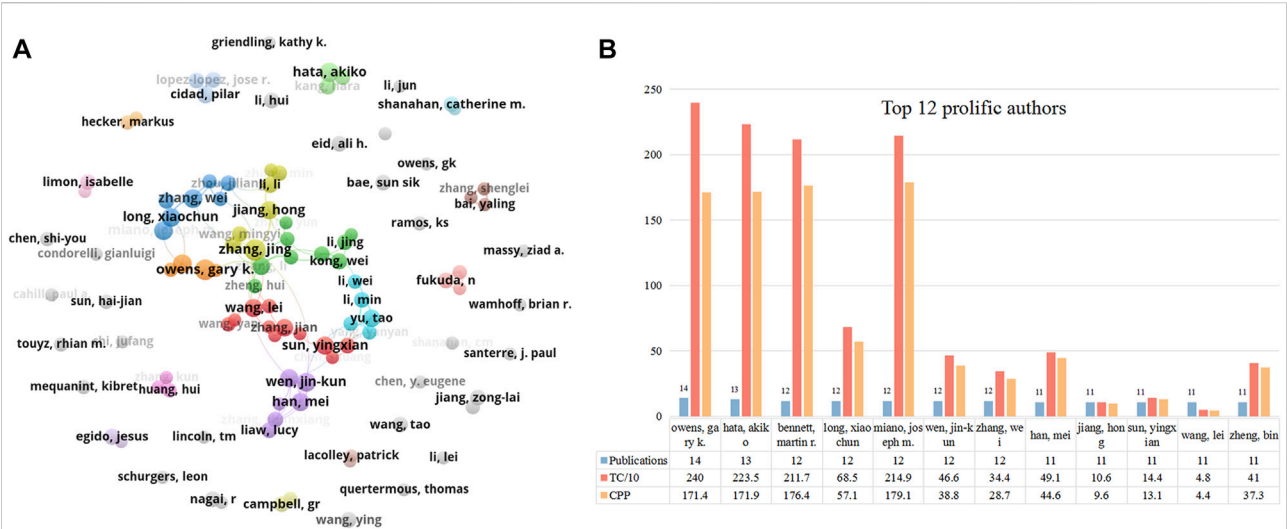


FIGURE 5 The top 12 most productive authors and collaboration network centered around these authors. (A) Collaboration among prolific authors who published more than 6 papers. Node size reflects the number of publications. The width of links represents the cooperative strength. (B) The number of publications, total citations (TC), and citations per publication (CPP) in the top 12 prolific authors.

TABLE 1 Top 10 prolific journals and co-cited journals.

Rank	Journal	Publications	TC	CPP	If (2021)	Co-cited journal	Co-citations	If (2021)
1	Arteriosclerosis Thrombosis and Vascular Biology	100	4977	49.8	10.5	Circulation Research	7444	23.2
2	Circulation Research	82	10169	124.0	23.2	Journal of Biological Chemistry	5741	5.5
3	Cardiovascular Research	55	4125	75.0	13.1	Arteriosclerosis Thrombosis and Vascular Biology	5737	10.5
4	Journal of Biological Chemistry	55	3329	60.5	5.5	Circulation	4030	39.9
5	Atherosclerosis	48	1493	31.1	6.8	PNAS	2569	12.8
6	Plos One	48	1609	33.5	3.8	Cardiovascular Research	2445	13.1
7	Biochemical and Biophysical Research Communications	43	943	21.9	3.3	Journal of Clinical Investigation	2307	19.5
8	International Journal of Molecular Sciences	35	473	13.5	6.2	Nature	2152	69.5
9	Scientific Reports	31	454	14.6	5.0	Cell	1577	66.9
10	Journal of Cellular Physiology	30	994	33.1	6.5	American Journal of Physiology-Heart and Circulatory Physiology	1541	5.1

Abbreviations: TC, total citations; CPP, citations per publication; IF, impact factors.

are listed in Figure 5B. Owens GK (University of Virginia, United States) ranked first with 14 papers, followed by Hata Akiko (University of California San Francisco) with 13 papers, and Bennett MR, Long Xiaochun, Miano, JM, Wen jin-kun, and Zhang wei with 12 publications. Regarding citations, Owens GK (2,400 TC, 171.4 CPP), Hata, Akiko (2,235 TC, 171.9 CPP), Bennett MR (2,117 TC, 176.4 CPP), and Miano JM (2,149 TC, 179.1 CPP) were the top four highest citation scholars in this area.

3.6 Journals

Table 1 lists the top 10 prolific journals and co-cited journals. Arteriosclerosis Thrombosis and Vascular Biology (ATVB) (IF (2021) = 10.5, Q1) was the most prolific journal, with 100 publications (4.5% of the total), followed by Circulation Research (IF (2021) = 23.2, Q1) with 82 publications, and Cardiovascular Research (IF (2021) = 13.1, Q1) with 55 publications. In terms of citations,

TABLE 2 Top 10 cited publications regarding VSMCPS.

Rank	Authors	Title	Document type	TC	Since 2013 usage count	Journal	Publication year
1	Owens, GK	Molecular regulation of vascular smooth muscle cell differentiation in development and disease	Review	2384	241	Physiol Rev	2004
2	Cordes, KR	miR-145 and miR-143 regulate smooth muscle cell fate and plasticity	Article	1201	132	Nature	2009
3	Jono, S	Phosphate regulation of vascular smooth muscle cell calcification	Article	1108	42	Circ Res	2000
4	Davis, BN	SMAD proteins control DROSHA-mediated microRNA maturation	Article	1039	98	Nature	2008
5	Griendling, KK	Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology	Review	753	34	ATVB	2000
6	Bennett, MR	Vascular Smooth Muscle Cells in Atherosclerosis	Article	751	186	Circ Res	2016
7	Johnson, RC	Vascular calcification - Pathobiological mechanisms and clinical implications	Review	674	98	Circ Res	2006
8	Rensen, SSM	Regulation and characteristics of vascular smooth muscle cell phenotypic diversity	Review	579	80	Neth Heart J	2007
9	Basatemur, G	Vascular smooth muscle cells in atherosclerosis	Review	564	228	Nat Rev Cardiol	2019
10	Cheng, YH	MicroRNA-145, a Novel Smooth Muscle Cell Phenotypic Marker and Modulator, Controls Vascular Neointimal Lesion Formation	Article	537	48	Circ Res	2009

Circulation Research ranked first (10,169 TC, 124 CPP), followed by *ATVB* (4,977 TC, 49.8 CPP) and *Cardiovascular Research* (4,125 TC, 75 CPP). However, it should be borne in mind that the number of publications may not represent its influence on a given field. Therefore, we used VOSviewer to identify the co-cited journals that frequently cited in the VSMCPS field. The top three co-cited journals were *Circulation Research* (7,444 co-citations), *Journal of Biological Chemistry* (5,741 co-citations), and *ATVB* (5,737 co-citations).

3.7 Top cited articles and co-cited references

To identify the most influential studies in this field, we extracted the top 10 most cited and most co-cited publications using VOSviewer and CiteSpace. Table 2 lists the top 10 most cited publications regarding VSMCPS, including five original articles and five reviews. The most cited paper (2,384 TC) was produced by Owens et al. (Owens et al., 2004) published in *Physiological Reviews* in 2004, entitled “Molecular regulation of vascular smooth muscle cell differentiation in development and disease”. In this review, the authors summarized the mechanisms that control the differentiated state of the VSMCs in normal conditions and how these regulatory processes are altered during the development of intimal lesions in atherosclerosis

or vascular injury. Additionally, three publications reported on the function of microRNA in VSMCs (Davis et al., 2008; Cheng et al., 2009; Cordes et al., 2009) and four on the role of VSMCs in atherosclerosis (Jono et al., 2000; Johnson et al., 2006; Bennett et al., 2016). Co-cited references are publications co-cited by at least two articles in a given field (Chen, 2006). Citation burst in CiteSpace was used to detect co-cited references that received relatively more attention from researchers during a specific time. Thirty seven co-cited references (more than 60 co-citations) were identified in Figures 6A,B, which showed the knowledge network evolution in this field. Papers that received more attention in the early years were colored dark blue, and publications primarily gained attention in the past few years were colored yellow. The publications with highest citation burst ($n = 21.48$) was Owens GK, 2004, *PHYSIOL REV*, V84, P767 (Owens et al., 2004). Publications with citation bursts ending in 2021 were as follows: Salabei JK, 2013, *BIOCHEM J*, V451, P375 (Salabei et al., 2013); Allahverdian S, 2014, *CIRCULATION*, V129, P1551 (Allahverdian et al., 2014); Feil S, 2014, *CIRC RES*, V115, P662 (Feil et al., 2014); Chistiakov DA, 2015, *ACTA PHYSIOL*, V214, P33 (Chistiakov et al., 2015); Shankman LS, 2015, *NAT MED*, V21, P628 (Shankman et al., 2015); Kapustin AN, 2015, *CIRC RES*, V116, P1312 (Kapustin et al., 2015); Vengrenyuk Y, 2015, *ARTERIOSCL THROM VAS*, V35, P535 (Vengrenyuk et al., 2015); Bennett MR, 2016, *CIRC RES*, V118, P692 (Bennett et al., 2016).

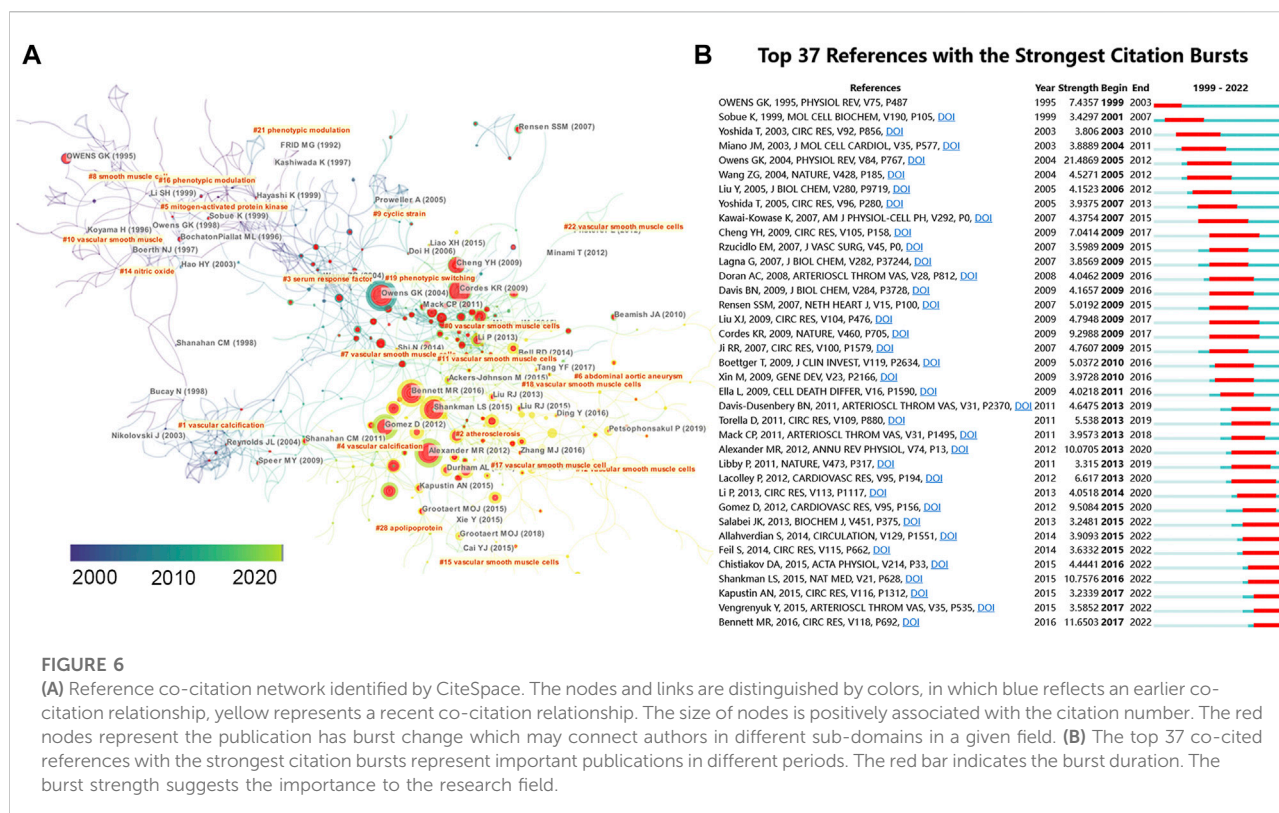


FIGURE 6

(A) Reference co-citation network identified by CiteSpace. The nodes and links are distinguished by colors, in which blue reflects an earlier co-citation relationship, yellow represents a recent co-citation relationship. The size of nodes is positively associated with the citation number. The red nodes represent the publication has burst change which may connect authors in different sub-domains in a given field. (B) The top 37 co-cited references with the strongest citation bursts represent important publications in different periods. The red bar indicates the burst duration. The burst strength suggests the importance to the research field.

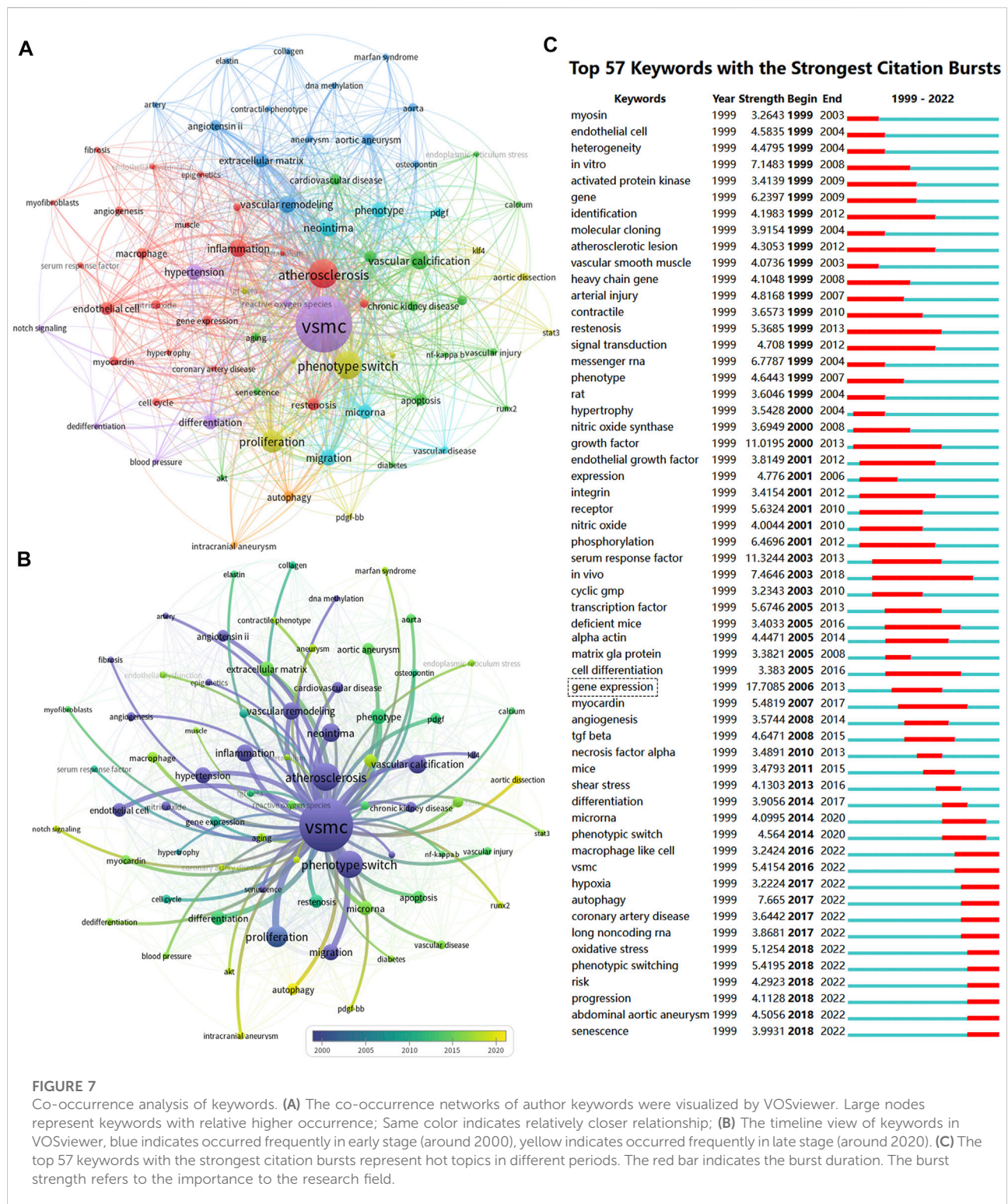
3.9 Keywords' evolution

Keyword co-occurrence analysis (VOSviewer) and keyword burst detection (CiteSpace) were used to identify research hotspots shifting and emerging topics. Keywords with similar meanings were merged, such as vascular smooth muscle cell, and vascular SMC were replaced by VSMC (Supplementary Table S1). Closely-related keywords were automatically identified by VOSviewer and labeled the same color. Nodes with larger sizes indicate keywords that occurred more frequently; lines with wider widths suggested they were linked closely together. As shown in Figure 7A, 73 keywords that appeared more than 10 times were grouped into six clusters. The top 10 keywords frequently used by scholars were “VSMC” ($n = 1003$), “atherosclerosis” ($n = 268$), “phenotype switch” ($n = 246$), “proliferation” ($n = 158$), “neointima” ($n = 114$), “vascular calcification” ($n = 111$), “inflammation” ($n = 100$), “vascular remodeling” ($n = 96$), “migration” ($n = 86$), “hypertension” ($n = 80$). Moreover, we used the timeline view of keywords to visualize the evolution of keywords in chronological order. In Figure 7B, keywords (e.g., vascular remodeling, vascular calcification, atherosclerosis, inflammation, and neointima) in dark blue implied that these topics were researched relatively early in this field. In contrast, keywords colored in yellow-green (e.g., apoptosis, aortic aneurysm, restenosis,

extracellular matrix, vascular injury, microRNA, reactive oxygen species, oxidative stress, autophagy, aortic dissection, intracranial aneurysm, aging, runx2, endoplasmic reticulum stress, macrophage) indicated that these topics gained more attention in recent years. Figure 7C lists the top 57 keywords with the strongest citation bursts in the field of VSMCPs. Red and blue represent active and inactive periods, respectively. Since 2014, keywords such as “micro RNA, macrophage-like-cell, hypoxia, autophagy, coronary artery disease, long noncoding RNA, oxidative stress, senescence, abdominal aortic aneurysm” were frequently used.

4 Discussion

In the era of big data, new researchers can quickly get insights into a particular research field through bibliometric analysis. However, no study has hitherto provided a comprehensive overview of research on VSMCPs, which is significant in understanding the pathogenesis of cardiovascular diseases. Therefore, in this study, we retrieved publications related to VSMCPs and identified the main contributors in this field. In addition, we visualized the evolution of hot topics in this field over the past 2 decades.



4.1 Current status and major contributing countries

The output of publications reflects the interest of researchers in a field (Durieux and Geveno, 2010). Overall, the number of publications in this field exhibited an upward trend. We found that until 2006, the number of publications was mostly less than 50. During this period, the most prolific authors were from the United States, with 20–30 publications (accounting for almost 50% of the total) per year before 2008. Since then, the number of publications from China has increased dramatically, from less than 10 before 2011 to 126 papers in 2020. In contrast, the annual publications from the United States stabled at 30 to 40 during this period. As of 2014, China became the most productive country in this field, as observed in the timeline view map of prolific authors and institutions (Supplementary Figure S1,2), where authors and institutions from the United States are colored dark blue, suggesting they were active relatively early in this field. Authors and institutions from China are colored yellow-green, indicating they were active in recent years. Moreover, the co-cited references analysis and co-occurrence keyword analysis showed that the frequency of keyword “gene expression” increased sharply after 2006 (the highest citation burst keyword with 17.7), and references with strong citation bursts were blossomed after 2007. This finding indicated that the VSMCPS field has flourished since 2007, which was validated in the publication trend map (Figure 2).

4.2 Active institutions and authors

To identify the most influential research teams, we used VOSviewer to identify the active institutions and authors, which may help scholars to choose potential partners in the VSMCPS field. Owens GK (University of Virginia, United States) was the most prolific and cited author. He and his colleagues summarized the mechanisms and regulatory processes of VSMCs in normal conditions and the formation of intimal lesions in atherosclerosis or vascular injury (Owens et al., 2004) in 2004. This study was the most cited paper and was the publication with the strongest citation burst (Table 2; Figure 6). Subsequently, they comprehensively reviewed and updated the significant role of VSMCs in various vascular diseases, including aortic aneurysm (Ailawadi et al., 2009), cerebral aneurysm (Starke et al., 2014) and atherosclerosis (Alexander and Owens, 2012; Bennett et al., 2016). New researchers should follow Owens GK and read these publications to obtain a theoretical basis in VSMCPS field. We also identified the most productive authors and institutions. There were six Chinese institutions, three United States institutions and one British institution in the top 10 prolific institutions. Moreover, there were seven active Chinese scholars (Wen jinkun, Zhang wei, etc.), four active United States scholars (Owens GK, Hata Akiko, Long

Xiaochun, Miano, JM) and one British author (Bennett MR) in the top 12 most productive authors. However, United States and British institutions and authors were associated with higher citations (TC and CPP) than Chinese ones. Several factors may explain China's contradiction between the quantity and quality of publications. First, compared with United States and British scholars, Chinese scholars explored this domain relatively late. In fact, China surpassed the United States in the number of publications for the first time in 2015, suggesting that Chinese publications may need more time to get cited. Besides, we found that the percentage of publications in *Circulation Research*, *ATVB*, and *Cardiovascular Research* (the highest citation journal) by authors from the United States and the United Kingdom were higher than their Chinese counterparts (Supplementary Table S2). Indeed, if Chinese scholars want to improve their influence in this field, they may deepen their research and choose more influential journals for their manuscript submission in the future.

4.3 Active journals and co-cited journals

Knowledge of the most prolific journals is essential to help researchers before submitting manuscripts, and top co-cited journals could be used as authoritative journals in a given field. *ATVB* was the most prolific journal with 100 publications, including 97 original research studies and seven reviews. *Circulation Research* was the second most prolific journal, including 75 original research studies and 7 reviews) with many citations (10,169 TC and 12.4 CPP). *Cardiovascular Research* ranked third with 55 publications (37 original research studies and 18 reviews), 4125 TC, and 75 CPP. To some extent, the relatively higher proportion of reviews in *Circulation Research* and *Cardiovascular Research* explained their greater number of citations.

4.4 Research hotspots and Frontier trend

The keyword co-occurrence analysis and co-cited references were used to visualize the knowledge network and Frontier research in a field (Chen, 2004). Over the past 20 years, the top ten frequently used keywords were “VSMC,” “atherosclerosis,” “phenotype switch,” “proliferation,” “neointima,” “vascular calcification,” “inflammation,” “vascular remodeling,” “migration,” “hypertension,” indicating that the mechanism of VSMCPS in vascular remodeling diseases (e.g., atherosclerosis, restenosis, and hypertension) was a major research hotspot.

We used the timeline view of VOSviewer and the keyword burst of CiteSpace to visualize the shifting of hot topics (Figures 7B,C). The research hotspots shifted from topics (e.g., vascular injury and restenosis, *in vivo* and *in vitro* models, activation,

transcription, inhibition, proliferation, apoptosis, gene expression, differentiation, and atherosclerosis) to topics regarding inflammation, macrophage, oxidative stress, autophagy, endoplasmic reticulum stress, aging, intracranial aneurysm, microRNA, and aortic dissection. CiteSpace's keyword burst analysis also yielded similar keyword shifts. Senescence, autophagy, oxidative stress, microRNA, macrophage-like-cell, and abdominal aortic aneurysms gained more attention in recent years. These new keywords are further discussed in the following section.

4.4.1 Emerging diseases

Over the years, many researchers have explored the function of VSMCs in common cardiovascular diseases, such as atherosclerosis (Bennett et al., 2016; Basatemur et al., 2019), hypertension (Lacolley et al., 2012; Touyz et al., 2018), in-stent stenosis (Wu et al., 2019). These diseases are characterized by artery injury, and thus arterial stiffness, neointimal formation, or stenosis. Corresponding drugs have been developed and used in clinics globally, including statins, anti-hypertensive drugs, and sirolimus. However, intracranial aneurysm, aortic dissection, aortic aneurysm, and Marfan syndrome, characterized arterial dilatation, often have poor prognoses due to the lack of efficient therapeutic drugs. Accordingly, researchers have focused on these diseases in recent years. Owens GK and his colleagues demonstrated that TNF- α could promote VSMCs pro-inflammatory/matrix-remodeling phenotype and accelerate the formation of intracranial aneurysms. Treatment with a TNF- α inhibitor *in vivo* could reverse aneurysmal change during the formation of intracranial aneurysms (Ali et al., 2013). Importantly, they summarized the function of VSMCs in intracranial aneurysm pathogenesis in 2014 and pointed out that targeting VSMCPs may retard the progression of intracranial aneurysm (Starke et al., 2014). Similarly, Rombouts et al. reviewed the function of VSMCs in aortic aneurysm and aortic dissection and pointed out that TGF- β signaling and its' regulatory RNA expression could modulate VSMCPs, and maybe potential targets for noninvasive AA and AD treatment options (Michel et al., 2018; Rombouts et al., 2022).

4.4.2 New phenotypes and mechanisms

The historical view of VSMCPs is that relatively quiescent VSMCs acquire the capability of hyper-proliferation and hyper-migration when spurred by various stimuli. However, the mechanisms underlying VSMCPs are largely unknown. Recently, researchers have shifted their focus to emerging phenotypes and new mechanisms. For example, Grootaert et al. reviewed the function of VSMCs in advanced atherosclerotic plaques and pointed out that defective VSMCs autophagy could accelerate the development of stress-induced premature senescence and atherogenesis (Grootaert et al., 2018). Starke et al. reported that cigarette smoke exposure initiated oxidative stress-induced VSMCPs and the formation and

rupture of intracranial aneurysm (Starke et al., 2018). Lu et al. (2021) summarized that the loss of VSMCs, VSMCPs, elevated reactive oxygen species, defective autophagy, and increased senescence could contribute to aortic aneurysm development. Additionally, non-coding RNA (e.g., microRNA, lncRNA, and circRNA) are also received much attention from researchers. Cordes et al. first demonstrated that miR-143 and miR-145 are cardiac and SMC-specific microRNAs. MiR-145 can control VSMCs fate, and miR-145 and miR-143 co-function to regulate VSMCPs. Song et al. (2018) reported that lncRNA MALAT1 regulates VSMCPs *via* activation of autophagy. Wang et al. reported that Hsa_circ_0031608 might be involved in the rupture of intracranial aneurysm *via* regulating VSMCPs (Wang et al., 2022). Arencibia et al. showed that lncRNAs regulate VSMCPs and participate in the pathological process of in-stent stenosis (Arencibia et al., 2022). Additionally, He et al. showed that circChordc1 could suppress vascular remodeling and reverse pathological aortic aneurysm progression through optimized VSMCPs and improved their growth (He et al., 2022). However, hitherto, no effective drugs targeting these non-coding RNA are available for clinical practice.

4.2.3 Cell-cell interactions

Macrophages and their interactions with VSMCs have become research hotspots in recent years. Pro-inflammatory (M1) and anti-inflammatory (M2) macrophages have been documented in various vascular diseases (Chen et al., 2020; Zhao et al., 2021). Barrett et al. reported that inflammation caused by the imbalance of M1/M2 might be a major factor affecting the VSMCPs and thus atherosclerosis plaque stability (Moore et al., 2013; Barrett, 2020). Moreover, VSMCs can switch to macrophage-like cells after cholesterol loading, and this alteration may play an important role in atherosclerosis advancement (Liu et al., 2017). In aneurysms, macrophages can infiltrate the aneurysm wall, secrete pro-inflammation factors, promote VSMCs apoptosis and death, weaken the vascular wall, and lead to the progression and rupture of the aneurysm (Hadi et al., 2018; Frosen et al., 2019). A study found that knock out of macrophage matrix metalloproteinases-9 can mitigate macrophage infiltration in blood vessels, reduce the abundance of inflammatory factors, and retard the formation and rupture of aneurysms (Longo et al., 2002). Additionally, VSMCs in neointima lesions could promote the maturation of macrophages and activate VSMCPs. Both processes can accelerate the progression of arterial stenosis after injury (Ostriker et al., 2014). However, the detailed crosstalk between macrophage and VSMCs remain to elucidate in the future.

4.2.4 New techniques

Recent single-cell sequencing and lineage tracing works have shown that VSMCs are more diverse than their previously recognized contractile or proliferative/synthetic phenotypes. VSMCs can adopt alternative phenotypes, including those resembling foam cells,

macrophages-like cells, mesenchymal stem cells and osteochondrogenic cells, which contribute positively and negatively to disease progression (Basatemur et al., 2019). Tang et al. conducted a lineage tracing with smooth muscle myosin heavy chain (as a marker) and found that in response to vascular injuries, multipotent vascular stem cells, instead of mature VSMCs, become proliferative and differentiate into VSMCs and chondrogenic cells, thus participating in vascular remodeling and neointimal formation (Tang et al., 2012). Using multicolor lineage labeling, Chappell et al. demonstrated that a low proportion of highly proliferative and plastic VSMCs rather than migration from nearby VSMCs could result in injury-induced neointimal lesions and progression of atherosclerotic plaques. Thus, further therapies could be developed targeting these hyperproliferating VSMCs without affecting vascular integrity to reduce vascular diseases (Chappell et al., 2016). Dobnikar et al. (2018) combined single-cell sequencing with lineage tracing to examine VSMCs heterogeneity in healthy mouse vessels. They found that a rare population of VSMC-lineage cells which express Sca1 (the multipotent progenitor marker), suggesting local VSMCs also have the ability of multilineage differentiation. It is worth exploring whether these markers could also be used as disease-relevant transcriptional signatures in VSMC-lineage cells, and be used for disease susceptibility, diagnosis and prevention in the future.

5 Limitations

Several limitations were found in this study due to the inherent nature of any bibliometric analysis. First, since the database updates, there may be incomplete literature retrieval, which may cause selection bias. Second, we only selected the Web of Science core collection-EXPANDED database as our data source. Indeed other databases, such as PubMed, Scopus, and Google Scholar, could also be used to increase the robustness of our findings. Third, only CPP and TC were used to measure the academic influence. G-index, H-index, SJR, CiteScore, and SNIP are also indicators of the quality of publications or journals. Fourth, our study only included English literature and excluded non-English publications. Finally, this study did not analyze or compare the funding of different studies.

6 Conclusion

This study analyzed and visualized the current status and research trends in the field of VSMCPS using VOSviewer and CiteSpace. Future research directions in this field include VSMCs function in dilated vascular diseases (aneurysm and aortic dissection), exploration of new mechanisms, new phenotypes and cell-cell interaction (e.g., micro RNA, macrophage-like-cell, hypoxia, autophagy, lncRNA, oxidative stress, endoplasmic reticulum stress, senescence, aging) and the implementation of

new techniques (single-cell sequencing and lineage tracing) for studying VSMCs function in various vascular diseases.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

YH and QZ conceived this study, participated in its design, and drafted the manuscript. LY and SL involved in study design, obtained data and contributed to interpretation, and helped to draft the manuscript. LX and CJ provided the theoretical frameworks, performed much of the editing of the manuscript and Funding acquisition. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.1004525/full#supplementary-material>

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A risk model developed based on necroptosis to assess progression for ischemic cardiomyopathy and identify possible therapeutic drugs

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Object: Ischemic cardiomyopathy (ICM), with high morbidity and mortality, is the most common cause of heart failure. Cardiovascular remodeling secondary to chronic myocardial ischemia is the main cause of its progression. A recently identified type of programmed cell death called necroptosis is crucial in the development of various cardiovascular diseases. However, the function role of necroptosis in cardiac remodeling of ICM has not been elucidated. Our study aimed to screen for genes associated with necroptosis and construct a risk score to assess the progression and evaluate the prognosis of ICM patients, and further to search for potentially therapeutic drugs.

Methods: The gene expression profiling was obtained from the GEO database. LASSO regression analysis was used to construct necroptosis-related gene signatures associated with ICM progression and prognosis. TF-gene and miRNA-gene networks were constructed to identify the regulatory targets of potential necroptosis-related signature genes. Pathway alterations in patients with high necroptosis-related score (NRS) were analyzed by GO, KEGG, GSEA analysis, and immune cell infiltration was estimated by ImmuneCellAI analysis. CMap analysis was performed to screen potential small molecule compounds targeting patients with high NRS. Independent risk analyses were performed using nomograms.

Results: Six necroptosis-related signature genes (STAT4, TNFSF10, CHMP5, CHMP18, JAK1, and CFLAR) were used to define the NRS, with areas under the ROC curves of 0.833, 0.765, and 0.75 for training test, test set, and validation set, respectively. Transcription factors FOXC1 and hsa-miR-124-3p miRNA may be regulators of signature genes. Patients with higher NRS have pathway enriched in fibrosis and metabolism and elevated nTreg cells. AZD-7762 may be an effective drug to improve the prognosis of patients with high NRS. A feature-based nomogram was constructed from which patients could derive clinical benefit.

Conclusion: Our results reveal 6 necroptosis gene signatures that can evaluate the progression and prognosis of ICM with high clinical value, and identify potential targets that could help improve cardiovascular remodeling.

KEYWORDS

necroptosis, ischemic cardiomyopathy, therapeutic drugs, cardiovascular remodeling, signature

1 Introduction

Ischemic cardiomyopathy (ICM) is among the most common causes of morbidity and mortality worldwide (Moroni et al., 2021). Ischemic cardiomyopathy is considered a special type of coronary heart disease (CAD) or a consequence of CAD in advanced stages. Its pathological process is long-term myocardial ischemia and hypoxia caused by atherosclerotic lesions (Panza et al., 2021). The severity of ischemic cardiomyopathy is mainly related to the grade of left ventricular (LV) dysfunction and the degree of coronary artery stenosis (Anversa and Sonnenblick, 1990).

Current diagnostic criteria for heart failure (HF) are based on the assessment of left ventricular ejection fraction (EF) which are the standard for grading patients with HF. Importantly, left ventricular systolic dysfunction is an independent predictor of adverse outcomes in patients with HF (Calderon-Dominguez et al., 2021). Increased mortality is strongly associated with reduced left ventricular ejection fraction during both hospitalized and readmitted patients. Randomized clinical trials have shown that severely reduced EF, below 15% at increased absolute risk of death due to arrhythmia and worsening HF (Gajanana et al., 2016). The prognosis of individuals with ICM can be determined with the aid of natriuretic peptides (NPs) and other indicators (Castiglione et al., 2022). However, these results lack specificity as there is a significant variability in NPs levels across the spectrum of HF (Volpe et al., 2016). Highly time-consuming imaging technique limited the assessment of the left ventricular systolic function in cardiac patients with LV dysfunction (Smiseth et al., 2016). Therefore, there is an urgent need to develop new, reliable tools for ICM risk stratification, which can guide the formulation of more effective and individualized therapeutic approaches for these patients.

Necroptosis is a type of non-apoptotic programmed cell death pathway, also called autophagy-induced cell death, which show have play an important role in the immune systems (Khoury et al., 2020; Bertheloot et al., 2021). A growing body of research has shown that necroptosis can trigger inflammation, which can contribute to the development of cardiovascular remodeling in ICM. This suggests that ICM may be diagnosed and treated by focusing on necroptosis (Piamsiri et al., 2021).

In this study, we performed a systematic bioinformatics analysis of ICM patients whose data were deposited in the

Gene Expression Omnibus (GEO) database. Screening of necroptosis-related prognostic genes by identifying differentially expressed necroptosis genes and least absolute shrinkage and selection operator (LASSO) regression. We constructed a necroptosis-related score to assess ICM progression and prognosis, and successfully divided ICM patients into two subtypes based on necroptosis-related scores. We explored differences between patients with high and low necroptosis-related score (NRS) in terms of pathway enrichment, immune cell infiltration, and identified targeted drugs for patients with high NRS. A nomogram was constructed to improve risk stratification for ICM patients.

2 Materials and methods

2.1 Data acquisition

The microarray data and clinical data of the GSE5406 dataset were obtained using the R package “GEO query” (Barrett et al., 2013). GSE5406 (Hannenhalli et al., 2006) containing 108 myocardial tissues from patients with ischemic cardiomyopathy and 16 non-failing donor myocardial tissues. The clinical data was obtained from Andreas et al. (Barth et al., 2011). The GSE57338 and GSE203160 datasets were used to validate the expression of the genes and the accuracy of the model.

2.2 Selection of necroptosis-related genes

Differentially expressed genes (DEGs) between ICM and NF were screened using the “limma” package, with an adjusted *p*-value of less than 0.05 as the threshold. Volcano and heat maps of DEGs were generated using the “ggplot2” package. Necroptosis-related genes were collected from the necroptosis pathway (hsa04217) from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Necroptosis-related differentially expressed genes (NRDEGs) were identified through an interactive website (www.interactivenn.net).

2.3 Screening of key genes

LASSO regression is used to select variables in linear regression by shrinking coefficient values and setting some

values to zero to improve prediction accuracy and data interpretation, which can improve data rationality and forecast accuracy. The “Caret” package was used to randomly allocated the samples into training and validation sets with a ratio of 7:3. LASSO regression was processed using the “glmnet” package, setting the observation value of patients with LVEF = 15% to 1 and patients with LVEF >15% to 0, and 21 necroptosis-related genes as independent variables to construct the correlation with LVEF genetic model. Receiver operating characteristic (ROC) curve and area under the curve (AUC) were used to evaluate the predictive effect of the model (Robin et al., 2011). The necroptosis-related score (NRS) was calculated using the following equation: $NRS = \sum(\beta_i * Exp_i)$ (β : coefficient, Exp : gene expression level). ICM patients were classified into low and high NRS subgroups based on the median NRS.

2.4 Functional enrichment analysis

The “limma” package was used to analyse the DEGs in patients with high and low NRS scores. The R package “clusterProfiler” was used to perform gene ontology (GO) and KEGG pathway enrichment analysis of DEGs. In addition, the “clusterProfiler” package was used to perform gene set enrichment analysis (GSEA) on potential mechanisms of c2 (c2. cp.v7.5.1. symbols.gmt) in the Molecular Signature Database (MSigDB) (Yu et al., 2012). Pathways with false discovery rates less than 0.05 were considered statistically significant.

2.5 Gene regulatory network analysis

We integrate information derived from two different databases (JASPAR and ChEA) for predicting gene interactions via Network Analyst v3.0 network tool (www.networkanalyst.ca). We took the intersections of TF-gene interactions obtained from JASPAR and ChEA. We used the miRTarBase, a database holding knowledge of miRNA targets from different organisms, to predict miRNA-gene interaction. The Cytoscape’s Cryotube plugin (Shannon et al., 2003) was used to provided topological analysis of this network and connectivity and retain the top 30 nodes of connectivity for further analysis.

2.6 Immune infiltration analyses

The Immune Cell Abundance Identifier (ImmuCellAI, bioinfo.life.hust.edu.cn/ImmuCellAI) (Miao et al., 2020) was used to calculate the immune cell abundance in myocardial tissue of ICM patients. ImmuCellAI is a gene

set signature-based immune cell abundance assessment method for accurate abundance estimation of 24 immune cell types (18 T cell subsets) from gene expression data. To find differences between immune cell infiltration levels between high and low NRS categories, the Wilcoxon rank sum test was performed. To investigate the relationship between important regulators and immune cells, Spearman correlation was used.

2.7 The connectivity map (CMap) analyses

The CMap (<https://clue.io/>) (Musa et al., 2018), a web-based database that exploits differences in gene expression following treatment of human cells with different disruptors to create a database of biological applications where small-molecule compounds, gene expression and disease are interrelated. CMap was used to interrogated the DEGs from patients with high and low NRS, and a correlation score was obtained based on the enrichment of DEGs in the reference gene expression profile. Enrichment scores >90 was considered promising candidate small molecule compounds, and the mode of action analysis (MoA) analysis was performed to predict their mode of action.

2.8 Establishment of a nomogram

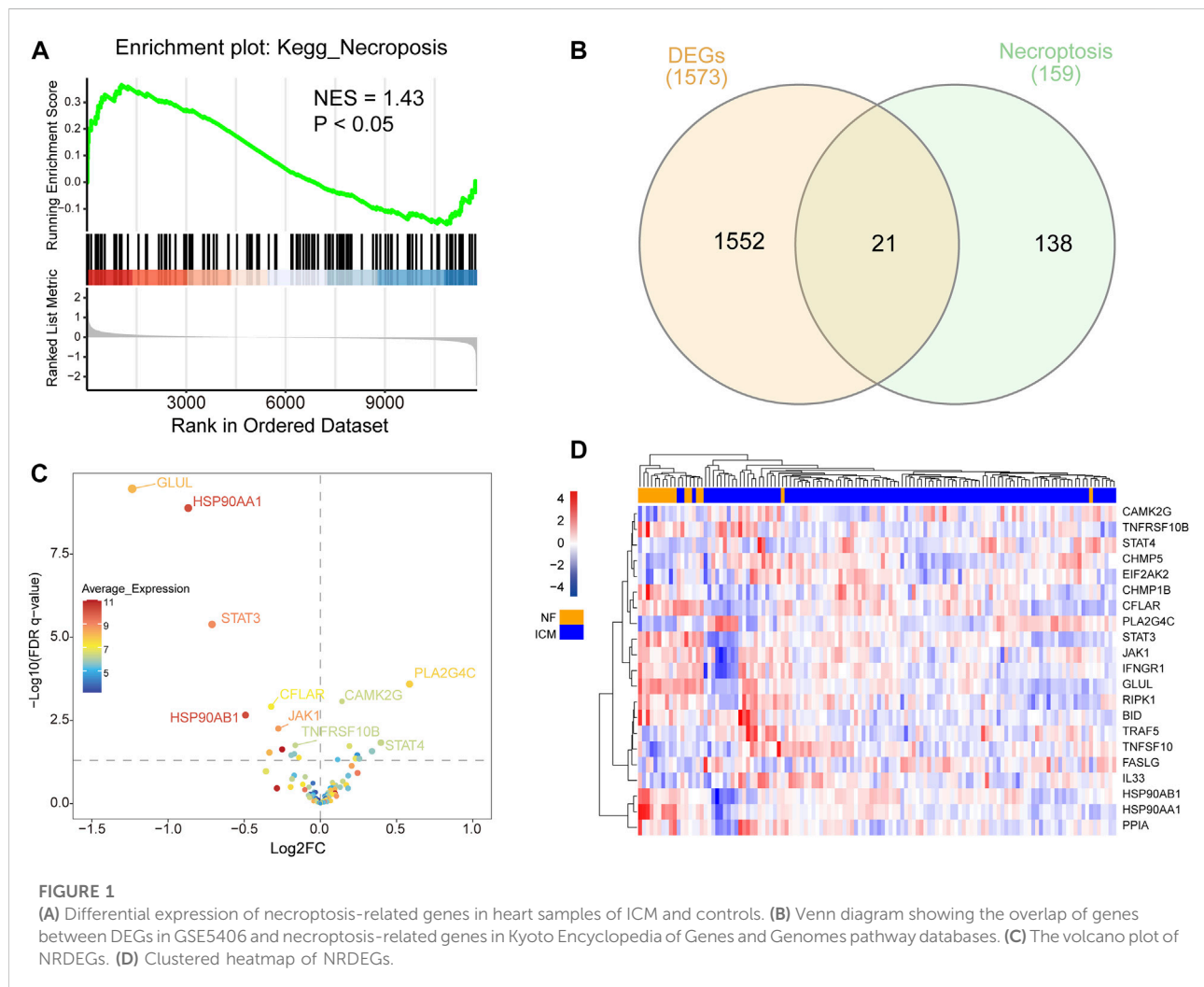
The “rms” package was used to incorporate NRS and clinical characteristics to create a nomogram. Calibration curves were used to assess the accuracy of the nomogram. The clinical usefulness of the nomogram was assessed by decision curve analysis.

2.9 Human samples

Six patients with ICM who had heart transplantation provided left ventricular myocardial tissue. At least 3 months before receiving a heart transplant, all patients had been diagnosed with ICM and an EF of less than 40%. Three organ donors whose hearts could not be transplanted owing to size concerns, ABO mismatches, or other factors were able to provide non-failing (NF) tissue. The study conformed with the Helsinki Declaration (revised 2013). The Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology evaluated and approved the study (Wuhan, China; approval number: UHCT-IEC-SOP-016-03-01). All patients or their families provided their written informed consent. The clinical data of the 6 ICM patients are shown in Supplementary Figure Table 1.

TABLE 1 The sequences of the primers used.

Gene	Forward (5' → 3')	Reverse (5' → 3')
STAT4	TGTTGGCCCAATGGATTGAAA	GGAAACACGACCTAACTGTTTCAT
TNFSF10	TGCGTGCTGATCGTGATCTTC	GCTCGTTGGTAAAGTACACGTA
CHMP5	AGATTCTCGATTGGATGCTGAG	TGTTGGGCAAGATTGTCCCG
CHMP1B	AAAGAACTGAGTAGGAGTGCCA	TGTATCCTCGCAACTTCCATGT
JAK1	CTTTGCCCTGTATGACGAGAAC	ACCTCATCCGGTAGTGGAGC
CFLAR	TGCTCTTTTGTGCCGGGAT	CGACAGACAGCTTACCTCTTTC
GAPDH	TGTGGGCATCAATGGATTG	ACACCATGTATTCCGGGTCAAT



2.10 Real-time polymerase chain reaction (PCR)

Total RNA was extracted from frozen heart tissue using TRIzol reagent (Invitrogen) and PrimeScript RT kit (TaKaRa

Biotechnology) was used to reverse transcribe the RNA to cDNA. Real-time fluorescent quantitative PCR was performed using SYBR green (Vazyme). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. GAPDH was used as a reference gene. The sequences of the primers are listed in Table 1.

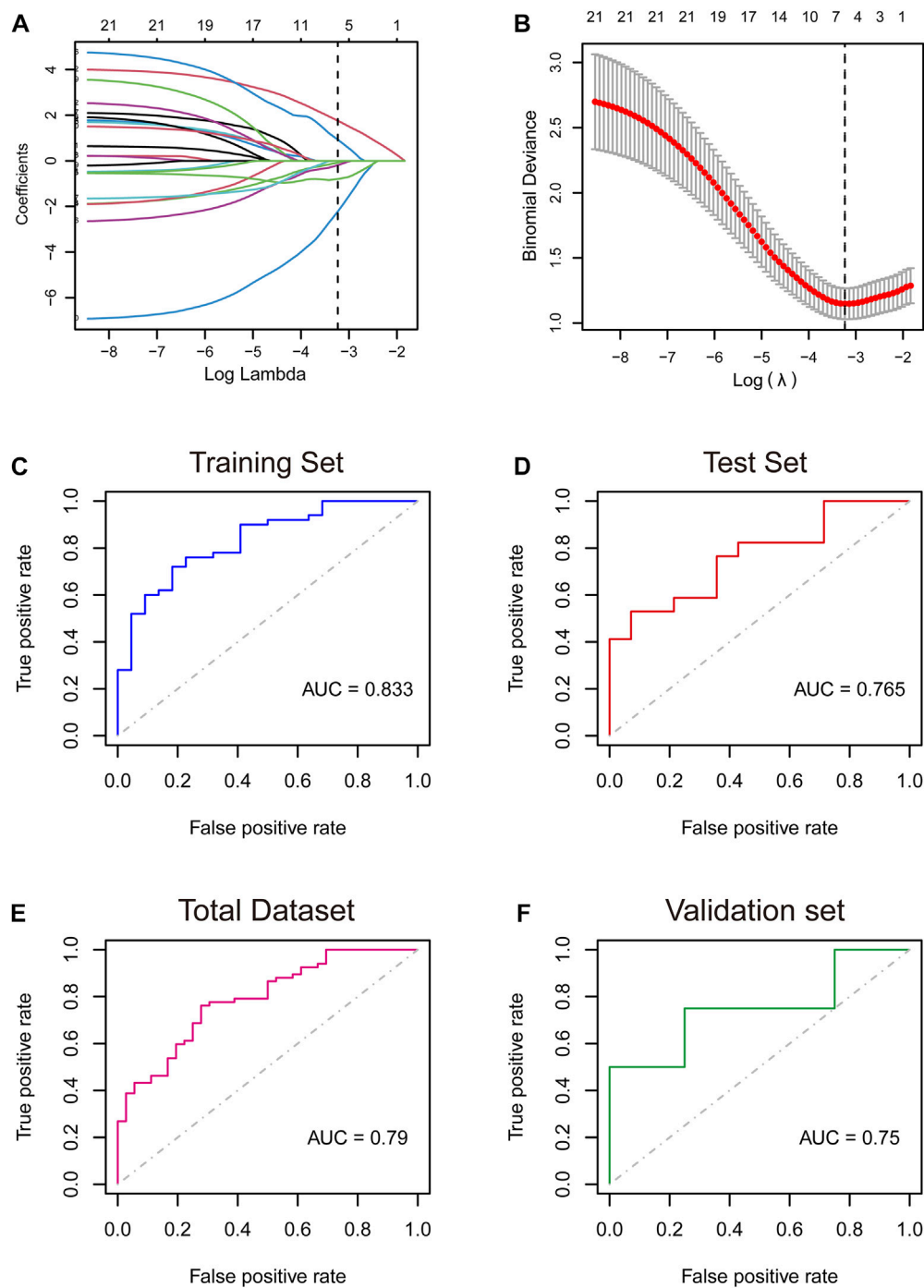


FIGURE 2

(A,B): Least absolute shrinkage and selection operator (LASSO) logistic regression algorithm to screen key genes. (C–F): Receiver operating characteristic (ROC) curves analysis of training set (C), testing set (D), total dataset (E), and validation set (F). AUC, area under the curve.

2.11 Statistical analysis

All statistical tests were implemented utilizing R software 3.6.1. Data are expressed as mean $\pm$ SD. Student's t-test was

utilized for analyzing the difference between the two groups. The correlation between the variables were determined using Spearman's correlation test. All statistical p -values were two-sided, and $p < 0.05$ was regarded as statistical significance.

3 Results

3.1 Identification of NRDEGs

Gene set enrichment analysis (GSEA) was performed to identify predominant signaling pathways between ICM patients and non-failing (NF) controls. As shown in Figure 1A, the necroptosis pathway was considerably enriched and predominantly upregulated (normalized enrichment score = 1.43, $p < 0.05$), indicating an over strong link between necroptosis and ICM. A total of 1573 DEGs between ICM and NF samples were analyzed, with 703 genes up-regulated and 870 genes down-regulated. NRDEGs were identified based on DEGs and necroptosis gene set. We overlapped necroptosis pathway-related genes with DEGs in GSE5406, and obtained 21 overlapping NRDEGs for further analysis (Figure 1B), of which 9 were up-regulated and 12 were down-regulated. The expression heatmap and volcano plot of NRDEGs are presented in (Figures 1C,D).

3.2 Construction of characteristic genes associated with progression and prognosis

LVEF is a key factor in the progression and prognosis of ischemic cardiomyopathy. In patients with heart failure, reduced LVEF is strongly associated with adverse cardiac outcomes. Randomized clinical trials have shown that when patients with EF are severely reduced, below 15%, the mortality rate is greater than 50%, which is much higher than that of patients with LVEF of 16%–45%. Therefore, we used EF = 15% as the boundary to divide the patients into EF ≤ 15% group ($n = 67$) and EF > 15% groups ($n = 36$). ICM patients were randomly divided into a training cohort and a validation cohort at a ratio of 7:3. We applied the LASSO regression algorithm with an optimal lambda value of 0.0394 to select characteristic genes in NRDEGs in ICM patients (Figures 2A,B). Six genes, including *CFLAR*, *JAK1*, *STAT4*, *TNFSF10*, *CHMP1B*, and *CHMP5* with coefficients of 0.929, −0.795, 1.759, −0.080, −2.222, and −0.190, were identified to construct the NRDEGs-related progressive and prognostic signature. Higher scores represent severe progression and worse prognosis of ICM patients. In training set, the AUC of the ROC curve was 0.833. In validation set, the AUC value of ROC curve was 0.765. In the overall set, the model achieved an ROC of 0.79 (Figures 2C–E). We did a validation using GSE203160 and found that the AUC reached 0.75 (Figure 2F), demonstrating that the NRDEGs-related signature possessed excellent diagnostic performance in predicting the progression of the progression and prognosis of ICM. In addition, we validated the expression of six NRS-related genes in ICM patients using two additional datasets (GSE57338 and GSE203160) and found that they

were mostly differential expressed (Supplementary Figures S1, S2).

3.3 Genes regulatory network identifies transcription factor and miRNA associated with signature genes

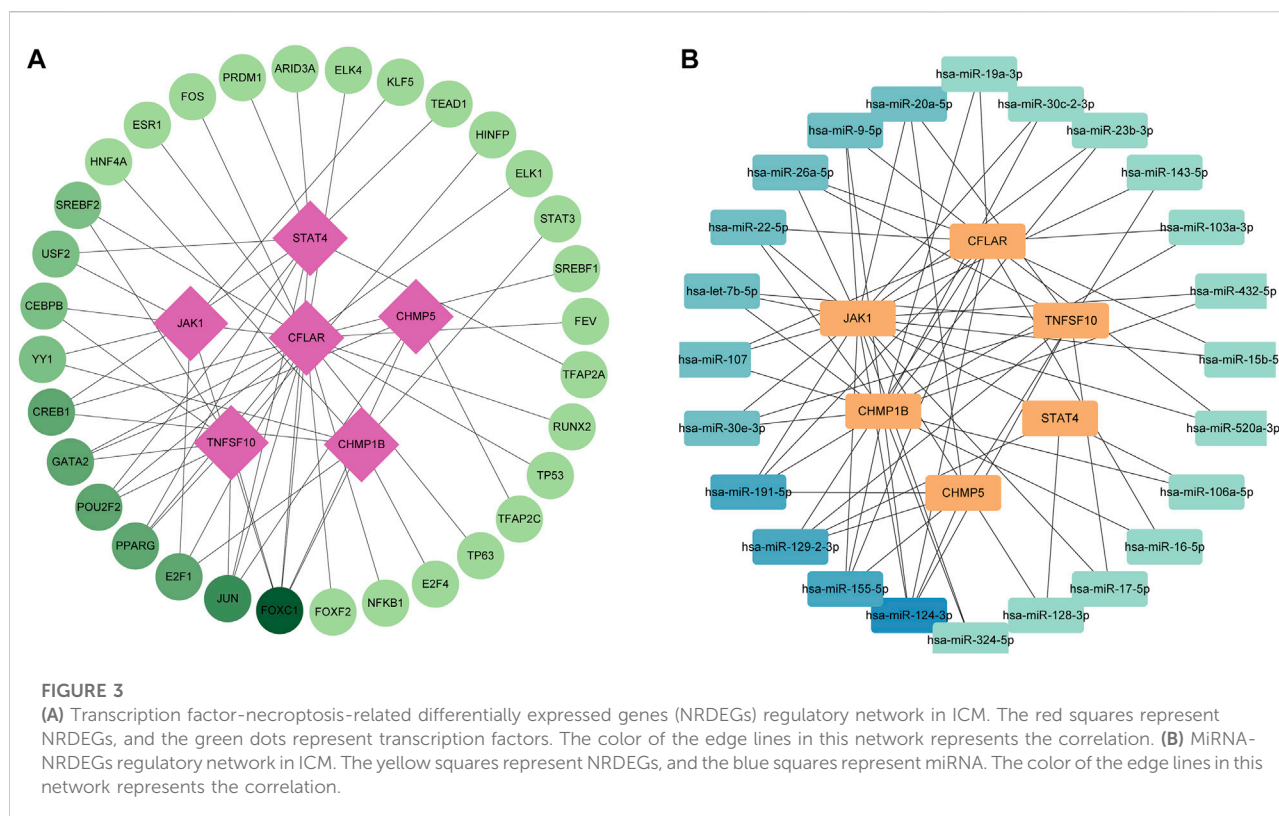
The circles in the figure represent TFs, while the squares represent characteristic genes. The color of the nodes reflects the degree. Nodes with higher degrees are considered important hubs of the network. We found that *JUN* and *FOX1* are major transcription factors (Figure 3A). Meanwhile We identified a miRNA-NRDEGs' interaction network, similar to previous analyses, we discover hsa-miR-20a-5p is a major regulator of signature genes (Figure 3B).

3.4 Identification of pathways associated with NRS

We identified 1573 DEGs associated with the necroptotic phenotype. The GO enrichment analysis revealed enrichment of phagocytosis, myeloid leukocyte activation, negative regulation of response to external stimulus, activation of immune response was downregulated in high-NRS group. Furthermore, enrichment of muscle system process, muscle contraction was found up regulated in high-NRS group (Figure 4A). Most importantly, the KEGG analysis revealed that DEGs tended to be enriched in the following terms: cardiac muscle contraction, hypertrophic cardiomyopathy (Figure 4B). GSEA analysis indicated assembly of collagen fibrils and other multimeric structures, neutrophil degranulation, Tyrobp causal network in microglia was down-regulated in high-NRS group. Meanwhile, respiratory electron transport was up-regulated in high-NRS group (Figure 4C).

3.5 Differences of immune infiltration in patients with high and low NRS

Among 24 immune cells, there were 5 cell types with significant differences in high-score groups compared with low-score groups. Among the 5 cell types, the score of nTreg cells in the low-score group was significantly enriched, whereas CD8-T cells, Gamma-delta cells, CD8-naïve cells, mucosa-associated lymphocytes (MALT) were noticeably enriched in the high-score group (Figures 5A,B; Supplementary Figure S3). The *STAT4* was predominantly favorably correlated with CD4-T cells, DC, Tfh, Th17, and Th2 cell infiltration and *CFLAR* was primarily positively correlated with central memory cell infiltration, meanwhile, *CHMP5* was negatively correlated with central memory cells (Figure 5C).



3.6 AZD-7762 as a potential therapeutic agent for patients with high NRS

To find targeted drugs for high-risk patients, Potential anti-ICM small molecule compounds were predicted by CMap analysis, as shown in (Figure 6A). Drugs AZD-7762, BMS-536924, PD-1 84352 scored higher, they were CHK inhibitor, IGF inhibitor, MEK inhibitor. The results showed that they might have an intervening effect on ICM progression.

3.7 Establishment of a signature gene-based nomogram for ICM progression and prognosis

We constructed a nomogram as a diagnostic tool for ICM progression. In the nomogram, each factor, including sex, age, coronary artery bypass grafting (CABG), inotropes, angiotensin converting enzyme inhibitor (ACEI), beta-block (BB), intra-aortic balloon counter pulsation (IABP), NRS, corresponds to a score, and the total score is obtained by adding the scores of all characteristic genes (Figure 6B). The total score corresponds to the different risks of the ICM. The calibration curve showed that the nomogram was able to accurately estimate the progression of ICM (Figure 6C). Patients diagnosed with ICM can be benefit from a nomogram, as shown by decision curve analysis (Figure 6D).

3.8 Validation of the key genes in the heart tissues

STAT4 were expressed at a higher level and *CFLAR*, *CHMP1B*, *JAK1* were expressed at lower levels in the ICM heart tissues than in the NF heart tissues ($p < 0.001$), whereas *CHMP5*, *TNFSF10* showed no significant differences in expression level between the two groups (Figure 7).

4 Discussion

Ischemic heart disease remains the leading causes of morbidity and mortality and a major burden on worldwide healthcare (Severino et al., 2020). Recent studies have shown the pathological process of ischemic cardiomyopathy includes chronic myocardial ischemia and ischemia/reperfusion injury, which was closely associated with immune cell infiltration and necroptosis (Antman and Braunwald, 2020).

First, results from our analysis confirm previous research indicating that necroptosis pathway was considerably enriched and predominantly upregulated in ICM samples. Then, we overlapped necroptosis pathway-related genes with DEGs and obtained 21 overlapping NRDEGs for further analysis. We applied the LASSO regression algorithm to select necroptosis-related characteristic genes in NRDEGs in ICM patients. Six

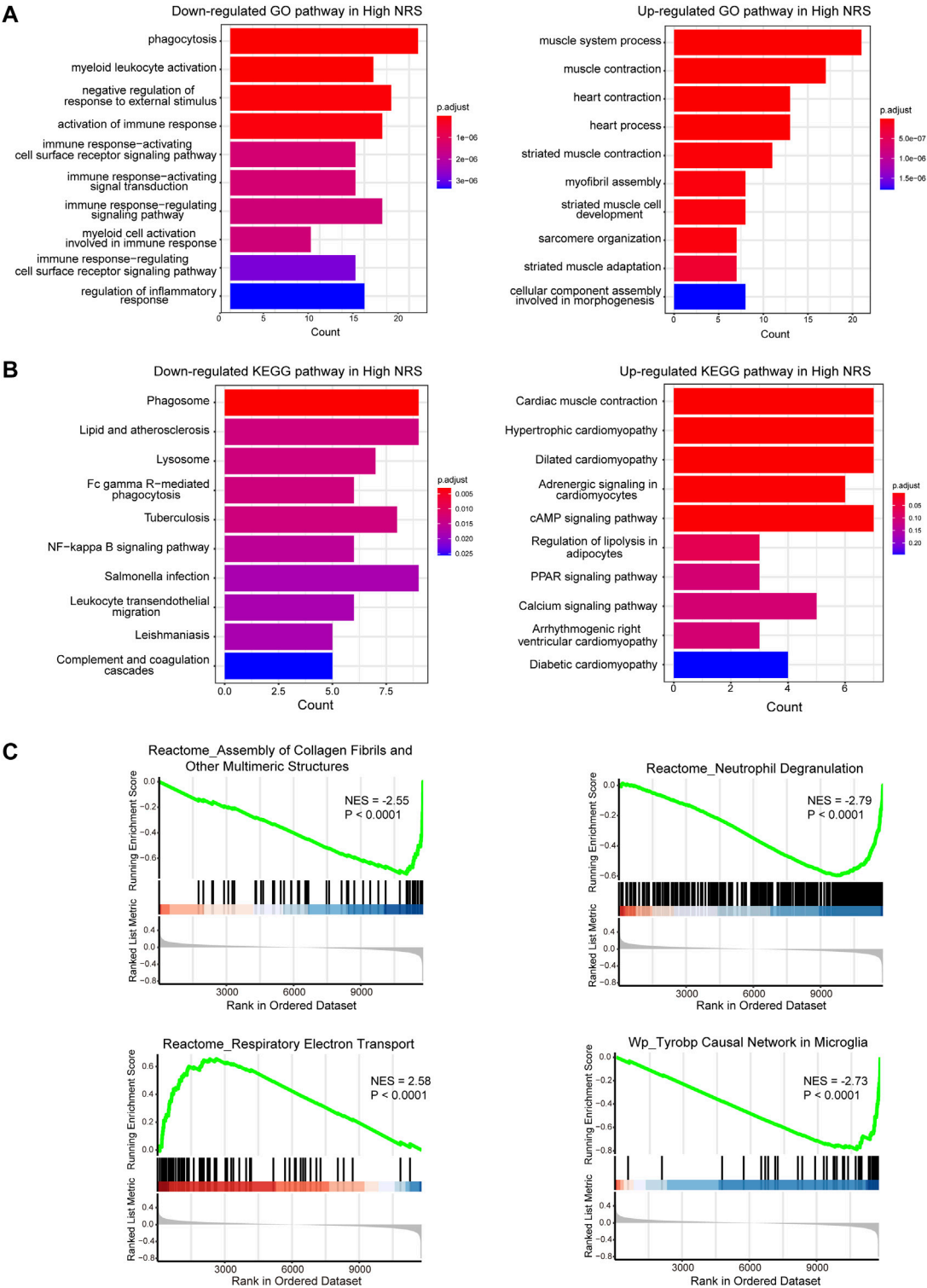
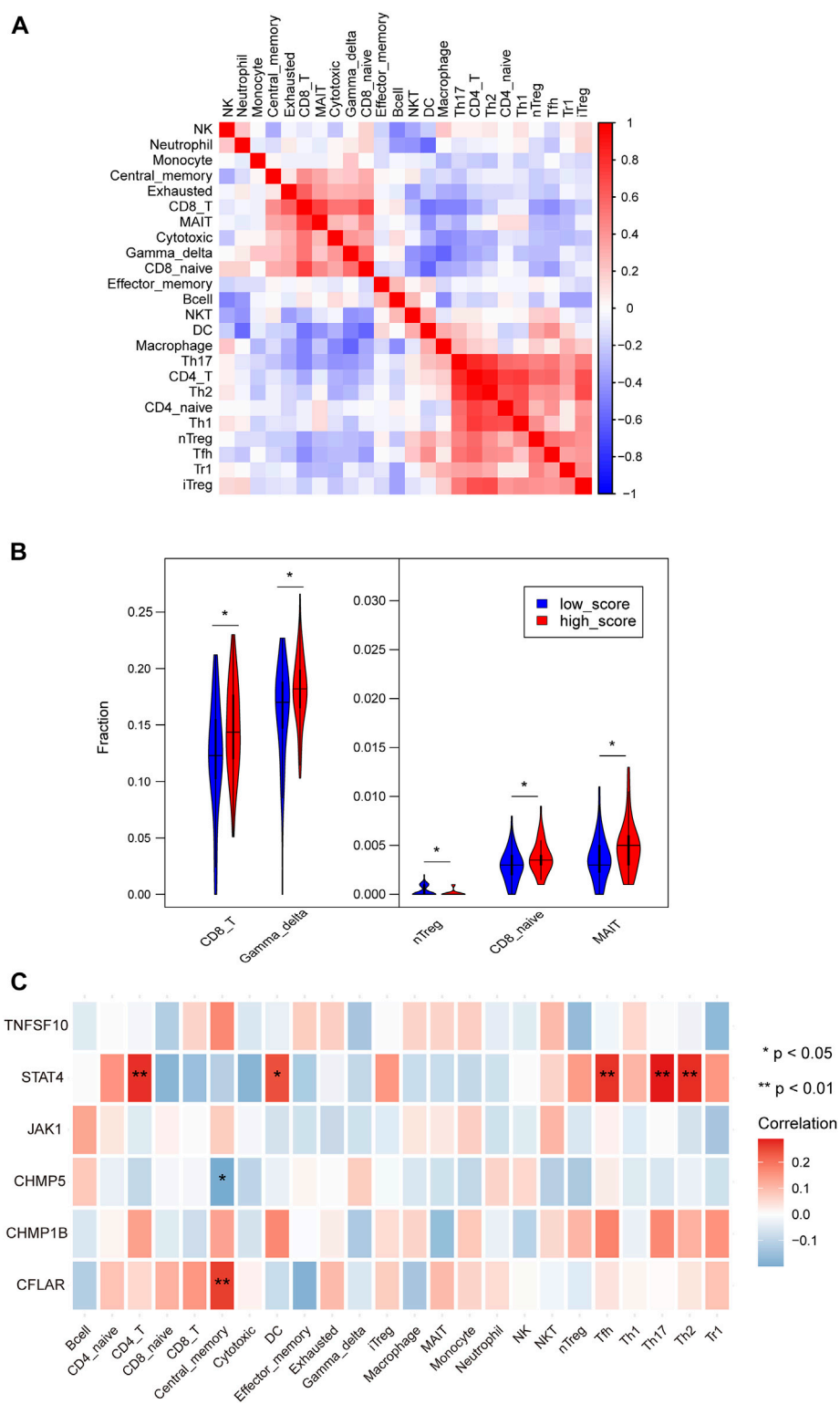


FIGURE 4
(A) GO functional analysis of genes in high NRS. (B) KEGG pathway analysis of genes in high NRS. (C) GSEA identifies signaling pathways involved in high NRS.



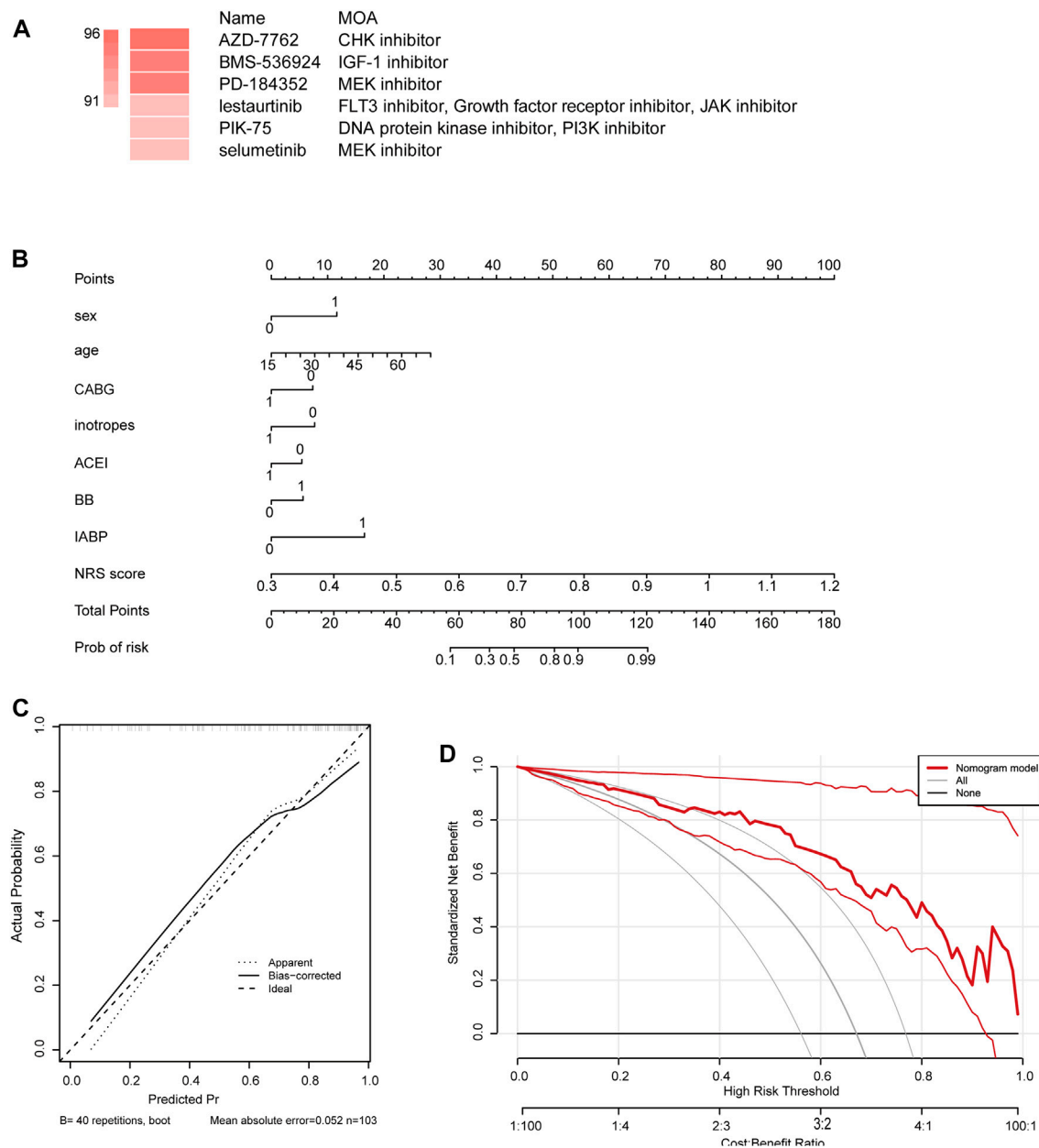


FIGURE 6

(A) Potential anti-ICM small molecule compounds were predicted by CMap analysis. (B) Establishment of a nomogram integrating characteristic genes for predicting ICM. In the nomogram, each variable corresponds to a score, and the total score can be calculated by adding the scores for all variables. (C) Calibration curve estimates the prediction accuracy of the nomogram. (D) Decision curve analysis shows the clinical benefit of the nomogram.

genes, including *STAT4*, *TNFSF10*, *CHMP5*, *CHMP1B*, *JAK1*, and *CFLAR* were identified to construct the necroptosis-related progressive and prognostic signature. We found that among these genes, *CFLAR*, *CHMP1B*, *JAK1*, and *STAT4* were the most prominent necroptosis-related signature genes between the myocardial tissue of the ICM patients and those of the NF control.

Our results show that *CHMP1B*, *CHMP5*, *JAK1*, and *TNFSF10* have minus coefficients in the model, suggesting that their expression is opposite to the severity of the disease. Given that necroptosis is elevated in ICM patients, we hypothesized that they may inhibit the necroptotic pathway. *CHMP1B* and *CHMP5*, both belong to the chromatin-modifying protein/charged multivesicular body

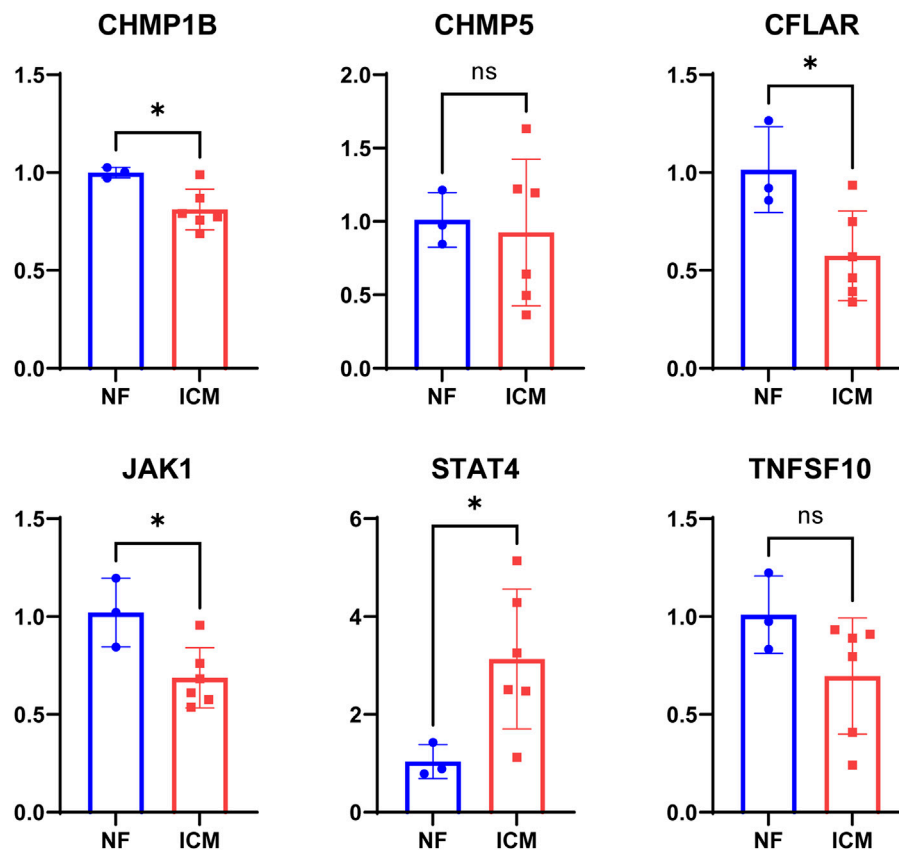


FIGURE 7

The levels of STAT4, TNFSF10, CHMP5, CHMP1B, JAK1, CFLAR mRNA expression in myocardium tissue from non-failing (NF) donor and ICM patients.

protein (CHMP) family, is required for both endocytic multivesicular bodies formation and regulation of cell cycle progression (Howard et al., 2001; Shim et al., 2006). It has been reported that in CHMP5-deficient glioma cells, both the granzyme B/perforin apoptotic pathway and the apoptosis-inducing factor-mediated necrosis pathway are activated, independently of the intrinsic and extrinsic apoptotic pathways (Shahmoradgoli et al., 2011; Wang et al., 2013). CHMP5 also promotes the stabilization of the intracellular pro-survival protein BCL2 in T cells and is rescued by the deletion of the pro-apoptotic protein BIM (Adoro et al., 2017). Similarly, the deletion of CHMP1 increased the susceptibility of renal tubular cells to death (Guan et al., 2021). This suggests that CHMP5 and CHMP1B may negatively regulate the necroptotic pathway in ICM patients. Previous studies on JAK1 have been focused mainly on the field of the proliferation and differentiation of tumor cells and some autoimmune disease. Sustained activation of signaling that inhibits JAK can lead to accelerated apoptosis of cells. In rheumatoid arthritis, continuous activation of JAK signaling in synovial joints results in the most pronounced “apoptosis

resistance” in apoptotic chondrocytes (Malemud, 2018). Tofacitinib or ruxolitinib, JAK small molecule inhibitor, downregulated myeloid cell leukemia-1 mRNA level and decreased its protein level, which enabled BAK to trigger necroptosis (Thapa et al., 2013; Li et al., 2021). Therefore, we speculate that the JAK1 is associated with the negative regulation of necroptosis in ICM. Current research on TNFSF10 is insufficient. TNFSF10 is a protein functioning as a ligand that induces the process of cell death called apoptosis, but has also been implicated as a pathogenic or protective factor in various pulmonary diseases (Wiley et al., 1995). Given the minute coefficient of *TNFSF10* (0.08), we suggest that it may have a minor role in necroptosis-induced ICM progression. Our study showed that *STAT4* and *CFLAR* have positive coefficients in the model. Previous studies have shown that *STAT4* plays a role in promoting apoptosis in hepatocellular carcinoma and ovarian granulosa cells (Jiang et al., 2020). A study in the vascular smooth muscle cell (VSMC) showed that the effect of *STAT4* on VSMC apoptosis was mainly mediated by the activation of the mitochondrial apoptotic pathway (Lv et al., 2014).

C-FLICE-like inhibitory protein (C-FLIP, CFLAR), a protein that not only inhibits apoptosis signaling but also modulates additional cell death pathways. Regulation of CFLAR expression represents a general tool to master cell death signaling pathways (Fulda, 2013). Therefore, STAT4 and CFLAR may contribute to the progression of ICM.

The identified GO category “myeloid leukocyte activation, negative regulation of response to external stimulus, activation of immune response” fits well with the concept emphasizing that Immunomodulatory is one of the most important factors predisposing to ventricular remodeling and coronary vascular occlusion in ICM (Geng, 2003; Abdel-Latif et al., 2015; El Bakry et al., 2017). The significantly enriched functions KEGG analysis were associated with cardiac muscle contraction, hypertrophic cardiomyopathy. The enriched GSEA pathways included the signaling pathways involved in inflammation, immunity, cell migration, and proliferation. These pathways included assembly of collagen fibrils and other multimetric structures, neutrophil degranulation, respiratory electron transport. These findings suggest that these signaling pathways play a critical role in the ventricular and immune responses in ICM.

Recent studies have shown that in the pathogenesis of chronic ischemic heart failure exposure of autoantigens in ischemic necrotic myocardial tissue activates immune cells and aggravates tissue damage and ventricular remodeling (Bansal et al., 2017). Hence, ICM is considered a chronic state of aberrant immune activation and inflammatory response in this study. We found that nTreg cells in the low-score group were significantly enriched, whereas CD8-T cells, Gamma-delta cells, CD8-naïve cells, mucosa-associated lymphocytes (MALT) were noticeably enriched in the high-score group. After acute MI, Treg cells delay ventricular remodeling by regulating macrophage phenotype, suppressing inflammatory responses, and promoting ventricular muscle repair (Bansal et al., 2019). Our results showed that STAT4 was positively correlated with Tfh, Th17, and Th2. However, their abundance values were close to zero, and we believe that these results may have relatively little practical significance.

Our analysis shows that ICM are widely involved in a series of pathological processes such as necroptosis, immunity, inflammation and cardiac fibrosis. Myeloid leukocyte activation, immune response and NF-kappa B signaling were down-regulated in high NRS, but cardiac muscle contraction and hypertrophic cardiomyopathy was up-regulated in high NRS. Meanwhile There was a few immune cell infiltrates showing significantly difference between the high NRS group and low NRS group. We speculated necroptosis may be more involved in myocardial fibrosis than inflammation.

AZD-7762 (AZD) (S)-55-(3-Fluorophenyl)-N-(piperidin-3-yl)-3-ureidothiophene-2-carboxamide is a small molecule inhibitor of WEE1 protein kinase. WEE1 controls the program of entry into mitosis after DNA damage, inhibiting the progression of cells from G2 to M phase, giving cells ample time to repair their damaged.

Research on the role of WEE1 kinase has mainly focused on the field of cancer and is limited in ICM. The current study provides evidence that damaged cells can activate WEE1 kinase, which prevents apoptosis and cell death by inducing cell cycle arrest at G2 phase. Therefore, inhibition of WEE1 kinase could sensitize cancer cells to chemotherapeutic drugs. Therefore, WEE1 kinase may be related to myocardial apoptosis and myocardial repair after myocardial infarction (Lv et al., 2014). Moreover, as depicted in our research curve analysis, the patients diagnosed with ischemic heart disease could clinically benefit from the nomogram.

5 Conclusion

In conclusion, we have described characteristic genes associated with necroptosis as a useful and non-invasive tool for LVEF classification in ICM patients, and identified pathways, immune cells and targeted drugs in high-risk patient groups. This is the first necroptosis-related feature in ICM that can evaluate progression and prognosis in ICM patients. However, some important limitations remain in our study. The sample size included in this study was insufficient, and there was an imbalance in the number of patients in both groups, which may affect the accuracy of the model. Larger cohorts are needed to validate the robustness and utility of the prognostic model in future clinical practice. In addition, more experiments will be performed to further elucidate the role of signature genes in ICM.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China; approval number: UHCT-IEC-SOP-016-03-01). The patients/participants provided their written informed consent to participate in this study.

Author contributions

YL and HY conceived and designed the study, plotted the figures, and wrote the manuscript. DW conducted data analysis and performed literature searches. YZ conducted data analysis, graphing, and manuscript revision. HY, JZ, and YD reviewed and

revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.1039857/full#supplementary-material>

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LncRNA MEG3: Potential stock for precision treatment of cardiovascular diseases

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The prevalence and mortality rates of cardiovascular diseases are increasing, and new treatment strategies are urgently needed. From the perspective of basic pathogenesis, the occurrence and development of cardiovascular diseases are related to inflammation, apoptosis, fibrosis and autophagy of cardiomyocytes, endothelial cells and other related cells. The involvement of maternally expressed gene 3 (MEG3) in human disease processes has been increasingly reported. P53 and PI3K/Akt are important pathways by which MEG3 participates in regulating cell apoptosis. MEG3 directly or competitively binds with miRNA to participate in apoptosis, inflammation, oxidative stress, endoplasmic reticulum stress, EMT and other processes. LncRNA MEG3 is mainly involved in malignant tumors, metabolic diseases, immune system diseases, cardiovascular and cerebrovascular diseases, etc., LncRNA MEG3 has a variety of pathological effects in cardiomyocytes, fibroblasts and endothelial cells and has great clinical application potential in the prevention and treatment of AS, MIRI, hypertension and HF. This paper will review the research progress of MEG3 in the aspects of mechanism of action, other systemic diseases and cardiovascular diseases, and point out its great potential in the prevention and treatment of cardiovascular diseases. LncRNAs also play a role in endothelial cells. In addition, LncRNA MEG3 has shown biomarker value, prognostic value and therapeutic response measurement in tumor diseases. We boldly speculate that MEG3 will play a role in the emerging discipline of tumor heart disease.

KEYWORDS

LncRNA MEG3, cardiovascular diseases, P53 signaling pathway, PI3K/akt signaling pathway, apoptosis, autophagy, inflammation, endoplasmic reticulum

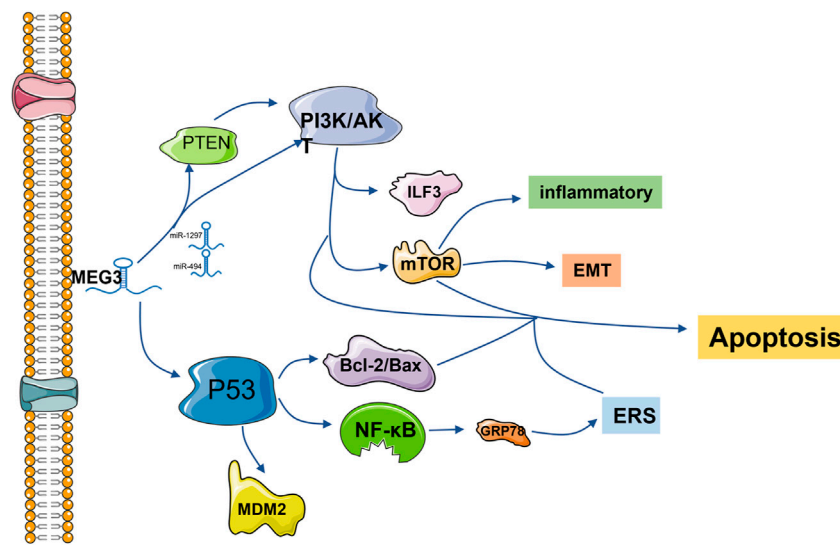


FIGURE 1

lncRNA MEG3 is frequently involved in endoplasmic reticulum stress, apoptosis, inflammation and epithelial-mesenchymal transformation via the P53 and PI3K/AKT pathways. The common downstream pathways are MDM2, NF-κB, Bcl-2/Bax, mTOR and other protein pathways. Regulation is also accomplished by competitively binding downstream protein pathways with miRNA.

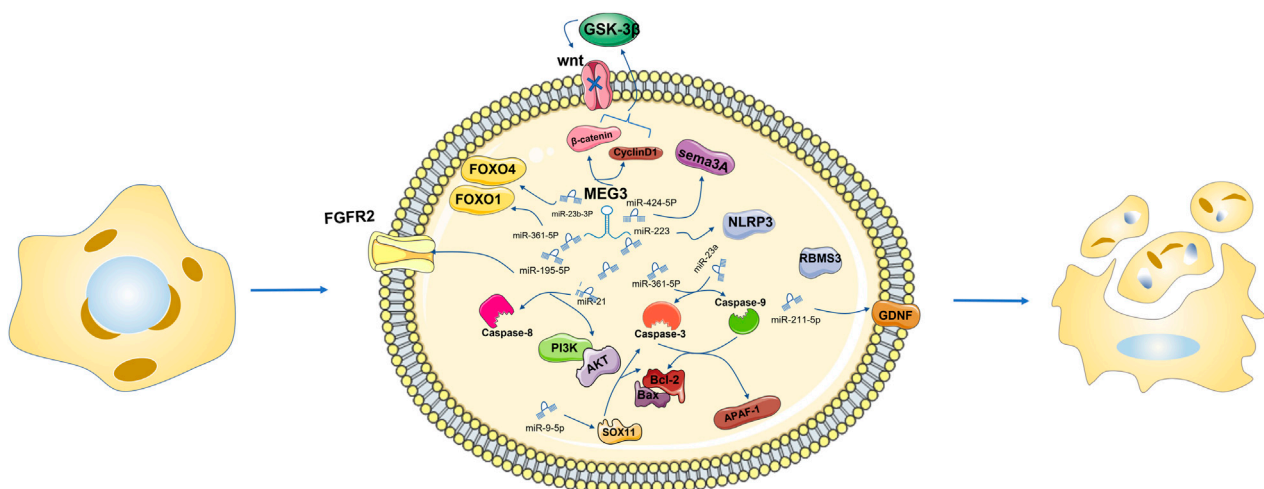


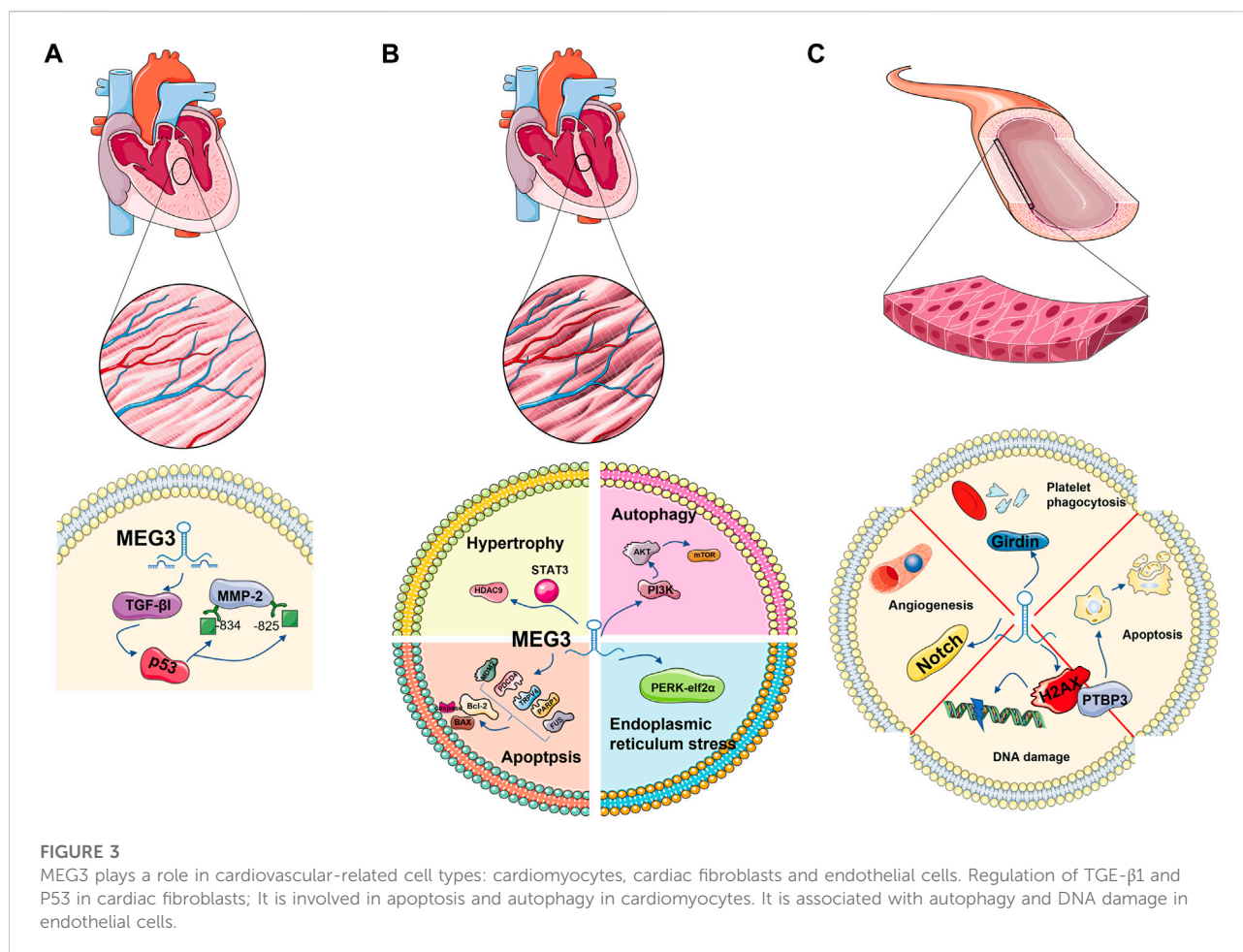
FIGURE 2

Downregulation of MEG3 can directly or through miR-361-5p mediate the downregulation of FOXO1, promote cell apoptosis, negatively regulate the expression level of miR-23b 3p, and negatively regulate the expression level of FOXO4 in the same family to promote cell apoptosis. MEG3 recovery can reduce β -catenin and CyclinD1, improve the level of GSK-3 β *in vitro* culture, block the activity of the Wnt signaling pathway, and then inhibit cell proliferation, migration and invasion, triggering cell apoptosis.

1 Introduction

The prevalence and mortality rates of cardiovascular diseases are on the rise, with an estimated 19 million people worldwide dying from cardiovascular diseases in 2020, an increase of 18.7% from 2010 (Tsao et al., 2022). 80% of deaths are related to CVD

(Global, 2017). By 2030, 23.6 million people are expected to die from cardiovascular disease, including heart disease and stroke (Dehghan et al., 2017), (Yusuf et al., 2014). New treatment strategies are urgently needed. From the perspective of basic pathogenesis, the occurrence and development of cardiovascular diseases are related to inflammation, apoptosis, fibrosis and



autophagy of cardiomyocytes, endothelial cells and other related cells (Hou et al., 2014; Zhang et al., 2019a; Lan et al., 2019; Shihabudeen Haider Ali et al., 2019). In recent years, precision medicine has been gradually applied to the cardiovascular field along with the development of gene medicine. Currently, the following diseases are considered: common cardiovascular diseases, such as hypertension (Sun et al., 2017a; Padmanabhan and Joe, 2017; Savoia et al., 2017); diagnosis and treatment of uncertain diseases, such as angina and coronary artery disease (Ladapo et al., 2017; Niccoli et al., 2017); high-mortality diseases and/or interventions are complex and expensive, such as dilated cardiomyopathy and cardiac resynchronization therapy (Halliday et al., 2017). It is worth emphasizing that these methods involve new molecular and genetic diagnostic methods (Padmanabhan and Joe, 2017; Savoia et al., 2017).

Maternally expressed gene 3 (MEG3) is an imprinted gene with maternal expression that encodes a noncoding RNA with a length of ~1,600 nt (Tian et al., 2015) located at chromosome 14q32 (Zhang et al., 2010). The involvement in human disease processes is

increasingly reported. For example, MEG3 functions as a competing endogenous RNA to regulate cancer progression, reduces mitochondrial-derived apoptosis (Wang et al., 2021a), participates in the regulation of tumor drug resistance (Yu et al., 2020), participates in glaucoma onset (Sun et al., 2018), etc., This noncoding RNA is not only involved in the pathogenesis of many diseases but also found in an increasing number of mechanisms. For instance, it participates in cell migration and proliferation, promotes cell apoptosis, inhibits cell autophagy activity, and inhibits inflammatory factors (Li et al., 2017; Han et al., 2020; Yang et al., 2022). Cardiovascular disease researchers are increasingly interested in MEG3. Hongchun Wu's group reported that MEG3 exists in mouse myocardial cells and myocardial fibroblasts and mainly plays a role in the myocardial nucleus (Wu et al., 2018). Thum's team reported that MEG3 plays a key role in promoting cardiac fibroblast fibrosis (Piccoli et al., 2017). The inhibition of Meg3 prevents cardiac fibrosis and diastolic dysfunction (Piccoli et al., 2017), and the downregulation of MEG3 protects myocardial cells against I/R-induced apoptosis through the miR-7-5p/PARP1 pathway (Zou et al., 2019) Chao

He et al. found in their study that MEG3, as a ceRNA, inhibits miR-9, affects the phenotype of MEG3-mediated vascular endothelial cells (He et al., 2017).

Currently, studies on lncRNA MEG3 in cardiovascular diseases are relatively rare, and there is still a large space for exploration. This paper will review the research progress of MEG3 in the functional mechanism, other systemic diseases and cardiovascular diseases and find its great potential in the prevention and treatment of cardiovascular diseases.

2 Functional mechanism

This paper focuses on the role of lncRNA MEG3 in cardiovascular disease. Although so far, there have been few studies on the effects of lncMEG3 on cardiovascular diseases, most of which focus on tumor diseases, from the perspective of functional mechanism, the regulatory mechanisms involved in MEG3 are also common in cardiovascular diseases, suggesting that MEG3 may be involved in the occurrence and development of cardiovascular diseases through these pathways. Therefore, the second part of this paper reviews MEG3 from the functional mechanism.

2.1 Signaling pathway

2.1.1 Regulation of the P53 signaling pathway

The p53 protein is a transcription factor that activates cell cycle arrest, DNA repair and apoptosis in response to stress and DNA damage (Kruiswijk et al., 2015). The 27 known splicing variants of MEG3 contain variable intermediate exons with common exons entraining at the 50 (E1-E3) and 30 (E10-E12) ends, and their ability to stimulate the p53 pathway varies (Yu et al., 2020). Based on this, MEG3 can regulate cell physiology by regulating p53 and downstream signaling pathways and play a role in a variety of diseases. In vascular endothelial cells, the p53 signaling pathway is the most significantly regulated pathway after MEG3 knockout. Downregulation of MEG3 leads to phosphorylation of p53 at serine 15 and weakens its binding with multiple p53 target gene promoters, such as MDM2, resulting in accumulation of p53. Meanwhile, MEG3 loss leads to high expression of P21, induces p53 cell cycle arrest, reduces cell proliferation, promotes cell apoptosis, and aggravates cardiac dysfunction after pressure overload in mice. Early studies have shown that the loss of p53 in the vascular endothelium can reduce apoptosis of endothelial cells and protect cardiac dysfunction after pressure overload in mice (Deng et al., 1995; Gartel and Radhakrishnan, 2005; Gogiraju et al., 2015; Shihabudeen Haider Ali et al., 2019). In tumor cells, lncRNA MEG3 inhibits the proliferation and metastasis of breast, gastric, liver and pancreatic cancer cells by activating the p53 signaling pathway (Chen et al., 2016; Hu et al.,

2016; Wei and Wang, 2017; Huang et al., 2022). Especially in hepatocellular carcinoma cells, MEG3 activates the NF- κ B signaling pathway and then activates the p53 pathway and upregulates the expression of the ER stress-related protein GRP78, resulting in ER stress (Chen et al., 2016). Xueling Li's group found that lncMEG3 was downregulated around the infarcted myocardial tissue, which directly acted on p53 and reduced its expression level. Meanwhile, the expression of ERS-related protein GRP78 decreased, which proved that lncRNA MEG3 in myocardial cells was awaiting ERS-mediated apoptosis through the p53 pathway (Li et al., 2019). On the other hand, inhibition of endogenous Meg3 in mouse fibroblasts reduced the expression of Mmp-2 at promoters by inhibiting p53 binding but had no effect on cell apoptosis or proliferation (Piccoli et al., 2017). Western blotting was used to detect the proteins in the pathway, and it was found that p53 was regulated by MeG3, which then regulated the apoptosis of adipose stem cells (adScs) through the Bcl-2/Bax pathway (Shi, 2020). In mouse neurons, overexpression of Meg3 induces p53 and enhances its transcriptional activity, leading to increased cell death (Yan et al., 2016) (Figure 1).

2.1.2 Regulation of the PI3K/Akt signaling pathway

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/AKT) signaling pathway is one of the core signaling pathways involved in regulating cell growth, proliferation, apoptosis and other processes (Yang et al., 2019a). Studies have shown that inhibition of the PI3K/AKT signaling pathway can inhibit cell proliferation and promote cell apoptosis (Okano et al., 2000; Oyama et al., 2007; Feng et al., 2016). lncRNA MEG3 has a negative regulatory effect on the PI3K/Akt signaling pathway. lncRNA MEG3 expression was significantly increased in the temporal cortex of rats with subarachnoid hemorrhage and in the cerebrospinal fluid of patients with subarachnoid hemorrhage, while the expression of the PI3K/Akt signaling pathway was decreased, which promoted neuronal apoptosis (Liang et al., 2018). At the same time, the upregulation of MEG3 can improve cognitive dysfunction by inhibiting the PI3K/Akt signaling pathway and then inhibiting the activation of hippocampal astrocytes (Yi et al., 2019). MEG3 overexpression of the inactivated PI3K/Akt signaling pathway can inhibit the growth of cervical cancer HeLa cells and breast cancer cells, thus achieving anticancer effects (Wang et al., 2017; Zhu et al., 2019).

PI3K/AKT can play a regulatory role alone and crosstalk with the mTOR signaling pathway to regulate various pathological processes (Rosenbloom et al., 2013). It is through this signaling axis that MEG3 inhibits the TNF- α -induced inflammatory response (Tang et al., 2021). In endometrial cancer cells, MEG3 regulates the PI3K/m-TOR signaling pathway to regulate cell cycle progression, thus regulating apoptosis (Sun et al., 2017b). MEG3 enhances adenosine-induced hepatocellular

carcinoma cytotoxicity by downregulating ILF3 and activating this signaling axis (Pu et al., 2019). MEG3 also plays a positive role as a potential target for the treatment of epilepsy by activating this pathway to reduce proinflammatory factors, oxidative stress and apoptosis of hippocampal neurons in rats (Zhang et al., 2020). In rat and cell models of diabetic retinopathy, overexpression of MEG3 inhibits endothelium-stromal transformation by inhibiting the PI3K/Akt/mTOR signaling pathway (He et al., 2021).

The tumor suppressor PTEN, a central negative regulator of the PI3K/Akt signaling pathway, is often mutated in cancer and loses its tumor suppressor function (Leslie, 2012; Wang et al., 2018). It has been reported that both MEG3 and PTEN are downregulated in ovarian cancer cells, which promotes tumor cell proliferation and inhibits cell apoptosis. LncRNA MEG3 regulates PTEN/PI3K/AKT to play an anti-proliferation role in hemangioma cells and testicular germ cell tumor cells through sponge adsorption of miR-494 and miR-1297, respectively.

Experiments have proven that upregulation of MEG3 can sponge miR-27a-3p, upregulate IGF1 and activate the PI3K/Akt signaling pathway, thus promoting osteogenic differentiation (Liu et al., 2019a) (Figure 1).

2.2 MEG3 induces apoptosis

Apoptosis is a programmed cell death that occurs after many stimuli, infections or injuries and plays a key role in normal physiological processes such as embryogenesis and adult tissue homeostasis (Morana et al., 2022). With the advancement of research on MEG3, it has been found that it has an obvious regulatory effect on the molecular mechanism of cell apoptosis, thus participating in a variety of diseases.

MEG3 down-regulation can regulate FOXO1 and FOXO4 to promote apoptosis through competitive binding of miR-361-5P and miR-23b-3p respectively (Tsao et al., 2022), (Wang et al., 2019a), (Wang et al., 2021b). H₂O₂ induces oxidative stress in adipose stem cells (adScs), thus increasing the apoptosis rate of adScs. MeG3 silencing can reduce H₂O₂-induced apoptosis, while MeG3 overexpression can aggravate apoptosis (Shi, 2020). MEG3 recovery can reduce β -catenin and cyclin D1 in melanoma cells, improve GSK-3 β levels *in vitro*, block Wnt signaling activity, inhibit cell proliferation, migration and invasion, and trigger cell apoptosis (Li et al., 2018a).

MEG3 functions as a ceRNA to regulate apoptosis by sponging different miRNAs (Moradi et al., 2019). Activation of Sema3A, a member of the Sema family, may disregulate a series of apoptosis-related regulatory factors, thus promoting apoptosis (Vadasz and Toubi, 2014; Cheng et al., 2019). MEG3 inhibition significantly increased the expression of miR-424-5p, decreased the expression of Sema3A, increased cell viability, and reduced cell apoptosis (Xiang et al., 2020).

As an endogenous sponge, MEG3 inhibits miR-223 function, increases NLRP3 expression and promotes endothelial cell apoptosis through sequence complementation (Zhang et al., 2018). Overexpression of MEG3 inhibits miR-141-3p, activates RNA binding motif single stranded protein 3 (RBMS3), and promotes apoptosis of breast cancer cells (Dong et al., 2021a). Overexpression of MEG3 and miR-376B-3p inhibits the tumorigenesis of PDFS cells and promotes cell apoptosis (Zhu et al., 2020). In the *In vitro* AP model, MEG3 promotes cell apoptosis through the MEG3/miR-195-5p/FGFR2 signaling axis (Chen and Song, 2021). MEG3 has been reported to regulate miR-21, target Caspase-8 to regulate proliferation and apoptosis of psoriatic epidermal cells, and inactivate the PI3K/Akt pathway to promote apoptosis in breast cancer cells (Jia et al., 2019; Zhu et al., 2019). Mir-361-5p. In the research of Shen B's group, it is suggested that MEG3 regulates miR-361-5p and promotes cell apoptosis (Shen et al., 2019). In laryngeal cancer cells, overexpressed MEG3 specifically binds and negatively regulates miR-23a, activates apoptotic protease activator factor-1 (APAF-1), and thus activates Caspase-9 and Caspase-3, leading to apoptosis (Zhang et al., 2019b). MEG3 overexpression leads to reduced apoptosis of intestinal ganglion cells and increased cell viability, and the upregulation of the MEG3 downstream gene miR-211-5p will counteract this effect. Further studies found that the increased expression of GDNF reversed the upregulation effect of miR-211-5p. In summary, hypoxia induced intestinal ganglion cell apoptosis through the MEG3/miR-211-5p/GDNF axis (Xia et al., 2019). MEG3 was found to be highly expressed in the blood and placental villus tissues of gestational diabetes mellitus (GDM). MEG3 knockdown significantly increased the viability of human chorionic trophoblast HTR-8/SVneo cells and reduced cell apoptosis. Inhibition of miR-345-3p negates all observed physiological effects of MEG3 downregulation on HTR-8/SVneo cells (Zhang, 2019). In HCC cells, MEG3 overexpression can sponge out miR-9-5p and upregulate the expression of SOX11, and then observe apoptosis-related changes of Bcl-2 and caspase-3, suggesting that MEG3 can promote the apoptosis of HCC cells (Liu et al., 2019b). For thyroid cancer cells resistant to 131I, low MEG3 expression is not good news. Downregulated MEG3 can promote cell apoptosis by upregulating miR-182 expression and reducing the therapeutic effect of 131I in TC cells (Liu et al., 2018). miR-205-5p has been identified as the downstream gene of MEG3 and is negatively regulated by MEG3, promoting cell apoptosis (Tao et al., 2020). MEG3 inhibition negatively regulates miR-145-5p, resulting in a decreased apoptosis ability of macrophages (Sun et al., 2020). In summary, MEG3, as a ceRNA, mostly has a negative regulatory effect on miRNA and then positively regulates cell apoptosis through the downstream signaling pathway of miRNA.

MEG3 inhibition significantly increases the expression of miR-424-5p, reduces the expression of Sema3A, increases cell

viability, reduces cell apoptosis, inhibits the function of miR-223, and increases the expression of NLRP3. Overexpression of MEG3 can inhibit miR-141-3p, activate RBMS3, and promote cell apoptosis. MEG3 promotes cell apoptosis through the MEG3/miR-195-5p/FGFR2 signaling axis. MEG3 can regulate miR-21, target Caspase-8 to regulate cell proliferation and apoptosis, and inactivate the PI3K/Akt pathway to promote cell apoptosis. Bcl-2 overexpression was detected in the siRNA-MEG3 and miR-361-5P inhibitor groups. MEG3 regulates miR-361-5p and promotes apoptosis. MEG3 overexpression and negative regulation of miR-23a activate APAF-1, thereby activating caspase-9 and caspase-3, leading to cell apoptosis. MEG3 gene knockout significantly increased the viability of HTR-8/SVneo cells and reduced cell apoptosis. MEG3 overexpression can sponge miR-9-5p and upregulate the expression of SOX11, and changes related to apoptosis of Bcl-2 and Caspase-3 were observed. Downregulation of MEG3 can promote cell apoptosis by upregulating the expression of miR-182 (Figure 2).

2.3 Cell autophagy

Autophagy refers to the transport of intracellular components to lysosomal chambers for degradation and circulation. To date, autophagy has been defined as three types: macrophage, microphage and chaperone-mediated autophagy, which can be “body autophagy” with nonselective degradation of autophagosome content and “selective autophagy” with elimination of single cell components (Saftig et al., 2008; He and Klionsky, 2009; Galluzzi et al., 2017; Dikic and Elazar, 2018; Gohel et al., 2020). Autophagy is an important function for maintaining cell and body homeostasis, and the basal level of autophagy is important for maintaining normal cellular homeostasis. Therefore, strict regulation of autophagy to induce it when needed plays a crucial role in health and disease (Botti et al., 2006; Meijer and Codogno, 2006; Yorimitsu and Klionsky, 2007; Cheng, 2019). The regulation of LncRNA MEG3 on autophagy is mainly positive regulation, and this effect has been verified in a variety of diseases and multiple pathways. FOXO1 belongs to the forkhead box protein family, which can regulate a variety of biological characteristics including apoptosis, autophagy, proliferation (Kitamura et al., 2005; Kobayashi et al., 2012; Zhang et al., 2016; Li et al., 2021). Downregulation of MEG3 or FOXO1 can lead to decreased autophagy of INS-1 cells induced by high glucose.¹During pulmonary fibrosis, downregulation of MEG3 activates the Hedgehog (Hh) signaling pathway and inhibits autophagy activity of A549 cells (Gao et al., 2022). Autophagy exists in tumor cells, and downregulation of MEG3 can partially inhibit autophagy in lung cancer cells and can also regulate ATG3 activity to inhibit autophagy in epithelial ovarian cancer cells. (Xia et al., 2018), (Xiu et al., 2017). MiR-7-5p

was found to play an active role in autophagy in cardiomyocytes of patients with ventricular septal defects, the binding sequence of MEG3 was found in the 3'UTR of miR-7-5p, and the predicted binding site of miR-7-5p was also found in the 3'UTR of EGFR. Upregulated MEG3 reverses myocyte autophagy through the miR-7-5p/EGFR axis (Kitamura et al., 2005; Kobayashi et al., 2012; Zhang et al., 2016; Li et al., 2021). Low expression of lncRNA MEG3 upregulates miR-543 to regulate autophagy in the IDO signaling pathway (Wang et al., 2021c). continued downregulation of MEG3 leads to IFN- γ induction of autophagy in infected macrophages (Pawar et al., 2016). LncRNA MEG3 enhances TNF- α -induced autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway while inhibiting autophagy through the Beclin-1 signaling pathway (Pu et al., 2019; Tang et al., 2021).

2.4 Inflammation

Inflammation is a double-edged sword with many functions that can be seen everywhere in the process of injury and the repair of disease. MEG3 regulates inflammation in a number of ways. KLF4, an important regulator of macrophage polarization, was decreased in M1 macrophages and significantly increased in M2 macrophages, and the expression of macrophage proinflammatory genes with KLF4 knockout was increased (Liao et al., 2011). Inhibition of MEG3 can regulate the secretion of microglia inflammatory cytokines by inhibiting M1 polarization and promoting M2 polarization through KLF4 (Li et al., 2020a). M2 macrophages themselves have certain anti-inflammatory activity. MEG3 can regulate the miR-20b-5p/CREB1 axis and induce M2 macrophage-derived extracellular vesicles (M2-EVs) to reduce the inflammatory response (Wang et al., 2021d). Upregulation of MEG3 in ulcerative colitis (UC) can enhance the protective effect of M2-macrophage-derived extracellular vesicles (M2-evs) against UC and reduce inflammation (Wang et al., 2021d). MEG3 upregulation in methylene blue (MB)-induced peripheral nerve axons alleviates pain and inflammation associated with obstructive sleep apnea by inhibiting P2X3 protein expression (Li et al., 2018b).

Inflammasomes are multimeric protein complexes that are assembled in the cytosol upon sensing pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (Strowig et al., 2012). AIM2 is one of the key proteins that regulates the formation of the inflammasome. Overexpression of MEG3 regulates the action of AIM2 on caspase1 signaling to trigger an inflammatory response (Liang et al., 2020). After sensing multiple molecular patterns, the n-terminal pyrin fragment (PYD) of NLRP3 serves as a scaffold to nucleate apoptosis-associated spotted proteins and recruit caspase preproteins to the inflammasome (Cai et al., 2014; Lu et al., 2014). MEG3 increases NLRP3-induced inflammation

by inhibiting miR-223 or miR-7A-5p (Zhang et al., 2018; Meng et al., 2021). Upregulated MEG3 increases the CXCL12/CXCR4/Rac1 axis and TLR4/NF- κ B pathway by inhibiting miR130A-5P, aggravating neuroinflammation (Dong et al., 2021b). Under the condition that IL-1 β induces chondrocytes to simulate OA, MEG3 regulates the miR-93/TGFBR2 axis to regulate the inflammatory response and delay the OA process (Chen et al., 2021). miR-181b is an important gene in many human diseases. Experiments verified that lncRNA MEG3 directly regulates the level of miR-181b by binding the 3'UTR of miR-181B and reduces the levels of TNF- α and IL-1B in the serum and cerebrospinal fluid of ICH rats (Xie et al., 2021). MEG3 upregulation inhibits the expression of miR-146a and LET-7I, and the inflammatory factors IL-1 β , IL-6 and TNF- α decrease synchronously, alleviating the inflammatory response in ankylosing spondylitis (Li et al., 2020b), (Ma et al., 2020). Acute pancreatitis (AP) is marked by severe inflammation. In the *In vitro* model of AP, the expression levels of MEG3 and FGFR2 are decreased, while the expression levels of miR-195-5p are increased. MEG3 promotes inflammatory responses through the MEG3/miR-195-5p/FGFR2 signaling axis (Chen and Song, 2021). MEG3 overexpression can downregulate miR-34a, enhance SIRT1 protein expression, inhibit the secretion of the inflammatory factors IL-1 β , IL-6 and TNF- α , and inhibit liver inflammation by targeting SIRT6 with EZH2 (Tong et al., 2019; Zou et al., 2022).

2.5 Endoplasmic reticulum

During a large amount of genetic and environmental damage, the ER of the cell is hindered in its ability to properly fold and translate postmodified proteins, leading to misfolding of proteins in the organelles, known as “ER stress”. When a large number of misfolded proteins are present, an intracellular signaling pathway called the “unfolded protein response” (UPR) performs transcription and translation functions, restoring homeostasis. Endoplasmic reticulum stress and UPR defects are increasingly becoming key factors in human diseases, and the regulatory role of MEG3 in this aspect has begun to emerge (Sevier and Kaiser, 2002; Tu and Weissman, 2004; Ron and Walter, 2007; Wang and Kaufman, 2012). After MEG3 is downregulated in rectal cancer cells and MEG3 is restored, UPR response-related proteins, including GRP78, ATF6 and CHOP, are highly induced, and the cell apoptosis rate increases. The above reactions can be alleviated by simulated administration of miR-103A-3p. It can be concluded that MEG3 induces ER stress to promote the apoptosis of rectal cancer cells, and the interaction between miR-103A-3p and MEG3 negatively regulates this process (Wang et al., 2021e). At the same time, MEG3 upregulation activates NF- κ B signaling to initiate ERS (Bao et al., 2020). As also mentioned above, MEG3 is involved in ER stress in HCC

cells and cardiomyocytes by activating the p53 signaling pathway.

2.6 Cell proliferation, migration and invasion

Genomic deletion of QKI-5, a member of the RNA (STAR) signal transduction activator protein family, promotes proliferation and dedifferentiation of cancer cells (Fu and Feng, 2015; He et al., 2016). Overexpression of MEG3 inhibits the expression of miR-9-5p and then upregulates QKI-5 to inhibit the proliferation, migration and invasion of prostate cancer cells (Wu et al., 2019). MEG3 overexpression inhibits the proliferation, invasion and migration of pituitary tumor cells, which is achieved by negatively regulating miR-23b -3p and miR-23b -3p negatively regulating FOXO4 (Wang et al., 2021b). MEG3, miR-376B-3p and HMGA2 form a signaling axis to regulate the invasion of pituitary tumor cells (Zhu et al., 2020). MEG3 and miR-361-5p regulate FoxM1 *in vitro* and inhibit the proliferation, migration and invasion of osteosarcoma cells (Shen et al., 2019). In a study of gastric cancer, MEG3 overexpression inhibits the expression of miR-21 and inhibits cell proliferation and metastasis (Dan et al., 2018).

For thyroid cancer cells resistant to 131I, low expression of MEG3 means increased expression of miR-182 and promotion of cell proliferation, which is detrimental to the treatment effect of 131I for thyroid cancer (Liu et al., 2018).

2.7 EMT

EMT was originally defined as the developmental program of epithelial cells during gastrula formation or movement of various cell types from the neural crest to distant sites in the embryo (Nieto et al., 2016; Dongre and Weinberg, 2019). In adult epithelial tissue, EMT is involved in the physiological process of wound repair and can be activated under certain pathological conditions, such as fibrosis and cancer (Nieto et al., 2016). In principle, cell phenotypic transitions engineered by EMT are reversible, so that individual cells previously activated by the EMT program can and often do revert to an epithelial state through mesenchymal to epithelial transition (MET) (Lu and Kang, 2019). EMT programs are initiated in cells by paracrine signaling factors to which they are exposed, most notably transforming growth factor- β (TGF β). In addition, there are various other signals, such as WNT proteins, cytokines, growth factors, and extracellular matrix (ECM)-integrin interactions (Lambert and Weinberg, 2021). In reports on U251 cells, MEG3 overexpression resulted in decreased EMT, decreased expression of n-cadherin, vimentin, snail-1 and β -catenin, and increased expression of e-cadherin in U87 and U251 cells (Gong and Huang, 2020). MEG3 silencing also

promotes EMT by regulating the miR-377/PTEN axis (Wang et al., 2019b). Yang et al. reported that the expression of MEG3 induced more mesenchymal cell morphology and increased zeb1/2 expression. Notably, autophagy inhibition can inhibit MEG3-induced EMT (Gao et al., 2018).

3 lncRNA MEG3 plays a role in cardiovascular disease

3.1 Cardiac fibrosis

Cardiac fibrosis is a pathologic process involved in almost all cardiac diseases. Cardiac fibroblasts (CFs) are the main coordinators of extracellular matrix (ECM) remodelling after left ventricular pressure overload and play a key role in maintaining cardiac ECM integrity and homeostasis. Upon injury, CFs are activated into myofibroblasts that produce matrix metalloproteinases (MMPs) (Eghbali et al., 1989; Spinale, 2007; Travers et al., 2016; Frangogiannis, 2021). As a member of the MMP family, the increase in MMP-2 leads to the aggravation of fibrosis. lncRNA MEG3 was significantly enriched in myocardial fibroblasts. MEG3 silencing inhibits transforming growth factor (TGF- β I), interferes with the binding of p53 to sites -843 and -825 on the MMP-2 promoter, inhibits the activity of MMP-2, and subsequently decreases the expression of the fibrosis marker Ctgf. Thus, lncRNA MEG3 is coupled with p53 and plays a role in myocardial fibrosis (Piccoli et al., 2017) (Figure 3A).

3.2 Myocardial cells

3.2.1 Cardiomyocyte hypertrophy

In the process of cardiac hypertrophy, the role of lncRNA MEG3 cannot be ignored. Jingchang Zhang et al. found that the expression of MEG3 was abundant in cardiomyocytes treated with 150 nM ANG-II, and SH-MEG3 reversed the increase in the surface area of cardiomyocytes induced by ANG-II. Meanwhile, the expression of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and β -myosin heavy chain (β -MHC) were decreased. This result preliminarily indicates that MEG3 positively regulates cardiac hypertrophy. After further research, Jingchang Zhang et al. found the promoter of MEG3-STAT3 with a binding relationship at site 2. Activated by STAT3, MEG3 competitively binds and inhibits miR-361-5p, upregulates downstream HDAC9 protein, and has a negative effect on cardiac hypertrophy (Zhang et al., 2019a).

3.2.2 Cardiac autophagy

Several studies have demonstrated that multiple upstream signals can regulate mTOR activity by regulating the phospholipid inositol three kinase protein (PI3K)/kinase B

(AKT) signaling pathway (Nishimoto, 2000; Sun et al., 2017b; Wang et al., 2018). Hou et al. (2014) reported that advanced glycation end products inhibited the PI3K/Akt/mTOR pathway to promote cardiac autophagy. miR-7-5p is an important regulator of autophagy in a variety of cells (Song et al., 2016; Cai et al., 2017; Gu et al., 2017). Yinyin Cao et al. reported that MEG3 directly targets miR-7-5p and regulates downstream mTOR signal activation, thus regulating cardiac autophagy (Cao et al., 2019).

3.2.3 Myocardial cell apoptosis

Hongchun Wu et al. reported that in hypoxic cardiomyocytes, p53 affects the expression of Meg3 by directly binding upstream of the Meg3 gene. Meg3 is upregulated by P53 and promotes cell apoptosis by directly binding to FUS protein. At the same time, after MEG3 inhibition, the expression of the pro-apoptotic gene Caspase-3 was decreased, and the expression of the anti-apoptotic gene Bcl-2 was increased, thus inhibiting myocardial cell apoptosis.²³ Ya Zhao et al. demonstrated that MEG3 regulates the expression of Bcl-2 and Caspase-3 proteins through miR-325 3p mediation, negatively regulating the expression of TRPV4 and thus alleviating myocardial cell injury and apoptosis (Zhou et al., 2021). In the study of Bin Yang et al., Bax expression was downregulated in H9C2 cells after oxygen and glucose deprivation (OGD) induction, caspase-3 and Caspase-9 were cleaved, and apoptosis occurred. In this process, MEG3 expression is increased, MDM2 depletion is increased, and the production of p53 is promoted, while the phosphorylation level of AMPK is reduced (Yang et al., 2019b). In the study of Yiwei Chen et al., lncRNA MEG3 expression was significantly increased in AC16 cardiomyocytes induced by high glucose (HG). Meanwhile, downregulation of MEG3s.

Three could restore the effects of decreased expression of Bcl-2/Bax and increased expression of Caspase-3, which had beneficial effects on cell viability and apoptosis. Further studies showed that miR-145 directly acted on MEG3 and targeted PDCD4 protein at the 3'UTR binding site, and inhibition of miR-145 would offset the beneficial effects of downregulated MEG3 on HG-treated AC16 cardiomyocytes (Chen et al., 2019). Through the study of H9C2 cardiomyocytes after I/R injury, it was confirmed that downregulation of MEG3 can directly and negatively regulate miR-7-5p, regulate the downstream PARP1 protein and caspase-3 protein of miR-7-5P, and save cell apoptosis (Zou et al., 2019).

ERS induced by myocardial ischemia can induce myocardial cell apoptosis (Wei et al., 2016). *In vivo* experimental studies have shown that inhibition of lncRNA MEG3 can reduce the upregulation of endoplasmic reticulum stress-related proteins after ischemia, including BIP/GRP78, ATF4, and C-EBP homologous proteins (CHOP) (Li et al., 2019). Decreased levels of reactive oxygen species (ROS) are downstream events

of endoplasmic reticulum stress after ischemia, and lenti-Si lncRNA MEG3 pretreatment can reduce ROS levels. Superoxide dismutase (SOD), an important antioxidant enzyme, is significantly reduced in ischemic myocardium, and downregulation of the MEG3 gene can negatively regulate this effect (Li et al., 2019). Previous studies have shown that the PERK-eIF2 α and Caspase 12 pathways play a dominant role in stress-mediated apoptosis (Groenendyk et al., 2013), and downregulation of lncRNA MEG3 can inhibit ER stress through this pathway. In conclusion, downregulation of MEG3 can inhibit endoplasmic reticulum stress in cardiomyocytes and protect cardiomyocytes (Li et al., 2019).

According to L.-Y. Zhao et al., lncRNA MEG3 can also promote the apoptosis of hypoxic cardiomyocytes through the FoxO1 signaling pathway. FoxO1 is activated in the form of phosphorylation, which affects cell stability, leads to cell disorder, and promotes apoptosis of cardiomyocytes (Liu et al., 2017a; Kinyua et al., 2018). However, MEG3 upregulation in hypoxic cardiomyocytes not only upregulates FoxO1 but also increases the expression of FoxO3a in the same family. The combination of the two can enhance autophagy of cardiomyocytes and aggravate apoptosis of cardiomyocytes (Zhao et al., 2019).

3.2.4 Survival and proliferation of cardiomyocytes

Jinwen Su et al. found that the survival and proliferation of cardiac progenitor cells (CPCs) were inhibited under hypoxia, and the level of MEG3 significantly increased. Moreover, the cell viability and proliferation potential of cardiomyocytes increased after MEG3 inhibition. With the inhibition of MEG3, the expression level of miR-22 is increased, the expression level of HMGB1 protein, the next level target of MEG3, is decreased, and cell viability and proliferation are restored. In summary, MEG3/miR-22/HMGB1 has been confirmed to play a role in the survival and proliferation of cardiomyocytes after hypoxia (Su et al., 2018) (Figure 3B).

3.3 lncRNA MEG and vasculature

3.3.1 Angiogenesis

Liu et al. showed that MEG3 silencing can negatively regulate the Notch pathway to promote endothelial cell proliferation and angiogenesis (Liu et al., 2017b). In line with this, Chao He et al. found in their study that MEG3, as a ceRNA, inhibits miR-9, affects the phenotype of MEG3-mediated vascular endothelial cells (VECs), and inhibits cell proliferation and *in vitro* angiogenesis. However, the restoration of miR-9 can only rescue part of the angiogenesis inhibition caused by MEG3, which means that other microRNAs are also involved in this process (He et al., 2017). The experiment of Liu, H. Z et al. reported that the interaction between MEG3 and miR-150-5p was also involved in the angiogenesis of endothelial progenitor

cells (Liu et al., 2016), which confirmed this point. In a report on anisomycin inhibition of angiogenesis, the spongy action of lncRNA MEG3 was inhibited and angiogenesis was inhibited via the miR-421/PDGFR α axis (Ye et al., 2019).

3.3.2 DNA damage of endothelial cells

DNA double-strand breaks (DSBs) are a serious form of DNA damage. Tail length and tail moment of DNA are positively correlated with DSB burden (Olive and Banáth, 2006; Ribas-Maynou et al., 2012). MEG3 is a DNA damage response gene mediated by the p53 pathway. ⁵According to a report, in endothelial cells, after Meg3 was knocked out by 10 nM GapmeRs (chemically modified antisense oligonucleotides), tail length and tail camber were increased by 1.9 times and 2.7 times, respectively. After Meg3 is reduced by 2 nM GapmeRs, tail length and tail camber are increased by 2.4 times and 2.9 times, respectively (Shihabudeen Haider Ali et al., 2019). Phosphorylation of histone H2AX at Ser-19, a marker of DSB damage and repair (Mah et al., 2010; Sharma et al., 2012; Turinetti and Giachino, 2015), also increases. These data suggest that MEG3 protects DNA from damage in endothelial cells. Further studies have found that knocking down PTBP3 induces the expression of the target gene p53, leading to cell apoptosis, while MEG3 can participate in the DSB process by regulating PTBP3's action on p53 or directly acting on p53, thus protecting endothelial function (Shihabudeen Haider Ali et al., 2019).

3.3.3 Endothelial cell senescence

lncRNA MEG3 also plays a role in endothelial cell senescence. It has been detected that the competitive adsorption of MEG3 in aging endothelial cells decreases, leading to the upregulation of miR-128 and the targeting of Girdin protein, reducing the expression of Girdin protein. In addition, these processes inhibit platelet phagocytosis of endothelial cells (Lan et al., 2019) (Figure 3C).

4 Conclusion

lnc1. The diseases involving lncRNA MEG3 mainly include osteosarcoma, laryngeal cancer, prostate cancer, lung cancer, liver cancer, breast cancer, endometrial cancer, oral squamous cell carcinoma, gastric cancer, pancreatic cancer, colorectal cancer, and other malignant tumors. Metabolic diseases such as diabetic retinopathy; rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus and other immune system diseases; heart failure, hypertension, stroke and other cardiovascular and cerebrovascular diseases. A comprehensive understanding of its mechanism is essential for its safety and efficacy in clinical application.

lncRNA MEG3 plays a preventive and/or therapeutic role in cardiovascular diseases by reducing inflammatory injury,

apoptosis and endoplasmic reticulum stress. lncRNA MEG3 is closely related to various pathologic effects of cardiomyocytes, fibroblasts and endothelial cells, and has great clinical application potential in the prevention and treatment of AS, MIRI, hypertension and HFLnc. Although some knowledge has been gained about the benefits of lncRNA MEG3 for cardiovascular disease from experimental data, it is worth noting that its specific underlying mechanisms are still relatively unknown. Therefore, available clinical and pharmacological data are insufficient to evaluate its efficacy.

In addition, lncRNA MEG3 has a profound research foundation in tumor diseases, first showing biomarker value in straight-colon cancer (Wang et al., 2019c), gastric cancer (Ghaedi et al., 2019), breast cancer (Ali et al., 2020) and other cancers (Duan et al., 2016; Zhao et al., 2018; Ali et al., 2020). Further research and technology development are needed to improve its bioavailability and overcome the challenges of its clinical application. Second, MEG3 expression is associated with WHO grade of tumor, old age at diagnosis, Karnofsky performance score (KPS), wild-type isocitrate dehydrogenase (IDH), tumor recurrence and overall survival, which is of prognostic value (Gong and Huang, 2017; Zhao et al., 2018; Buccarelli et al., 2020). Third, enhanced MEG3 expression can increase chemotherapy sensitivity to cisplatin, while SI-RNA silencing of MEG3 can induce cisplatin resistance (Ma et al., 2017), which can be used to measure therapeutic response. At the same time, MEG3 has gradually gained attention in cardiovascular disease, a major human public health problem. It can be a worthy object of study. Based on the research basis of lncRNA MEG3 in cancer diseases, we boldly speculate that MEG3 will play a role in the emerging discipline of cancer heart disease (Momtazmanesh and Rezaei, 2021).

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Author contributions

In this paper, ZL is the first author and JG is the co-first author. ZL was responsible for the compilation, drawing and submission of the article; JG was responsible for the design of the article; DS, QJ, JM, SL and Xu Fan were responsible for the collation of the literature; YL and WC were responsible for the proofreading; HL was the corresponding author.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Peripheral vascular remodeling during ischemia

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About 230 million people worldwide suffer from peripheral arterial disease (PAD), and the prevalence is increasing year by year. Multiple risk factors, including smoking, dyslipidemia, diabetes, and hypertension, can contribute to the development of PAD. PAD is typically characterized by intermittent claudication and resting pain, and there is a risk of severe limb ischemia, leading to major adverse limb events, such as amputation. Currently, a major progress in the research field of the pathogenesis of vascular remodeling, including atherosclerosis and neointima hyperplasia has been made. For example, the molecular mechanisms of endothelial dysfunction and smooth muscle phenotype switching have been described. Interestingly, a series of focused studies on fibroblasts of the vessel wall has demonstrated their impact on smooth muscle proliferation and even endothelial function via cell-cell communications. In this review, we aim to focus on the functional changes of peripheral arterial cells and the mechanisms of the pathogenesis of PAD. At the same time, we summarize the progress of the current clinical treatment and potential therapeutic methods for PAD and shine a light on future perspectives.

KEYWORDS

ischemia, endothelial cells, smooth muscle cells, fibroblasts, vascular disease

Introduction

Peripheral arterial disease (PAD) is a kind of vascular disease in which the blood flow is restricted due to stenosis or obstruction of arteries, resulting in tissue ischemia. There are various disease included in PAD, such as visceral artery aneurysm, arteritis, carotid artery stenosis and atherosclerosis obliterans (ASO). In this review, PAD mainly refers to ASO. With increasing incidence and mortality, PAD has affected more than 230 million people worldwide (Song et al., 2020). Traditional cardiovascular risk factors, including advanced age, hypertension, diabetes, and smoking, are strongly associated with the risk of PAD (Selvin and Erlinger, 2004; Allison et al., 2006; Fowkes et al., 2013; Whelton et al., 2018). Patients who suffer from PAD often have coronary or cerebrovascular disease as well. The typical symptom of PAD is intermittent claudication, mainly being manifested in the lameness of the lower limbs after walking for a certain distance. The above symptoms can be relieved by a short rest (Gerhard-Herman et al., 2017; Golledge, 2022). As the disease worsens, the patient develops resting pain (Jones and Farber, 2020). This is

due to the aggravation of the lesion, resulting in occlusion of arteries, leading to ischemia and hypoxia of the skeletal muscle. After tissue ischemia, peripheral arteries display a positive enhancement of vascular remodeling, in which atherosclerosis is the main cause of peripheral vascular obstruction (Newman et al., 1993; Stock, 2022). On the other hand, ischemia can also trigger a series of cellular events that lead to peripheral vascular remodeling.

At the present, the exploration of the pathogenesis of peripheral artery disease is limited. We do not yet fully understand the complex cellular functional changes and the mechanisms underlying cell-to-cell interactions that drive PAD. Under physiological conditions, vascular cells, such as endothelial and smooth muscle cells (SMCs), and fibroblasts, are essential for maintaining stable vascular structure and function. When ischemia occurs, dysfunction of vascular cells leads to peripheral arterial remodeling. Currently, drug usage and revascularization approaches are the main treatments for PAD (Wiseman et al., 2017; Farb et al., 2021; Sogaard et al., 2021), although the effectiveness is limited. In this article, we aim to discuss the mechanism of peripheral vascular cell dysfunction under ischemic conditions, summarize the clinical treatment and potential therapeutic methods and highlight the potential future research focusing on PAD.

The role of endothelial cells in peripheral arterial disease

Vascular endothelium regulates the cellular and molecular transport between blood and tissues and plays an important role in maintaining the stability of vessel wall (Bazzoni and Dejana, 2004; Trimm and Red-Horse, 2022). Tissue ischemia leads to lower oxygen supply, free radical production, and other cellular response in most tissues. In response to tissue ischemia of the limb, the inflammatory response of endothelial cells (ECs) is closely related to the progression of vascular lesion development (Gimbrone and Garcia-Cardena, 2016; Duan et al., 2021). As a critical enhancer, tissue ischemia not only induced the expression of Vcam-1 and Icam-1 on ECs, but also upregulated the levels of inflammatory markers (IL-1 β , IL-6 and TNF- α). Meanwhile, tissue ischemia also results in production of platelet-derived exosomes in serum that play an important role in a variety of pathological mechanisms (Li et al., 2017). Exosomes are mainly involved in immune response and cell-to-cell communication in the pathogenesis of atherosclerosis (Bu et al., 2021). As an intercellular communication carrier, exosomes can wrap miRNA for transfer. It has been found that miRNA regulate the expression of related target genes by matching the target sites of the 3' untranslated region (3'utr) of target genes (Ambros, 2004; Bartel, 2004). Evidence showed that ECs could endocytose exosomes to upregulate miR-25-3p, and suppress levels of α -smooth muscle actin (α -SMA), collagen I α 1, Collagen III α 1, IL-

1 β , IL-6, and TNF- α . miR-25-3p inhibits endothelial inflammation by inhibiting the expression of Adam10, and reducing the phosphorylation level of NF- κ B signaling pathway (Yao et al., 2019). Exosomes containing miR-155 have been reported to translocate from smooth muscle cells (SMCs) to ECs, causing endothelial dysfunction and ultimately aggravating lesion formation of atherosclerosis (Zheng et al., 2017). Furthermore, miR-155 ameliorates ox-LDL-induced inflammation in human umbilical vein endothelial cells (HUVECs) by inhibiting SOCS1-dependent NF- κ B signaling (Zhang et al., 2017), in which miR-19 attenuates endothelial dysfunction induced by ischemia by negatively regulating KLF10 and inhibiting TGF- β 1/Smad signaling pathway (Xu et al., 2018). In addition, Jiang et al. (2019) found that miR-449a was significantly up-regulated in atherosclerosis lesions and also was able to promote proliferation and migration of ECs. Besides, it could also induce expression of α -SMA and Smad3, and inhibits E-cadherin expression in ECs. Eventually, it was found that miR-449a enhances the expression of VACM-1 and ICAM-1 induced by TNF- α in ECs (Jiang et al., 2019). These results indicate that miRNA is an important regulator of endothelial inflammation in response to ischemia, where exosomes are one of the important carriers for miRNA transport.

Ischemia-induced EC dysfunction can also lead to the overexpression of endothelin-1 (ET-1). As a potent vasoconstrictor, endothelin-1 (ET-1) is mainly secreted by ECs and interacts with two different G-protein-coupled receptors, ETA and ETB (Yanagisawa et al., 1988; Miyauchi and Masaki, 1999). SMCs express ETA and ETB receptors to mediate vasoconstriction, whereas ETB receptors on ECs mediate vasodilation through the release of nitric oxide (NO) and prostacyclin (Kirchengast and Munter, 1998; Luscher and Barton, 2000). ET-1 has been reported to play an important role in neointimal formation and atherosclerosis (Bacon et al., 1996; McKenna et al., 1998). ET-1 expression is upregulated in diseased vessels when stimulated by shear stress. It was found that loss of ET-1 was associated with decreased expression of VCAM-1 and MCP-1 in endothelial cells and decreased recruitment of inflammatory cells to the vessel wall (Anggrahini et al., 2009). *In vitro* studies also showed that ET-1 enhanced the expression of VCAM-1 in TNF- α -stimulated ECs (Ishizuka et al., 1999). ET-1 has been reported to induce proliferation of SMCs through ERK, p38-activated protein kinase and c-Myc-dependent signaling (Chen et al., 2006). ETA receptor signaling mediates SMC proliferation (Hafizi et al., 1999). Numerous studies revealed that ETA and ETB expression were decreased and SMC proliferation was attenuated in ET-1 null diseased vessels (Anggrahini et al., 2009). These results suggest that EC-derived endothelin-1 is involved in mediating SMCs proliferation and endothelial inflammation in a paracrine manner, thereby promoting vascular remodeling (Figure 1).

Moreover, inflammation can induce endothelial-to-mesenchymal transition (EndMT), which leads to pathological

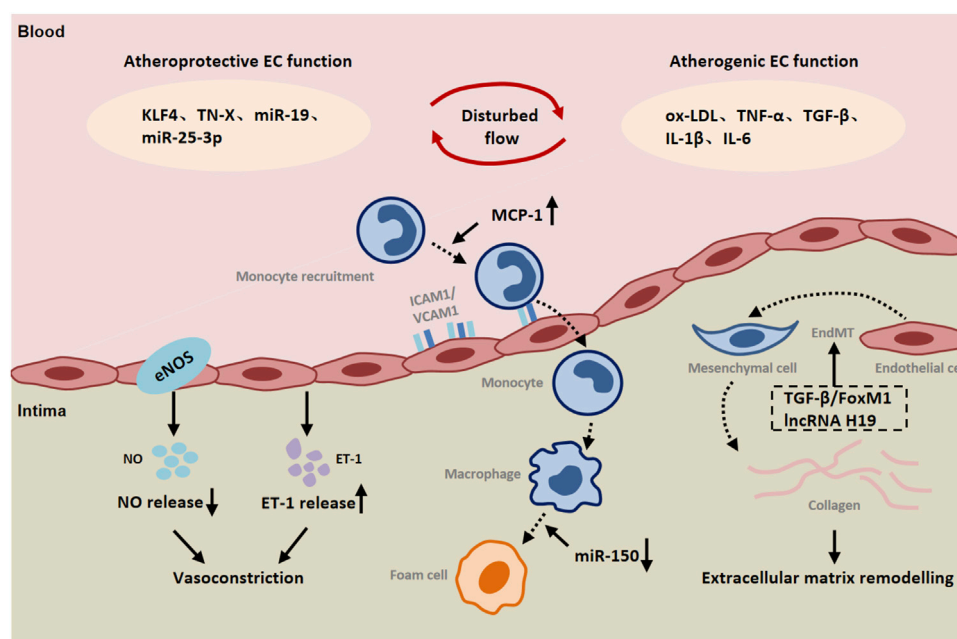
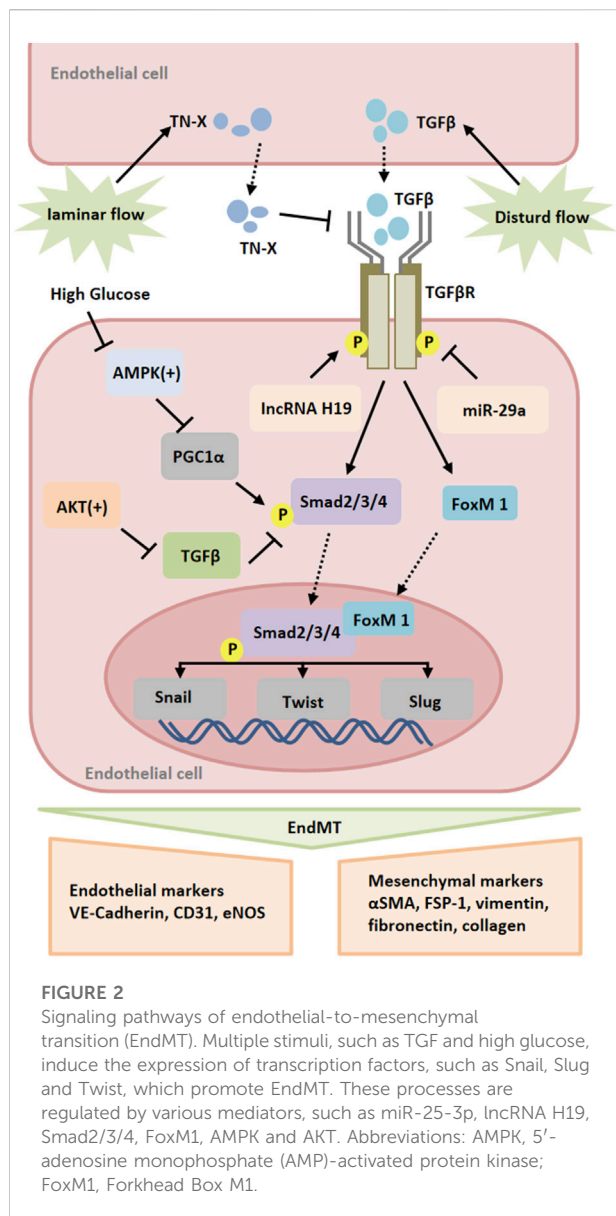


FIGURE 1

The role of endothelial cells (ECs) in peripheral arterial disease (PAD). Both atheroprotective and atherogenic mechanisms are operative in endothelial cells (ECs) exposed to disturbed flow. Endothelial cells are involved in communication with immune cells and smooth muscle cells by releasing a variety of inflammatory cytokines, chemokines and ET-1. Endothelial-to-mesenchymal transition (EndMT) contributes to vascular remodeling. Abbreviations: KLF4, Kruppel like factor 4; TN-X, Tenascin-X; oxLDL, oxidized low-density lipoprotein; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; TGF- β , transforming growth factor- β ; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; FoxM1, Forkhead Box M1; ET-1, endothelin-1.

vascular remodeling (Cho et al., 2018; Sanchez-Duffhues et al., 2019). EndMT is a phenomenon that ECs lose their endothelial markers and acquire mesenchymal characteristics. During EndMT, endothelial markers such as VE-cadherin and CD31 are lost, while the expression of mesenchymal markers such as fibroblast specific protein-1 (FSP-1), α -SMA, N-cadherin, and fibronectin is increased (Li et al., 2018; Kovacic et al., 2019). TGF- β is a key mediator of EndMT. Studies have shown that TGF- β promotes the phosphorylation of Smad2/3, and then regulates the expression of Snail, Twist, Slug and other transcription factors and promotes EndMT (Medici et al., 2011). Recently, increasing evidence highlights the potential role of Foxm1 in vascular fibrosis/remodeling (Balli et al., 2013). According to the report, TGF- β could induce the expression of Foxm1 in ECs. Foxm1 promoted the expression of TGF- β -induced vimentin, α -SMA and FSP1 in ECs through Smad2/3 signaling pathway, and inhibited the expression of VE-cadherin and CD31 (Song et al., 2019). FoxM1 has been reported to interact with Smad3 to maintain activation of the Smad2/Smad3/Smad4 complex in the nucleus. The complex translocated to the nucleus and activated the expression of ECM-related genes as well as Snail, and Twist genes (Xue et al., 2014; Sabbineni et al., 2018). It has been found that Snail inhibits VE-cadherin

expression by directly binding to the E-box sequence within the VE-cadherin promoter (Cano et al., 2000). Activation of AMP-activated protein kinase (AMPK) upregulates the level of peroxidase proliferator-activated receptor- γ coactivator-1 α (PGC1 α), resulting in a decrease in phosphorylated Smad2 and the nuclear translocation of Snail, thereby inhibiting EndMT (Mao et al., 2022). The intracellular signaling pathway mediated by protein kinase Akt is closely related to the regulation of endothelial cell survival, proliferation, migration, glucose metabolism and gene expression (Shiojima and Walsh, 2002). Here, it was found that silencing Akt1 in endothelial cells resulted in increased expression of mesenchymal genes (e.g FN1 and KRT7) and decreased expression of eNOS, accompanied by activation of p38 MAP kinase. AKT1 can negatively regulate the expression of TGF β 2 and Smad2/3 phosphorylation, as well as the expression of Snail1 and FoxC2 (Sabbineni et al., 2019). In addition to the expression of mesenchymal markers, EndMT is also characterized by changes in EC morphology (Krenning et al., 2016). It was observed that normal ECs had a polygonal cobblestone shape, whereas AKT1-null ECs changed into a more spindle-like fibroblast shape. Inhibition of β -catenin reverses EndMT induced by loss of Akt1 (Sabbineni et al., 2019). Taken together, ischemia can directly and indirectly lead



to endothelial-to-mesenchymal transition that plays a part in vascular remodeling in peripheral arteries.

It has been found that different forms of blood flow activate different signal transduction pathways in ECs, leading to vascular remodeling (Zhou et al., 2014; Nakajima and Mochizuki, 2017). In terms of peripheral arteries during ischemia, often there is atherosclerotic lesions resulting altered blood flow. Disturbed flow induces endothelium mesenchymal transition (EndMT). Unidirectional laminar flow inhibited TGF- β signaling as well as TGF- β -induced EndMT compared with disturbed flow (Moonen et al., 2015; Lee et al., 2017). The study found that laminar flow increased endothelial TN-X expression in a KLF4-dependent manner. TN-X expression in endothelial cells was negatively correlated with the expression of mesenchymal

marker genes and inflammatory genes, such as FN1, CCL2, VACM-1, and the phosphorylation of Smad. TN-X directly interacts with TGF- β through its fibrin-like domain, thereby interfering with its binding to TGF- β receptors (Liang et al., 2022). Elevated TGF- β levels were also found in the plasma of TN-X deficient patients (Morissette et al., 2014). In addition, lncRNA H19 was significantly upregulated in TGF- β -induced EndMT. The expression of endothelial marker CD31 was up-regulated, and the inhibition of mesothelial cell marker FSP-1 was related to the inhibition of lncRNA H19. Moreover, the increased expression of lncRNA H19 could alleviate the inhibitory effect of miR-29a on EndMT-related gene expression (Shi et al., 2020). These results indicate that lncRNA H19 can compete with miR-29a and participate in the regulation of EndMT development through TGF- β /SMAD3 pathway (Figure 2).

Tissue ischemia results in accumulation of free radicals locally, especially in peripheral arteries. In fact, oxidative stress is one of the important causes of vascular endothelial dysfunction (Cai et al., 2003). Elevated vascular reactive oxygen species (ROS) production is considered to be an early trigger mechanism for endothelial dysfunction, including oxidative stress, activation of proinflammatory signaling cascades, and reduction of endothelial nitric oxide (NO) bioavailability (Hsieh et al., 2014; Blaser et al., 2016; Wu et al., 2018). The NOX protein family contains several isoforms, including NOX1, NOX2, and NOX4, whose sole function is to produce ROS (Bedard and Krause, 2007). Under physiological conditions, NOX1 and NOX2 are expressed at low levels in the peripheral vascular, but up-regulated in pathological situations such as hypertension, diabetes and hyperlipidemia (Mollnau et al., 2002; Wendt et al., 2005). Increased NOX1 expression in ECs promotes the expression of MCP-1, VCAM-1, CTGF and collagen I/III/IV, which contributes to lesion formation (Gray et al., 2013). Moreover, there was a study demonstrates that NOX2 expression was upregulated in ECs of diseased vessels. Further studies revealed that decreased arterial superoxide production could significantly reduce lesion formation and increased NO bioavailability when NOX2 was deficient (Judkins et al., 2010). Endothelium-derived NO plays a protective role in vasculature by inhibiting the contraction and proliferation of vascular SMCs. NOX derived ROS can not only reduce the bioavailability of NO, but also directly react with NO to produce the oxidizing substance peroxynitrite (Judkins et al., 2010). Peroxynitrite has been reported to oxidize tetrahydrobiopterin (BH4), a cofactor of eNOS, leading to uncoupling of eNOS (Laursen et al., 2001). Compared with NOX1 and NOX2, NOX4 mainly plays a protective role in the cardiovascular system. NOX4 is predominantly expressed in ECs. Multiple recent reports indicate that Nox4 produces primarily H_2O_2 rather than superoxide (Schader et al., 2020). Low concentration of H_2O_2 contributes to vascular protection. H_2O_2 can not only maintain

vasodilatation by activating protein kinase G, but also activate and induce eNOS expression (Indra et al., 1999; Garcia-Prieto et al., 2019). It has been found that H₂O₂ promoted vasodilation by enhancing Ca²⁺ release from endoplasmic reticulum stores, which in turn promoted the opening of Ca²⁺-activated K⁺ channels (Hecquet et al., 2008). Katrin et al. proved that endogenous Nox4 accelerated the expression of eNOS, the formation of NO, and the expression of the antioxidant HO-1. HO-1 expression is controlled by the transcription factor Nrf-2. NOX4 reduces Nrf-2 degradation by producing H₂O₂, thereby promoting HO-1 expression. Loss of NOX4 not only exacerbates Ang II-induced vascular stenosis but also increases the rate of endothelial cell apoptosis, which is an important stimulator of vascular inflammation (Schroder et al., 2012). Moreover, NOX4 in endothelial cells can inhibit the expression of MCP-1, IL-1 β , TGF- β and other proinflammatory factors, and limit vascular inflammation and macrophage infiltration. It has been reported that TGF- β /SMAD3 signaling pathway is involved in the regulation of CTGF expression and vascular fibrosis (Mao et al., 2020). The expression of EC-NOX4 was negatively correlated with the phosphorylation level of SMAD3 and the expression of CTGF (Gray et al., 2016). Thus, the balance between NOX-mediated free radical generation and eNOS pathway-generated NO production can be disturbed during ischemia, resulting endothelial dysfunction and eventually peripheral vascular remodeling.

The role of smooth muscle cells in peripheral arterial disease

In normal arteries, SMCs remain quiescent and highly differentiated. However, they have dedifferentiation potential and plasticity compared with skeletal muscle and cardiomyocytes. Under pathological conditions, SMCs can switch from a contractile to a synthetic phenotype, which is also known as SMC phenotypic switching. During SMC phenotypic transition, the cell loses specific markers, such as smooth muscle actin and SMMHC (Hao et al., 2003; Gomez and Owens, 2012). This type of SMCs serves as main player for intima hyperplasia that is a key event for peripheral vascular remodeling (Owens et al., 2004). In response to ischemia and ischemia-mediated cytokines released from ECs, SMC undergo metabolic reprogramming upon activation to rapidly provide high energy and meet the biosynthetic requirements for cell proliferation (Kim et al., 2017). Increased glycolysis is essential for bioenergetic transfer during SMC proliferation and migration (Park et al., 2021). Pyruvate kinase (PKM2) is an important regulatory element in glycolysis and is mainly expressed in highly proliferating cells, such as stem cells and tumor cells (Xie et al., 2016; Liu et al., 2017). Emerging studies show that PKM2 is activated and nuclear translocated in neointimal SMC during neointimal formation. Nuclear PKM2 interacts with STAT3 and

promotes STAT3 phosphorylation, thereby regulating MEK5 transcription in SMCs (Jain et al., 2021). At the same time, Liao et al. (Liao et al., 2015) found that STAT3 can regulate SMC phenotypic switching by interacting with myocardin. Furthermore, PKM2 was found to interact with β -catenin and enhance the expression of cyclin D1, GLUT1, and LDHA (Jain et al., 2021). Up-regulation of cyclin D1 and LDHA plays a key role in cell cycle progression and lactate production (Kim et al., 2017). Overexpression of glucose transporter GLUT1 in SMCs mediates their proliferation and phenotypic switching by regulating glucose transport/glucose metabolism (Pyla et al., 2013). It has been found that lactic acid, as a metabolite of glycolysis, can promote the synthetic phenotype of SMCs. Lactate-induced phenotypic modulation of SMCs is mediated by monocarboxylic acid transporters and N-myc downstream regulated gene (Yang et al., 2017). These results indicate that energy metabolism is an important factor influencing SMC functional transformation during tissue ischemia leading to vascular remodeling.

Concerning the molecular mechanisms of SMC phenotype changes, epigenetic inheritance also plays an important role in the process (Alexander and Owens, 2012). Epigenetic inheritance refers to heritable changes in the function of a gene without changes in its DNA sequence, which ultimately lead to changes in the phenotype. Epigenetic regulatory mechanisms include DNA methylation, histone acetylation, and noncoding RNA (Yao et al., 2016; Carter and Zhao, 2021). Increased histone acetyltransferase (HAT) activity stimulated SM22 α expression, while increased histone deacetylase (HDAC) inhibited SM22 α expression (Gomez et al., 2015). PDGF-BB and oxidized phospholipids induce KLF4 to recruit HDAC in the CArG box region. KLF4, ELK-1 and HDAC form a complex, which reduces the histone H3 acetylation level in the SM22 α promoter by binding to G/C repressor elements, resulting in down-regulation of SM22 α expression (Salmon et al., 2012). MicroRNA also play a key role in the regulation of SMC phenotype, especially the miR-143/145 cluster, which is a potent promoter for the contraction of SMC phenotype. miR-145 maintained the contractile phenotype of SMCs by inducing the expression of contractile genes, while down-regulating the expression of KLF4 and Elk-1 (Cordes et al., 2009; Lovren et al., 2012). Therefore, the mechanism of SMC phenotype switching involves not only different signal pathways but also epigenetic modification that leads to changes of gene expression profile.

There is an animal model of arterial ligation where artery ischemia occurs. The study has shown that increased expression of CyPA secreted by SMCs leads to intimal hyperplasia by promoting cell proliferation and migration. In ischemic arteries, CyPA can induce the increase of VCAM-1 expression, thereby recruiting inflammatory cells to the vessel wall. Additionally, ROS produced locally by inflammatory cells will further promote the release of CyPA from SMCs, which will form a proinflammatory cycle that promotes vascular

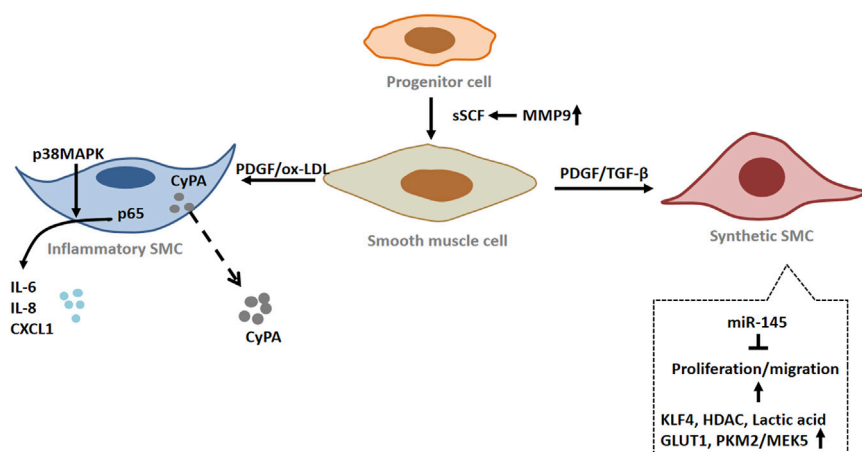


FIGURE 3

The role of smooth muscle cells (SMCs) in peripheral arterial disease (PAD). Under pathological conditions, smooth muscle cells (SMCs) undergo phenotypic switching. The proliferation and migration of SMC with synthetic phenotype were enhanced. Inflammatory SMCs produce and secrete a variety of inflammatory factors. When blood vessels are injured, vascular progenitor cells become activated and differentiate into SMCs. Abbreviations: SCF, stem cell factor; MMP9, matrix metalloproteinase 9; PDGF, platelet derived growth factor; IL-6, interleukin-6; IL-8, interleukin 8; CXCL1, C-X-C motif chemokine ligand 1; p38 MAPK, p38 mitogen activated protein kinase; HDAC, histone deacetylase; PKM2, pyruvate kinase M2; MEK5, mitogen-activated protein kinase 5; CyPA, cyclophilin A; p65, nuclear factor- κ B p65; GLUT1, glucose transporter 1.

remodeling (Sato et al., 2008). Moreover, activated SMCs synthesize and secrete growth factors, proinflammatory factors and matrix proteins to form an extracellular environment, thereby triggering the infiltration and activation of surrounding proinflammatory cells and progenitor cells and further aggravating intimal hyperplasia (Geary et al., 2002). Concomitantly, it was found that neointimal hyperplasia occurred where there were breaches in the internal elastic lamina, and speculated that elastin disruption would promote neointimal hyperplasia (Lin et al., 2021). Elastin depletion in SMCs promotes their transition from a contractile to a synthetic phenotype (Rebello et al., 2021). What's more, extracellular matrix glycoprotein biglycan enhances vascular smooth muscle cell proliferation through cdk2- and p27-dependent pathways (Shimizu-Hirota et al., 2004). This may be a reactive response to ischemia, which triggers transcriptional and phenotypic changes in SMCs, causing them to migrate into the intima and proliferate.

As mentioned above, ischemia alters the balance of redox process in the arterial wall. Redox signaling helps maintain the SMC contractile phenotype and proliferative response associated with vascular remodeling (Tong et al., 2010; Tong et al., 2015). As a member of the p38MAPK family, MAPK14 is a redox regulatory element. MAPK14 is considered to be a key regulator of SMC inflammation, proliferation and migration. Previous studies have found that SMC-MAPK14 can regulate MKL1 nuclear translocation and is a negative regulator of SMC contractile phenotype (Long et al., 2013). Some studies showed that in the neointima of mouse carotid arteries, SMCs in the neointima region showed high expression level of

MAPK14 protein, accompanied by a decrease in the expression of MYH11. NOX4 is a key mediator of MAPK14 regulatory of SMC contractile gene expression (Wu et al., 2019). At the same time, knockdown of MAPK14 in SMC would lead to down-regulation of PDGF α and PDGF β expression, thereby inhibiting the proliferation of SMCs. SMC-MAPK14 in neointima activates proinflammatory gene programs through a p65/NF- κ B-dependent pathway. MAPK14 phosphorylates p65, which induces the expression of proinflammatory genes such as IL6, IL8, and CXCL1. Interestingly, in the neointimal region, high expression of MAPK14 was also observed in some CD45-positive inflammatory cells in addition to SMCs. The infiltrating inflammatory cells interact with inflammatory SMCs, leaving the vascular in a “hyperinflammatory” state (Wu et al., 2019). These results suggest that high expression of MAPK14 in SMCs in neointimal region regulates the pro-proliferative and pro-inflammatory properties of SMCs through two different pathways. Taken together, these studies suggest that SMCs play a central role in driving lesion development and vascular remodeling via “phenotypic switching” (Figure 3).

Mechanistically, there is a study found that stem cell factor (SCF)/c-Kit tyrosine kinase signaling pathway can regulate neointima formation. The investigators found that vascular injury not only resulted in local upregulation of membrane-bound SCF (mSCF) expression, but also increased circulating SCF (sSCF) levels. SCF not only promotes CD34⁺ cell mobilization, but also promotes progenitor adhesion to the exposed matrix on injured vessels, and mediates the differentiation of c-Kit⁺ cells into SMCs (Wang et al., 2006).

The researchers found that MMP-9 is activated and cleaves mSCF in response to vascular injury, increasing circulating SCF levels. Some studies have reported that activation of MMP-9 can also promote the release of soluble Kit ligand (sKitL), thus playing a role in progenitor mobilization (Heissig et al., 2002). By using genetic cell lineage tracing techniques, the direct evidence from our group demonstrated that c-Kit⁺ cells can differentiate into SMCs *in vitro* and *in vivo* (Ni et al., 2019). In this aspect, detailed review has been published (Zhang et al., 2018). Thus, progenitor cells may be a source of SMCs during vascular remodelling.

The role of fibroblasts in peripheral arterial disease

Adventitia is the outermost connective tissue of blood vessels. In recent years, some reports have suggested that the adventitia is a key regulator that directly or indirectly modulates the structure and function of the vascular wall (Patel et al., 2000; Hu and Xu, 2011). Fibroblasts are the most important cell types in the adventitia of blood vessels, which may be the first cells of vascular to be activated in response to ischemia and hypoxia. When being activated, fibroblasts will produce more collagens, secrete cytokines and be more actively proliferating (Anwar et al., 2012). Activation of fibroblasts by various stimuli can lead to differentiation of fibroblasts into a myofibroblast phenotype. The expression of α -SMA is an important signal for fibroblasts to differentiate into myofibroblasts. Myofibroblasts are key players in vascular remodeling due to their ability to respond promptly to changes in the local environment *in vivo*, including the production of extracellular matrix proteins, as well as various cytokines and ROS (Gabbiani, 2003). In addition, myofibroblasts can migrate from the adventitia to the media and even to the intima, thereby promoting vascular pathological remodeling (Li et al., 2000).

15-hydroxyeicosanoic acid (15-HETE) is known as an important factor in vessel remodeling under ischemia/hypoxia, participating in the mediation of cell proliferation, migration and other biological processes (Ma et al., 2011). Zhang et al. (Zhang et al., 2016) found that 15-HETE induced fibroblasts to differentiate into myofibroblasts and promoted FGF-2 expression in adventitial fibroblasts by activating the p38MAPK/EGR-1 pathway. FGF2 not only increased the expression of α -SMA, but also promoted the proliferation of fibroblasts by inhibiting p27kip1 (Zhang et al., 2016). As a CDK inhibitor, p27kip1 negatively regulates the cell cycle by inhibiting cyclin activity and prevents the G1/S phase transition of cells, thereby inhibiting cell proliferation (Goncalves et al., 2015). Other studies have found that FGF-2 mediated cell phenotypic changes and increased nuclear FoxO3a through PI3K-Akt and ERK pathways during hypoxia-induced vascular remodeling (Khalil et al., 2005). At the same time, 15-HETE

promotes the migration of adventitial fibroblasts and induces the up-regulation of STAT3 expression in fibroblasts. JNK1-mediated CREB activation was required for 15-HETE-induced STAT3 phosphorylation in VAFs. Phosphorylated STAT3 binds to the MMP2 promoter and induces the expression of MMP-2, thereby promoting the migration and phenotypic changes of fibroblasts (Zhang et al., 2015).

Fibroblast proliferation, migration, differentiation and collagen production are energy-dependent processes. Fatty acid oxidation is a process of energy metabolism that occurs in mitochondria by converting fatty acids into various products [e.g. acetyl coenzyme A (acetyl-CoA)] to generate energy (Rodgers et al., 2020). Fatty acid binding protein 3 (FABP3) plays an important role in fatty acid oxidation, controlling fatty acid transport to maintain lipid metabolism and energy homeostasis (Storch and Corsico, 2008). An emerging study showed that FABP3 expression was increased in the adventitia of patients with arteritis and was positively correlated with adventitial fibroblast proliferation and ECM production such as FN1 and collagens (Wu et al., 2022). Elevated serum FABP3 levels have been reported to be associated with increased collagen1 expression and myocardial fibrosis (Maneikyte et al., 2020). Further studies revealed that FABP3 upregulation in adventitial fibroblast promoted the expression of carnitine palmitoyl transferase 1A (CPT1A) and carnitine/acylcarnitine carrier protein (CACT), two key enzymes in FAO, as well as the upregulation of adenosine triphosphate (ATP) (Wu et al., 2022). This suggests that FABP3-mediated progression of vascular fibrosis may be achieved by promoting fatty acid oxidation and ATP production in fibroblasts. Extracellular ATP plays an important role in vascular remodeling. It has been found that hypoxia stimulates the release of exogenous ATP, which can stimulate fibroblast proliferation by autocrine or paracrine pathways (Gerasimovskaya et al., 2002).

Adventitia plays a crucial role in the initiation and maintenance of vascular inflammation (Moreno et al., 2002). Vascular inflammation has traditionally been considered an inside-out response centered on the recruitment of leukocytes to the intima of vessels. However, a growing body of experimental evidence supports an outside-in hypothesis that vascular inflammation begins and persists in the adventitia and progresses toward the intima (Okamoto et al., 2001). After ischemia/hypoxia, activated adventitial fibroblasts promote the expression of chemokines and adhesion molecules and attract macrophages to the perivascular tissue and adventitia (Hu and Xu, 2011). There is a study revealed that JE/MCP-1 expression was up-regulated in fibroblasts at the early stage of atherosclerosis (Xu et al., 2007). Vascular endothelial growth factor (VEGF) is highly expressed in vascular endothelial cells and exerts its effects mainly through two tyrosine kinase receptors, Flt-1 and Flk-1 (Carmeliet, 2000). It has been found that Flt-1 is increased in adventitial fibroblasts of damaged

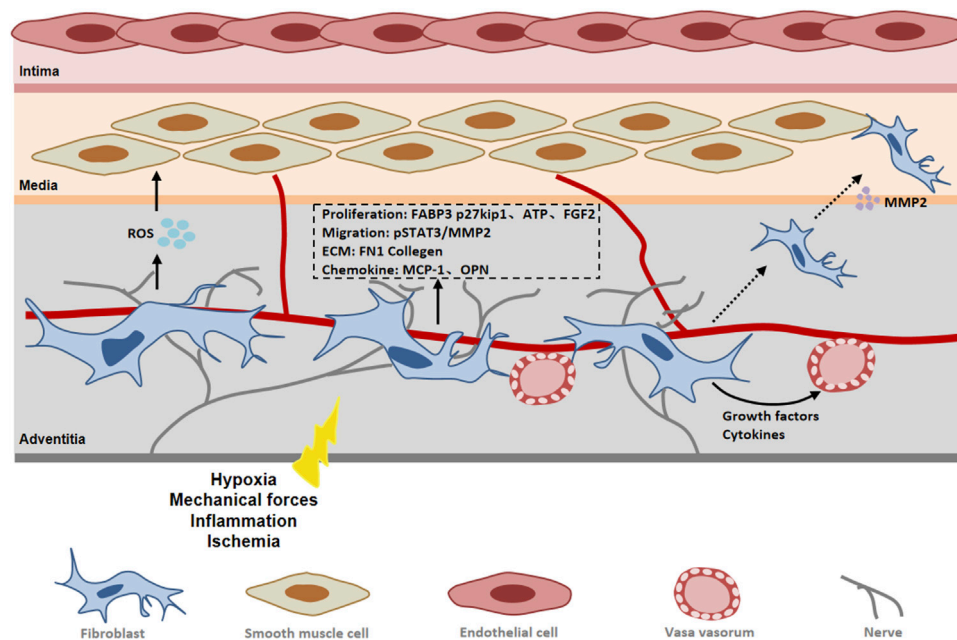


FIGURE 4

The role of fibroblast in peripheral arterial disease (PAD). In response to vascular injury, adventitial fibroblasts become activated and differentiate into myofibroblasts. Fibroblasts contribute to vascular remodeling through proliferation, migration and extracellular matrix synthesis. Abbreviations: FGF2, fibroblast growth factor 2; FABP3, fatty acid binding protein 3; MMP2, matrix metalloproteinase 2; pSTAT3, phosphorylated signal transducer and activator of transcription 3; ROS, reactive oxygen species; FN 1, fibronectin 1; OPN, osteopontin.

arteries (Jin et al., 2007). Exogenous VEGF was found to regulate OPN protein expression by promoting ERK1/2 phosphorylation by acting on outer membrane fibroblast Flt-1. AF-derived OPN is a key mediator of VEGF/FLT-1-induced macrophage chemotaxis (Li et al., 2012). OPN can attract inflammatory cells to migrate and adhere to diseased blood vessels, and regulate cytokine production in macrophages, dendritic cells and T cells (Scatena et al., 2007). Further studies demonstrated that antagonistic Flt-1 could inhibit the proliferation of fibroblasts and neointima formation. It has been found that adventitia fibroblasts can migrate through the media to the neointima in damaged blood vessels. This implies that the contribution of Flt-1 to VEGF-mediated neointima formation may be due to inflammatory activation leading to adventitial cell proliferation and migration. In response to inflammatory stimulation, adventitial fibroblasts produce a large amount of NOX-derived ROS. Casino et al. found that adventitia-derived hydrogen peroxide enhanced vasoconstriction by activating p38 MAP kinase in medial SMC as well as inhibiting SHP-2 phosphorylation (Cascino et al., 2011). It was found that fibroblast-NOX2 modulates the growth of SMC in a GDF6-dependent manner. GDF6 is a member of TGF- β superfamily (Harrison et al., 2021) (Figure 4). We hypothesized that in vascular ischemia, leukocytes recruited by adventitial fibroblasts may infiltrate into the media through vasa

vasorum, thereby promoting the development of inflammation. At the same time, fibroblasts are associated with peripheral vascular remodeling via secretion of cytokines that influence SMCs and even endothelium.

General treatment

Improved treatment for PAD must begin with lifestyle interventions, including smoking cessation, diet, and exercise. Smoking is an important risk factor for the development of PAD. It promotes thrombosis through effects on platelets, ECs, and the coagulation system by inducing oxidative stress and lowering nitric oxide (Celermajer et al., 1993; Anand, 2017). Therefore, quitting smoking can reduce the damage to vessels caused by nicotine in cigarettes and slow down the progression of PAD. Epidemiological studies connect healthy dietary patterns with lower incidence of clinical PAD. Improving the quality of diet is important for people with PAD, including fiber and fruit intake (Kulezic et al., 2019). Exercise therapy can maintain and improve the walking ability and quality of life of people with PAD through a series of supervised and systemic exercises (Hamburg and Balady, 2011). Supervised exercise training has been found to improve painless walking distance and maximum walking

TABLE 1 Summary of PAD treatment.

Tharepy	Intervention	Effectiveness
General treatment	smoking	Reduced mortality and amputation rates in patients with PAD
	diet	Improve intermittent claudication and severe limb ischemia
	exercise	improve painless walking distance and maximum walking distance
Drugs	statin	Reduce the incidence of intermittent claudication and amputation in patients with PAD
	rivaroxaban/aspirin	Reduce vascular obstruction and the mortality of PAD patients
	cilostazol	Increase the maximum walking distance and reduced the risk of amputation (HR 0.42, 95%CI 0.27–0.66) and repeat revascularization (relative risk (RR) 0.44, 95% CI 0.37–0.52) in patients with PAD

distance in patients with intermittent claudication (Parmenter et al., 2015). Another trial showed that weekly high-intensity home training, which stimulates moderate-to-severe ischemic leg pain, also significantly improved walking distance (McDermott et al., 2021). This suggests that PAD patients need to make exercise plans according to their own conditions and make timely adjustments to adapt to high-intensity exercise.

Therapy with drugs

The American College of Cardiology (ACC)/American Heart Association (AHA) recommends high-strength statins for all patients with PAD (Gerhard-Herman et al., 2017). Statin therapy is the most recognized cholesterol-lowering drug in PAD drug therapy. Over the median 5.4-year follow-up period, the incidence of carotid murmurs and claudication was reduced in the simvastatin group (Pedersen et al., 1998). In a retrospective cohort study of diabetic patients with and without PAD, Sohn et al. (2013) compared outcomes in patients taking statins with those taking non-statin lipid-lowering drugs and found that statin therapy reduced amputation rates in patients with PAD. Anticoagulant therapy and antiplatelet therapy are also important to prevent vascular obstruction in patients with PAD. During a median of 23 months of follow-up, the combination of low-dose rivaroxaban and aspirin reduced mortality in patients with PAD as compared with aspirin alone (Eikelboom et al., 2021). In addition, Cilostazol has vasodilatation and antiplatelet function. By inhibiting phosphodiesterase activity in platelets and SMCs, cilostazol can increase the concentration of cAMP in platelets and SMCs and exert antiplatelet and vasodilatation effects (Hashimoto

et al., 2006). There is a study showed that cilostazol not only significantly increased the maximum walking distance on the treadmill in PAD patients, but also reduced the risk of amputation (higher risk (HR) 0.42, 95% CI 0.27–0.66) and repeat revascularization (relative risk (RR) 0.44, 95% CI 0.37–0.52) in patients with PAD who underwent revascularization (Desai et al., 2021). Although these drug treatments can improve the symptoms of PAD, it is far from optimal. Understanding the molecular mechanism of the vascular disease development could offer some potential targets for the therapy (Table 1).

Summary and perspectives

In summary, the incidence of PAD is increasing year by year, which requires more clinical attention and early intervention. Current studies have shown that multiple mechanisms are involved in peripheral vascular remodeling in PAD, including EndMT, phenotypic transition of SMCs, and activation of fibroblasts. However, the current research is relatively limited, and we need to further explore the underlying mechanism. Firstly, endothelial functional changes in the development of PAD have not been fully understood, although some progress in the field was achieved, including NO/ROS balance, which was used as drugs for treatment of vascular disease. Current techniques of single cell RNA and protein sequencing combining with genetic approaches might be powerful tool for further understanding the mechanisms of cell dysfunctions. Secondly, for SMC biology, most studies focus on phenotype switching from contractile to secretory cells that proliferate and secrete matrix proteins. However, accumulating evidence indicates resident stem/progenitor cells may differentiate into SMCs where they participate in neointima hyperplasia (Wang et al., 2015). Finally, fibroblasts in the vessel wall have been paid attention recently. In this aspect, there is a large amount of work being to do. If researchers in these fields could make studies thoroughly, some major progress for the pathogenesis of PAD may be seen in near future.

At the present, the main treatment methods for PAD are drug therapy and surgery. However, patients with arterial occlusion who cannot undergo revascularization can only undergo amputation. In recent years, great progress has been made in the research of vascular stem cells. Animal studies have demonstrated that vascular stem cells can significantly improve blood supply to ischemic limbs. Novel therapies that promote tissue regeneration and stimulate vasculogenesis and angiogenesis are urgently needed for the treatment of the patients. Cell-based therapies have great potential for the treatment of ischemic diseases (Sprengers et al., 2008; Gupta and Losordo, 2011). Therefore, stem cell

therapy could be a potential approach for the treatment of PAD in the future.

Author contributions

QX and YH conceived and designed the study. SL synthesized literature and drafted the review. HZ and RL participated in manuscript editing. All authors have read and approve the published version of the manuscript.

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Glossary

PAD peripheral arterial disease	ATP adenosine triphosphate
ASO atherosclerosis obliterans	KLF4 Kruppel like factor 4
SMCs smooth muscle cells	TN-X Tenascin-X
ECs endothelial cells	Ox-LDL oxidized low-density lipoprotein
α-SMA α -smooth muscle actin	IL-1β interleukin 1 β
HUVECs human umbilical vein endothelial cells	IL-6 interleukin 6
NO nitric oxide	IL-8 interleukin 8
EndMT endothelial-to-mesenchymal transition	ICAM-1 intercellular cell adhesion molecule-1
AMPK AMP-activated protein kinase	VCAM-1 vascular cell adhesion molecule-1
eNOS endothelial nitric oxide synthase	MCP-1 monocyte chemotactic protein-1
ROS reactive oxygen species	FoxM1 Forkhead Box M1
HDAC histone deacetylase	ET-1 endothelin-1
15-HETE 15-hydroxyeicosaenoic acid	SCF stem cell factor
FABP3 fatty acid binding protein 3	MMP9 matrix metalloproteinase 9
CPT1A carnitine palmitoyl transferase 1A	PDGF platelet derived growth factor
CACT carnitine/acylcarnitine carrier protein	TGF-β transforming growth factor- β
CXCL1 C-X-C motif chemokine ligand 1	p38 MAPK p38 mitogen activated protein kinase
PKM2 pyruvate kinase M2	MEK5 mitogen-activated protein kinase 5
CyPA cyclophilin A	p65 nuclear factor-kB p65
GLUT1 glucose transporter 1	FGF2 fibroblast growth factor 2
MMP2 matrix metalloproteinase 2	pSTAT3 phosphorylated signal transducer and activator of transcription 3
FN 1 fibronectin 1	OPN osteopontin



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Farrerol prevents Angiotensin II-induced cardiac remodeling *in vivo* and *in vitro*

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Cardiovascular disease has become the primary disease that threatens human health and is considered the leading cause of death. Cardiac remodeling, which is associated with cardiovascular disease, mainly manifests as cardiac hypertrophy, fibrosis, inflammation, and oxidative stress. Farrerol plays an important role in treating conditions such as inflammation, endothelial injury and tumors, and we speculated that Farrerol may also play an important role in mitigating cardiac hypertrophy and remodeling. We established a model of myocardial remodeling using Angiotensin II (Ang II) with concurrent intraperitoneal injection of Farrerol as an intervention. We used cardiac ultrasound, immunohistochemistry, Immunofluorescence, Wheat Germ Agglutinin, Dihydroethidium, Western Blot, qPCR and other methods to detect the role of Farrerol in cardiac remodeling. The results showed that Farrerol inhibited Ang II-induced cardiac hypertrophy; decreased the ratio of heart weight to tibia length in mice; reduced inflammation, fibrosis, and oxidative stress; and reduced the size of cardiomyocytes *in vivo*. Farrerol inhibited Ang II-induced cardiomyocyte hypertrophy, levels of oxidative stress, and the proliferation and migration of fibroblast *in vitro*. Our results revealed that Farrerol could inhibit Ang II-induced cardiac remodeling. Farrerol may therefore be a candidate drug for the treatment of myocardial remodeling.

KEYWORDS

Farrerol, myocardial remodeling, inflammation, fibrosis, oxidative stress

1 Introduction

Cardiovascular disease has become the primary disease that threatens human health and is considered a leading cause of death (Zhang et al., 2019). Heart failure is the final outcome in a variety of cardiovascular diseases, such as hypertension, coronary heart disease, and cardiomyopathy. Although existing clinical drugs, device therapies and adjuvant treatments can delay the progression of heart failure, the overall prognosis of patients with heart failure is still very poor. Myocardial remodeling is a major component of heart injury and is a pathophysiological process that occurs when cardiovascular disease progresses to heart failure (Aimo et al., 2019; Maki and Takeda, 2020).

Cardiac remodeling refers to adaptive and compensatory changes in the structure, morphology and function of the heart in response to various pathophysiological factors (Montiel et al., 2020; Nadruz, 2015; Porter and Turner, 2009). It is a serious pathological change in the development of heart failure. Cardiac remodeling mainly entails structural remodeling and electrical remodeling. The main features are myocardial interstitial fibrosis, cardiomyocyte hypertrophy, reduced ventricular compliance and abnormal ventricular electrical conduction, which together can directly affect cardiac function and even lead to death (Dixon and Spinale, 2011). Therefore, the prevention and treatment of heart failure through the mitigation of myocardial remodeling holds great importance and potential.

Epidemiology and molecular biology studies have shown that flavonoids can reduce the risk of cardiovascular disease in the diet, and that their biological functions may be related to antioxidant effects (Dludla et al., 2017). Farrerol is the main ingredient isolated from the traditional Chinese herbal medicine Manshanhong (Yin et al., 2019).

Recent studies have shown that Farrerol has various biological functions such as scavenging free radicals, regulating enzyme activity, inhibiting cell proliferation and inflammatory responses. Farrerol has some potential therapeutic effects in a variety of diseases including cardiovascular disease, neurological disease, metabolic disease and cancer (Chae et al., 2021; Duan et al., 2021; Li et al., 2019). Studies have shown that Farrerol can inhibit oxidative stress and inflammatory reactions, thereby reducing microglial cell damage (Cui et al., 2019). Farrerol can reduce the damage of renal mesangial cells induced by high sugar (Chen et al., 2020). Farrerol can relieve collagenase-induced tendinopathy by inhibiting ferroptosis (Wu et al., 2022). Farrerol effectively inhibit cisplatin-induced inflammation and renal fibrosis by activating Nrf2 and PINK1/Parkin-mediated mitophagy (Ma et al., 2021). Farrerol suppress the migration, invasion, and induce the apoptosis of LSCC cells *via* the mitochondria-mediated pathway (Zhang et al., 2021). Farrerol can reduce the oxygenation damage of the endothelium in the blood duct (Yan et al., 2020). However, there have been no studies published on cardiac remodeling.

One biologically active octapeptide involved in remodeling is Angiotensin II (Ang II), which is found in the renin-angiotensin aldosterone system (RAAS). Ang II functions mainly through angiotensin type 1 receptor (AT1 receptor, AT1R) and angiotensin type 2 receptor (AT2 receptor, AT2R). AT1R mediated most of the physiological and pathological effects of Ang II. The AT1R is the most well studied angiotensin receptor. When activated by Ang II, AT1R activates phospholipase C (PLC) and increases Ca^{2+} concentration, or inhibits adenylate cyclase, which activates a series of cellular reactions, like activating reactive oxygen species (ROS)-dependent molecular signaling pathways or TAK1-mediated cardiac inflammation

(Dai et al., 2022; Ding et al., 2020; Rao et al., 2020). Activation of AT1 receptor can cause vasoconstriction, aldosterone synthesis and secretion, increase vasopressin secretion, promote myocardial hypertrophy, increase peripheral norepinephrine activity, proliferation of vascular smooth muscle cells, inhibit renin secretion, affect cardiac contractility and extracellular matrix synthesis (Lin et al., 2022; Bhullar and Dhalla, 2022; Booz et al., 2021; Mondaca-Ruff et al., 2022).

The aim of the experiment in the present study was to determine the effect of Farrerol on cardiac remodeling and cardiac hypertrophy induced by Ang II. The results showed that Farrerol could inhibit Ang II-induced cardiac hypertrophy and reduce HW/TL, inflammation, fibrosis, oxidative stress, the volume of cardiomyocytes and the proliferation and migration of fibroblast. The results from this experiment suggest that Farrerol may represent a new therapeutic target for cardiac remodeling.

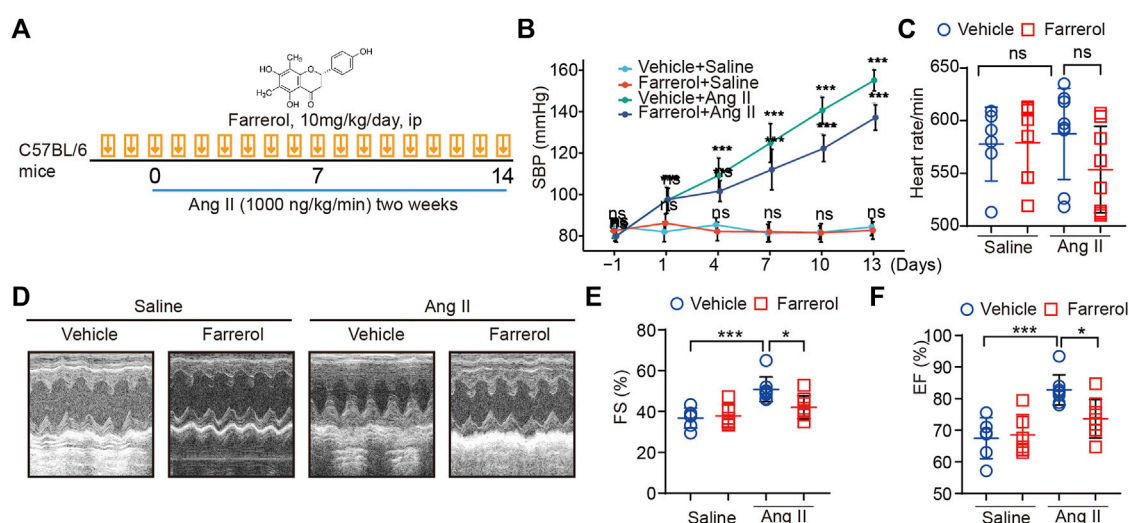
2 Materials and methods

2.1 Animals

Male wild-type C57BL/6J mice, 8-weeks-old, purchased from Aikesaisi Biotechnology Co., Ltd., kept in an environmentally controlled room with a 12-h light/dark cycle. The animal experiment was approved by the Animal Research Committee of Dalian Medical University (AEE20029). Farrerol (Selleck, S9552, China) was dissolved in dimethyl sulfoxide (DMSO, Sigma), diluted with DMSO and normal saline, and injected intraperitoneally at a dose of 10 mg/kg/day (Ma et al., 2021; Zhou et al., 2022). 2 days after administration, Ang II (1,000 ng/kg/min, 4474-91-3, aladdin) was subcutaneously infused to establish a cardiac remodeling model for 2 weeks *via* implanted osmotic minipumps (Model 1007D, Alzet, Cupertino, CA, United States). This method is a recognized method to establish cardiac remodeling.

2.2 Echocardiography

The anesthetized mice lay on their backs on a platform with their paws placed on electrodes. Ensure appropriate ECG, body temperature was around 37°C. Vevo 2100 high-resolution imaging system (VisualSonics Inc.) was used to perform chest echocardiography on mice to measure ejection fraction (EF%), fractional shortening (FS%) and other indicators. Induce anesthesia with 3% isoflurane and maintain at 1%–2% during the procedure. Mice were immobilized and supine. A parasternal short-axis image of the left ventricle was obtained at the level of the papillary muscle in M-mode.

**FIGURE 1**

Farrerol inhibits Ang II-induced hypertension and cardiac dysfunction in mice. (A) Graphical flow chart; (B) Systolic blood pressure of mice in different treatment groups; (C) Heart rate of mice in different treatment groups; (D–F) Representative M-mode echocardiography of left ventricular (LV) chamber and echocardiographic assessment of FS(%), EF (%) (Saline = 6, Farrerol = 6, Ang II = 8, Ang II + Farrerol = 8).

2.3 Blood pressure and heart rate measurement in mice

Blood pressure was measured by the tailcuff method before starting treatment and every 2 days after Ang II infusion as previously described (Wang et al., 2016) (Figure 1A). Within a week before the official assay, the mice underwent training assays to acclimate them. Briefly, awake and calm mice were placed on the immobilizer with the tail fully exposed. When the sine wave is displayed on the screen of the non-invasive sphygmomanometer, press the pressure measurement button to start the measurement, record the heart rate, systolic blood pressure and diastolic blood pressure. Each mouse is measured 5 times and averaged for statistical analysis.

2.4 Wheat germ agglutinin (WGA) and dihydropyrimidine (DHE)

Slides were dewaxed and hydrated, washed with phosphate buffered solution (PBS) 3 times, incubated with WGA for 1 h or DHE for 30 min at room temperature, washed with PBS 3 times, seal the cover glass. Images were captured by the Nikon microscope and analysis by ImageJ.

2.5 Hematoxylin-eosin (H&E)

Slides were dewaxed and hydrated, washed with PBS 3 times. Placed in hematoxylin staining solution for 5 min,

hydrochloric acid aqueous solution was differentiated, ammonia solution returned to blue, running water removed residual staining solution, and completed 5 min of counterstaining in eosin staining solution. Seal the cover glass. Images were captured by the Nikon microscope and analysis by ImageJ.

2.6 Masson's trichrome

Slides were dewaxed and hydrated, washed with PBS 3 times, Hematoxylin staining for 5–10 min, Wash with running water for a while, and differentiate with 1% hydrochloric acid alcohol. Rinse with running water for several minutes, dye with Ponceau for 5–10 min, rinse with distilled water for a while, treat with 1% phosphomolybdic acid solution for about 5 min, counterstain with aniline blue for 5 min, seal the cover glass. Images were captured by the Nikon microscope and analysis by ImageJ.

2.7 Immunohistochemistry (IHC)

Slides were dewaxed and hydrated, washed with PBS 3 times, antigen retrieval, washed with PBS 3 times, 3% H_2O_2 10 min, washed with PBS 3 times, blocking buffer 30 min, the primary antibody (CD68 (ARG10514) and Nitrotyrosine (ARG22269) were purchased from arigo) incubation overnight, the secondary antibody incubation at RT for 1 h. Hemotaxlylin 5 min,

TABLE 1 The sequence of primers.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>nppa</i>	GCTTCCAGGCCATATTGGAG	GGGGGCATGACCTCATCTT
<i>nppb</i>	AGTCCTTCGGTCTCAAGGCA	CCGATCCGGTCTATCTTGTGC
<i>cola1</i>	TAAGGGTCCCCAATGGTGAGA	GGGTCCCTCGACTCCTACAT
<i>cybb</i>	TGAATGCCAGAGTCGGGATTT	CGAGTCACGGCCACATACA
<i>actb</i>	GGCTGTATTCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

dehydration with ethanol and xylene. Images were captured by the Nikon microscope and analysis by ImageJ.

2.8 Lactate dehydrogenase (LDH)

LDH were measured by Solarbio (Beijing, China) according to the manufacturer's suggestions at 450 nm. The LDH were calculated according to the manufacturer's suggestions.

2.9 Real-time PCR analysis

According to the manufacturer's protocol, total RNA was extracted with TRIzol and RNA (500–1,000 ng) transcribed into cDNA. cDNA was used for PCR. The mRNA expression of target genes was analyzed with the $2^{-\Delta\Delta C_t}$ method and β -actin was used as an internal control. The sequence is showed in Table 1.

2.10 Western blot analysis

Radio-Immunoprecipitation Assay (RIPA) buffer was used to extract the protein in the heart tissue. The protein lysate is separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with the antibody NOX2 (A19701) and β -actin (A3718) were purchased from ABclonal, α -SMA (ARG66381) were purchased from arigo, ERK 1/2 (11257-1-AP), p-ERK 1/2 (28733-1-AP), AKT (10176-2-AP) and p-AKT (28731-1-AP) were purchased from Proteintech), The antibodies against stat3 (ARG54150) were purchased from arigo Biolaboratories. The antibodies against p-STAT3 (9145) and NFAT2 (8032) were purchased from Cell Signaling Technology, overnight at 4°C, and then incubated with the horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. All blots are developed and

labeled using electrochemiluminescence (ECL) Plus chemiluminescence system. The intensity of protein bands was measured by ImageJ. β -actin was used as an internal control.

2.11 Neonatal rat cardiomyocytes (NRCM) and fibroblast separation

NRCM was isolated from Sprague Dawley (SD) rats within 24 h after birth. The heart is removed and digested. The dispersed cells were then incubated on a 100 mm³ Petri dish in 5% CO₂ at 37°C for 90 min. Collect unattached cardiomyocytes and transfer them to the well plate of the desired experiment. Fibroblasts continue to be cultured in 100 mm³. The medium required for cardiomyocytes and fibroblasts is Dulbecco's Modified Eagle Media F12medium (DMEM/F12), 10% fetal bovine serum (FBS) and penicillin. The cells are divided into different groups according to the experiment. After Farrerol (20 μ M) treatment for 24 h, Ang II (2 μ M) treatment for 24 h.

2.12 Immunofluorescence (IF)

NRCMs or slides were permeabilized with .1% Triton X-100 for 5 min, 3% bovine serum albumin (BSA) 30 min, the primary antibody (α -actinin (A8939) was purchased from ABclonal, collagen III (22734-1-AP) was purchased from Proteintech) overnight, secondary antibody 1 h, seal the cover glass. Images were captured by the Nikon microscope and analysis by ImageJ.

2.13 Mitochondrial ROS measurement

Mitochondrial ROS levels were quantified by measuring the fluorescence of MitoSOX™ Red (M36008, Thermo Fisher Scientific). After treatment, cells were washed once with PBS. Then, cells were incubated with 5 μ M MitoSOX™ reagent working solution at 37°C for 10 min, protected from light.

Finally, the cells were rinsed three times with PBS and observed with an inverted fluorescence microscope.

2.14 Cell-counting-kit-8 (CCK-8)

Farrerol and Ang II was treated with fibroblast for 24 h, 48 h, 72 h, and 96 h in 96 wells. CCK-8 (K1018, APEX BIO) was added and incubated for 3 h at 37°C. Optical density (OD) value was detected at 450 nm.

2.15 Wound-healing assay

The cells were fused to 95%–100% culture and the wound healing experiment was performed by scraping off the monolayer of cells with the tip of .1–10 μ l pipette. After Farrerol and Ang II treatment, scratches were observed and photographed. ImageJ software was used for quantitative analysis of scratches.

2.16 Statistical analysis

Graphpad prism 9.3 software was used for statistical analysis, and all measurement data were expressed as $\bar{x} \pm$ Standard Deviation (SD). Student's *t*-test was used for comparison between the two groups (Vehicle vs. Farrerol), and one-way ANOVA analysis (Bonferroni's test) was used for comparison between multiple groups (Vehicle vs. Ang II, Ang II vs. Ang II + Farrerol). Blood pressure was measured by Pairwise Comparisons of Estimated Marginal Means (www.xiantao.love/). **p* < .05, ***p* < .01, ****p* < .001, *****p* < .0001 were considered statistically significant.

3 Results

3.1 Farrerol inhibits Ang II-induced hypertension and cardiac dysfunction in mice

A graphical flow chart was shown in Figure 1A. In this paper, we used Ang II to create a mouse model of cardiac hypertrophy. Ang II is known to increase blood pressure in mice. Therefore, we first detected the blood pressure of the mice in each group in this experiment. The results showed that the blood pressure of the mice was significantly increased in the Ang II group, while the blood pressure of mice was significantly decreased in Farrerol + Ang II group (Figure 1B). There was no significant difference in heart rate between groups (Figure 1C). After 14 days of Ang II treatment, cardiac function of each group was detected by ultrasound. The results showed that FS (%) and EF (%) were increased in Ang II group compared to Vehicle group, which suggested that we established a successful myocardial hypertrophy mold in mice. FS

(%) and EF (%) were significantly decreased in Ang II + Farrerol group compared to Ang II group (Figures 1D–F).

3.2 Farrerol inhibits Ang II-induced hypertrophy in mice

We first tested whether Farrerol was toxic to the heart by means of LDH testing. The results showed no significant difference between the control group and Farrerol group (Figure 2A). We found that heart weight/tibia ratio (HW/TL) was significantly increased in the Ang II group compared to control group. The HW/TL was decreased in Farrerol + Ang II compared to Ang II group (Figure 2B). Then, WGA staining was used to observe the size level of cardiomyocytes in each group. It was found that the area of cardiomyocytes was significantly increased in the Ang II group compared with control group. The size of cardiomyocytes was decreased in Farrerol + Ang II group compared to Ang II group (Figure 2C). The mRNA expressions of cardiac hypertrophy markers *nppa* and *nppb* were further investigated in each group. It was found that the mRNA expressions of *nppa* and *nppb* were significantly increased in Ang II group compared to the control group. Farrerol could significantly reduce the mRNA expression levels of *nppa* and *nppb* induced by Ang II compared to Ang II group (Figure 2D).

3.3 Farrerol inhibits Ang II-induced fibrosis and inflammation in mice

Masson staining of myocardial tissue was used to observe the difference level of cardiac fibrosis in each group. It was found that the degree of cardiac fibrosis was significantly increased in the Ang II group compared to the control group. The level of cardiac fibrosis was decreased in Farrerol + Ang II group compared to Ang II group (Figures 3A, B). Next, we detected the fluorescence expression level of Collagen III, and the results showed that the fluorescence level of Collagen III was significantly increased in the Ang II group compared to the control group. The fluorescence level of Collagen III was significantly decreased in the Farrerol + Ang II group compared to Ang II group (Figures 3C, D). The mRNA expression of *clo1a1*, a marker of cardiac fibrosis, was further investigated in each group, and it was found that the mRNA expression of *clo1a1* was significantly increased in Ang II group compared to the control group. The mRNA expression of *clo1a1* was significantly decreased in the Farrerol + Ang II group compared to Ang II group (Figure 3E). Next, H&E staining were used to observe the inflammation level in cardiac tissues of each group. As shown in Figure 3F, the degree of cardiac inflammation was significantly increased the level of nuclear aggregation in the Ang II group compared to control group. The level of nuclear aggregation was decreased in Farrerol + Ang II group compared to Ang II group. We also

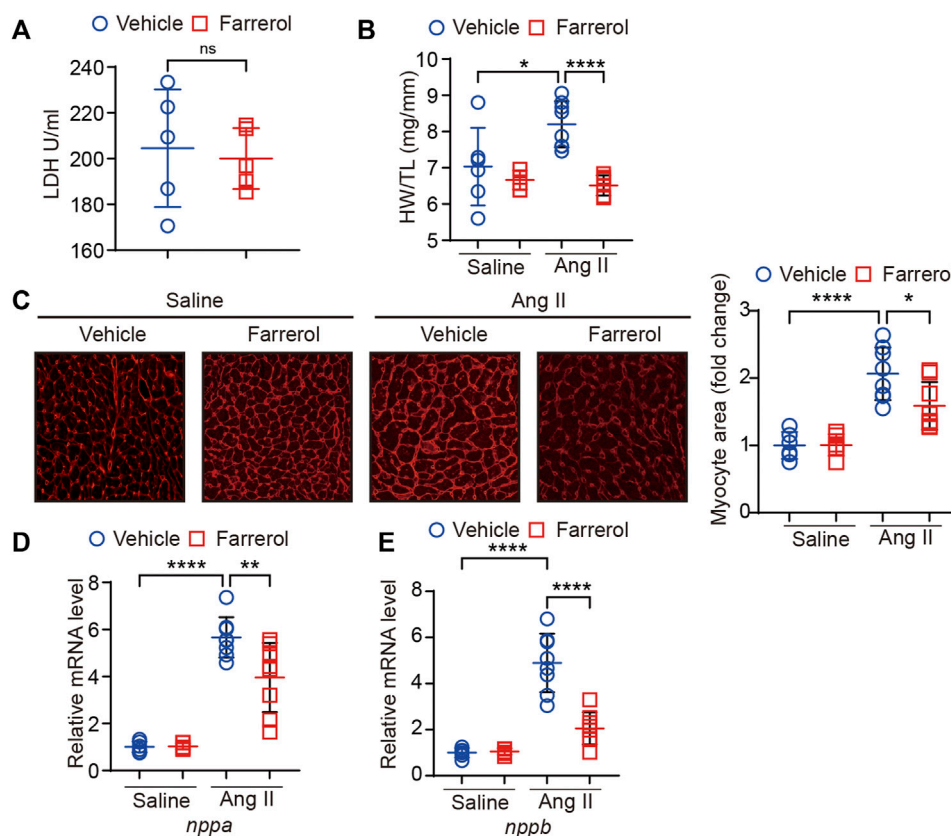


FIGURE 2

Farrerol inhibits Ang II-induced hypertrophy in mice. (A) The level of LDH; (B) Ratio of heart weight to tibia length in experimental groups; (C) Representative images of hearts stained with WGA and the relative value of myocardial cell size of mice in each group; (D,E) The mRNA level of *nppa* and *nppb* in each group (Saline = 6, Farrerol = 6, Ang II = 8, Ang II + Farrerol = 8).

detected the expression of CD68 by IHC in different group. The result showed that the expression of CD68 was increased in the Ang II group compared to control group. The expression of CD68 was decreased in Farrerol + Ang II group compared to Ang II group (Figures 3F, G).

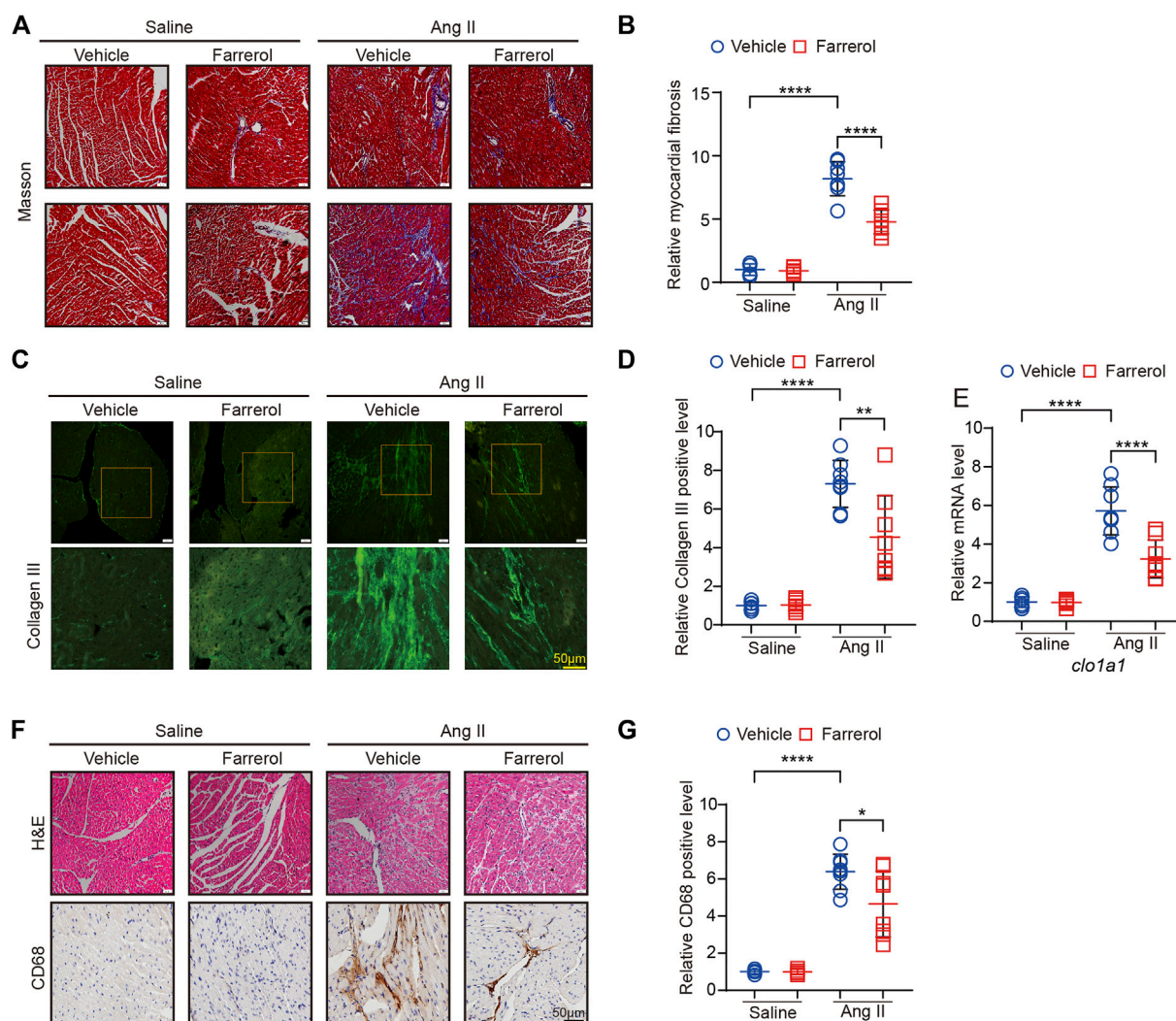
3.4 Farrerol inhibits Ang II-induced oxidative stress in mice

DHE staining was used to observe the oxidative stress level of heart tissues in each group. The positive rate of DHE staining fluorescence signal was significantly higher in Ang II group compared to control group, while Farrerol could reduce the positive rate of DHE staining signal in Farrerol + Ang II group compared to Ang II group (Figures 4A, B). Further we detected the mRNA expression of cardiac oxidative stress marker *cybb* in each group. The result showed that the mRNA expression of *cybb* was significantly increased in Ang II group compared to control group. Farrerol significantly reduced the mRNA level of

cybb in Farrerol + Ang II group compared to Ang II group (Figure 4C). We also detected the expression of nitrotyrosine by IHC. The result showed that the expression of nitrotyrosine was increased in the Ang II group compared to control group. The expression of nitrotyrosine was decreased in Farrerol + Ang II group compared to Ang II group (Figures 4D, E). These results suggested that Farrerol could reduce the oxidative stress level and improve the antioxidant capacity of heart tissue.

3.5 Effect of Farrerol on signaling pathways of cardiac hypertrophy, oxidative stress, fibrosis, and inflammation

Next, we further evaluated the expression of related-proteins in hypertrophy, inflammation and ROS signaling pathways. The results showed that the expression of p-Extracellular signal-regulated kinase [p-ERK1/2 (The202/204)], NADPH Oxidase Type 2 (NOX2) and α -smooth muscle actin (α -SMA) were

**FIGURE 3**

Farrerol inhibits Ang II-induced fibrosis and inflammation in mice. (A) Representative images of hearts stained with Masson; (B) The relative value of cardiac fibrosis of mice in each group; (C) Representative images of hearts stained with Collagen III; (D) The relative value of Collagen III of mice in each group; (E) The mRNA level of *col1a1* in each group; (F) Representative images of hearts stained with H&E and CD68; (G) The relative value of CD68 of mice in each group (Saline = 6, Farrerol = 6, Ang II = 8, Ang II + Farrerol = 8).

significantly increased in Ang II group compared to control group. The expression levels of these proteins were significantly inhibited in Farrerol + Ang II group compared to Ang II group (Figure 5).

3.6 Farrerol inhibits Ang II-induced cardiac remodeling *in vitro*

We have established that Farrerol regulated cardiac remodeling *in vivo*, and then we studied the effect of Farrerol (20 μ M) on NRCMs and fibroblast *in vitro*. The cell area was significantly larger in Ang II group compared to control group in

NRCM. Farrerol reduced cell surface area in Farrerol + Ang II group compared to Ang II group in NRCM (Figure 6A). In addition, we used a MitoSOX Red probe to measure mitochondrial ROS levels in NRVMs. The results showed that Farrerol attenuated the increase in Ang II-induced mitochondrial ROS levels (Figure 6B). Next we examined the expression of hypertrophy and oxidative stress related proteins. The results showed that, the expression levels of p-AKT (Ser473) and NOX2 were significantly increased in Ang II group compared to the control group in NRCM. The expression levels of p-AKT (Ser473) and NOX2 were significantly decreased in Farrerol + Ang II group compared to Ang II group in NRCM (Figure 6C). We also examined the expression of downstream cardiac

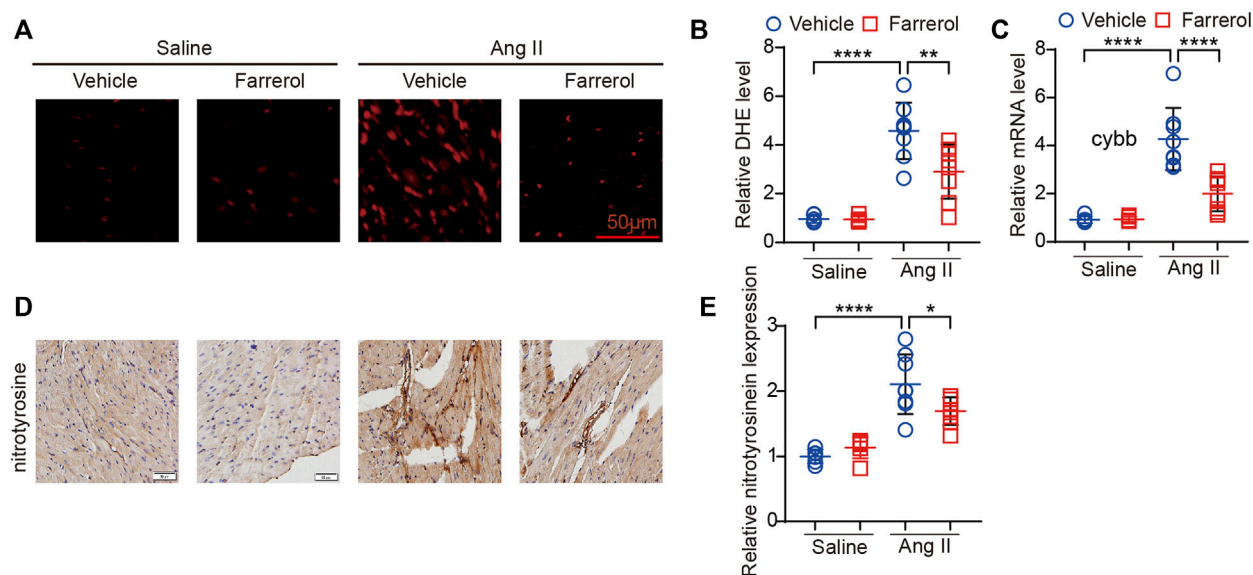


FIGURE 4

Farrerol inhibits Ang II-induced oxidative stress in mice. (A) Representative images of hearts stained with DHE; (B) The relative value of DHE of mice in each group; (C) The mRNA level of *cybb* in each group; (D) Representative images of hearts stained with nitrotyrosine; (E) The relative value of nitrotyrosine of mice in each group (Saline = 6, Farrerol = 6, Ang II = 8, Ang II + Farrerol = 8).

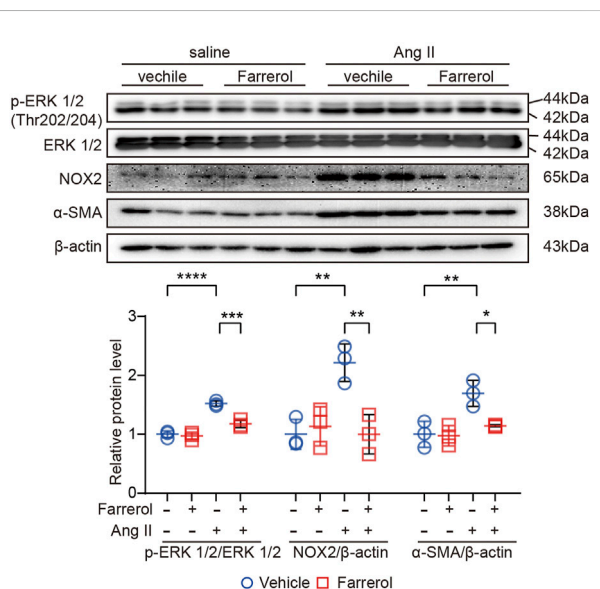


FIGURE 5

Effect of Farrerol on signaling pathways of cardiac hypertrophy, oxidative stress, fibrosis and inflammation. The protein expression of p-ERK (The202/204), ERK, NOX2, α -SMA and β -actin in the heart of each group of mice, and the relative value of the statistical chart (Saline = 3, Farrerol = 3, Ang II = 3, Ang II + Farrerol = 3).

hypertrophy signaling proteins through MAPK-ERK pathway. The results showed that the expressions of p-ERK 1/2 were significantly increased after treatment with an inhibitor of

ERK (SCH772984, S7101, SELLECK), but not p-STST3 and NFAT2. The expressions of p-ERK 1/2, p-STST3 and NFAT2 were significantly increased after the addition of Ang II stimulation, while the expressions of these proteins were significantly decreased after Farrerol treatment compared with Ang II group. After treatment with SCH772984, the expression of these proteins was further decreased in NRCM (Figure 6D).

Cardiac remodeling includes proliferation and migration of fibroblasts in addition to myocardial hypertrophy. We next examined the effect of Farrerol on fibroblasts. The migration ability of fibroblasts was detected by Wound-healing assay. The results showed that it was significantly promoted the migration of fibroblasts in Ang II group compared with the control group, while Farrerol significantly inhibited the migration of fibroblasts in Farrerol group compared to control group (Figure 6E). We detected the effect of Farrerol on fibroblast proliferation by CCK-8. The results showed that it was significantly promoted the proliferation of fibroblasts in Ang II group compared to the control group, while Farrerol significantly inhibited the proliferation of fibroblasts in Farrerol group compared to the control group (Figure 6F). Next, Western Blot was used to detect the expression of Collagen III and α -SMA in each group. The results showed that Ang II significantly promoted the expression of Collagen III and α -SMA compared to control group, while the expression of Collagen III and α -SMA were significantly inhibited in Ang II + Farrerol compared to Ang II group (Figure 6G).

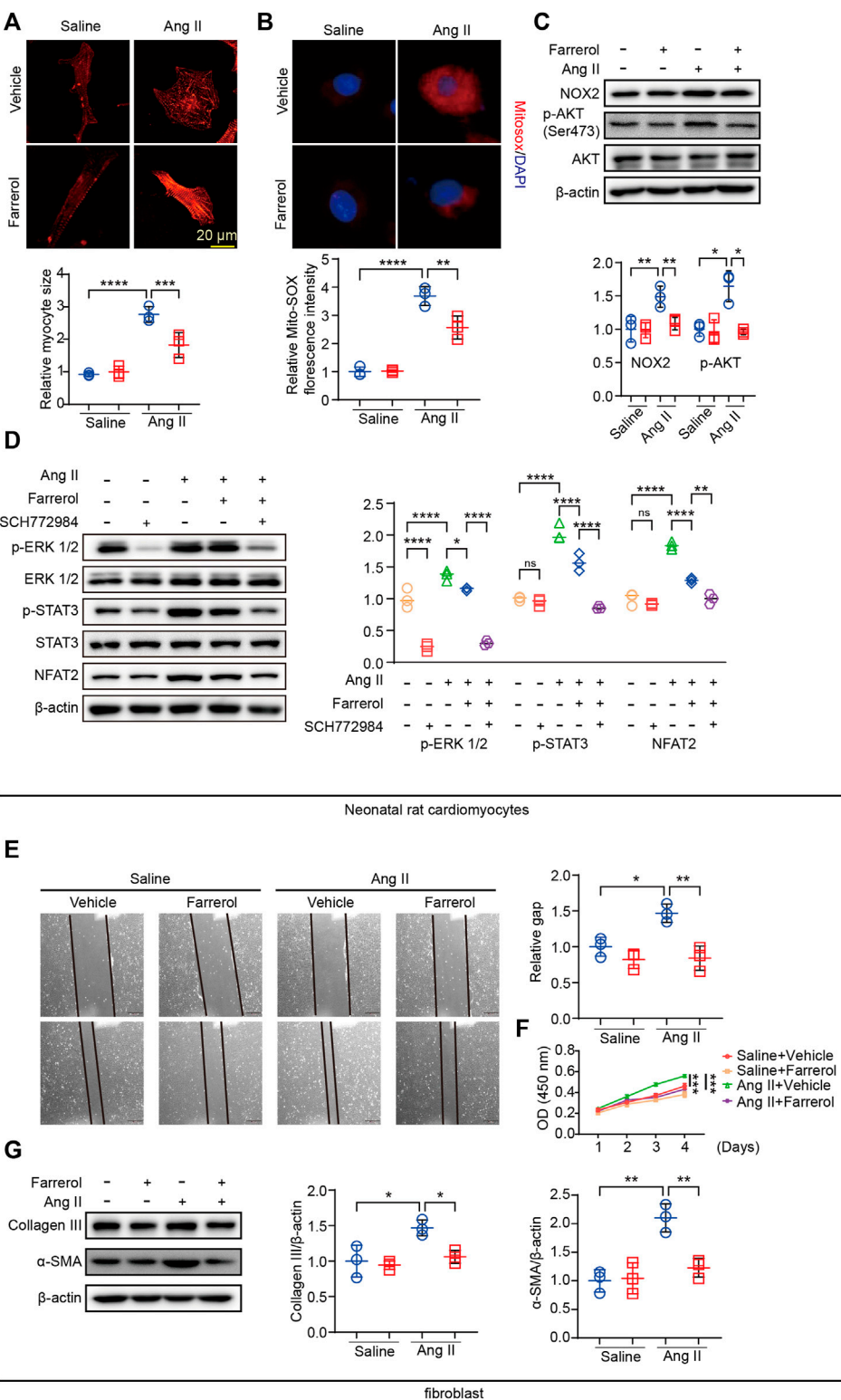


FIGURE 6 Farrerol inhibits Ang II-induced cardiac remodeling *in vitro*. **(A)** Representative images of Myocardial cells stained with α-actinin in each group and the relative value of the statistical chart; **(B)** Mitochondrial ROS levels in NRVMs were determined by MitoSOX Red staining; **(C)** The expression of p-AKT (Ser473), AKT, NOX2 and β-actin detected by Western Blot; **(D)** The expression of p-ERK 1/2, ERK 1/2, p-STAT3, STAT3, NFAT2 and β-actin detected by Western Blot; **(E)** the migration ability of fibroblasts by Wound scratch assay in each group; **(F)** The proliferation ability of fibroblasts was detected by CCK-8; **(G)** The expression of p-AKT (Ser473), AKT, NOX2 and β-actin detected by Western Blot (Saline = 3, Farrerol = 3, Ang II = 3, Ang II + Farrerol = 3).

4 Discussion

The process of cardiac remodeling is carried out when the heart is damaged or has an abnormal hemodynamic condition. This reconstruction process leads to changes in the heart's size, shape, and function. (Verdejo et al., 2012). During the process of myocardial reconstruction, interstitial fibrosis develops can reduce the heart's cardiac compliance. It also affects the heart's contractile function. This condition can lead to a reduction in the number of myocardial cells and to apoptosis (Pauschinger et al., 2004; Turner, 2018). In addition, collagen fibers can also wrap around the cardiomyocytes, which can lead to electrical conduction disorders and cause arrhythmia. This condition can also decrease the blood vessel density (Widiapradja et al., 2017; Zhang and Shah, 2007). Therefore, it is necessary to deeply study the molecular regulatory mechanism of cardiac remodeling in order to discover new targets and to improve heart failure treatment.

Despite a large number of studies on cardiac remodeling, the specific mechanism that underlies the phenomenon is still unclear. Apoptosis of cardiomyocytes and proliferation of non-cardiomyocytes (fibroblasts, endothelial cells, smooth muscle cells, etc.), as well as myocardial inflammation, oxidative stress, fibrosis, and intramyocardial angiogenesis have all been shown to be associated with myocardial remodeling.

Clinical and experimental studies have shown that Ang II is a major effector of the RAAS and plays an important role in the biological process leading to cardiac remodeling. In addition to its physiological role in the regulation of arterial blood pressure (that is, through vasoconstriction and sodium-water retention), Ang II also directly induces cardiac remodeling by activating oxidative stress, inflammation, hypertrophy, fibrosis, and extracellular matrix accumulation (Liu et al., 2017; Zhang et al., 2017).

In the past few decades, there has been evidence that certain Chinese herbal medicines are effective in treating human cardiovascular diseases, which can promote the activation of cellular inflammation, oxidative stress and other related signaling pathways involved in human cardiovascular disease at the molecular level (Chang et al., 2020; Zhao et al., 2020).

Farrerol is a kind of plant polyphenol compound that is abundant in some fruits, vegetables and herbal plants. According to past studies, it has many therapeutic effects on various pathological conditions, such as cancer, muscle atrophy, inflammation, microbial infections, and oxidative stress (Guo et al., 2022; Wu et al., 2022). These therapeutic effects of Farrerol are mainly due to free radical scavenging, antioxidant and anti-inflammatory properties. Farrerol alleviates myocardial ischemia/Reperfusion injury *in vivo* (Zhou et al., 2022). We believe that Farrerol plays the same role in cardio-protection from hypertrophy and remodeling.

In this study, we found that Farrerol can inhibit the expression of *nppa* and *nppb*, which are markers of cardiac

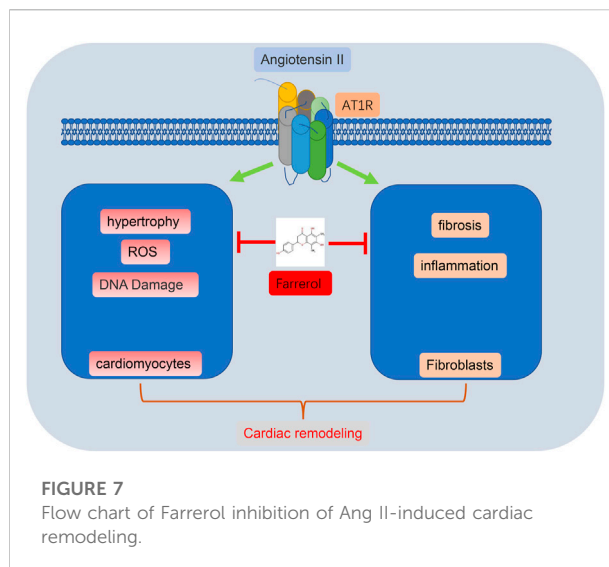


FIGURE 7
Flow chart of Farrerol inhibition of Ang II-induced cardiac remodeling.

hypertrophy. Therefore, it can be concluded that Farrerol may effectively protect against cardiac hypertrophy caused by Ang II. Cardiomyocyte hypertrophy is an important indicator of cardiac remodeling in mice. We have demonstrated that Farrerol can inhibit the increase in size of cardiomyocytes induced by Ang II *in vivo* and *in vitro*.

Inflammation and fibrosis are common pathological attributes of cardiac remodeling, and are related to structural damage and impaired function in the left ventricle (Fu et al., 2022). Studies have shown that the increased expression of proinflammatory factors promotes cardiac remodeling. The initiation of the inflammatory response causes the accumulation of extracellular matrix and accelerates the process of myocardial fibrosis (Cooper et al., 2022). Oxidative stress is a key element that affects cardiac remodeling (Mahmoudi et al., 2008). The relationship between Farrerol and oxidative stress has been studied. Farrerol can affect the occurrence and development of diseases by regulating NOX4/ROS/ERK/TGF- β signaling pathway, NRF2/Keap1 signaling pathway, HO-1 and NQO1 signaling pathway (Qin et al., 2022; Chen et al., 2020; Ma et al., 2019). In this paper, we found that Farrerol could inhibit the expression of DHE, mitoSOX and the expression of NOX2. Thus, we found that Farrerol protected heart tissue from inflammation, fibrosis and oxidative stress.

Studies have shown that activation of MAPK signaling induces cardiac hypertrophy. Specific MAPK/ERK inhibitors attenuate this process. STAT3 and NFAT2 play key roles in the development of cardiac hypertrophy. As shown in the results above, Ang II can promote the expression of p-ERK, p-STAT3, and NFAT2, while Farrerol and ERK inhibitors can significantly inhibit their expression, and we found that Farrerol may exert an anti-cardiac hypertrophy effect through MAPK/ERK. In subsequent experiments, we could overexpress the expression levels of MAPK/ERK pathway-related proteins, which further

proved that Farrerol may exert its anti-cardiac hypertrophy effect through MAPK/ERK.

In summary, Farrerol could effectively inhibit Ang II-induced myocardial remodeling and myocardial hypertrophy in mice, indicating that Farrerol may be a candidate drug for the treatment of myocardial remodeling (Figure 7).

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee in Dalian Medical University.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by JH, DX, and LW. The first draft of the manuscript was written by JH and XY. All authors

commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comparative analysis of thoracic and abdominal aortic aneurysms across the segment and species at the single-cell level

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Introduction: Aortic aneurysm is a life-threatening disease resulted from progressive dilatation of the aorta, which can be subdivided into thoracic and abdominal aortic aneurysms. Sustained subcutaneous angiotensin II infusion can induce aortic aneurysms in mice. However, the relevance of using angiotensin II induction model to study aneurysm disease and the degree of commonality between species remain elusive.

Methods: We utilized scRNA-seq to infer aortic cell sub-structures and transcriptional profiles in clinical patient TAAs and AAAs, as well as mouse models of corresponding diseases (Ang II induction) and in healthy mouse aorta. Unbiased comparison between mice and humans explored the possible reasonability and utility of mouse Ang II-induced aortic aneurysm as a model for human aortic aneurysm diseases. Meanwhile, we performed comparative analysis of aortic aneurysms between TAA and AAA in both organisms.

Results and Discussion: We demonstrated similarities and differences of changes in the components of human and mouse cell types, and our unbiased comparison between mouse and human identified well conserved subpopulations of SMCs and macrophages. Furthermore, the results of our comparative analyses suggested different biological functions and distinct potential pathogenic genes for thoracic and abdominal aortic aneurysms. MIF and SPP1 signaling networks participated in aortic aneurysm in both organisms. This study maps aortic aneurysm and offers opportunities for future researches to investigate the potential of subpopulations or marker genes as therapy targets.

KEYWORDS

thoracic aortic aneurysm, abdominal aortic aneurysm, species homology and diversity, single-cell RNA sequencing, comparative analysis

Introduction

Aortic aneurysm is a life-threatening disease that results from progressive dilatation of the aorta and weakness of the blood vessel walls (Mizrak et al., 2022), which usually goes unnoticed until incidental detection during imaging examination or until rupture of the aorta, a disastrous event with a mortality rate of up to 50%–80% (Kuivaniemi et al., 2015). On the basis of anatomic locations, aortic aneurysms are subdivided into two categories: the thoracic aortic aneurysm (TAA, which occurs above the diaphragm) and abdominal aortic aneurysm (AAA, which occurs below the diaphragm). Although the two diseases share several etiological similarities, such as inflammation, proteolytic elastic tissue, degeneration of the extracellular matrix (ECM), and smooth muscle cell (SMC) apoptosis, there are also significant differences in the population prevalence rate, genetic patterns, and susceptibility genes. AAAs are more common than TAAs because they affect 4%–7% males over the age of 65 and 1%–2% in females (Lederle, 2003), whereas the prevalence of TAA is estimated to be around 1% of the general population (Verstraeten et al., 2017). In addition, approximately 20%–25% of TAA patients are estimated to have familial TAAs, and considerable proportion (~25%) of the familial cases exhibit an autosomal dominant pattern of inheritance of the disease within the family, i.e., thoracic aortic disease caused by a mutation in a single gene (Marfan syndrome due to *FBN1* mutations, *COL3A1* mutations related to vascular Ehlers–Danlos syndrome) (Pinard et al., 2019), whereas AAA does not typically demonstrate such inheritance. Nonetheless, studies discover the involvement of genetic determinants linked to AAAs and identified several risk genes such as *AGTRI* and *MMP3*. Importantly, risk factors for AAA also include manageable smoking, hypertension, and hyperlipidemia (Vardulaki et al., 2000). The human aortic wall is composed of a variety of cell types and exhibits significant regional heterogeneity corresponding to their differing embryology (Cheung et al., 2012; Sawada et al., 2017), which greatly restricts our better knowledge of the pathogenesis of TAAs and AAAs. However, both TAAs and AAAs are complex, multifactorial, and highly heterogeneous diseases. An improved knowledge of the cellular mechanism networks that trigger the development and succeeding expansion of aneurysms is critical to discovering innovative therapeutic targets.

The second primary challenge in investigating aortic aneurysms is the potential discrepancy between mice and humans. The mouse remains the dominant research model for studying disease pathogenesis, partly not only because of their scalability and reproducibility compared to human investigations but also due to their utility value in the study of mechanism regulatory networks (Zilionis et al., 2019). Three experimental techniques, CaCl_2 application, elastase perfusion, and angiotensin II (Ang II) infusion, can induce aortic aneurysms in mice (Lu and Daugherty, 2017; S  n  maud et al., 2017). The Ang II subcutaneous perfusion model has advantages of simulating several features of human aortic aneurysms, such as medial

degeneration, thrombosis, accompanying with atherosclerosis, ECM degradation, endothelial permeability, and phagocyte and inflammatory cell infiltration (S  n  maud et al., 2017). Importantly, mouse Ang II-induced aneurysms generally occur in the ascending or the suprarenal aorta, whereas aortic aneurysms in humans usually take place in the infrarenal aortic segment (S  n  maud et al., 2017). The differences have aroused practical concerns and interests in the different gene expressions and pathological processes between mice and humans; therefore, it becomes crucial to link population structures of humans and mice.

To address both difficulties, single-cell RNA sequencing (scRNA-seq) provides an opportunity to sample the entire transcriptome of a single cell, thus clustering similar cells independently of any previous assumptions on cellular marker genes or species conservation and allowing for unbiased comparisons between organisms (Briggs et al., 2018). Here, we used scRNA-seq to infer aortic cell sub-structures and transcriptional profiles in clinical patient TAAs and AAAs, as well as mouse models of corresponding diseases (Ang II induction), and in healthy mouse aorta. Unbiased comparison between mice and humans explored the possible reasonability and utility of mouse Ang II-induced aortic aneurysm as a model for human aortic aneurysm diseases. Meanwhile, comparative analysis of aortic aneurysms of different cross sections revealed distinct pathogenic genes between TAAs and AAAs in both organisms. Our findings discovered commonalities in the pathogenesis and gene expression profiles of aortic aneurysms between mice and humans, opening up the possibility of using specific sub-populations as potential therapeutic targets and exploring them in mice.

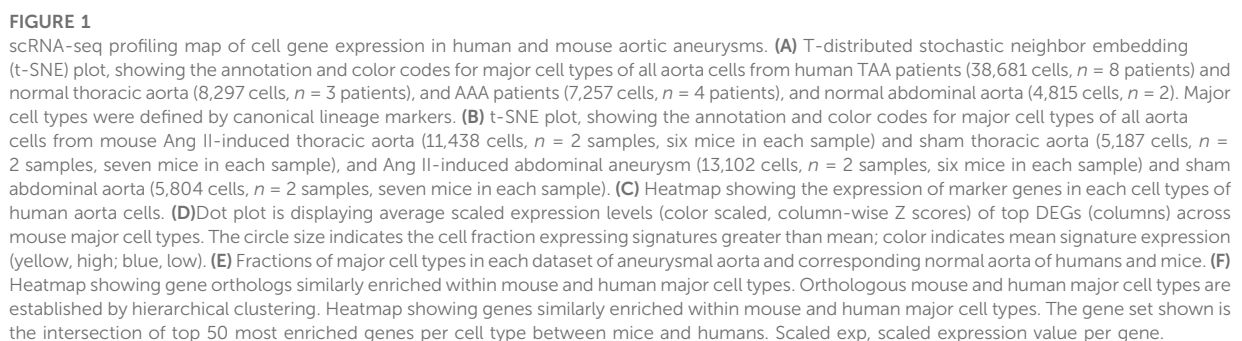
Materials and methods

Collection of human samples for histological analysis

The protocol for collecting human tissue samples was approved by the Research Ethics Committees of the First Affiliated Hospital of Zhejiang University School of Medicine. Normal ascending aortic tissue samples were collected from recipients of heart transplants or lung donors, and diseased thoracic aortic tissue specimens were collected from patients with sporadic TAAs; one abdominal aneurysmal aortic tissue was collected from patients with AAA. These tissues were collected for histopathological analysis. Clinical characteristics of the study groups of AAA and TAA are summarized in [Supplementary Dataset S1](#).

Animal and ethics statement

All animal procedures were in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institute of Health (8th edition, 2011) and were approved by the Institutional Animal Care and Use



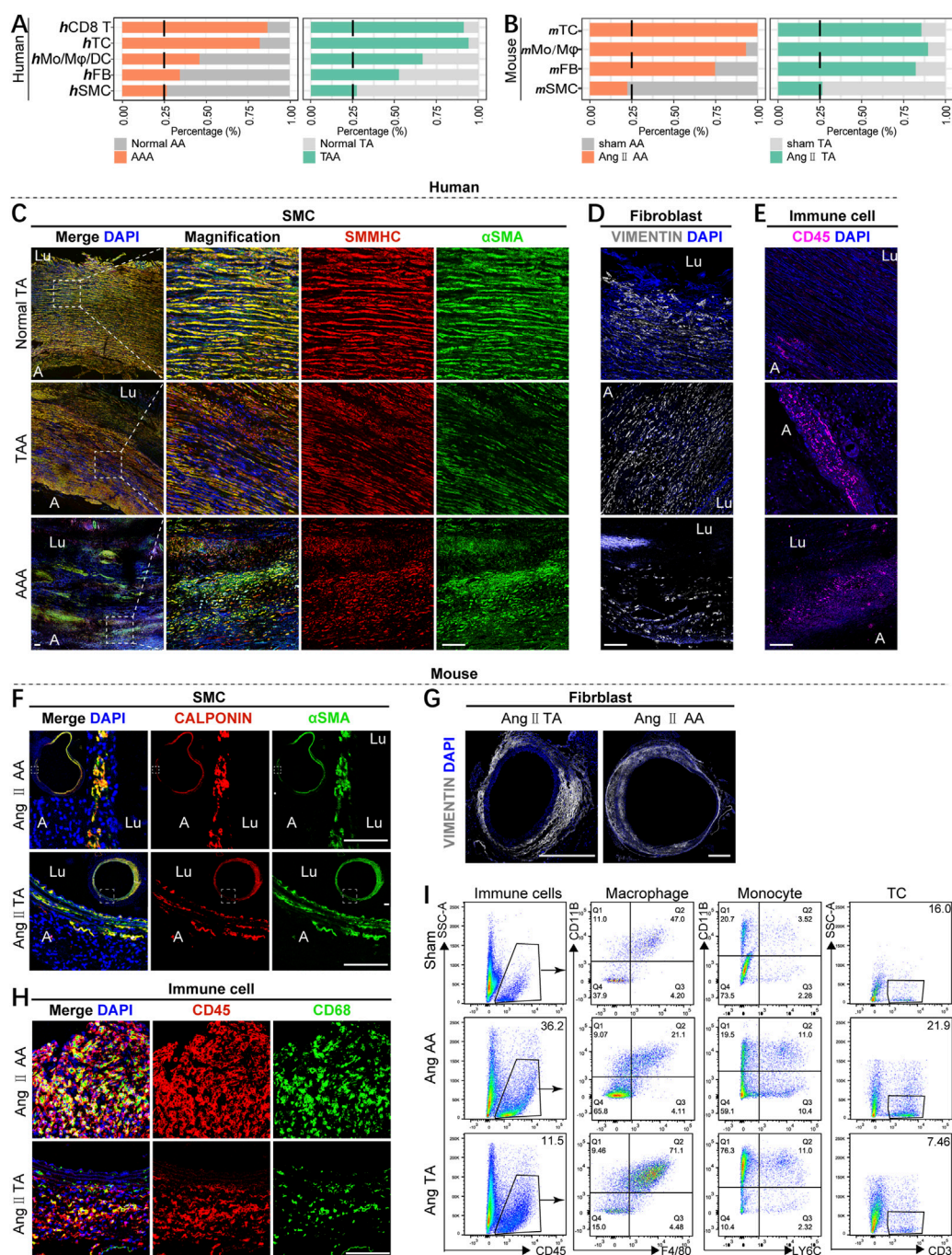


FIGURE 2

Alteration of cellular composition in the diseased status of mice and humans. (A,B) Fractions of major cell types in aneurysmal aorta vs. the corresponding normal aorta of humans (A) and mice (B). (C) Immunofluorescence analysis for SMMHC (red), αSMA (green), and overlays with DAPI-labeled nuclei (blue) showing smooth muscle cells in the human vascular media (scale bars: 100 μm). Lu means the lumen side of the vascular wall, and A means the adventitia side of the vascular wall. (D,E) Immunofluorescence analysis for vimentin (white) (D) and CD45 (purple) (E) and overlays with DAPI-labeled nuclei (blue) showing fibroblasts and immune cells in the human aorta (scale bars: 100 μm). (F) Immunostaining for calponin (red), αSMA (green), and overlays with DAPI-labeled nuclei (blue) showing smooth muscle cells in the mouse aortic media (scale bars: 100 μm). (G) Immunostaining for vimentin (white) and DAPI overlay in the thoracic and abdominal aorta from mice subjected to 4 weeks of Ang II induction (scale bars: 500 μm). (H) Immunostaining for CD45 (red), CD68 (green), and overlays with DAPI-labeled nuclei (blue) showing macrophages in the mouse aorta (scale bars: 100 μm). (I) Flow cytometry of the proportion of macrophages (F4/80⁺CD11b⁺), monocytes (LY6C⁺CD11b⁺), and T cells (CD3⁺) in CD45⁺ immune cells among three groups (n = 3–4, per group).

Committee of the Zhejiang University School of Medicine. ApoE^{-/-} mice were purchased from the Shanghai Model Organisms Center at the age of 8 weeks. Here, 12-week male ApoE^{-/-} mice were continuously treated with subcutaneous Ang II infusion for 4 weeks (1,000 ng/kg/min, MCE, Cat. No.: HY-13948) by using an osmotic pump (Alzet model 1004, ALZA Corporation).

Immunofluorescence staining

Human aortic tissue specimens were dehydrated, embedded, and cut into 3- μ m-thick sections using a Leica RM2235 manual rotary microtome. Tissue sections were deparaffinized and hydrated in gradient xylene and ethanol. The antigen retrieval solution used was prepared with 10 mM Tris, 1 mM EDTA, and .05% Tween 20 (pH 9.0) by placing in 98°C water bath for 25 mins. After cooling down to room temperature (RT), tissue was blocked and permeabilized with 5% BSA+.1% Triton X-100 (Sigma, T8787) for 1 h at RT, stained with primary antibodies overnight at 4°C, then incubated with Alexa Fluor-conjugated secondary antibodies (1:500, Invitrogen) for 1 hour, and stained with DAPI (Servicebio, G1012).

Immunofluorescence staining of frozen sections from mouse aortic artery specimens was performed, arteries were first harvested, washed in PBS and fixed in 4% paraformaldehyde for 2 h at 4°C, and then dehydrated at 4°C in 30% sucrose solution overnight until fully penetrated. Tissues were then embedded in optimal temperature (O.C.T., Sakura, 4583) and cut into 8- μ m sections by a cryostat (Leica CM1950). The frozen sections were air-dried for about 30 mins at room temperature, permeabilized and blocked in 5% donkey serum (Solarbio® 41, SL050) for 1 h, incubated with primary antibodies at 4°C overnight, and then stained with Alexa Fluor-conjugated secondary antibodies (1: 500, Invitrogen) for 1 hour, followed by DAPI (Servicebio, G1012) staining.

Primary antibodies were used as listed: SMA-FITC (1:500, Sigma, F3777), SM22 alpha (1:200, Abcam, ab14106), SM-MHC (1:200, Abcam, ab53219), CD45 (1:50, R&D, AF114), CD68 (Abcam, ab125212, 1:200), and vimentin (Abcam, ab8978, 1: 200). Secondary antibodies were used as listed: donkey anti-rabbit IgG Alexa Fluor 555 (Invitrogen, A-31572, 1:500), donkey anti-rat IgG Alexa Fluor 488 (Invitrogen, A-21208, 1:500), donkey anti-mouse IgG Alexa Fluor 488 (1:500, Invitrogen, A-21202), donkey anti-goat IgG Alexa Fluor 488 (Invitrogen, A-11055, 1:500), and donkey anti-goat IgG Alexa Fluor 647 (1:500, Invitrogen, A-21447) antibodies. Isotype control primary antibodies (Invitrogen, Cat No. 31933, 02-6102, 31903, and 31245) for each host species were used as negative controls, together with secondary antibodies, and only control to validate specificity of antibodies and to eliminate the background signal. The images were taken with a Leica SP5 confocal microscope and analyzed with LAS AS software (Leica).

Isolation of single cells from the aorta

We digested the aortic vessels according to the method reported by our group (Deng et al., 2020; Jiang et al., 2021). Mice were euthanized and perfused with PBS, and aortic arteries were harvested and placed in a Petri dish containing DMEM (ATCC, 302002) with 10% fetal bovine serum (FBS, Gibco, 10099141) on ice. The perivascular connective tissue and adipose tissue were carefully removed. After collecting all arteries, arteries were washed with PBS, cut into pieces, and digested with 0.75 mg/ml papain digesting solution. After 1–2 digestions, the rest tissues were digested with 0.75 mg/ml papain and 1 mg/ml collagenase I in Hank's solution (HBSS, Gibco, 14025092). The whole process of digestion was carried out in 37 water baths with reciprocating shaking. The detached cells were collected in DMEM containing 20% FBS, and fresh digestion solutions were replaced every 10 mins until the tissues were digested completely. At the end of the digestion process, all the cells digested from one sample were collected together. After thorough digestion, we filtered the cells with a 40- μ m cell filter and centrifuged at 500 g speed at 4°C for 8 mins. Subsequently, the cell pellet was resuspended in relevant solutions for further experiments.

Flow cytometric analyses

For isolated aortic single cells, the harvested cells were incubated with red blood cell lysis buffer, and then, single-cell suspensions were obtained using a 40- μ m cell strainer. The cells were stained with conjugated antibodies (1 μ g per 10,634 cells) for 30 min at 4°C. Conjugated antibodies used include CD45-FITC (Invitrogen, 11-0451-82), F4/80-PE-Cyanine7 (Invitrogen, 25-4801-82), CD11B-PE (Invitrogen, 12-0112-82), LY6C-APC (17-5932-82), and CD3-PE (Invitrogen, 12-0031-82). An Alexa Fluor® 38488 conjugated rat IgG2a, κ isotype control (BD Pharmingen™, 557676) was used to validate specificity of antibodies. Then, the cells were washed and suspended and stained with a LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (1:1000, Invitrogen, L34975) for 5 mins. The cells were then washed and re-suspended in PBS with 1% FBS for FACS analyses. All prepared samples were analyzed by using a BD LSR Fortessa II flow cytometer (BD Biosciences). FlowJo v10 software (BD Biosciences, United States) was used to analyze the flow cytometric data.

ScRNA-seq of artery cells with 10x chromium and data analysis

Human scRNA-seq datasets for TAA (GSE155468) and AAA (GSE166676) were collected from public repositories. We

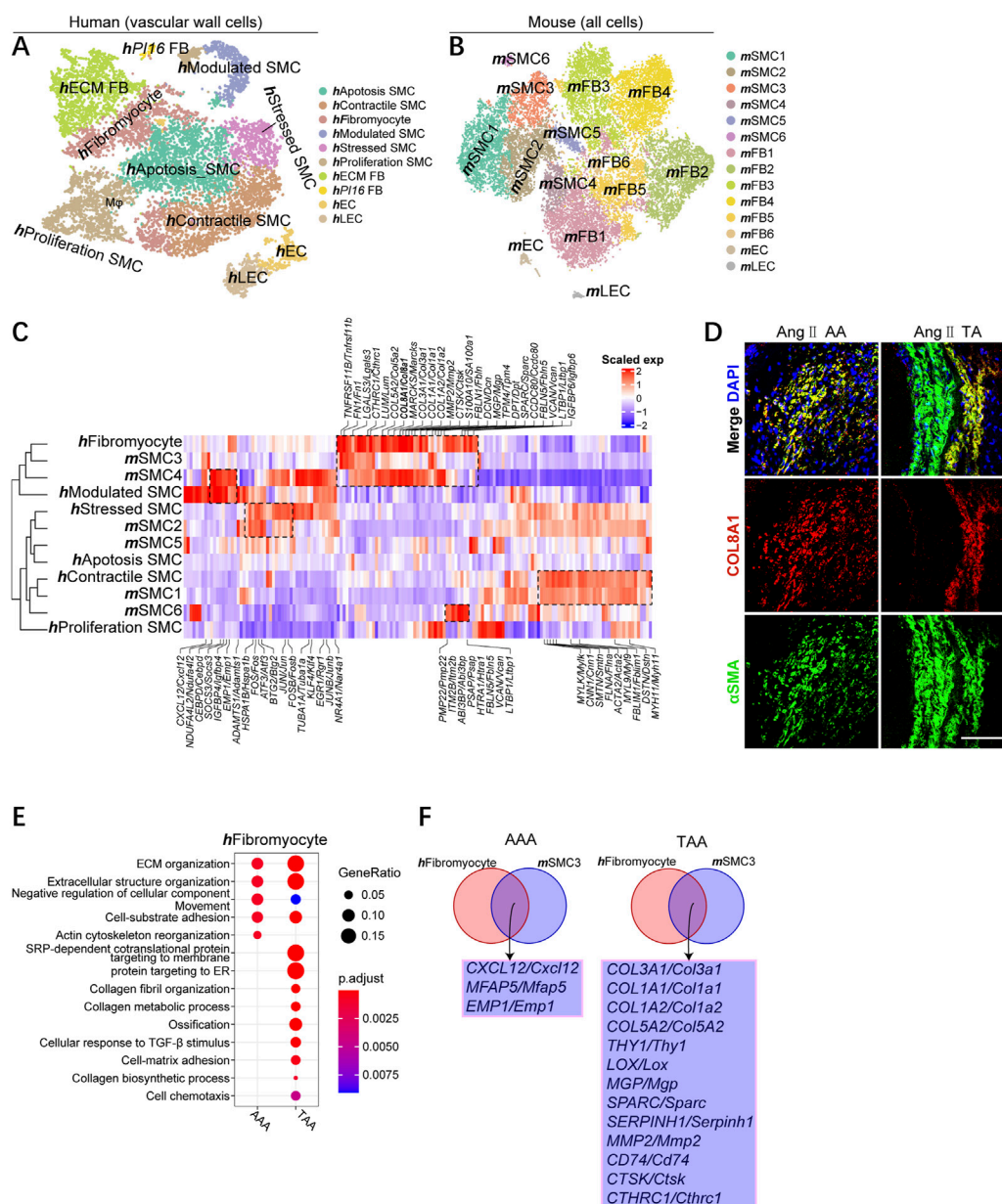


FIGURE 3

Identifying well-conserved SMC subsets and distinct marker genes for TAA and AAA across species. **(A)** T-SNE plot showing the annotation and color codes for human vascular wall cells. **(B)** t-SNE plot, clustering mouse aortic cells, showing cell clusters by color. **(C)** Comparison of mouse and human SMC subsets. Orthologous mouse and human SMC subsets established by hierarchical clustering. Heatmap showing genes similarly enriched within mouse and human SMC subsets. The gene set and scaled expression defined, as shown in Figure 1F. **(D)** Representative immunofluorescence images: COL8A1 (red), αSMA (green), and DAPI (blue), scale bar 100 μm. **(E)** Representative GO terms and pathways enriched in upregulated DEGs of hFibromyocytes between TAA and AAA groups. TAA group: TAA vs. Normal TA; AAA group: AAA vs. Normal AA. **(F)** Integrated comparative analysis of upregulated DEGs of AAA (left) and TAA (right) between hFibromyocytes and mSMC3s.

performed scRNA-seq of thoracic and abdominal aorta cells, respectively, from Ang II-induced mice and the control group. After full digestion, the cells were suspended with PBS and stained with a LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit and Hoechst 33342 (1:1000, Invitrogen, H3570) for

20 mins on ice. We then sorted single nucleated live cells into PBS containing .04% BSA by BD FACS ARIA II flow cytometry (BD Biosciences). The samples were subjected to scRNA-seq using the Chromium™ Single Cell 3' Reagent Kit v2 or v3 chemistry (10x Genomics) and followed a standard protocol. The library was

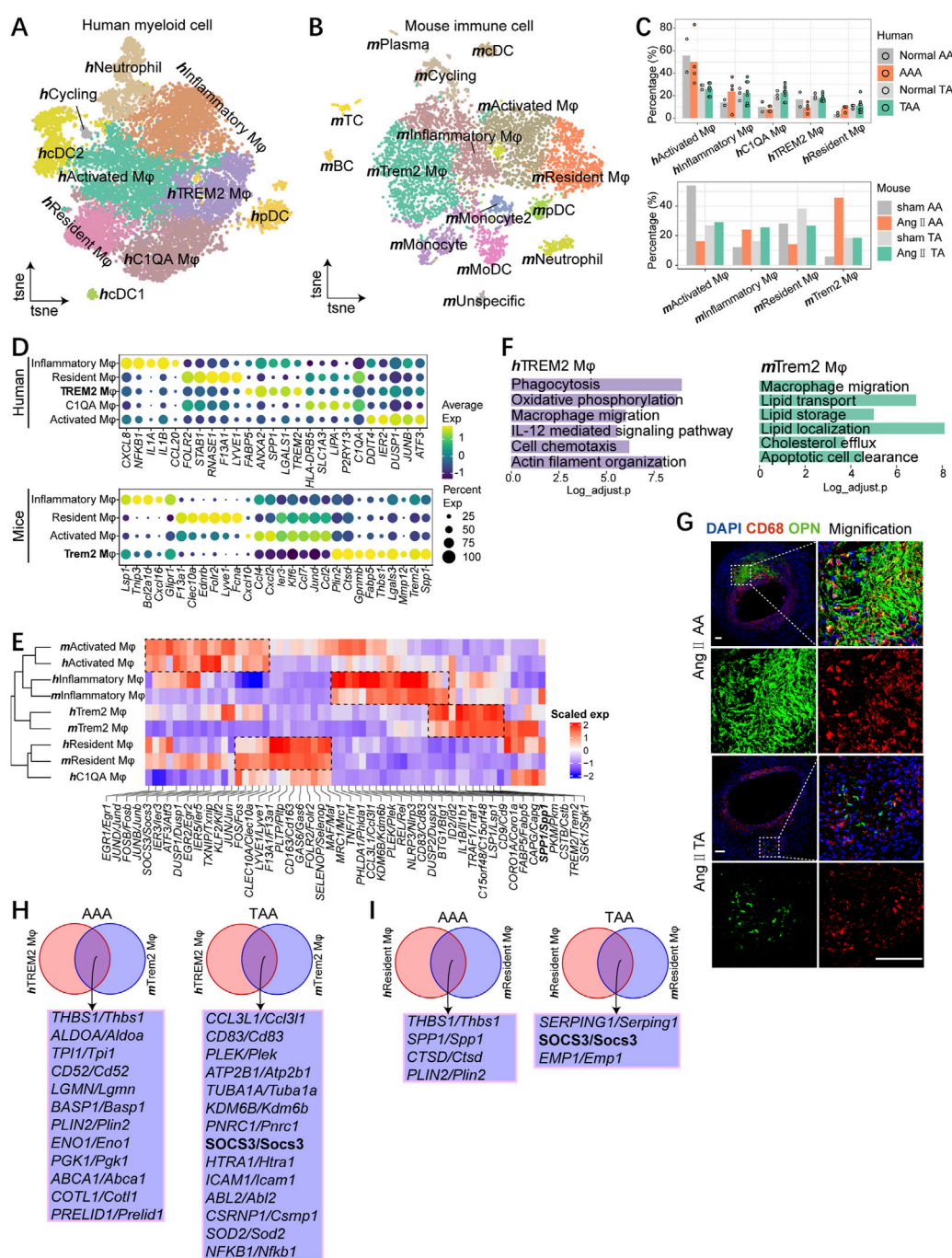
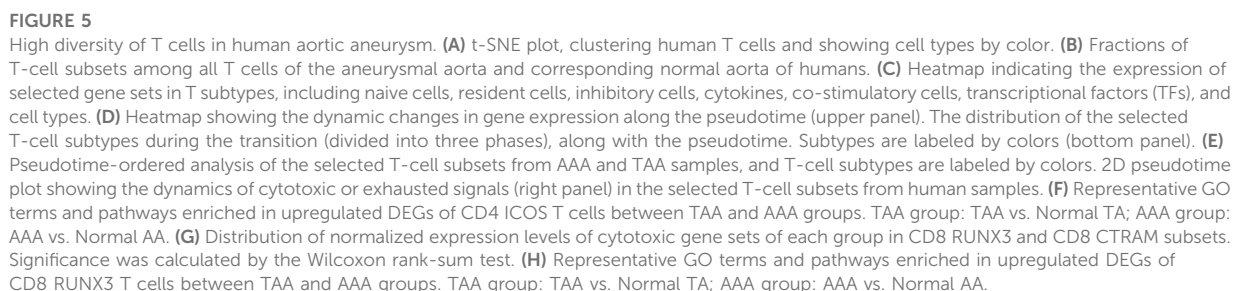


FIGURE 4

Macrophages contain common subsets across species. (A) t-SNE plot, clustering human myeloid cells and showing cell types by color. (B) t-SNE plot, clustering mouse immune cells and showing cell types by color. (C) Fractions of macrophage subsets among all macrophages of aneurysmal aorta and the corresponding normal aorta of humans (upper) and mice (bottom). (D) Dot plot displaying average scaled expression levels (color scaled, column-wise Z scores) of top DEGs (columns) across mouse (bottom) and human (upper) macrophage subsets. The circle size indicates the cell fraction expressing signatures greater than mean; color indicates mean signature expression (yellow, high; blue, low). (E) Heatmap showing gene orthologs similarly enriched within mouse and human macrophage subsets. Orthologous mouse and human major cell types established by hierarchical clustering. (F) Representative GO terms and pathways enriched in *hTREM2* macrophages (left) and *mTREM2* macrophages (right). (G) Immunostaining for CD68 (red), OPN (green), and overlays with DAPI-labeled nuclei (blue) showing *mTREM2* Mφ in the Ang II-induced mouse aorta (scale bars: 100 μm). (H) Integrated comparative analysis of upregulated DEGs of AAA (left) and TAA (right) between *hTREM2* Mφ and *mTREM2* Mφ. (I) Integrated comparative analysis of upregulated DEGs of AAA (left) and TAA (right) between *hResident* Mφ and *mResident* Mφ.



Cell filtration, data normalization, dataset integration, and further cell clustering and visualization were performed with the R package Seurat (version 4.0.1) with default parameters, unless otherwise specified (Stuart et al., 2019). Briefly, gene signatures

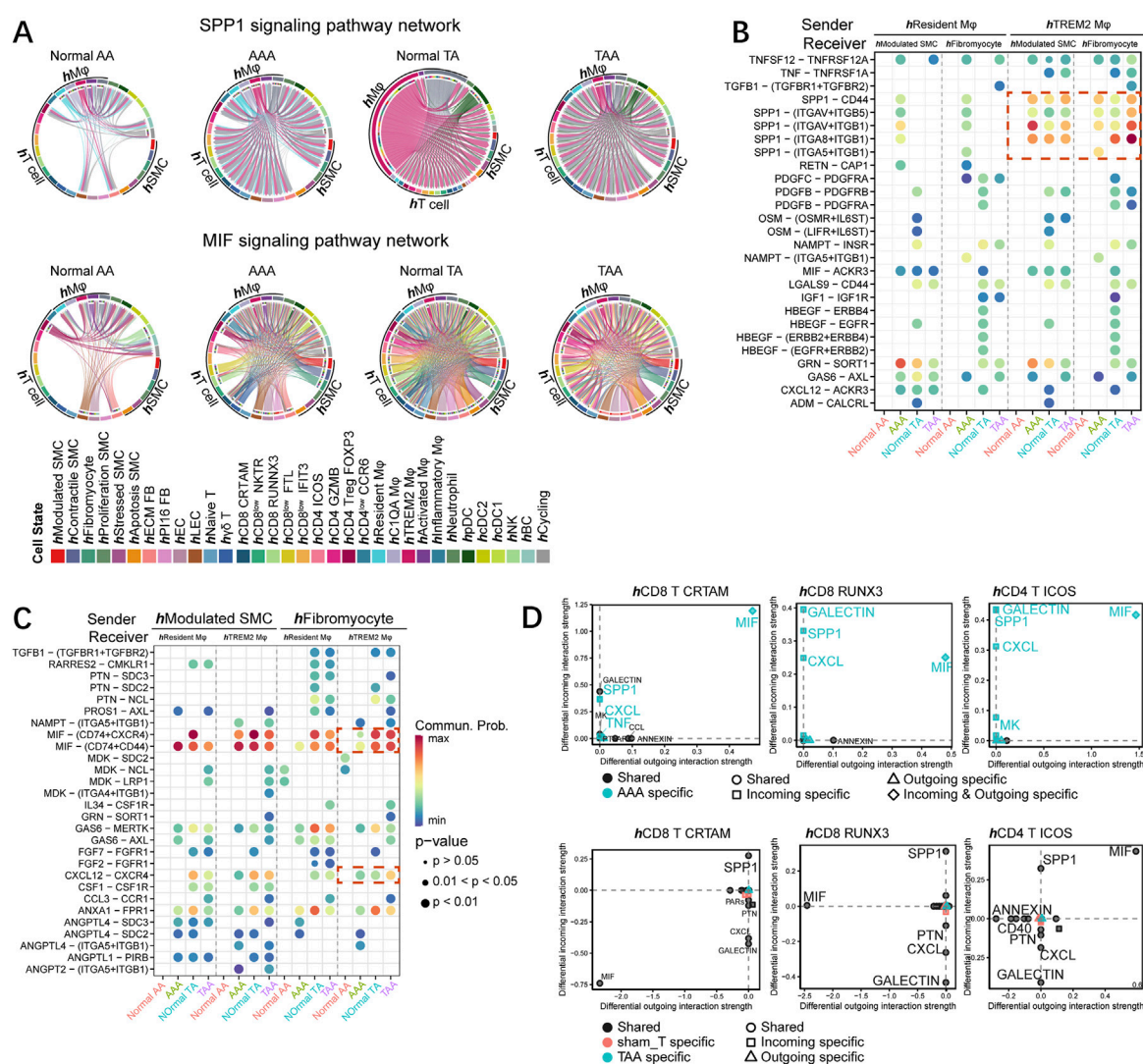


FIGURE 6

MIF and SPP1 signaling pathways commonly altered among different species. (A) Chord diagram of SPP1 (upper) and MIF (bottom) signaling networks in human samples (normal AA, AAA, normal TA, and TAA). (B) Comparison of the significant ligand-receptor pairs between normal AA, AAA, normal TA, and TAA, which contribute to the signaling from hResident Mφ and hTREM2 Mφ to hModulated SMC and hFibromyocyte. The dot color reflects communication probabilities, and the dot size represents computed *p*-values. Empty space means the communication probability is zero. *p*-values are computed from the one-sided permutation test. (C) Comparison of the significant ligand-receptor pairs between normal AA, AAA, normal TA and TAA, which contribute to the signaling from hModulated SMC and hFibromyocyte to hResident Mφ and hTREM2 Mφ. (D) Signaling changes of T-cell subpopulations (hCD8 CRTAM, hCD8 RUNX3, and hCD4 ICOS) in AAA (upper) and TAA (bottom) compared to the respective control group.

expressed in cells with fewer than three cells and cells expressing fewer than 100 or greater than 4,000 genes were excluded for filtering out non-cell or cell aggregates. Moreover, the cells expressing mitochondrial gene percentages greater than 5% were also excluded. Then, the DoubletFinder R package was performed to filter out doublet cells (McGinnis et al., 2019). After alignment and quality control, 8,297 cells from non-diseased thoracic aortic wall tissue (normal TA, $n = 3$), 38,681 cells from aneurysmal thoracic aortic wall tissue (TAA, $n = 8$), 4,815 cells

from the normal abdominal aorta group (normal AA, $n = 2$), and 7,257 cells from the abdominal aortic aneurysm group (AAA, $n = 4$) were combined and included in the subsequent analysis; mouse Ang II-induced thoracic aorta (11,438 cells, $n = 2$ samples), sham thoracic aorta (5,187 cells), Ang II-induced abdominal aneurysm (13,102 cells, $n = 2$ samples), and sham abdominal aorta (5,804 cells) were aggregated and included in the subsequent analysis. After log normalization, the top 2,000 highly variable genes were chosen and scaled by

“ScaleData.” Datasets were integrated in Seurat using first 30 dimensions. The dimension was then reduced with 30 principal components, and the t-distributed stochastic neighbor embedding (t-SNE) was used to visualize clusters with the resolution set at .5.

Gene enrichment analyses

Gene ontology and KEGG pathway analyses of DEGs were performed using the R package clusterProfiler (<https://github.com/YuLab-SMU/clusterProfiler>) (Yu et al., 2012). The biological process of gene ontology was annotated by the enrichGO function with annotation databases org.Hs.eg.db and org.Mm.eg.db. Biological process expression levels referred to the calculation of the average expression of gene sets in BP annotation using the online database (geneontology.org) and AddModuleScore in the Seurat R package.

T-cell score definition

Genes related to CD4/CD8 T-cell resident markers, exhausted markers, cytotoxic markers, and co-stimulatory score markers are from published studies (Guo et al., 2018; Zhang et al., 2020). The average expression levels of these gene sets were used to determine immune states for T cells.

Pseudotime trajectory analyses

Pseudotime trajectory analyses were conducted with the R package Monocle (version 2.14) with default settings. The top 250 (arranging by avg log2 fold change) differentially expressed genes (DEGs) were used for pseudotime by the function differentialGeneTest with fullModelFormulaStri set as pseudotime. For dimension reduction and subsequent cell ordering along the pseudotime trajectory, the DDRTree method was used.

Cell–cell communication

CellChatDB is a literature-supported database of mouse and human ligand–receptor interactions (Jin et al., 2021). We eliminated cycling cells and unspecific cells for cell–cell communication analysis. Then, Seurat-preprocessed human and mouse datasets were separately loaded to the CellChat package to analyze and visualize the cell–cell interaction. The interesting network pathways among source cells and target cells were selected and visualized by the hierarchy plot. Comparison of contribution of significant ligand–receptor pairs in the signaling

between indicated cell types among each group was analyzed and displayed by the dot plot.

Data availability

The data and R scripts that linked to the results of the study are available on reasonable request. ScRNA-seq data on the study are available in the Gene Expression Omnibus dataset. The scRNA-seq datasets for human TAAs (GSE155468) and AAAs (GSE166676) were collected from public repositories.

Statistics

All statistical analyses and presentations were conducted with the R package ggsignif. Comparisons of the expression levels of gene sets or gene signatures between the two groups were carried out by Wilcoxon rank-sum tests. Statistical tests used in figures are shown in corresponding figure legends. The precise value of *n* is displayed in the figure legends, and the meaning of *n* is also displayed in the figure legends.

Results

scRNA-seq delineated cellular landscapes in mouse and human aortic aneurysms

Our study set out to determine aortic aneurysm cell sub-clusters from human TAAs and AAAs and in a mouse Ang II-induced aortic aneurysm model and to find homologies and discrepancies across aortic segments and organisms. We respectively collected human scRNA-seq datasets for TAAs (GSE155468) and AAAs (GSE166676) from public repositories (Li et al., 2020; Davis et al., 2021). After alignment and quality control, 8,297 cells from non-diseased thoracic aortic wall tissue (Normal TA, *n* = 3), 38,681 cells aneurysmal thoracic aortic wall tissue (TAA, *n* = 8), 4,815 cells from the normal abdominal aorta group (Normal AA, *n* = 2), and 7,257 cells from the abdominal aortic aneurysm group (AAA, *n* = 4) were combined and included in the subsequent analysis. The population structure of these human cells was analyzed and annotated according to acknowledged molecular markers, as shown in [Supplementary Figures S1A–C](#). All cell types were classified as vascular wall cells [vascular smooth muscle cells (*hSMCs*), endothelial cells (*hECs*), and fibroblast (*hFBs*)], myeloid immune cells [*hNeutrophils*, monocytes/macrophages/dendritic cells (*hMo/Mφ/DC*), and *h mast* cells], lymphocytes [*hT* cell, *hCD8⁺* T cells, natural killer cells (*hNK*), *hB* cells, and *h plasma* cells], and others (*hepithelial* cells, *h cycling* cells, and *h unspecific* cells) (Figure 1A).

The matched dataset of mouse cells was acquired from the Ang II-induced aneurysmal abdominal aorta (Ang II AA) and thoracic aorta (Ang II TA), as well as the corresponding sham group (sham AA and sham TA). Mouse cells were partitioned with t-SNE into 22 clusters and were identified as eight major cell types *via* the expression of canonical lineage markers (Supplementary Figures S1D, E). Similarly, all mouse cells consisted of three major classifications, including vascular wall cells (*mSMC*, *mEC*, and *mFB*), myeloid immune cells (*mMo/Mφ*, *mNeutrophil*, and *mcDC*), and lymphocytes (*mTC* and *mBC*) (Figure 1B), each containing cells from all four groups (Supplementary Figure S1F).

Both mouse and human cells were divided into major cell types, some of which contained complex subpopulation structures. To further uncover the characteristics of the cells, we identified differentially expressed genes (DEGs) of each cell clusters and displayed top five DEGs arranged by log2 fold change (Figures 1C, D). Albeit each cell type identified here possesses sub-cluster, the gene expression patterns annotated on the basis of acknowledged gene signatures reflected many known markers. Regardless of humans or mice, thoracic or abdominal aorta, the aortic SMC percentage was significantly lower than that of the corresponding normal group, as confirmed by quantification of the SMC composition of each sample. Fibroblasts apparently expanded in different segments of mouse aneurysmal aorta, while the proportion of fibroblasts in the human aorta was low and did not alter much in the diseased state. Moreover, the increase of inflammatory cells was predominantly lymphocytes in human aortic aneurysm specimens, whereas the enhancement of myeloid immune cells was dominated in mice (Figure 1E). To comprehensively compare the gene expression profiles of mouse and human cells, we quantified the homologies between average transcriptome of cell types recognized in the two organisms by visualizing the intersection of top 50 DEGs. We observed that the consistency of cell types, rather than source species, determined the homologies of gene expression patterns, as indicated by examining the expression of unique cell type genes in both mice and humans, as well as by hierarchical clustering tree of the major cell lineages. Except for *mcDCs*, every major cell type clustered with its homolog (Figure 1F). However, the gene expression profiles of *mcDCs* were more similar to neutrophils and *Mo/Mφ/DCs*. An extensive conservation of primary gene expression profiles in aortic cells was observed through unbiased comparison of mouse and human cells.

Collectively, there are both differences and similarities in the composition of cellular components and the changes in the aortic aneurysm disease status across segments and species. More importantly, this cell type correspondence reaffirms that the existence of consistent gene expression patterns between mice and humans and justifies an inspection of similarities and discrepancies within each cell lineage.

Alteration of cellular composition in the diseased status of mice and humans

Our analysis revealed the remarkable reduction of SMC proportion and increase of immune cells including T cells and monocytes/macrophages (*Mo/Mφ*) in aneurysmal aorta compared with corresponding controls both in mice and humans, although the absolute number of T cells in the mouse aorta was very small; different from human aortic aneurysm, the number and percentage of mouse *Mo/Mφ* elevated significantly on the pathological condition (Figures 2A, B). To further validate our analysis, we collected clinical diseased aortic wall specimens from thoracic and abdominal aorta aneurysm patients and non-dilated ascending aortic wall tissues from lung donors. Histological analysis confirmed elastin degradation manifested by increased elastin fragmentation and even elastin loss, as well as collagen deposition, especially in focal areas with severe disruption of elastin (Supplementary Figure S2A). Immunofluorescence (IF) staining of the mature SMC marker SMMHC and the SMC contractile marker α SMA demonstrated a significant decrease of SMC density in aortic vascular media and phenotypic switches in abdominal aortic aneurysm indicated by SMC morphology alteration (Figure 2C). Moreover, the number of fibroblasts increased in thoracic aortic aneurysm patients, which might be inconsistent with scRNA-seq data, could be due to tissue digestion and individual differences; fibroblasts did not enhance in the AAA group (Figure 2D). Meanwhile, CD45⁺ inflammatory cells were obviously infiltrated in the diseased group (Figure 2E).

In line with humans, mice Ang II-induced aortic aneurysm displayed remarkable elastin break and reduction of number of SMCs (Figure 2F; Supplementary Figure S2B). However, different from human samples, mouse thoracic and abdominal aortic adventitia were markedly thickened and increase of fibroblast proportion, especially in Ang II-induced thoracic aorta (Figure 2G; Supplementary Figure S2B). In addition, IF staining indicated a lot of inflammatory cells and macrophages accumulated in the aortic wall after Ang II stimulation (Figure 2H). The infiltration of macrophages, monocytes, and T cells was verified by flow cytometry (Figure 2I). Generally, the Ang II-induced aortic aneurysm model showed similar pathological changes of SMCs and immune cells but different fibroblast alterations with clinical human thoracic and abdominal aortic aneurysm diseases.

Aortic aneurysms contain well-conserved SMC subsets across species and distinct marker genes for TAAs and AAAs

We then analyzed the sub-clusters of each classification, focusing on SMCs, macrophages, and T cells. Clustering human vascular wall cells identified six SMC subpopulations,

two subset fibroblast subtypes, endothelial cells (ECs), and lymphatic endothelial cells (LECs), which were named based on previously reported human SMC subgroups and the corresponding functional enrichment analysis (Li et al., 2020; Mizrak et al., 2022) (Figure 3A; Supplementary Figures S3A–D). Clustering mouse vascular wall cells uncovered six *mSMC* subclusters, six *mfibroblast* subpopulations, *mECs*, and *mLECs* (Figure 3B; Supplementary Figures S3A, C).

Unbiased comparison of mouse and human vascular wall cells also indicated the aggregation of the same cell type rather than species except for *hfibroblast*, *hModulated SMC*, *mSMC3*, and *mSMC4* (Supplementary Figure S4A). On the other hand, *hfibroblast*, *hModulated SMC*, *mSMC3*, and *mSMC4* were more similar to fibroblasts than SMC, reflecting their proximity in gene expression and had undergone phenotypic transformation into synthetic SMC (Supplementary Figure S4A). The percentage of *hfibroblast* and *hModulated SMC* in human aneurysm and the proportion of *mSMC3* and *mSMC4* in mouse aortic aneurysm consistently enhanced in contrast to other subsets of SMCs (Supplementary Figure S4B).

To define how SMC subgroups associate with each other across organisms, we compared mouse and human SMC subtypes comprehensively on the entire transcriptome levels and the single-cell levels. Species conservation of several aspects of SMC subpopulation structures between humans and mice were observed, which could be demonstrated by an unsupervised comparison (Figure 3C). In both organisms, we observed highly similar subpopulations. *hContractile SMC* and *mSMC1* highly expressed plenty of canonical contractile SMC markers (*MYH11*, *DSTN*, *MYL9*, *CNN1*, and *MYLK*; omitting mouse gene symbols with synonymous lowercase here and later), whereas *hStressed* and *mSMC2* displayed high expression of stress response-related genes, such as *ATF3*, *FOS*, *JUN*, and *HSPA1B*, suggesting the stress response state in this type of SMC. *mSMC6* was most closely associated with their *mSMC1* counterparts (e.g., *mSMC6* had low expression of contractile SMC markers), although *mSMC6* uniquely expressed *Vcan* and *Ltbp1*. More importantly, *mSMC3* are more similar to *hfibroblast* than *mSMC4* according to the hierarchical clustering tree, but *mSMC4* also closely associated with these two subgroups because all three groups possessed the expression of classic synthetic SMC markers [collagen genes, such as *Col1a1*, *Col1a2*, and *Col3a1*; fibrosis-associated genes (*Fln*, *Sparc*, and *Fbln*); and matrix metalloproteinase (MMP) gene (*Mmp2*)]. Meanwhile, we identified several innovative marker genes well-conserved across species, including *CTHRC1*, *COL8A1*, and *LGALS3*, involved in phenotypic switching into synthetic SMCs. In addition, we observed that the gene expression of *mSMC4* resembled *hModulated SMC*, and both of them highly expressed cytokines *CXCL12*, and *CEBPD* and *SOC3*, further suggesting they were phenotypically transformed SMCs (Figure 3C). IF staining demonstrated the

colocalization of *COL8A1* and α SMA in phenotypic-transformed SMCs in mouse thoracic and abdominal aortic aneurysms, but *COL8A1* was not expressed in contractile smooth muscle (Figure 3D; Supplementary Figure S4C).

We next focused on *hfibroblast* and *mSMC3* subgroups due to their phenotypic transformation and their increase percentages among SMCs during the pathological process of aneurysm. By conducting comparative GO analysis, we studied the biological implication of TAA and AAA-related upregulated DEGs and found that both *hfibroblast* and *mSMC4* enriched for cell chemotaxis and cell–cell adhesion in the TAA group compared with AAA group; *mSMC4* in TAA and AAA exhibited similar enrichment for collagen fibril organization, ossification, and cell adhesion (Figure 3E; Supplementary Figure S4D). In both organisms, we uncovered that *CXCL12*, *MFAP5*, and *EMPI* might participate in the pathogenesis of AAA, among which it had been reported that the blockade of *CXCL12*/*CXCR4* protected against AAA formation (Michineau et al., 2014), whereas several collagen genes (*COL1A1*, *COL1A2*, *COL3A1*, and *COL5A2*); some reported virulence genes for TAA (*LOX*, *COL3A1*, and *MMP2*) (Longo et al., 2002; Shen et al., 2015; Pinard et al., 2019; Chen et al., 2022); and potential causative genes (*CTHRC1*, *SERPINH1*, *SPARC*, *THY1*, and *CTSK*) were identified involving in the pathology of TAAs (Figure 3F).

In all, we first identified conserved modules of aortic SMC gene expression within human and mouse SMCs, including contractile SMCs, stressed SMCs, and synthetic SMCs (fibroblasts and modulated SMCs). Second, our analysis revealed distinct causative genes for TAA and AAA across species in well-conserved SMC subsets (*hfibroblast* and *mSMC3*).

Macrophages contain common subsets across species

Spectral clustering of patient myeloid cells (without *h*mast cell) identified five macrophage subsets, namely, *hActivated Mφ*, *hResident Mφ*, *hTREM2 Mφ*, *hInflammatory Mφ*, and *hC1QA Mφ*, which were defined by unique feature genes and previous publications (Cochain et al., 2018; Weinberger et al., 2020) (Figures 4A, D). The macrophage subsets were all present in each sample, albeit the low cell numbers in the normal group (Supplementary Figure S5A). *hTREM2 Mφ* is characterized by higher expression of *SPPI*, *TREM2*, *LGALS1*, *FABP5*, and *ANXA2*, a subpopulation newly identified in atherosclerotic disease (Cochain et al., 2018); *hResident Mφ* expressed genes characteristic of adventitia macrophages (*LYVE*, *1 F13A1*, *RNASE1*, *STAB1*, and *FOLR2*); *hInflammatory Mφ* was defined by the high expression of chemokines (*CCL20*, *IL1A*, *IL1B*, *CXCL8*, and *NFKB1*); *hActivated Mφ* expressed stress response genes including *ATF3*, *JUNB*, *DUSP1*, *IER2*, and

DDIT4; *hC1QA* Mφ is defined by the high expression of complement genes (*C1QA*) (Figure 4D).

In mice, we found four distinct macrophage subsets, which were also annotated by the high expression of characteristic genes (Figures 4B, D; Supplementary Figure S5B). Although the proportion of similar subpopulations varied across species (Figure 4C), these four subpopulations could correspond one-to-one with those found in humans except for *hC1QA* Mφ, first by detecting unique markers for each cell type and followed by unsupervised hierarchical clustering using homologous variable genes (Figures 4D, E). *mResident* Mφ mirrored *hResident* Mφ and shared common gene expression patterns (*Lyve1*, *F13a1*, *Pltp*, *Cd163*, *Gas6*, *Folr2*, *Selenop*, *Maf*, and *Mrc1*), whereas *mTrem2* Mφ mirrored *hTREM2* Mφ and featured the high expression of *Spp1* and *Trem2* (Figure 4E). We next analyzed the representative biological functions of each sub-clusters (Supplementary Figure S5C). More importantly, GO term analyses suggested highly specialized functional features of *mTrem2* Mφ, for example, in lipid handling processes, and *hTREM2* Mφ showed enrichment of phagocytosis and cell chemotaxis; both *mTrem2* Mφ and *hTREM2* Mφ enriched for macrophage migration (Figure 4F). We observed accumulative *OPN*⁺ macrophages (*mTrem2* Mφ) in Ang II-induced abdominal aortic aneurysm and several *OPN*⁺ macrophages (*mTrem2* Mφ) in Ang II-induced thoracic aorta (Figure 4G).

To further compare TAA and AAA, we performed comparative analysis and found the TAA group enriched for response to oxidative stress both in *mTrem2* Mφ and *hTREM2* Mφ (Supplementary Figure S5D). In *hResident* Mφ and *mResident* Mφ, the AAA group displayed enrichment for antigen processing and presentation compared with the TAA group (Supplementary Figure S5E). In *Trem2* and resident macrophage subsets, we identified different intersected genes between mice and humans. The upregulation of *THBS1* and *PLIN2* both in the *Trem2* macrophage and resident macrophage during AAA pathogenesis was observed. Meanwhile, the expression of *SOCS3* upregulated in the TAA group both in *Trem2* and resident macrophages, indicating *SOCS3* might be a potential causative regulator gene that participated in the pathological process of TAA.

Generally, four macrophage subpopulations are well-conserved between mice and humans. We identified a subpopulation of the *Trem2* macrophage featured biological function on macrophage migration and expression of *SPPI*, *TREM2*, *FABP5*, and *ANXA2* in both organisms during the pathology of aortic aneurysm. Moreover, our analysis indicated *THBS1* and *PLIN2* might implicate in AAA, and *SOCS3* might participate in TAA progression.

High diversity of T cells in human aortic aneurysm

The large and increased proportion of *hT* cells revealed its essential role during the aortic aneurysm pathogenesis, and the

number of *mT* cells is low for further analysis; thus, we performed unsupervised clustering of *hT* cells. The re-clustering of *hT* cells indicated 11 sub-clusters, containing naïve T cells, two subsets of *CD8*⁺ T cells (*CD8 CRTAM* and *CD8 RUNX3*), three *CD4*⁺ T-cell subsets (*CD4 ICOS*, *CD4 GZMB*, and *CD4 Treg FOXP3*), four *CD8*^{low} T-cell subpopulations (*CD8*^{low} *NKTR*, *CD8*^{low} *FTL*, *CD8*^{low} *IFIT3*, and *CD8*^{low} *IFNG*), and *CD4*^{low} *CCR6* cells (Figure 5A). Except for the *CD4 GAMB* subset, other subsets were shared across aneurysm and normal groups, albeit in variable proportions possibly because of the low cell numbers in the normal group (Figure 5B; Supplementary Figures S6A, B).

The diversity of T cells in aneurysm specimens was more abundant than that in non-dilation aortic samples (Figure 5). Naïve T cells with high expression of naïve markers, such as *SELL*, *CCR7*, *LEF1*, and *TCF7*, preferentially enriched in abdominal aorta including physiological and pathological conditions. *Treg FOXP3* possessed special gene expression properties resembling both *Treg* and naïve T-cell traits (Figure 5C) with expression of unique *Treg* hallmarks *FOXP3*, *IL2RA*, *TIGIT*, *TNFRSF14*, and *CTLA4* and blood T-cell-related genes *SELL*, *LEF1*, and *CCR7*, suggesting it as a subset of blood *Treg* that might exist in aneurysm dissection (Figure 5C). *FOXP3*⁺ *Treg* cells were abundant in aortic aneurysms, especially in AAA groups (Figure 5B; Supplementary Figure S6B) and displayed high expression of exhausted genes *CTLA4*, *TIGIT*, *TNFRSF14*, *ICOS*, and *CD28* (Supplementary Figures S6C, D). Among *CD4*⁺ T cells, *CD4 ICOS* specifically expressed *ICOS*, *PDCD1*, and *CD28*, suggestive of the state of exhausted *CD4* T cells. The fraction of *CD4 ICOS* was enhanced in TAA but reduced in AAA compared with their corresponding control groups. *CD4 GZMB* showed low percentage in each group and expressed both cytotoxic (*GZMB*) and exhausted *CTLA4*-related genes. *CD4*^{low} *CCR6* cells might be a cluster of memory-like, tissue-resident T cells on account of the highest expression level of *CD69*, *RUNX3*, and *NR4A1*. In addition, *CD8 CRTAM* cells highly expressed genes involved in cellular cytotoxicity (*NKG7*, *IFNG*, *GZMK*, and *GZMH*) and exhibited high expression of exhaustion-related markers (*PDCD1*, *TIGIT*, *TNFRSF9*, and *LAG3*) and chemokine genes (*CCL3*, *CCL4*, and *CCL5*) (Figure 5C; Supplementary Figure S6D). *CD8 RUNX3* cells displayed enhanced expression of exhaustion-related markers (*PDCD1*, *HAVCR2*, *TNFRSF9*, and *LAG3*) and were typical of the high expression of cytotoxic genes *GNLY*, *NKG7*, and *GZMH* and also displayed a high level of the tissue-resident gene *RUNX3* and a moderate level of tissue-resident genes *NR4A1* and *CD69* (Sun et al., 2021). *CD8 CRTAM* cells exhibited an escalating trend of proportions both in TAA and AAA than their corresponding control samples, whereas *CD8 RUNX3* cells exhibited an opposite trend, namely, decreased proportions in aortic aneurysm compared with the normal group. We also identified that the gene expression patterns of *CD8*^{low} *FTL* cells was similar to the identified

CD8 CX3CR1 cells (Zheng et al., 2017). CD8^{low} NKTR and IFIT3 cells showed the intermediate expression level of cytotoxic-related genes *GZMH*, *GZMK*, *NKG7*, and *IFNG* and also expressed low levels of the checkpoint genes (*GTLA4*, *HAVCR2*, and *TIGIT*), indicating that they might be precursors of cytotoxic T cells. CD8^{low} IFNG resembled tissue-resident CD8⁺ effector cells due to high abundance of effector cell hallmark *IFNG* and resident genes *CD69* and *NR4A1*, and the mediate expression of the cytotoxic genes. CD8^{low} IFNG also highly expressed genes linked to stress response, such as *FOS*, *JUN*, and genes encoding heat shock proteins (HSPs), including *HSPA1B*, *HSPA1A*, and *HSP90AA1*.

We next explored the dynamic immune state and cell transitions in naïve, CD4^{high} (except for blood Treg), and CD8^{high} T cells by using Monocle to infer cell state pseudotime trajectories. We excluded CD4 Treg on account of the high expression of blood T-cell markers. This analysis indicated that naïve T cells existed at the start of the pseudotime trajectory path (phase 1), typical of upregulated expression of *SELL*, *CCR7*, *TCF7*, *IL7R*, and *KLF2*, whereas the CD4 GZMB cells and partial CD4 ICOS cells at a terminal state (phase 3); CD8 CTRAM and RUNX3 cells were predominately at the intermediate pseudotime (phase 2) and distributed in another branch (Figures 5D, E), indicating that CD4 and CD8 T cells exhibit distinct differentiating trajectory. Pathway analysis demonstrated that signaling pathways associated with T-cell-mediated cytotoxicity and killing, lymphocyte differentiation, cytokine and chemotaxis production, and response to tumor necrosis factor were enriched in phase 2. Phase 3 cells were characterized by high expression of transcriptional factors *CEBPB*, *HOPX*, and stress response genes *DUSP5* and *ENO1*, and enriched for biological functions on T-cell activation, proliferation, and migration (cell–cell adhesion), T-cell receptor signaling pathway, and oxidative phosphorylation (Figure 5D). Both CD4 and CD8 T cells expressed the upregulated cytotoxic signature during the transition process, and CD4 cells expressed little higher exhausted gene set scores than CD8 cells (Figure 5E). To further characterize the transition state linked to CD4 and CD8 T cells in aneurysm groups, we inferred the movement trajectories of CD4 and CD8 T cells in the TAA and AAA groups, respectively. Significantly, early-stage CD4/CD8⁺ T cells were primarily present at AAA groups, with few cells found at the terminal point of the state transition path, while T cells in TAA groups were mainly distributed at the ends of both branches (Figure 5E).

To further compare TAA and AAA in different CD4/CD8 T-cell subsets, we conducted and compared GO analysis in the CD4 ICOS subgroup and uncovered common biological function on T-cell activation and migration supported by enrichment in cell–cell adhesion, cytoplasmic translation, and actin polymerization/depolymerization; the TAA group uniquely enriched in response to INF γ , T cell-mediated cytotoxicity,

chemotaxis, and cell killing (Figure 5F). Among CD8 T cells, CD8 CTRAM in the AAA group exhibited abundance in cytotoxic genes compared with the normal AA group, while TAA significantly exacerbated cytotoxicity in CD8 RUNX3 T cells (Figure 5G). Consistently, CD8 RUNX3 T cells in the TAA group displayed upregulation of cytokine production and NF- κ B signaling (Figure 5H).

We conclude that CD4⁺ and CD8⁺ cells in thoracic and abdominal aortic aneurysm samples showed distinct transition trajectories and subsets and displayed distinct inflammatory and gene transcriptional situations, indicating that targeted treatment strategies should be taken into consideration for the therapy of TAA and AAA.

MIF and SPP1 signaling pathways commonly altered among different species

MIF and SPP1 signaling involved both in TAA and AAA progress among different species based on cell–cell communication. SPP1 signaling mainly sent from macrophages and became much more abundant in the aortic aneurysm group in both organisms, although *hTREM2* M ϕ in the normal group showed strong SPP1 signaling (Figure 6A; Supplementary Figure S7A). MIF signaling originated from *hSMC* and *hT* cell appeared in the AAA group compared with the normal AA group (Figure 6A, bottom). Meanwhile, MIF signaling was produced by different cell populations and received by different cell populations in the human TAA group and Ang II-induced aortic aneurysm (Figure 6A; Supplementary Figure S7A). Among the ligand–receptor pairs between the macrophage subset (*hResident* M ϕ and *hTREM2* M ϕ) and SMC subsets (*hModulated* SMC and *hFibromyocyte*), consistently, several ligand–receptor pairs (SPP1–CD44, SPP1–ITGAV+ITGB5, SPP1–ITGA8+ITGB1, and SPP1–ITGA5+ITGB1) of SPP1 signaling sent from *hTREM2* M ϕ , received by SMC subsets, were obviously upregulated both in TAA and AAA compared with their respective normal groups (Figure 6B). On the other hand, MIF–(CD74+CXCR4) and MIF–(CD74⁺CD44) ligand–receptor pairs were significantly enriched in signaling sent from SMC subsets and received by macrophage subsets in the AAA group compared with the normal AA group, while the enrichment of these two ligand–receptor pairs in thoracic aortic aneurysm only presented in *hFibromyocyte* and *hTREM2* M ϕ (Figure 6C). In line with previous research (Michineau et al., 2014), the CXCL12–CXCR4 ligand–receptor pair was abundant among T-cell subsets, macrophage subsets, and SMC subsets (Figure 6C; Supplementary Figures S7C, D). In mice, we also observed the upregulation of MIF secreted by *mSMC3* in TAA, whereas the difference with human samples was that the elevation of the ligand SPP1 was observed in *mResident*

M ϕ , *mSMC3*, and *mSMC4* but not *mTrem2* M ϕ after Ang II stimulation across segments (Supplementary Figure S7B).

We then focused on the T-cell subset and found the specific increase of both incoming and outgoing MIF in CD8 CRTAM, CD8 RUNX3, and CD4 ICOS T cells in the AAA group (Figure 6D). Incoming MIF signaling received by T-cell subsets mainly originated from *hModulated SMC* and *hFibromyocyte*, and MIF- (CD74+CXCR4) and MIF- (CD74+CD44) ligand-receptor pairs were more abundant across T-cell subsets in AAA than those in normal AA (Supplementary Figure S7C). Moreover, SPP1 signaling received by these three T-cell subsets was specifically enhanced in the AAA group; further analysis indicated the SPP1 secreted by *hTREM2* M ϕ significantly potentiated. SPP1-CD44 ligand-receptor pairs participated in the interaction of *hTREM2* M ϕ and T-cell subsets (Figure 6D; Supplementary Figure S7D). Different from AAA, SPP1 and MIF signaling also enriched in the normal TA group; thus, both TAA and normal TA groups shared SPP1 and MIF incoming and outgoing in *hCD8 CRTAM*, *hCD8 RUNX3*, and *hCD4 ICOS* T-cell subsets (Figure 6D, bottom). However, MIF- (CD74+CXCR4) and MIF- (CD74+CD44) ligand-receptor pairs sent from *hModulated SMC* and *hFibromyocyte*, received by T-cell subsets showed increased expression in TAA compared with normal TA (Supplementary Figure S7C). SPP1-CD44 ligand-receptor pairs, originated from *hTREM2* M ϕ , received by T-cell subsets also displayed enhanced expression in TAA compared with normal TA (Supplementary Figure S7D).

In all, SPP1 and MIF signaling between macrophage and SMC subsets might exert an essential role during aneurysm progress among different species. Furthermore, although the alteration of SPP1 and MIF signaling pathways in T-cell subsets was distinct between TAA and AAA, the common increase of ligand-receptor pairs containing MIF-(CD74+CXCR4), MIF- (CD74+CD44), and SPP1-CD44 was observed across segments.

Discussion

In this study, we compared aneurysmal aortic cell cluster structures between humans and mice by scRNA-seq, without biasing the notions of pre-defined marker genes and cell states. In doing so, our group depicted the aortic aneurysm transcriptional profiles in each organism, displaying both known and unappreciated aortic aneurysm disease-associated biological functions and marker genes. In discussing this work, we begin with our findings regarding aortic aneurysm homogeneity and dissimilarity across species and segments, conservation of SMC and macrophage subsets between organisms, and distinct marker genes for TAA and AAA. Both SMCs and macrophages contained conserved subpopulations across species. We additionally uncovered different biological functions and

distinct causative genes for TAA and AAA. Moreover, high diversity of T cells in human aortic aneurysms was described. MIF and SPP1 signaling networks participated in aortic aneurysm in both organisms. Thus, we provide basic information for understanding the mechanisms of cellular compositions and gene profiles for both TAA and AAA.

SMCs are the main cell components of the aorta wall, and their loss by necroptosis or apoptosis is a common decisive feature of both TAA and AAA (Quintana and Taylor, 2019). Consistently, our data demonstrated that the fraction of SMCs was remarkably reduced both in TAA and AAA among different species. Similar to AAA, TAA is characterized by ECM abnormalities that disrupt the structural integrity of the aorta. Accumulative studies have shown that genetic variants in proteins (COL3A1, FIBRILLIN-1, and so on) directly affect the mechanical properties of the aorta, ultimately leading to TAA (Malfait, 2018), reflecting that adventitial fibroblasts, as the main producer of ECM, might be related to the pathogenesis of aneurysm. In Ang II-induced mouse aortic aneurysm, we observed significant fibroblast expansion in pathological conditions. However, the proportion of fibroblasts was reduced in human AAA, which might be caused by the low number of AAA samples and the small cell counts. As for immune cells, a hallmark of abdominal aortic aneurysm formation is a strong inflammatory response, including almost all classical inflammatory cell components, as well as resident inflammation in the arterial wall, e.g., neutrophils, macrophages, and T-cell infiltration (Eliaison et al., 2005; Rateri et al., 2011; Ait-Oufella et al., 2013). T cells and macrophages existed in the media of human aneurysmal thoracic aorta, although there is less data supporting the function for inflammatory cells in TAA (He et al., 2008). Different from human samples, the number of T cells in mouse samples is very low, although the disease group is more numerous than the normal group. Our findings, to some extent, illustrate the similarity between thoracic and abdominal aortic aneurysms in terms of inflammatory infiltration, as well as the inadequacy of the Ang II-induced aneurysm model in studying T cells.

Although the single-cell map of SMCs and macrophages in aortic aneurysm has been reported (Zilionis et al., 2019; Davis et al., 2021; Liu et al., 2022; Yu et al., 2022), we reveal an innovative conservation of gene expression profiles between mice and humans. First, we see coherence of SMC subpopulations. The *hFibromyocyte* and *mSMC3* highly express innovative marker genes well conserved across species, including *CTHRC1*, *COL8A1*, and *LGALS3*, which involves in phenotypic switching into synthetic SMC. The high similarities of gene expression profiles between *hContractile SMC* and *mSMC1*, *hStressed* and *mSMC2*, and *hModulated SMC* and *mSMC4* are observed. Second, macrophages show striking coherence between organisms. Unsupervised clustering identifies five macrophage subsets *hActivated M ϕ* , *hResident M ϕ* , *hTREM2 M ϕ* , *hInflammatory M ϕ* , and *hC1QA M ϕ* in humans and four subsets in mice, and we, indeed, observe

four subpopulations showing a one-to-one correspondence between mice and humans. Both *mTrem2* M ϕ and *hTREM2* M ϕ are characterized by high expression of *SPP1* and *TREM2*, respectively, and enrich for macrophage migration. Furthermore, in TAA and AAA, we identify distinct marker genes as potential regulators participating in the pathological process in both organisms. For example, both in *hFibromyocyte* and *mSMC3*, we identify potential causative genes for AAA (*CXCL12*, *MFAP5*, and *EMP1*), among which the blockade of *CXCL12/CXCR4* protects against AAA formation (Michineau et al., 2014), and distinct pathogenic genes for TAA [collagen genes (*COL1A1*, *COL1A2*, *COL3A1*, and *COL5A2*), some reported virulence genes for TAA (*LOX*, *COL3A1*, and *MMP2*) (Longo et al., 2002; Shen et al., 2015; Pinard et al., 2019; Chen et al., 2022), and other unreported genes (*CTHRC1*, *SERPINH1*, *SPARC*, *THY1*, and *CTSK*)]. Among the genes we enriched, the presence of these proven disease-causing genes to some extent supported the validity of our analysis, and the role of these unreported genes deserved further investigation. Generally, the emergence of the congruent expression patterns in two different species encourages us to establish a treatment approach for the aneurysm biological relation between the laboratory and clinics.

A limited number of reports suggest T-cell depletion attenuates AAA formation (Xiong et al., 2004). Regulatory T cells protect against AAA formation by secreting inflammatory cytokines IL-10 and TGF- β (Wang et al., 2010; Yodoi et al., 2015; Zhou et al., 2015). Conflicting reports suggest that CD4⁺ T-cell-derived IFN- γ exerts the function on inducing AAA and preventing from AAA formation, and the exact function of CD8⁺ T cell in AAA remains elusive (Li et al., 2018). Patients treated with doxycycline for 2 weeks prior to open AAA surgery experienced a 95% decrease in cytotoxic T-cell counts on AAA biopsies compared to the control group (Lindeman et al., 2009). Furthermore, little is known about the function of T cells on TAA. Here, we defined T-cell subsets by the unique expression genes. CD4⁺ T cells and CD8⁺ T cells showed distinct transition trajectories and subsets and displayed different immune and transcriptional states in thoracic and abdominal aortic aneurysm samples. CD8 CTRAM and CD8 RUNX3 might have exerted opposite functions during the process of thoracic and abdominal aortic aneurysms because of the opposite trend on fraction alterations. In addition, except for shared biological functions, T-cell subsets in TAA and AAA exhibited distinct functions, for example, CD4 ICOS in the TAA group uniquely enriched for cellular response to INF- γ compared with the AAA group. Therefore, the findings could shed light on using recognized sub-populations as potential innovative therapeutic targets and investigating them in mice.

We note three limitations to this study. First of all, profiling more AAA and normal AA patients could be more convincing. We could only collect two normal AA and four AAA scRNA-seq datasets. The heterogeneity of human samples and the small number of cells may lead to the deviation of the results of cell

composition analysis. Second, unbiased comparison analysis of T-cell subsets between mice and humans are not performed due to the very small absolute number of mouse T cells. Third, in comparison with humans, one mouse model system of Ang II induction is used, and other mouse aneurysm models were not compared. Although there is a considerable degree of commonality between species, the utilization of animal models to study the pathological mechanisms of human aneurysm disease is still inadequate. Therefore, the unbiased comparison method developed here between different species can be applied to other mouse models, which helps select and/or prove animal models associated with human aortic aneurysm pathogenesis.

Despite these technical challenges and limitations, our study yielded some innovative and interesting findings. We revealed similarities and differences of changes in the components of human and mouse cell types, and our unbiased comparison between mice and humans identified well-conserved subpopulations of SMCs and macrophages. Furthermore, the results of our comparative analyses suggested different biological functions and distinct marker genes for TAA and AAA. These discoveries expand our understanding of thoracic and abdominal aortic aneurysm pathogenesis in both organisms and may contribute to the development of novel treatments.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material. To review GEO accession GSE221789: Go to <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE221789> Enter token ihgbyaaynjgtmp into the box.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committees of the First Affiliated Hospital of Zhejiang University School of Medicine. The patients/participants provided their written informed consent to participate in this study. All animal procedures conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institute of Health (8th edition, 2011) and were approved by the Institutional Animal Care and Use Committee of the Zhejiang University School of Medicine.

Author contributions

HW, QX, and HZ conceived and designed the study. HW, CX, and JC analyzed the data and drafted the manuscript. QX and JC acquired the financial support. All authors agreed to

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Activation of the sirtuin silent information regulator 1 pathway inhibits pathological myocardial remodeling

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Myocardial remodeling refers to structural and functional disorders of the heart caused by molecular biological changes in the cardiac myocytes in response to neurological and humoral factors. A variety of heart diseases, such as hypertension, coronary artery disease, arrhythmia, and valvular heart disease, can cause myocardial remodeling and eventually lead to heart failure. Therefore, counteracting myocardial remodeling is essential for the prevention and treatment of heart failure. Sirt1 is a nicotinamide adenine dinucleotide⁺-dependent deacetylase that plays a wide range of roles in transcriptional regulation, energy metabolism regulation, cell survival, DNA repair, inflammation, and circadian regulation. It positively or negatively regulates myocardial remodeling by participating in oxidative stress, apoptosis, autophagy, inflammation, and other processes. Taking into account the close relationship between myocardial remodeling and heart failure and the involvement of SIRT1 in the development of the former, the role of SIRT1 in the prevention of heart failure via inhibition of myocardial remodeling has received considerable attention. Recently, multiple studies have been conducted to provide a better understanding of how SIRT1 regulates these phenomena. This review presents the progress of research involving SIRT1 pathway involvement in the pathophysiological mechanisms of myocardial remodeling and heart failure.

KEYWORDS

sirtuins, oxidative stress, myocardial remodeling, NLRP3 inflammasome, autophagy

1 Introduction

Cardiovascular diseases (CVDs) are the leading cause of death globally. According to the World Health Report, an estimated 17.9 million people died from CVDs in 2019, representing 32% of all global deaths. Out of the 17 million premature deaths (under the age of 70) due to non-communicable diseases in 2019, 38% were caused by CVDs. Heart failure is a type of CVD. Almost all types of heart failure are associated with myocardial remodeling. Myocardial remodeling is a compensatory process caused primarily by obesity, hypertension, heart valve disease, and cardiovascular disease. In the early phase, myocardial remodeling is characterized by the thickening of the ventricular wall and improvement in myocardial systolic function. However, in the long-term, myocardial remodeling is accompanied by interstitial fibrosis, systolic dysfunction, and abnormalities in gene expression, protein expression, energy metabolism, and electrophysiological characteristics, eventually leading to decompensated heart failure (Frey et al., 2004). The mechanisms of myocardial remodeling have not been fully elucidated and are mainly related to the activation of various cellular signaling pathways. The

most well-known mechanisms of myocardial remodeling are related to the activation of the renin-angiotensin-aldosterone system, sympathetic stimulation, apoptosis, inflammation, oxidative stress, myocardial fibrosis, etc (Sciarretta et al., 2018). Sirtuin silent information regulator 1 (SIRT1) is a nicotinamide adenine dinucleotide (NAD⁺)-dependent histone deacetylase (HDAC) that plays an important role in biological processes, such as inflammation, apoptosis, and oxidative stress response (Hajializadeh and Khaksari, 2022). This article reviews the role and molecular mechanisms of Sirt1 protein in myocardial remodeling and outlines the specific mechanisms by which Sirt1 improves different types of myocardial remodeling and heart failure.

2 Sirtuins and cardiovascular regulation

Sirtuins are highly conserved class III histone deacetylases. Seven sirtuin-encoding genes (*SIRT1-7*) have been identified and characterized in mammals. They are localized in different cellular sites and play different roles: regulation of metabolism, oxidative stress, apoptosis, inflammation, and senescence. Sirtuins perform their functions by deacetylating target

proteins at different sites. They play an important role in cardiovascular biology and may modulate cardiovascular health and age-dependent cardiovascular diseases (Cencioni et al., 2015), different members of the family playing different roles (Table 1). Recently, it has become clear that SIRT1 deacetylates histone and non-histone proteins to participate in multiple cellular process, including apoptosis, autophagy, calorie restriction, energy metabolism, transcriptional regulation, cell survival, DNA repair, inflammation, and circadian regulation (Luo et al., 2019). SIRT1 is not only an important regulatory mechanism involved in the pathologic occurrence of myocardial remodeling, but also involves many therapeutic targets for improving myocardial remodeling with drugs. Based on SIRT1, it has a broad prospect in revealing the pathological mechanism of myocardial remodeling and developing new clinical drugs.

3 SIRT1-mediated apoptosis in pathological myocardial remodeling

Apoptosis is a distinct type of cell death characterized by a series of typical morphological events such as cell shrinkage, fragmentation

TABLE 1 Type, location, and function of various sirtuins.

Sirtuins	Location	Model	Function
SIRT1	Cytoplasm, nucleus	Hypoxic mouse model	Promotes autophagy, inhibits apoptosis Luo et al. (2019)
		Ischemia/reperfusion injury	Inhibits apoptosis, reduces oxidative stress Zhang et al. (2018)
		Doxorubicin-induced cardiotoxicity	Reduces oxidative stress, inhibits apoptosis, improves the ejection function Hu et al. (2020)
		Myocardial hypertrophy	Inhibits inflammation and aging Zhang et al. (2021b)
SIRT2	Cytoplasm, nucleus	Myocardial hypertrophy	Reduces oxidative stress, inhibits apoptosis Ren et al. (2021)
		Dilated cardiomyopathy	Reduces oxidative stress Zhao et al. (2018a), Zhao et al. (2018b)
		Myocardial hypertrophy	Inhibits fibrosis, improves the ejection function Tang et al. (2017)
SIRT3	Mitochondria	Dilated cardiomyopathy	Inhibits inflammation Sun et al. (2022a)
		Sepsis-induced myocardial injury	Improves mitochondrial biogenesis Xin and Lu (2020)
SIRT4	Mitochondria	Myocardial hypertrophy	Reduces oxidative stress, improves endothelial dysfunction Dikalova et al. (2020)
		Ischemia/reperfusion injury	Inhibits apoptosis, improves mitochondrial biogenesis Zeng et al. (2018)
		Sirt 4 ^{-/-} rats	Reduces oxidative stress, promotes cardiac hypertrophy, promotes pulmonary fibrosis Luo et al. (2017)
SIRT5	Mitochondria	Doxorubicin-induced cardiotoxicity	Reduces apoptosis and autophagy He et al. (2022b)
		Heart failure	Inhibits inflammation, reduces oxidative stress, improves mitochondrial dysfunction Chang et al. (2021)
SIRT6	Nucleus	Hyperlipidemia	Inhibits inflammation, reduces atherosclerosis Grootaert et al. (2021)
		Diabetic cardiomyopathy	Increases autophagy, inhibits mitochondrial dysfunction Yu et al. (2021)
		Myocardial infarction	
		Doxorubicin-induced cardiotoxicity	Reduces oxidative stress, reduces apoptosis Wu et al. (2021)
		Aortic constriction-induced cardiopathy	Inhibits inflammation, reduces fibrosis, regulates telomere shortening Li et al. (2017)
SIRT7	Nucleus	Aortic constriction-induced cardiopathy	Reduces myocardial fibrosis, improves cardiac hypertrophy Yamamura et al. (2020)
		SIRT7 ^{-/-}	Increases lifespan, inhibits inflammation Vakhrusheva et al. (2008)
			Promotes endothelial formation, increases smooth muscle proliferation Kimura et al. (2021)
		Hypoxia/reoxygenation injury <i>in vitro</i>	Reduces apoptosis Sun et al. (2018)

into membrane-bound apoptotic vesicles, and rapid phagocytosis of neighboring cells without inducing an inflammatory response (Kerr et al., 1972). Apoptosis is thought to be the main cause of cell death within the first few hours after acute myocardial infarction (Palojoki et al., 2001). Sirt1 activates or inactivates apoptosis-associated proteins through deacetylation, thereby inhibiting apoptosis to ameliorate myocardial remodeling and delay the progression of heart failure. The activation of Sirt1 signaling pathway mediates apoptosis by various target signal.

3.1 Regulation of FoxO transcription factor

The transcription factor FoxO belongs to the forkhead protein O family, being one of the important transcription factors in the human body. It regulates the expression of genes that modulate glucose and lipid metabolism, oxidative stress, apoptosis, autophagy, and endoplasmic reticulum stress (Xing et al., 2018). It plays a key role in the development of cardiac remodeling (Zhang et al., 2021a). The effect of SIRT1 on FoxO function is complex and depends on FOXO target genes (Karbasforooshan and Karimi, 2017). Treatment with Ang II increased the acetylation of FoxO1 *in vivo* and *in vitro*, and subsequently the pro-apoptotic protein Bim was upregulated. Sirt1 overexpression deacetylated FoxO1, inhibiting Ang II-induced FoxO1 acetylation and reducing the level of the pro-apoptotic protein Bim. However, these beneficial effects were not observed after SIRT1 knockdown (Li et al., 2019b). Diabetic cardiomyopathy (DCM) is one of the main causes of myocardial remodeling. Notably, SIRT1-FoxO1 pathway was significantly inhibited in DCM mice, accompanied by increased myocardial apoptosis. So, Inhibition of SIRT1-FoxO1 pathway may be an important mechanism mediating diabetic induced cardiomyopathy. To prevent ambiguity, we have changed the description. Curcumin, a natural polyphenol isolated from turmeric root, has antioxidant, anti-inflammatory, anti-apoptotic, and anti-cancer effects. Curcumin partially restores SIRT1-FoxO1 pathway activity and inhibits apoptosis both *in vitro* and *in vivo*. Furthermore, the effect of curcumin on improving apoptosis disappeared after using EX527, a Sirt1 inhibitor (Ren et al., 2020). In addition, Sirt1 reduces p53 expression and apoptosis by targeting the FoxO3 transcription factor, and it deacetylates the DNA repair protein Ku70, which binds to and inactivates the pro-apoptotic factor Bax (Vahtola et al., 2008).

3.2 Regulation of the tumor suppressor gene p53

Overexpression of protein p53 owing to mutations of the tumor suppressor gene p53 leads to rapid loss of cell viability, which is characteristic of apoptosis. Increased levels of tumor suppressor p53 are associated with left ventricular hypertrophy and remodeling (Veeroju et al., 2020). MicroRNAs (miRNAs) are highly conserved, non-coding, single-stranded RNAs in eukaryotes. They can specifically recognize and complementarily bind to target mRNAs, leading to the post-transcriptional degradation of target genes (Henning, 2021). Downregulation of miR-128 in mice with heart failure significantly improved myocardial remodeling and counteracted Ang II-induced apoptosis, by targeting the SIRT1/p53 signaling pathway. Conversely, treatment with EX527 abolished the beneficial effect of miR-128 downregulation

(Zhan et al., 2021). Taurine, a free intracellular β -amino acid, has cardioprotective effects. In mice with aortic constriction (TAC)-induced cardiomyocyte hypertrophy, compared to control, taurine significantly decreased the levels of acetylated (at Lys382) p53/p53. Taurine increased the NAD⁺/NADH ratio, promoted SIRT1 expression, inactivated p53 deacetylation, and inhibited cardiomyocyte apoptosis. Furthermore, EX527 mitigated the beneficial effects of taurine on cardiac function, levels of natriuretic peptide, and apoptosis (Liu et al., 2020). Anthracycline chemotherapeutic agents, such as doxorubicin (DOX), cause cardiotoxicity. DOX treatment leads to apoptosis through p53 activation. Thus, by regulating the levels of p53 expression, SIRT1 activation significantly reduces DOX-induced fibrosis, hypertrophy, and apoptosis in H9c2 cardiomyocytes (Lohanathan et al., 2022). CTRP3 preserves DOX-induced cardiac dysfunction and alleviates DOX-induced cardiac inflammation and apoptosis by activating SIRT1. So, sirt1 is an important therapeutic target for correcting DOX induced cardiac remodeling (Yuan et al., 2018).

3.3 Upregulation of SIRT1 expression via adenosine 5'-monophosphate-activated protein kinase (AMPK) modulates apoptosis

AMPK is considered cellular fuel. Cellular energy is a key regulator of the AMPK system (Carling, 2017). The cellular energy imbalance that occurs during stress leads to an increase in the intracellular AMP/ATP ratio, resulting in AMPK activation. This helps cells survive without oxygen (Horman et al., 2012). In myocardial tissue, under pathological conditions, such as hyperglycemia, hypoxia, and pressure overload, AMPK activation is significantly downregulated (Li et al., 2019a; Zhang et al., 2020). In diabetic rats, treatment with metformin and atorvastatin reduced the levels of expression of caspase-3 and increased the Bcl-2/Bax ratio. Furthermore, in diabetic rats, the levels of expression of p-AMPK and Sirt1 were downregulated, and this was reversed by the combination treatment, which subsequently increased NAD⁺ levels and reduced apoptosis in cardiomyocytes (Jia et al., 2021; Laddha and Kulkarni, 2021). It's worth noting that AMPK is a trimeric serine/threonine protein kinase comprising a catalytic α subunit and non-catalytic β and γ subunits, which are encoded by seven different high-homologous genes (α 1, α 2, β 1, β 2, γ 1, γ 2, γ 3). However, in the existing literature, the differences in the regulation of SIRT1 by AMPK coding from different high-homologous genes have not been clarified.

4 SIRT1 regulates oxidative stress in pathological myocardial remodeling

An imbalance between the production of ROS and the ability of the body to detoxify reactive intermediates results in oxidative stress. Prolonged pathological stimulation, release of inflammatory cytokines, and activation of mitogen-activated protein kinases lead to the generation of high levels of oxygen free radicals. Detoxification of ROS is ensured by the activity of antioxidant enzymes such as Mn-SOD, catalase, glutathione reductase, and peroxidase (Rababa'h et al., 2018). Oxidative stress is a major stimulus for signal transduction in cardiac myocytes. It causes abnormal Ca²⁺ metabolism associated with subcellular remodeling, defective energy production, inflammation, apoptosis, fibrosis, and cardiomyocyte loss, all of which are thought to

contribute to myocardial remodeling and heart failure (Shah et al., 2021). Sirt1 suppresses oxidative stress, preventing pathological myocardial remodeling by.

4.1 Regulation of FoxO1 transcription factors

In an angiotensin-induced hypertension model, SIRT1 overexpression attenuated Ang II-induced ROS formation. Sirt1-mediated deacetylation of FoxO1 directly controlled the expression of catalase and MnSOD, thereby promoting the breakdown of ROS and suppressing oxidative stress (Li et al., 2019b). In H9C2 cells, Ang II increased the concentration of malondialdehyde and decreased SOD activity, thus inducing oxidative stress. Furthermore, it reduced the levels of expression of SIRT1 and FoxO1. Conversely, addition of a SIRT1 agonist attenuated the Ang II-induced oxidative stress index (Jiang et al., 2021). Fibroblast growth factor 20 (FGF20) is a member of the fibroblast growth factor family and is involved in apoptosis, senescence, inflammation, and autophagy. FGF20 upregulates SIRT1 expression, leading to FOXO1 deacetylation, which promotes the transcription of downstream antioxidant genes, thereby suppressing oxidative stress. It has anti-hypertrophic effect which is greatly counteracted in SIRT1 knockout mice. Furthermore, these mice also presented an increase in oxidative stress (Chen et al., 2022b).

4.2 Regulation of NF- κ B

NF- κ B, a classical signaling pathway, is involved in the development of myocardial remodeling, and inhibition of its phosphorylation can help treat myocardial hypertrophy (Yu et al., 2013). A major component of ginseng, ginsenoside Rg3, has anti-aging effects [27] and ameliorates Ang-II-induced myocardial remodeling. The underlying mechanism consists of the regulation of the Sirt1/NF- κ B pathway leading to downregulation of the expression of superoxide dismutase, malondialdehyde, heme oxygenase-1 (HO-1), and nuclear factor (erythroid-derived 2)-like2 (Nrf2). This reduction of oxidative stress ameliorates myocardial remodeling, and the phenomenon is counteracted by the administration of Sirt1 inhibitor AGK2 (Ren et al., 2021).

4.3 Regulation of transforming growth factor beta (TGF- β)

In an animal model of DCM, Sirt1 decreased the activity of TGF- β and prevented myocardial remodeling by inhibiting p300. Sirt1 deacetylates p65 subunit of NF- κ B, leading to reduced binding of NF- κ B-P65 to DNA. Subsequently, this reduces cardiac hypertrophy and oxidative stress by diminishing the transcription of subunits of NADPH oxidase (NOX1 and NOX2) (Karbasforooshan and Karimi, 2017). Epithelial mesenchymal transition (EndMT) is closely associated with pathogenesis of myocardial remodeling. EndMT is strongly induced by TGF- β . SIRT1 pathway inhibits EndMT by inhibiting the TGF β /Smad pathway, thereby reducing cardiac fibrosis and reversing myocardial remodeling (Liu et al., 2019). Thus, ellagic acid, a phytochemical found mainly in nuts

and some fruits (e.g., raspberries, grapes, and pomegranates), activated SIRT1 and inhibited TGF- β , suppressing oxidative stress and inhibiting myocardial remodeling caused by DCM (Altamimi et al., 2020). TGF- β promotes the development of fibrotic disease by enhancing collagen expression and inducing cell differentiation into myofibroblasts. And SIRT1 binding to Smad2/3 inhibited Smad2/3 nuclear translocation, thereby regulating myocardial remodeling via the TGF- β /Smad2/3 pathway in cardiac fibroblasts (Liu et al., 2019).

4.4 Regulation of the expression of peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1)

Mitochondrial biogenesis is a central player in the pathophysiology of many cardiovascular diseases, including myocardial remodeling (Forte et al., 2021). Oxidative stress is closely associated with mitochondrial biogenesis. One major regulator of this phenomenon is PGC-1 α which is activated by Sirt1 through deacetylation. Subsequently, mitochondrial function improves and ROS production is reduced (Waldman et al., 2018). Neuraminidase-1 (NEU1) is involved in the response to multiple signals and regulates a variety of cellular metabolic processes. Furthermore, it is closely associated with the onset and progression of cardiovascular disease. NEU1 knockdown attenuates cardiomyocyte injury by regulating the SIRT1/PGC-1 α signaling pathway, thereby promoting mitochondrial biogenesis and function. Canagliflozin is used to treat type 2 diabetes and improves myocardial remodeling, and therefore being recommended in heart failure guidelines. This effect is mediated by the activation of the AMPK/SIRT1/PGC-1 α signaling pathway, upregulating PGC-1 α expression and reducing cardiac hypertrophy, fibrosis, and oxidative stress (He et al., 2022a).

Additionally, the levels of expression of SIRT1 are modulated by AMPK, which thus, is implicated in the control of ROS levels. During the development of cardiac remodeling, energy deficits can exacerbate cardiac insufficiency. Elevated expression of antioxidants (SOD1, catalase, and MnSOD) and reduced mitochondrial ROS production mitigates myocardial remodeling. AMPK is an upstream regulator of Sirt1. As mentioned before, in reality, AMPK and Sirt1 regulate each other and share many common target molecules; AMPK increases NAD⁺ levels and activates Sirt1 (Wang et al., 2020b). The expression levels of p-AMPK and SIRT1 were reduced in diabetic mice and in H9C2 cells exposed to high concentrations of glucose. Combined treatment with metformin and atorvastatin activated the AMPK/SIRT1 signaling pathway, thereby attenuating cardiomyocyte fibrosis, hypertrophy, and oxidative stress (Jia et al., 2021). Aldehyde dehydrogenase 2 (ALDH2) is an essential mitochondrial enzyme that controls cardiac function. It exacerbates aging-induced cardiac hypertrophy, oxidative stress, and mitochondrial damage. AMPK/SIRT1 activation (resveratrol and SRT1720) prevented ALDH2-induced contractile dysfunction in cardiomyocytes. AMPK enhances SIRT1 activity by increasing cellular NAD⁺ levels, leading to deacetylation and regulation of the activity of downstream targets of SIRT1, including PGC1 α , thereby reducing oxidative stress in cardiomyocytes (Zhang et al., 2014).

In summary, the main molecular mechanism of SIRT1-mediated regulation of oxidative stress is the modulation of the levels of expression of catalase and MnSOD through SIRT1-mediated

FoxO1 deacetylation, which leads to the inhibition of the production of ROS and suppression of oxidative stress. PGC-1 α and NF- κ B are activated by SIRT1 through deacetylation, thereby improving mitochondrial function and counteracting the pro-oxidant effect of cell stress, thereby reversing cardiomyocyte remodeling.

5 SIRT1 signaling mediates inflammatory responses in pathological myocardial remodeling

Inflammatory cytokines play an important role in the pathophysiology of adverse myocardial remodeling and are significantly elevated in both heart failure and adverse myocardial remodeling (Hanna and Frangogiannis, 2020). The early inflammatory response after myocardial infarction may increase myocardial fibrosis and remodeling (Gao et al., 2019). The prevalence of DCM in diabetes is approximately 17%, and patients with type 1 and type 2 diabetes have a significantly increased risk of heart failure. DCM progresses in part through inflammation, leading to structural changes in the diabetic heart (Elmadbough and Singla, 2021). SIRT1 signaling mediates inflammatory responses in pathological myocardial remodeling by various mechanisms, the main one being regulation of transcriptional co-activator PGC-1 α . Obese mice show significant cardiac hypertrophy, inflammatory cell infiltration, reduced SIRT1 activity, altered mitochondrial signaling and oxidative homeostasis, and overexpression of inflammatory markers. Melatonin prevents cardiac remodeling caused by obesity by activating SIRT1, which regulates cellular metabolic signaling by acetylating and activating the coactivator PGC-1 α . This induces mitochondrial transcription factors, thereby enhancing mitochondrial content and cellular metabolic oxidative capacity, reducing oxidative stress and inflammation. Nrf2, a transcription factor regulated by PGC-1 α , is significantly reduced in the hearts of obese mice, leading to a significant decrease in the levels of HO-1 expression and an increase in lipid peroxidation and the levels of the pro-inflammatory markers NLRP3, tumor necrosis factor- α , and IL-6. The SIRT1/PGC-1 α /Nrf2/HO-1 pathway is a key for preventing adverse obesogenic myocardial remodeling (Favero et al., 2020). Tongguan capsule dramatically decreased the expressions of TNF- α , IL-1 β , and IL-6 Post-myocardial Infarction Remodeling through Sirt1 Activation. And the induction of Sirt1 by TGC was inhibited by the specific inhibitor EX527. In the presence of EX527, TGC-induced autophagy-specific proteins were down-regulated, while inflammatory factors were upregulated (Mao et al., 2018). And in streptozotocin-induced diabetic mouse models, phloetin exerts anti-inflammatory effects by docking with SIRT1, thereby protecting against cardiac injury and remodeling (Ying et al., 2019). Therefore, SIRT1-mediated inflammatory response is also an important therapeutic target for myocardial remodeling.

6 SIRT1 signaling regulates cellular autophagy in pathological myocardial remodeling

Besides necrosis and apoptosis, autophagy is another type of cellular death (Glick et al., 2010). Autophagy involves the formation of autophagic vesicles, which encapsulate degraded or long-lived proteins and organelles and then fuse with lysosomes. Autophagy plays multiple roles in myocardial hypertrophy. In hypertrophic cardiomyocytes, autophagy can ensure the degradation of excess harmful substances, reduce cytotoxic damage caused

by misfolded protein aggregation, mitigate oxidative stress, and maintain cell survival. Because cardiomyocytes are terminally differentiated cells, their survival is overly dependent on autophagy to self-clean abnormal substances; therefore, effective autophagy is essential for the stability of the cardiovascular internal environment (Bravo-San Pedro et al., 2017). Increasing evidence suggests that dysregulation of cardiomyocyte autophagy is associated with the progression of myocardial remodeling (Shirakabe et al., 2016). Excessive autophagy can exacerbate mitochondrial damage and impair energy metabolism by non-selectively degrading normal mitochondria and mitochondria-associated proteins, thereby resulting in energy disorders. In summary, basal levels of autophagy are essential for ensuring a proper functioning of cardiomyocytes and dysregulated autophagy can lead to cardiomyocyte hypertrophy (Zheng et al., 2021). SIRT1 signaling regulates cellular autophagy in pathological myocardial remodeling by various mechanisms.

6.1 Interaction with AMPK

Among drugs that prevent myocardial remodeling, angiotensin-converting enzyme inhibitors (ACEIs) are commonly used in clinical practice. Furthermore, they promote autophagy in cardiomyocytes (Wu et al., 2013) owing to the activation of the AMPK pathway (Hernández et al., 2014). Metformin is an activator of AMPK and is used clinically for the treatment of diabetes. It can reduce the serious complications of diabetes, including myocardial remodeling and heart failure by upregulating SIRT1 and AMPK and subsequently, promoting autophagy (Xie et al., 2011). Sodium-glucose cotransporter (SGLT2) is not expressed in the heart but SGLT2 inhibitors are recommended by the latest treatment guidelines in patients with heart failure, with or without diabetes mellitus. SGLT2 inhibitors may directly bind to SIRT1, thus inhibiting autophagy (Osataphan et al., 2019).

6.2 Regulation of FoxO1 transcription factor

Treatment of rat cardiomyocyte cell lines with Ang II results in insufficient cardiomyocyte autophagy and interferes with the expression of the autophagy-associated proteins beclin1 and p62. Ginkgolide B Protects Cardiomyocytes from Angiotensin II-Induced Hypertrophy via Regulation of Autophagy through SIRT1-FoxO1 (Jiang et al., 2021). Another study showed that SIRT1-dependent deacetylation of the transcription factor Foxo1 is involved in cardiac senescence: SIRT1 activates FoxO1, promoting its nuclear localization, and Akt inhibits it by phosphorylation, preventing nuclear translocation. The inhibition of SIRT1-Foxo1-mediated autophagy in aged mice can be counteracted by the ablation of Akt2, an enzyme that has the opposite effect (Ren et al., 2017).

6.3 Modulation of FGF21 expression

FGF21 is a novel peptide ligand involved in a variety of physiological and pathological processes, including regulation of glucose and lipid metabolism and reduction of atherosclerotic plaque formation in large blood vessels. It also plays a

cardioprotective role in myocardial infarction, cardiac ischemia-reperfusion injury, cardiac hypertrophy, and DCM (Geng et al., 2020). In animal models of DCM, diabetes-induced oxidative damage and inflammation inhibited cardiac autophagy, suggesting that these phenomena contribute to the pathogenesis of diabetic heart disease (Zhang et al., 2016). Fenofibrate (FF), a peroxisome proliferator-activated receptor- α (PPAR α) agonist, is used clinically to treat hypertriglyceridemia. In a mouse model of diabetic cardiomyopathy, pre-treatment with FF partially restored autophagy in diabetic hearts. Furthermore, the cardioprotective effect of FF in type 1 diabetes mellitus is dependent on FGF21. The upregulation of cardiac FGF21 expression may increase SIRT1-mediated autophagy, which plays a key role in preventing diabetes-induced cardiac inflammation, oxidative stress, fibrosis, and dysfunction (Zhang et al., 2016). *In vitro* studies using H9C2 cells also showed that exposure to high glucose (HG) significantly increased inflammatory responses, oxidative stress, and pro-fibrotic responses, and significantly inhibited autophagy. The effects of HG were inhibited by treatment with FF. Inhibition of autophagy by 3-methyladenine (3 MA) or SIRT1 by sirtinol (SI) counteracted the beneficial effects of FF. Thus, FF may prevent cardiac pathology and functional abnormalities caused by type 1 diabetes by increasing FGF21 levels, which may upregulate SIRT1-mediated autophagy. Moreover, FGF21 is involved in multiple disease processes through SIRT1-dependent autophagy, including wound healing (Chen et al., 2022a), osteoarthritis (Lu et al., 2021b), and acute liver injury (Yang et al., 2022), which overlaps with the pathological mechanism of myocardial remodeling. So, SIRT1-dependent autophagy mediated by FGF21 may be an important mechanism involved in pathological myocardial remodeling.

In summary, SIRT1-dependent deacetylation of FoxO1 prevents cardiac senescence and enhances cellular autophagy. SIRT1 plays a key role in preventing diabetes-induced cardiac inflammation, oxidative stress, and dysfunction. Ang-II induces cardiac hypertrophy by suppressing SIRT1 expression, whereas ginkgolide B counteracts it by enhancing autophagy through the activation of the SIRT1-FoxO1 pathway.

7 SIRT1 improves mitochondrial dysfunction mitigating myocardial remodeling

The heart is the most metabolically active organ, accounting for approximately 8% of ATP consumption daily (Brown et al., 2017). Impaired mitochondrial function is involved in the development and progression of maladaptive cardiac hypertrophy and heart failure (Tham et al., 2015). In myocardial remodeling and heart failure, mitochondrial production of energy gradually decreases. This leads to increased production of ROS and cytoplasmic release of cytochrome c, which promote programmed cell death, cardiomyocyte injury, and ultimately, heart failure (Elorza and Soffia, 2021). Histidine attenuates pressure overload and phenylephrine (PE)-induced myocardial hypertrophy through upregulation of SIRT1, which prevents mitochondrial dysfunction and oxidative damage in response to hypertrophic stimuli and maintains mitochondrial respiratory function and ATP synthesis. Inhibition of SIRT1 could reverse the protective effect of histidine on myocardial hypertrophy (Wang et al., 2020b). SIRT1 was shown to

regulate mitochondrial energy transduction, ATP synthesis, and biogenesis by upregulating the activity of PPAR α and PGC-1 (Planavila et al., 2011).

ATP deficiency can cause myocardial contractile dysfunction, whereas adenosine monophosphate activated protein kinase (AMPK) activity is regulated by the ADP/ATP ratio. SIRT1 is required for the activity of AMPK (Price et al., 2012). It has been demonstrated that activation of SIRT1 can prevent the decrease in ATP while promoting the transcription of energy metabolism-related genes (Um et al., 2010). Meanwhile, Sirt1 stimulates the ability of PGC-1 α to coactivate hepatocyte nuclear factor 4 α and to inhibit glycolytic genes in response to pyruvate, thereby positively regulating gluconeogenic genes in response to pyruvate in hepatic cells (Rodgers et al., 2005). Additionally, Loss of Sirt1 activity led to dilated cardiomyopathy in adult hearts, which is accompanied by mitochondrial dysfunction (Planavila et al., 2012). However, another study has shown that Sirt1 is upregulated in failing hearts and inhibits the expression of genes associated with mitochondrial function (Oka et al., 2011). Therefore, the effects of Sirt1 on cardiac mitochondrial function and metabolism are also complex and may be dose-dependent or even bidirectional.

8 Inducers and inhibitors of SIRT1

8.1 Inducers of SIRT1

Since SIRT1 and its regulation play an important role in human diseases, there is an increasing interest in discovering small molecules that modulate its activity. Common agonists of SIRT1 are reviewed here. The polyphenol resveratrol (RSV), a natural compound, was the first SIRT1 agonist. Resveratrol exhibits a wide range of physiological and biochemical activities, including antioxidant, anti-inflammatory, antiplatelet, and anticoagulant activities, suggesting that its administration is beneficial for cardiovascular diseases (Bonnefont-Rousselot, 2016). RSV inhibits cell membrane lipid oxidation, protects low-density lipoprotein from oxidation, and increases the concentration of high-density lipoprotein (Berrougui et al., 2009). RSV has antithrombotic effects and inhibits thrombosis by inhibiting prostaglandin and thromboxane synthesis and platelet activity (Snopek et al., 2018). In the cardiovascular system, disturbances in intracellular calcium homeostasis lead to cardiovascular system dysfunction, including cardiac systolic dysfunction, arrhythmias, remodeling, and apoptosis. RSV differentially regulates Ca²⁺ handling by stimulation of NO production or antioxidant activity, maintaining Ca²⁺ homeostasis under normal and pathological conditions (Liu et al., 2017). Other SIRT1 agonists include small molecules that are structurally different from RSV, but hundreds of times more potent, such as SRT1720 and SRT501.

8.2 SIRT1 inhibitors

SIRT1 agonists are considered beneficial in a variety of diseases, as demonstrated in various animal models. However, SIRT1 inhibitors might be beneficial in cancer, Parkinson's disease, and infection with human immunodeficiency virus (HIV) (Pagans et al., 2005; Alcaín and Villalba, 2009). Sirtinol induces senescence-like growth arrest in human breast cancer MCF-7 and lung cancer H1299 cells (Ota

TABLE 2 Inducers and inhibitors of Sirt-1.

Number	Name	Data source	References
Sirt1 inducers			
1	Resveratrol	A review	Bhullar and Hubbard (2015)
2	Salvianolic acid	A rat model of chronic alcoholic liver disease	Zhang et al. (2017b)
3	Quercetin	A rat model of osteoarthritis	Qiu et al. (2018)
4	Fisetin	3T3-L1 cells model	Kim et al. (2015)
5	Panaxtriol saponins	PC12 cells and zebrafish	Zhang et al. (2017a)
6	Ginsenoside Rg3	Aged rats model	Yang et al. (2018)
7	Ginsenoside Rb2	H9C2 cell line	Huang et al. (2019)
8	Ginsenoside Rc	HEK293T cell line	Kim et al. (2014)
9	Ophiopogonin D	H9c2 cell line	Wang et al. (2016)
10	SRT1720	Obese mice model	Minor et al. (2011)
11	Strigolactone analogue GR24	Rat L6 skeletal muscle cell line	Modi et al. (2017)
12	SIRT1460	Obese mice model	Milne et al. (2007)
13	SRT2183	sirt1 ^{-/-} mice model	Gurt et al. (2015)
14	A03	Alzheimer's disease mouse model	Campagna et al. (2018)
15	MHY2233	db/db mice model	Kim et al. (2018)
16	SRT2104	Clinic trial	Lin et al. (2019)
17	SRT3025	Clinic trial	Venkatasubramanian et al. (2013)
Sirt1 inhibitors			
1	Sirtinol	Rats model	Safari et al. (2017)
2	cambinol	RPMI8226 and U266 cells	Lu et al. (2021a)
3	inauhzin	HCT116 and DLD1 cells	Sun et al. (2022b)
4	EX527	U87MG and LN-299 glioma cell lines	Wang et al. (2020a)
5	AGK2	A549 and H1299 non-small cell lung cancer cells	Ma et al. (2018)
6	Suramin	Structure-activity Study	Trapp et al. (2007)
7	Tenovin	HCT116 cells	Ueno et al. (2013)
8	Salermide	BxPC-3 pancreatic cancer cell line	Yar Saglam et al. (2016)

et al., 2006). Sirt1 activation promotes chronic granulocytic leukemia (CML) cell survival, and proliferation is associated with the deacetylation of multiple SIRT1 substrates, including FoxO1, p53, and KU70. Treatment of mice with the SIRT1 inhibitor tenovin-6 prevents cancer progression (Yuan et al., 2012). Table 2 shows both the inducers and inhibitors of SIRT1.

SIRT1 activators have been proposed as a therapeutic strategy for treating and preventing vascular disease. A clinical trial proved that the defective Sirt1 may be correlated to the abnormal IFN γ expression in severe aplastic anemia patients, and activation of Sirt1 signaling by SRT3025 may help improve the inflammatory status of severe aplastic anemia (Lin et al., 2019). And Sowmya found SRT2104, an activator of SIRT1, appears to be safe and well tolerated and associated with an improved lipid profile without demonstrable differences in vascular or platelet function in otherwise healthy cigarette smokers based on a

double-blind trial (Venkatasubramanian et al., 2013). At present, more ongoing clinical trials are underway to investigate the efficacy, pharmacokinetics, and safety of Sirtuin modulator compounds in several diseases (<http://clinicaltrials.gov>). Unfortunately, there are still reports about the use of sirt modulators in clinical trials. So, future research should aim to elucidate the role of Sirt1 completely and to develop pharmacological strategies that can allow its action to be modulated (Table 2).

9 Discussion

(4) Myocardial remodeling is a common pathophysiological process in heart failure, and its amelioration is a cornerstone of chronic heart failure treatment. However, drugs capable of

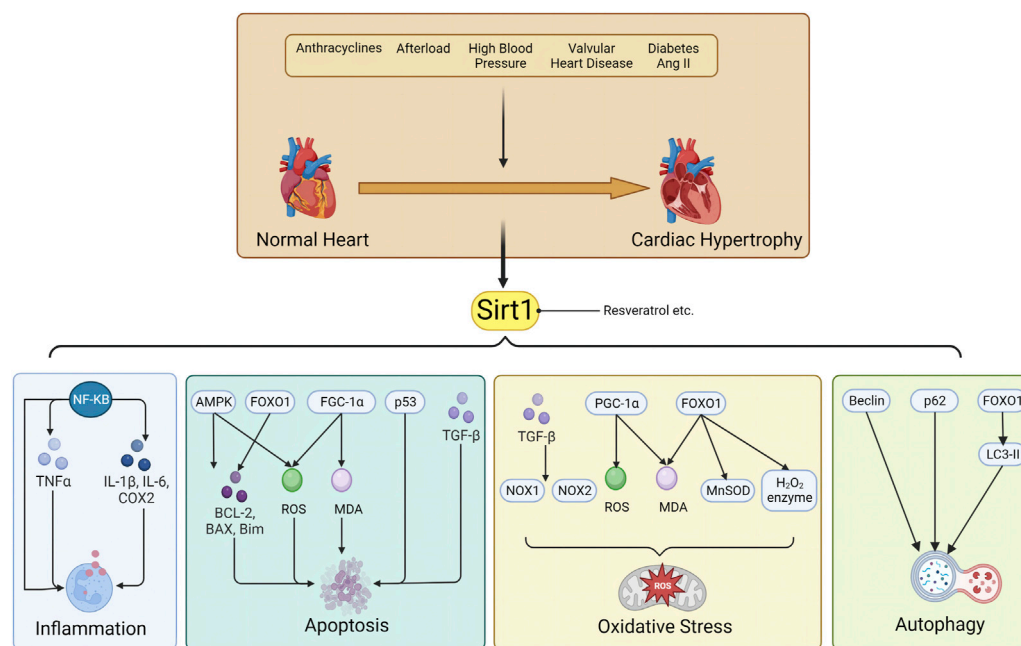


FIGURE 1

Sirt1 is involved in the pathological mechanism of myocardial remodeling through inflammation, apoptosis, oxidative stress and autophagy.

reversing myocardial remodeling are scarce; thus, representing a current clinical unmet need. SIRT1 plays a key role in the pathogenesis of cardiovascular diseases. It mediates oxidative stress, apoptosis, autophagy, inflammation, and mitochondrial dysfunction in cardiomyocytes. Its activation reverses myocardial remodeling and other cardiac diseases, such as coronary atherosclerosis, which accelerates the onset of heart failure and increases heart failure-related morbidity and mortality. We have summarized the role of SIRT1 in cardiac remodeling of various etiologies, and the underlying mechanisms, including 1) SIRT1-mediated apoptosis by FoxO transcription factor, p53 and AMPK pathway; 2) SIRT1 regulates oxidative stress by FoxO1 transcription factors, NF- κ B, TGF- β and PGC-1 pathway. 3) SIRT1 regulates inflammatory responses through acetylation and activation of the coactivator PGC-1 α ; 4) SIRT1 signaling regulates cellular autophagy by interaction with AMPK, regulation FoxO1 transcription factor and modulation of FGF21 activity. The specific molecular pathways involved are shown in Figure 1.

Although the specific mechanisms of myocardial remodeling have not yet been fully elucidated, some critical elements have been identified. These might represent relevant therapeutic targets that are associated with the pathogenesis of myocardial remodeling. Preclinical data indicate that SIRT1 is a promising target - increasing evidence suggests that SIRT1 activation ameliorates or prevents myocardial remodeling, delaying the progression of heart failure.

Author contributions

WY drafted this manuscript by consulting and summarizing relevant literature. The contents of each study were summarized and tabulated. The protective mechanism of myocardial remodeling

was described graphically. Both ZR and WC are involved in this work. LX and HL completed the rework during review. Corresponding authors WL and WX oversaw the development of the entire manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Differences in right ventricular function and response to targeted therapy between patients with IPAH and PAH-CHD

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Background and aims: Pulmonary arterial hypertension (PAH) is a chronic pulmonary vascular disorder characterized by elevated pulmonary vascular resistance (PVR) and pulmonary arterial pressure (PAP). Right heart failure is a life-threatening complication of PAH and predicts a poor prognosis. PAH associated with congenital heart disease (PAH-CHD) and idiopathic PAH (IPAH) are two prevalent PAH subtypes in China. In this section, we set out to explore baseline right ventricular (RV) function and its response to targeted agents between IPAH and PAH-CHD.

Methods and results: Consecutive patients diagnosed with IPAH or PAH-CHD by right heart catheterization (RHC) in the Second Xiangya Hospital from November 2011 to June 2020 were included. All patients received PAH-targeted therapy and the RV function was assessed by echocardiography at baseline and during follow-up. A total of 303 patients (age, 36.23 ± 13.10 years; women, 213 (70.3%); mean PAP [mPAP], 63.54 ± 16.12 mmHg; PVR, 14.74 ± 7.61 WU) with IPAH ($n = 121$) or PAH-CHD ($n = 182$) were included in this study. Compared with PAH-CHD, patients with IPAH had worse baseline RV function. As of the latest follow-up, forty-nine patients with IPAH and six patients with PAH-CHD died. Kaplan-Meier analyses showed better survival in PAH-CHD versus IPAH. After PAH-targeted therapy, patients with IPAH had less improvement in 6 MWD, World Health Organization functional class, and RV functional parameters compared with patients with PAH-CHD.

Conclusion: Compared with patients with PAH-CHD, patients with IPAH had worse baseline RV function, unfavourable prognosis, and inadequate response to targeted treatment.

KEYWORDS

congenital heart disease, pulmonary arterial hypertension, pulmonary hypertension, right ventricle, right ventricular function, targeted therapy, treatment

1 Introduction

Pulmonary arterial hypertension (PAH) is a chronic pulmonary vascular disease characterized by elevated pulmonary vascular resistance (PVR) and pulmonary arterial (PA) pressure (PAP). Currently, the incidence and prevalence of PAH are approximately 5/million and 25/million, respectively (Hoepfer et al., 2016). According to the updated 2022 European Society of Cardiology/European Respiratory Society (ESC/ERC) guideline, PAH is pre-capillary pulmonary hypertension defined as mean PAP (mPAP) > 20 mmHg, PVR > 2 Wood Units and PA wedge pressure

(PAWP) ≤ 15 mmHg as measured by right heart catheterization (RHC) (Humbert et al., 2022). Clinically, PAH is divided into the following subgroups based on etiology, including idiopathic PAH (IPAH), heritable PAH, drug- and toxin-induced PAH, PAH associated with other conditions [such as connective tissue disease (CTD), congenital heart disease (CHD)], PAH with features of venous/capillaries involvement, and persistent PAH of the newborn (Humbert et al., 2022). At present, the pathogenesis of PAH remains unclear, and pulmonary arterial remodeling is an important pathological hallmark. Similar to malignancy, patients with PAH have an extremely poor prognosis (Ruopp and Cockrill, 2022). Previous studies demonstrated that the median survival of treatment-naïve patients with IPAH was only 2.8 years, and the 1- and 5-years survival rates were 68% and 34%, respectively (D'Alonzo et al., 1991). During the past few decades, advances in understanding of the pathogenesis of PAH have led to significant progress in PAH-targeted medications, including phosphodiesterase-5 inhibitors (PDE-5i), endothelin receptor antagonists (ERAs), prostacyclin analogues and prostacyclin receptor agonists (PGI₂), and soluble guanylate cyclase stimulators (sGCs). Notably, although targeted drugs improve the symptoms and haemodynamics, the 5-years survival rate of PAH has only increased to over 60% (Farber et al., 2015), which has not yet been satisfied.

Elevated PVR and PAP increase the right ventricular (RV) afterload and promote adaptive or non-adaptive RV remodeling, which triggers RV dysfunction and right heart failure. Studies have revealed that RV function is closely related to functional class and prognosis in patients with PAH, and deteriorating cardiac function portends a poor prognosis. To our knowledge, there were significant survival differences among different subgroups of PAH. The survival of patients with IPAH was inferior to that of patients with PAH associated with CHD (PAH-CHD) (Manes et al., 2014), but superior to that of patients with CTD-associated PAH (CTD-PAH) (Chung et al., 2010; Chung et al., 2014; Ramjug et al., 2017). In addition, significant survival differences were also observed between different subtypes within the same subgroup. Chung and colleague confirmed that patients with systemic sclerosis (SSc)-associated PAH (SSc-PAH) have a higher mortality rate than those with CTD other than SSc-associated PAH (Chung et al., 2010). In the PAH-CHD cohort, when compared with patients with Eisenmenger syndrome (ES) or PAH-CHD associated with systemic-to-pulmonary shunts (SPs), patients with small defects (SDs) or PAH after defect correction (CDs) had the worse prognosis (Manes et al., 2014). Previous studies have shown that diverse RV function and response to PAH-targeted therapy are associated with differences in prognosis between patients with CTD-PAH and patients with IPAH (Rhee et al., 2015; Ramjug et al., 2017). In China, PAH-CHD and IPAH are two prevalent types of PAH, and their baseline right heart function and response to targeted drugs are less studied. Given that RV dysfunction are closely associated with poor prognosis, we intend to explore baseline RV function and its response to targeted agents between IPAH and PAH-CHD.

2 Patients and methods

2.1 Study designs and subjects

We retrospectively collected and studied adult treatment-naïve patients with IPAH or PAH-CHD who were admitted to the Second

Xiangya Hospital from November 2011 to June 2020. All recruited patients with IPAH or PAH-CHD met the following diagnostic criteria: mPAP ≥ 25 mmHg, PVR > 3 WU and PAWP ≤ 15 mmHg during resting RHC (Galiè et al., 2016). Except for IPAH and PAH-CHD, patients with other types of PAH or PH belonging to groups 2–5 were also excluded. All recruited patients were prescribed PAH-specific therapy based on multiparameter risk stratification of contemporaneous guidelines, and cardiac function and haemodynamics parameters were assessed by means of echocardiography. In the 2022 ESC/ERS guideline for pulmonary hypertension, it was recommended to use a three-strata risk-assessment model to classify patients as low, intermediate, or high risk at initial assessment, and to use a four-strata model to classify patients as low, intermediate-low, intermediate-high, or high risk during follow-up (Humbert et al., 2022). Risk stratification can help guides treatment decisions in patients with PAH. Briefly, initial monotherapy with PDE-5i or ERAs was recommended for patients with PAH and cardiopulmonary comorbidities. In patients with PAH without cardiopulmonary complications, initial dual combination therapy of ERAs and PDE-5i was recommended for patients with low- or intermediate-risk of death, and triple combination therapy of ERAs, PDE-5i, and prostacyclin analogue is recommended for patients with high-risk of death. At follow-up, patients who reach low-risk status continued the initial regimen, and patients with medium-low risk were suggested to add prostacyclin receptor agonist or replace PDE-5i to sGC. In addition, intravenous or subcutaneous prostacyclin or evaluation for lung transplantation was recommended for patients who had insufficient treatment response and were still at intermediate-high or even high risk. PAH-specific medications in this study included PDE-5i (e.g., sildenafil and tadalafil), ERAs (e.g., bosentan, ambrisentan, and macitentan), prostacyclin analogues (e.g., intravenous or subcutaneous treprostinil, intravenous epoprostenol, and inhaled iloprost), prostacyclin receptor agonists (slexipag), and sGCs (e.g., riociguat). The study was approved by the Ethics Committee of the Second Xiangya Hospital.

2.2 Data collections

The following electronic medical record data were collected: age, gender, heart rate (HR), arterial oxygen saturation (SaO₂), 6-min walking distance (6 MWD), World Health Organization functional class (WHO FC), and application of PAH-targeted drugs.

Transthoracic echocardiography (TTE) was performed according to the guidelines (Mitchell et al., 2019), and the following parameters were recorded: tricuspid regurgitation velocity (TRV, m/s), RV systolic pressure (RVSP), PA systolic pressure (PASP), left ventricular ejection fraction (LVEF), tricuspid annular plane systolic excursion (TAPSE), RV fractional area changes (RVFAC), and pericardial effusion. PASP is equal to RVSP in the absence of right ventricular outflow tract and pulmonary artery stenosis. RVSP was estimated according to the simplified Bernoulli's equation, i.e., $4 \times \text{TRV}^2 + \text{right atrial pressure}$. The TAPSE/PASP ratio was used to assess the RV-PA coupling (Tello et al., 2019).

A haemodynamic assessment was performed by RHC *via* the right femoral vein. Pressure in the right atrium, right ventricle,

TABLE 1 Demographic and clinical characteristics at baseline in IPAH and PAH-CHD.

Variables	Total population (n = 303)	IPAH (n = 121)	PAH-CHD (n = 182)	p-value
Female sex, n (%)	213 (70.30)	80 (66.12)	133 (73.08)	0.194
Age, y	36.23 ± 13.10	37.78 ± 13.27	35.20 ± 12.93	0.094
HR, bpm	85.30 ± 13.90	85.64 ± 13.49	85.08 ± 14.21	0.735
SaO ₂ , %	93.92 ± 4.03	95.15 ± 3.01	93.10 ± 4.40	<0.001
NT-ProBNP, ng/mL	1017 (325.36, 2813.56)	2059.62 (898.99, 4029.09)	713.56 (223.78, 1403.30)	<0.001
6 MWD, m	380.80 ± 78.61	355.31 ± 92.32	397.75 ± 62.73	<0.001
WHO FC, n (%)				0.621
I-II	130 (42.90)	54 (44.63)	76 (41.76)	
III-IV	173 (57.10)	67 (55.37)	106 (58.24)	
Echocardiography				
TRV, m/s	4.45 ± 0.72	4.40 ± 0.72	4.48 ± 0.72	0.386
PASP, mmHg*	90.74 ± 25.96	90.08 ± 26.59	91.18 ± 25.59	0.720
LVEF, %	61.69 ± 7.74	61.36 ± 6.08	61.90 ± 8.68	0.527
TAPSE, mm	13.12 ± 2.67	11.98 ± 2.25	13.88 ± 2.66	<0.001
RVFAC, %	29.42 ± 4.51	28.45 ± 4.92	30.06 ± 4.11	0.003
TAPSE/PASP, mm/mmHg	0.16 ± 0.06	0.15 ± 0.06	0.17 ± 0.06	0.009
Pericardial effusion, n (%)	71 (23.43)	49 (40.50)	22 (12.09)	<0.001
Right heart catheterization				
mPAP, mmHg	63.54 ± 16.12	58.31 ± 12.57	67.02 ± 17.28	<0.001
mRVP, mmHg	42.96 ± 11.85	41.77 ± 12.30	43.76 ± 11.50	0.152
mRAP, mmHg	11.27 ± 6.14	12.93 ± 6.15	10.17 ± 5.91	<0.001
PAWP, mmHg	8.62 ± 2.09	8.55 ± 1.71	8.66 ± 2.30	0.635
Qp, L/min	4.46 ± 1.89	3.21 ± 1.17	5.295 ± 1.82	<0.001
Qs, L/min	3.67 ± 1.54	3.21 ± 1.17	3.98 ± 1.67	<0.001
Qp/Qs	1.27 ± 0.50	1	1.45 ± 0.58	<0.001
CI, L/min/m ²	3.06 ± 1.36	2.09 ± 0.73	3.71 ± 1.30	<0.001
PVR, WU	14.74 ± 7.61	17.69 ± 7.89	12.79 ± 6.75	<0.001
SvO ₂ , %	60.75 ± 9.13	57.96 ± 9.90	62.60 ± 8.09	<0.001
Treatment				0.007
Monotherapy	75 (24.75)	20 (16.53)	55 (30.22)	
ERA	43 (61.43)	13 (65)	35 (60)	
PDE-5i	15 (21.43)	5 (25)	10 (20)	
PGI2	12 (17.14)	2 (10)	10 (20)	
Combination therapy	228 (75.25)	101 (83.47)	127 (69.78)	
ERA + PDE-5i	143 (62.72)	55 (54.46)	88 (69.29)	
ERA + PDE-5i + PGI2	38 (16.67)	24 (23.76)	14 (11.02)	
PDE-5i + PGI2	25 (10.96)	14 (13.86)	11 (8.67)	
ERA + PGI2	16 (7.02)	6 (5.94)	10 (7.87)	
ERA + sGCs	6 (2.63)	2 (1.98)	4 (3.15)	
Follow-up (months)	66.04 ± 24.53	60.60 ± 23.52	68.05 ± 23.90	0.222

PAH, pulmonary arterial hypertension; IPAH, idiopathic PAH; CHD, congenital heart disease; PAH-CHD, PAH associated with CHD; HR, heart rate; bpm, beats/min; NT-proBNP, N-terminal pro hormone brain natriuretic peptide; 6 MWD, 6-min walking distance; WHO FC, World Health Organization functional class; TRV, the velocity of tricuspid valve regurgitation; PASP, pulmonary artery systolic pressure; LVEF, left ventricular ejection fraction; TAPSE, tricuspid annular plane systolic excursion; RVFAC, right ventricular fractional area changes; mPAP, mean pulmonary artery pressure; mRVP, mean right ventricular pressure; mRAP, mean right atrial pressure; PAWP, pulmonary artery wedge pressure; Qp, pulmonary artery blood flow; Qs, systemic artery blood flow; CI, cardiac index; PVR, pulmonary vascular resistance; WU, Wood units; SvO₂, oxygen saturation of mixed venous blood. ERA, endothelin receptor antagonists; PDE-5i, phosphodiesterase type-5, inhibitors; sGCs, soluble guanylate cyclase stimulators; PGI2, prostacyclin analogues and prostacyclin receptor agonists.

and pulmonary artery was recorded by fluid-filled catheters connected to pressure transducers. Pulmonary (Qp) and systemic (Qs) flow was calculated by the Fick method (Miranda et al., 2022). PVR was calculated as (mPAP-PAWP) divided by Qp. Cardiac index (CI) was calculated as cardiac output divided by body surface area.

2.3 Statistical analysis

In the present study, the follow-up time was defined as the interval from baseline time of RHC diagnosis to 1 November 2022. Continuous variables with a normal or abnormal distribution were expressed as mean ± standard deviation (SD) or median

[interquartile range (IQR)], respectively, and independent samples *t*-test or Mann-Whitney *U* test was used to compare continuous variables between two groups. Categorical variables were expressed as frequency (percentage) and chi-square test was used for comparisons between two groups. The overall survival distribution was analysed by the Kaplan-Meier method. Log-rank test was used to compare differences in survival between groups. All statistical analyses were performed by Statistic Package for Social Science (SPSS, version 19; IBM Corp., Armonk, NY, United States) and GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, United States). Differences were considered statistically significant when *p*-value < 0.05.

3 Results

3.1 Demographic and clinical characteristics

According to the inclusion and exclusion criteria, a total of 303 adult treatment-naïve patients (age, 36.23 ± 13.10 years; women, 213 (70.3%); mPAP, 63.54 ± 16.12 mmHg; PVR, 14.74 ± 7.61 WU) with IPAH (*n* = 121) or PAH-CHD (*n* = 182) were included in this study. The overall follow-up time was 66.04 ± 24.53 months. There was no significant difference in follow-up time between the two cohorts.

Baseline demographic and clinical characteristics, echocardiographic and hemodynamic parameters were presented in Table 1. As illustrated in Table 1, no significant differences were observed in age, gender, HR, WHO FC, and follow-up time in patients with IPAH versus PAH-CHD. Patients with IPAH had higher SaO₂, NT-ProBNP and worse 6 MWD as compared to patients with PAH-CHD (all *p* < 0.05).

TTE showed no significant differences in baseline LVEF, TRV, and PASP between the two groups. Patients with IPAH had a higher incidence of pericardial effusion, lower TAPSE, lower RVFAC, and worse RV-PA coupling (TAPSE/PASP) than patients with PAH-CHD (all *p* < 0.05). RHC revealed that patients with IPAH had more severe PAH than those with PAH-CHD, with a lower mPAP, CI, and SvO₂, and higher PVR and mRAP (all *p* < 0.05), without statistical difference in PAWP and mRVP. In summary, IPAH group had worse baseline RV function and more severe hemodynamic status.

3.2 Treatment strategy, survival analysis, and treatment response

Treatment strategies for patients with PAH were also evaluated and summarized in Table 1. In terms of treatment strategies, combination therapy and parenteral targeted medication were more common in IPAH. As of the latest follow-up, forty-nine (40.50%) patients with IPAH and six (3.3%) patients with PAH-CHD had died, and two (1.65%) IPAH patients had received lung transplantation. The survival was significantly shorter in patients with IPAH than in patients with PAH-CHD (log-rank *p* < 0.05) (Figure 1). Right heart failure [48 (87.27%)] was the main cause of death. In the remaining seven cases, six (10.91%) patients suffered sudden unexpected death, and one (1.82%) patient died after transthoracic needle biopsy of pulmonary nodules.

Targeted therapy significantly improved WHO FC, PASP, RV-PA coupling and right heart function without affecting left heart function (Table 2; Figure 1). After targeted treatment, 189 (62.38%) patients WHO FC reaches I or II, PASP decreased from 90.74 ± 25.96 to 77.50 ± 32.96 mmHg, TAPSE increases from 13.12 ± 2.67 to 14.77 ± 4.23 mm, RVFAC increased from 29.42 ± 4.51 to 32.01 ± 6.42 mmHg, and TAPSE/RVSP increases from 0.16 ± 0.06 to 0.29 ± 0.30 . Remarkably, despite the 6 MWD after targeted treatment increased from 380.44 to 391.54 m, the difference was not statistically significant. Further subgroup analysis demonstrated that these improvements were primarily in patients with PAH-CHD rather than IPAH patients. In PAH-CHD group, 6 MWD increased from 397.75 ± 62.73 to 431.33 ± 104.85 m, TAPSE increased from 13.88 ± 2.66 to 16.30 ± 3.83 mm, PASP decreased from 91.18 ± 25.59 to 69.08 ± 33.76 mmHg, RVFAC increased from $30.06\% \pm 4.11\%$ to $33.91\% \pm 5.90\%$, and TAPSE/PASP increased from 0.17 ± 0.06 to 0.37 ± 0.35 . 131 (71.98%) patients WHO FC reached I-II, the number of patients with pericardial effusion decreased from 22 (12.09%) to 11 (6.04%). Fortunately, thirty-six PAH-CHD patients underwent successful closure by interventional or surgical methods. In patients with IPAH, RV function parameters (such as TAPSE and RVFAC) were slightly increased but did not reach statistical difference.

Considering that the baseline RV function between the two cohorts was not matched, we used TAPSE (cut-off value 12 mm) as a reference to match the RV function of the two cohorts, so as to further analysed the treatment response to targeted drugs in patients with IPAH and PAH-CHD with different baseline RV functions. The clinical characteristics, echocardiographic, and hemodynamic parameters of RV function-matched patients were summarized in Supplementary Table S1. As presented in Supplementary Table S1, patients with IPAH had a higher proportion (44.63%) of severe (i.e., TAPSE < 12 mm) RV dysfunction. There were no statistical differences in age, gender, HR, PASP, LVEF, mRVP, and PAWP between IPAH and PAH-CHD under the new classification. Compared with patients with PAH-CHD, patients with IPAH had higher NT-ProBNP and PVR, and lower mPAP and CI. In patients with severe RV dysfunction, no statistical differences were observed in 6 MWD, WHO FC, and TAPSE between IPAH and PAH-CHD. Targeted therapy significantly improved the WHO FC and TAPSE in patients with severe RV dysfunction, whereas there was no statistical difference in Δ 6MWD, Δ TAPSE, and Δ RVFAC between IPAH and PAH-CHD (all *p* > 0.05; Supplementary Table S2). For patients with TAPSE ≥ 12 mm, 6 MWD, WHO FC, and TAPSE in the IPAH group were inferior to those in the PAH-CHD group, and targeted drugs significantly improved the WHO FC, PASP, TAPSE, RVFAC, and RV-PA coupling in patients with PAH-CHD, but had little effect on patients with IPAH (Supplementary Table S2). Collectively, compared with IPAH patients, patients with PAH-CHD were more likely to benefit from targeted drugs with greater improvement than those with IPAH.

4 Discussion

Right heart failure is a life-threatening complication of PAH and is associated with increased mortality. In the present study, we retrospectively analysed the differences in baseline RV function and

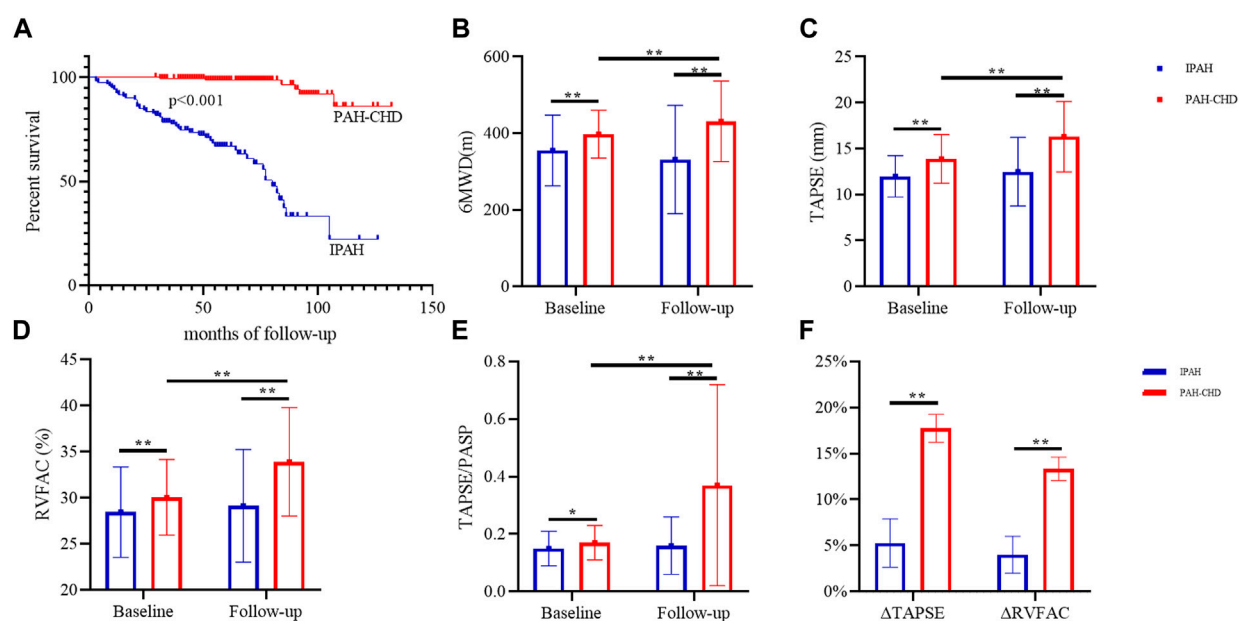


TABLE 2 Changes in WHO FC and right ventricular parameters for IPAH or PAH-CHD.

Variables	Total patients (n = 303)		p-value	IPAH (n = 121)		p-value	PAH-CHD (n = 182)		p-value
	Baseline	Follow-up		Baseline	Follow-up		Baseline	Follow-up	
6 MWD, m	380.80 ± 78.61	391.54 ± 130.08	0.219	355.31 ± 92.32	331.70 ± 141.37	0.125	397.75 ± 62.73	431.33 ± 104.85	<0.001
WHO FC, n (%)									
I-II	130 (42.90)	189 (62.38)	<0.001	54 (44.63)	58 (47.93)	0.606	76 (41.76)	131 (71.98)	<0.001
III-IV	173 (57.10)	114 (37.62)		67 (55.37)	63 (52.07)		106 (58.24)	51 (28.02)	
TRV, m/s	4.45 ± 0.72	4.02 ± 1.06	<0.001	4.40 ± 0.72	4.43 ± 0.76	0.766	4.48 ± 0.72	3.75 ± 1.14	<0.001
PASP, mmHg*	90.74 ± 25.96	77.50 ± 32.96	<0.001	90.08 ± 26.59	90.17 ± 27.35	0.981	91.18 ± 25.59	69.08 ± 33.76	<0.001
LVEF, %	61.69 ± 7.74	61.17 ± 6.76	0.377	61.36 ± 6.08	61.71 ± 6.10	0.658	61.90 ± 8.68	60.80 ± 7.15	0.188
TAPSE, mm	13.12 ± 2.67	14.77 ± 4.23	<0.001	11.98 ± 2.25	12.48 ± 3.73	0.212	13.88 ± 2.66	16.30 ± 3.83	<0.001
RVFAC, %	29.42 ± 4.51	32.01 ± 6.42	<0.001	28.45 ± 4.92	29.14 ± 6.12	0.330	30.06 ± 4.11	33.91 ± 5.90	<0.001
TAPSE/PASP	0.16 ± 0.06	0.29 ± 0.30	<0.001	0.15 ± 0.06	0.16 ± 0.10	0.133	0.17 ± 0.06	0.37 ± 0.35	<0.001
Pericardial effusion, n (%)	71 (23.43)	68 (22.44)	0.772	49 (40.50)	57 (47.11)	0.300	22 (12.09)	11 (6.04)	0.045

IPAH, idiopathic pulmonary arterial hypertension; PAH-CHD, pulmonary arterial hypertension associated with congenital heart disease; 6 MWD, 6-min walking distance; WHO FC, World Health Organization functional class; TRV, the velocity of tricuspid valve regurgitation; PASP, pulmonary artery systolic pressure; LVEF, left ventricular ejection fraction; TAPSE, tricuspid annular plane systolic excursion; RVFAC, right ventricular fractional area changes.

response to targeted therapy between patients with IPAH and patients with PAH-CHD admitted to our centre over the past 10 years. First, the average age of these enrolled population was

36.23 years old and female was predominant. The overall female-to-male ratio was 2.37:1, and the ratio in IPAH group was approximately 1.95:1, which was similar to what we observed in

COMPERA (Comparative, Prospective Registry of Newly Initiated Therapies for Pulmonary Hypertension) Registry (Hoeper et al., 2013).

The normal right ventricle is thin-walled, low afterload, and high-compliance distinct from the left ventricle. Anatomic and functional characteristics determine that the right ventricle is more capable of accommodating the volume overload rather than pressure overload (Edward et al., 2022). In PAH, as a consequence of persistently elevated PAP, the RV afterload and wall stress are increased resulting in RV hypertrophy, dilation, and dysfunction under suitable conditions, and the severity is affected by the duration of the pressure overload. Interestingly, abundant literature demonstrated that RV structure and function in people lacking cardiopulmonary disease vary with age, gender, race, exercise (Kawut et al., 2011; Cornwell et al., 2020; Thenappan and Weir, 2020; Edward et al., 2022). Therefore, it is easy to understand the divergent responses of RV to PH subtypes with different pathogenesis and pathophysiology. For example, Emami and colleagues found that patients with IPAH had better RV function compared to those with PH secondary to chronic heart failure (PH-HF) (Emami et al., 2020). This study showed that patients with IPAH had higher peak systolic velocity of tricuspid annulus, higher dp/dt, and better RV free wall stiffness, but other RV parameters such as TAPSE and FAC are only slightly increased without reaching statistical significance. In subgroup analysis of PAH, multiple studies have demonstrated that patients with SSc-PAH had worse resting and reserve RV function than patients with IPAH (Overbeek et al., 2008; Tedford et al., 2013; Hsu et al., 2016; Hsu et al., 2018). This RV dysfunction in SSc-PAH was associated with impaired intrinsic myocardial contractility, decreased calcium cycling, higher RV fibrosis and diastolic stiffness, and RV-PA decoupling (Tedford et al., 2013; Hsu et al., 2016; Hsu et al., 2018).

In our study, the mPAP in IPAH group was significantly lower than that in PAH-CHD group, but a higher baseline PVR implied more severe pulmonary vascular disorders in the IPAH cohort. Pericardial effusion, a relatively common complication of PAH, is associated with increased mortality (Fenstad et al., 2013; Shimony et al., 2013; Chandel et al., 2021). In this study, the incidence of pericardial effusion (40.5%) in patients with IPAH was similar to that in Shimony's study (47.1%), which may be related to RV dysfunction and subsequent increased right atrial filling pressure (Shimony et al., 2013). Subsequent analysis showed that the RV function (TAPSE, RVFAC) in patients with IPAH was significantly inferior to that in patients with PAH-CHD. As discussed above, RV structure and function are subject to multiple factors, and following conditions may account for the difference in RV function at baseline: firstly, the clinical manifestations in patients with IPAH are non-specific with insidious onset, and some patients present with RV dysfunction or even decompensation at initial diagnosis, which is confirmed by the higher proportion of severe RV dysfunction in the IPAH group in our study. Secondly, pathogenesis participates in this difference. Anomalous systemic-to-pulmonary shunt increases volume-load or pressure-load, which plays an important role in the development of PAH-CHD. In the early stage of PAH-CHD, PVR is only moderately elevated. As PAH-CHD progresses, which may take decades, irreversible pulmonary artery lesions such as plexiform lesions and necrotising arteritis appear, leading to loss of pulmonary vascular beds and subsequent progressive elevation of

PVR and PAP with bidirectional or reverse shunt (Van der Feen et al., 2017). In the long-term chronic course of PAH-CHD, right ventricle can better adapt to the elevated afterload and maintain better RV function (Giusca et al., 2016; Brida and Gatzoulis, 2018). Whereas, the mechanism of RV dysfunction in IPAH is more mysterious and complex, including genetics, afterload overload, metabolic transformation, myocardial ischemia, and other aspects (van der Bruggen et al., 2016; Sun et al., 2017; Sanz et al., 2019; Sree et al., 2021). Finally, RV histological features contribute to these differences. Adaptive RV remodelling in PAH-CHD (such as ES) patients is characterized by RV hypertrophy, increased RV contractility, lower RV fibrosis and RV diastolic stiffness (Giusca et al., 2016; van der Bruggen et al., 2017). However, the adaptive RV remodelling in IPAH patients is consistent with a combination of RV hypertrophy, increased RV contractility, higher RV fibrosis and RV diastolic stiffness (van der Bruggen et al., 2017). These features suggest that IPAH patients have poor RV adaptation and are more susceptible to deteriorate from RV hypertrophy to RV failure. Notably, patients with PAH-CHD who met operative indications at initial diagnosis were not included in this study, and this design may narrow the actual gap in baseline RV function between the two cohorts.

At present, assessing the severity of PAH patients by multiparameter risk stratification is an important principle for selecting treatment strategies. A large number of literature has demonstrated that PAH-specific medications, especially combined therapy, improve activity tolerance, quality of life, hemodynamics, and RV structure and function in patients with IPAH and patients with PAH-CHD (D'Alto et al., 2020; Fathallah and Krasuski, 2018; Ruopp and Cockrill, 2022; Vonk et al., 2022). Given that vasodilators are expensive, PAH patients often bear a heavy economic burden. Therefore, we dynamically adjusted treatment strategy based on contemporaneous guidelines and economic conditions. In our study, patients with IPAH had a higher proportion of combination therapy and parenteral prostacycline than patients with PAH-CHD. Targeted therapy remarkably improved overall 6 MWD, WHO FC, PASP, RV function, and survival. Further subgroup analysis showed that vasodilator therapy substantially improved hemodynamic status and RV function in patients with PAH-CHD, and 36 patients successfully repaired the defect. However, although survival in IPAH cohort was improved, RV function was only slightly increased and did not have statistical difference, and 6 MWD even witnessed a downward trend, which was inconsistent with previous studies. To our knowledge, targeted agents relieve symptoms by dilating PA and reducing RV afterload. Patients' response to targeted therapy was influenced by RV remodelling and treatment strategies, which had also been observed in other subtypes of PH. For instance, patients with CTD-PAH had a worse therapeutic response than those with IPAH (Rhee et al., 2015; Argula et al., 2017; Hsu et al., 2018). Goh and colleagues evaluated RV remodelling by cardiac magnetic resonance (CMR) and demonstrated that the high-volume-low-mass (HVLM, i.e., maladaptive RV remodelling) group had a worse prognosis than the low-volume-low-mass (LVLM, i.e., adaptive RV remodelling) group (Goh et al., 2022). After treatment, a higher proportion (73.5%) of LVLM patients remained in this group, and only 17.4% of HVLM patients converted to LVLM. In our study, IPAH patients had more

severe baseline RV dysfunction, RV-PA coupling, and pulmonary vascular disorders. Considering that the baseline RV function was not matched between IPAH and PAH-CHD, we matched the RV function and subsequently analysed the patients' response to targeted drugs. The results indicated that targeted drugs improved WHO FC and TAPSE in patients with severe RV dysfunction (i.e., TAPSE < 12 mm), and there was no significant difference in Δ TAPSE and Δ RV FAC between PAH-CHD and IPAH. Notably, for patients with non-severe RV dysfunction (i.e., TAPSE $\geq$ 12 mm), patients with PAH-CHD had a better response to treatment than patients with IPAH. The limited effect of targeted agents on PA dilation may partially account for the different treatment responses between two cohorts. Additionally, deterioration of treatment-naïve PAH patients has progressed over time. Targeted therapy effectively increases the time to clinical deterioration and improves survival in IPAH patients. However, the follow-up period in our study was relatively longer (up to 132 months), it is necessary to further verify whether the improvement in RV function in IPAH patients receiving treated therapy will continue to improve in the next few years.

Lung transplantation is effective in treating end-stage PAH. However, given the difficulty in obtaining donors and long waiting time, patients with a high or intermediate-high risk of death or with an inadequate response to treatment should be considered as lung transplantation candidates earlier. Several small sample studies (Boucly et al., 2021; D'Alto et al., 2020) have proved that triple combination therapy including parenteral prostacyclin significantly increases transplant-free survival and reverses RV remodelling, suggesting that this may be a good transition for critical patients.

This study still had the following limitations. Firstly, this was a single-center retrospective study. During long-term follow-up, patients' treatment strategies, especially combination therapy, were dynamically adjusted according to contemporaneous guidelines and economic condition, and this study reflected the effectiveness of the overall treatment strategy. Secondly, we chose conventional parameters to evaluate RV function. Emerging evidence demonstrated that CMR was also effective evaluation tools, and TTE combined with these new indicators may have more important value. Finally, the included population was relatively small, and there were large differences between different groups after matching RV function. Prospective studies with larger populations will be required in the future.

In conclusion, we analysed RV function and its response to PAH-targeted therapy between IPAH and PAH-CHD cohorts. Compared with patients with PAH-CHD, patients with IPAH had worse baseline RV function, unfavourable prognosis, and inadequate response to targeted treatment. Patients with

inadequate response to treatment may be considered as candidates for lung transplantation earlier.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#), further inquiries can be directed to the corresponding author.

Ethics statement

The study was approved by the Ethics Committee of the Second Xiangya Hospital.

Author contributions

TZ recruited patients, analysed the data, and wrote the manuscript. TZ, PW, ZT, QJ, YC, ZC, LL, YT, and JL planned the project, enrolled patients, and performed the research. ZF designed and supervised the whole study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2023.1124746/full#supplementary-material>

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Extracellular vesicles mediate biological information delivery: A double-edged sword in cardiac remodeling after myocardial infarction

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Acute myocardial infarction (AMI) is a severe ischemic disease with high morbidity and mortality worldwide. Maladaptive cardiac remodeling is a series of abnormalities in cardiac structure and function that occurs following myocardial infarction (MI). The pathophysiology of this process can be separated into two distinct phases: the initial inflammatory response, and the subsequent longer-term scar revision that includes the regression of inflammation, neovascularization, and fibrotic scar formation. Extracellular vesicles are nano-sized lipid bilayer vesicles released into the extracellular environment by eukaryotic cells, containing bioinformatic transmitters which are essential mediators of intercellular communication. EVs of different cellular origins play an essential role in cardiac remodeling after myocardial infarction. In this review, we first introduce the pathophysiology of post-infarction cardiac remodeling, as well as the biogenesis, classification, delivery, and functions of EVs. Then, we explore the dual role of these small molecule transmitters delivered by EVs in post-infarction cardiac remodeling, including the double-edged sword of pro- and anti-inflammation, and pro- and anti-fibrosis, which is significant for post-infarction cardiac repair. Finally, we discuss the pharmacological and engineered targeting of EVs for promoting heart repair after MI, thus revealing the potential value of targeted modulation of EVs and its use as a drug delivery vehicle in the therapeutic process of post-infarction cardiac remodeling.

KEYWORDS

myocardial infarction, cardiac remodeling, extracellular vesicles, cargo delivery, drug delivery

1 Introduction

Among the leading causes of death and disability worldwide are acute myocardial infarction (AMI) and end-stage heart failure (HF), causing approximately 17 million deaths annually and accounting for 30% of all deaths globally (Ong et al., 2018; Roth et al., 2020). Coronary atherosclerosis underlies the development of myocardial infarction (MI), which

contributes to vessel lumen narrowing and ultimately elicits myocardial necrosis due to hypoxia/ischemia. Currently, the primary goal of clinical treatment for such disease is to promptly restore flow in the occluded vessel and restore the ischemic myocardium, commonly performed by direct percutaneous coronary intervention. Intriguingly, however, such interventions can contribute to cardiomyocyte death and myocardial injury (Schirone et al., 2022). Consequently, novel therapies and interventions are urgently required to attenuate adverse ventricular remodeling and mitigate HF.

The heart undergoes a series of structural and functional alterations to adapt to cardiac injury induced by MI, which is known as cardiac remodeling (Zhao et al., 2020). Specifically, cardiac remodeling is a complex pathophysiological process, including inflammation, mitochondria dysfunction, oxidative stress, cardiac hypertrophy, and cardiac fibrosis. The entire process can be divided into two stages, the inflammatory phase and the fibrotic scar revision phase (Schirone et al., 2017; Liu H et al., 2020). The initial phase involves the exposure of intracellular contents into the extracellular environment due to cell necrosis, called damage-associated molecular patterns (DAMPs), triggering and exacerbating sterile inflammation. While the latter phase is attributed to the transdifferentiation of fibroblasts into myofibroblasts stimulated by multiple factors, including inflammatory factors, angiotensin II, and mechanical stress stimulation, which secrete collagen and facilitate scar repair in the infarcted region and maintain the structural integrity of the ventricle (Prabhu and Frangogiannis, 2016; Peet et al., 2020).

During cardiac remodeling, numerous immune cells recruitment to the infarct area, interact with cardiac fibroblasts (CFs) which crucially coordinates the balance between profibrotic and antifibrotic effects (Zaidi et al., 2021). In the initial inflammatory phase of AMI, the recruitment and activation of immune cells, such as macrophages, neutrophils, and monocytes, not only secrete inflammatory mediators to aggravate the inflammation but also shift towards an anti-inflammatory phenotype, which can prevent the release of their contents into the extracellular environment by removing necrotic cellular debris from the MI zone to attenuate inflammation (Ong et al., 2018; Andreadou et al., 2019; Doran et al., 2020). Besides this, the crosstalk between other diverse cells, including mesenchymal stem cells, endothelial cells (ECs), and cardiomyocytes is also essential for maintaining dynamic balance throughout the whole process. Interestingly, however, these cells secrete large numbers of EVs, which carry numerous bioactive substances, including miRNAs, lipids, and proteins. The bioactive substances not only have similar roles to parental cells but are also essential regulators of intercellular communication (Xiong et al., 2021). In general, there are three major subtypes of EVs, apoptotic bodies (ABs), microvesicles (MVs), and exosomes (Sanchez-Alonso et al., 2018), of which the most comprehensively explored are exosomes. In contrast, the least well-studied are ABs.

This review summarizes the dual roles of EVs derived from diverse cells in postinfarction cardiac remodeling, including pro-inflammatory and anti-inflammatory effects in the initial postinfarction inflammatory response and profibrotic and antifibrotic effects in the scar repair phase. Importantly, we discuss the role of crosstalk between these EVs in achieving a

coordinated dynamic balance between the inflammatory response and the fibrotic process, which is significant for maladaptive cardiac remodeling after MI. In parallel, we also discuss therapeutic opportunities for targeting EVs that play a significant role in post-infarction cardiac remodeling, including pharmacological preconditioning and engineered modification. These elements will be beneficial in expanding therapeutic strategies for post-infarction cardiac remodeling.

2 Pathophysiological process of cardiac remodeling after myocardial infarction

AMI results from myocardial blood flow obstruction due to atherosclerotic plaque rupture or erosion (Ibanez et al., 2018). AMI is currently treated mainly by revascularization procedures such as thrombolysis and coronary intervention. However, recanalizing occluded vessels under long-term ischemic conditions causes additional myocardial damage, termed “I/R injury” (Yellon and Hausenloy, 2007). Such a process is accompanied by a blockage of blood flow, a sudden and dramatic interruption of oxygen and nutrient supply in the blood, a short cessation of mitochondrial oxidative phosphorylation (Frank et al., 2012), a dramatic decrease in available intracellular adenosine triphosphate (ATP), and a reduction in cardiomyocyte contractile function and cardiac stiffness and contracture minutes after the onset of ischemia (Jennings and Reimer, 1991). In the meantime, the myocardial ischemic injury may contribute to substantial cardiomyocyte death *via* different mechanisms. At the outset of reperfusion, cytosolic calcium overload and massive reactive oxygen species (ROS) generation rupture the mitochondrial membrane and release the contents, which in turn activate the caspase-mediated intrinsic pathway of apoptosis (Schirone et al., 2022). On the other hand, phosphorylation of receptor-interacting protein kinase 3 (RIPK3) is recruited and activated by the receptor-interacting protein kinase 1 (RIPK1), which in turn triggers necroptosis and exacerbate the pathological process after MI (Rodriguez et al., 2016).

The large volume of dead and injured cardiomyocyte contents (including heat shock proteins, organelle debris, and nuclear debris) enter the interstitial matrix, namely, DAMPs (Frangogiannis, 2012; Timmers et al., 2012). Simultaneously, innate immunity is triggered when pattern recognition receptors (PRRs) on leukocytes recognize DAMPs (Suetomi et al., 2019; Silvis et al., 2020). Immediately afterwards, the Toll-like receptor signaling pathway and other numerous inflammatory pathways are activated, thereby inducing proinflammatory cytokine expression, including interleukin 6 (IL-6), interleukin 1 β (IL-1 β), and tumor necrosis factor α (TNF- α), as well as the expression of chemokines, including monocyte chemoattractant protein-1 (MCP-1/CCL2) (Frangogiannis, 2007). Additionally, neutrophils and inflammatory monocytes recruited by chemokine CCL2 activation are recruited to the infarcted heart (Dewald et al., 2005; Ley et al., 2007). On the other hand, when tissues are damaged, nuclear factor κ B (NF- κ B) is activated in ROS-dependent way and increases the expression of specific cellular genes, which can also eventually stimulate the growth of cells associated with inflammation and immune responses and exacerbate the inflammation even further (Kabe et al., 2005; Sun, 2009).

Short-term inflammatory responses contribute to debris removal, while long-term inflammation can facilitate extracellular matrix degradation and cell death, resulting in infarct size expansion. Consequently, timely resolution of the inflammation is essential for maintaining post-infarction remodeling. At the same time, apoptosis and clearance of neutrophils in the infarcted area is a hallmark that inflammation is beginning to subside (Frangogiannis, 2012; Westman et al., 2016; Ong et al., 2018). As leukocyte infiltration removes dead cells and matrix debris from the infarct and represses inflammatory mediator release, the anti-inflammatory monocyte subsets become dominant (Frangogiannis, 2008; Prabhu and Frangogiannis, 2016). Furthermore, suppressive subsets of other immune cells like lymphocytes, monocytes, and anti-inflammatory macrophages can suppress inflammation in the infarcted heart. Macrophages, in particular, play a crucial role in this process by shifting from a pro-inflammatory to an anti-inflammatory phenotype (Yan et al., 2013). In this way, the post-infarction pathological process moves from the pro-inflammatory response phase to the anti-inflammatory response phase, collectively referred to as the inflammatory response phase (within 3–7 days after MI), and eventually to the scar repair phase (7 days after MI) (Liu S et al., 2020).

In the cardiac tissue remodeling phase, fibroblast proliferation, extracellular matrix secretion, and eventually fibrotic scar maturation (Kologrivova et al., 2021). During the scar proliferation phase, subpopulations of monocytes and macrophages activate and recruit mesenchymal repair cells and primarily fibroblast-like cells by secreting growth factors (Frangogiannis, 2014). There is evidence that CFs originate from circulating progenitor stem cells (PSCs) recruited into the ventricular myocardium after birth and distribute throughout the normal myocardium as strands and sheets between myocardial muscle fibers and are critical effector cells in the scar repair process (Visconti and Markwald, 2006). In the normal physiological state, CFs account for 60%–70% of total cardiac cell numbers (Porter and Turner, 2009). They secrete collagen, which provides structural scaffolding for cardiomyocytes, coordinates myocardial motion, and mediates the onset of electrical activity (Bretherton et al., 2020). Under pathological conditions, fibroblasts transdifferentiate into myofibroblasts (MFBs) in response to various stimuli, including renin-angiotensin system activation, mechanotransduction signaling, and release of transforming growth factor β (TGF- β), inflammatory factors (Frangogiannis, 2015; Han et al., 2022).

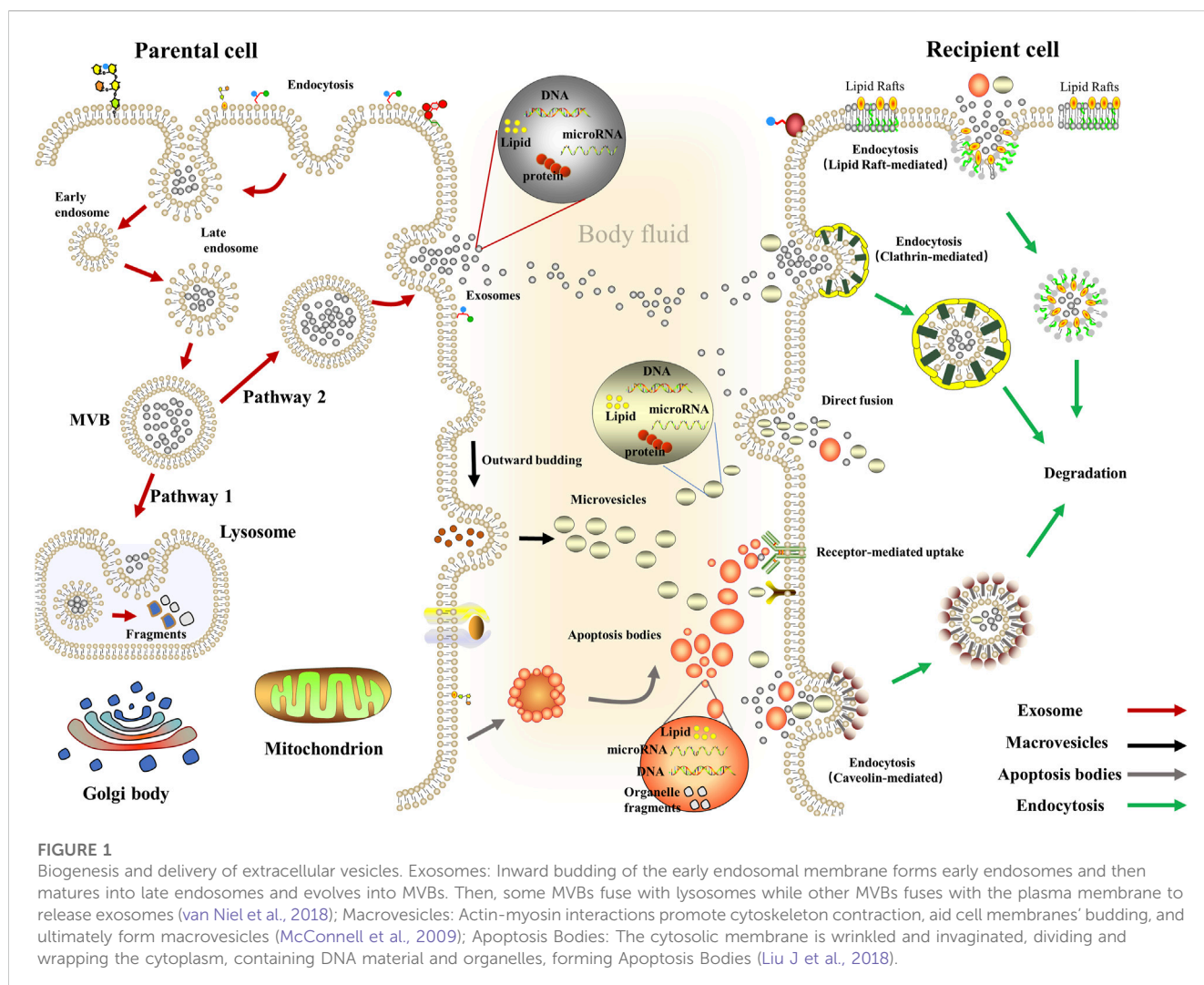
MFBs possess characteristics of both fibroblasts and smooth muscle cells, and secrete collagen to promote myocardial fibrosis. Meanwhile, these cells are analogous to smooth muscle cell contraction (as present in the vascular system), which can be modulated by circulating factors and neurohormones, including angiotensin II (van den Borne et al., 2010). Apoptosis of most repair cells indicates the end of the proliferative phase, and as the development of MI pathophysiology, the scar matures *via* a steady increase in collagen cross-linking (Frangogiannis, 2012). However, persistent MFBs differentiation and activation, as well as extracellular matrix (ECM) deposition, contribute to a decreased compliance of the myocardium (Umbarkar et al., 2021). On the other hand, progressive decline in the delivery of oxygen and nutrients induces atrophy and cell death in the cardiomyocytes,

contributing to progressive acute left ventricular dilatation and, ultimately, dysfunction and heart failure (Gibb et al., 2020). Thus, controlling the fibrosis process and achieving a dynamic balance between pro- and anti-fibrosis are of great significance for post-infarction repair. Given the complexity of post-infarction cardiac remodeling, this review focuses on two significant aspects: inflammatory response and fibrotic scar repair.

3 Biogenesis, sorting, delivery, and outcome of extracellular vesicles

EVs are nanoscale lipid particles generated by the endosomal system of eukaryotes and secreted into the extracellular environment (Borges et al., 2013; van der Pol et al., 2014; Yanez-Mo et al., 2015; Zaborowski et al., 2015). Currently, there is still no exact criteria on the nomenclature of EVs. Various names are used, such as exosomes, microparticles, MVs, ABs. Remarkably, the nomenclature of “extracellular vesicles” proposed by Chistiakov mainly used in this review (Chistiakov et al., 2015), which means that EVs are typically categorized subsets by their size, formation, marker proteins, release mechanisms, and lipid composition, including three types of plasma membrane exfoliated vesicles: exosomes (30–150 nm), MVs (1,000–1,000 nm), and ABs (500–2,000 nm) (Liu H et al., 2021). Exosomes are phospholipid bilayers membrane-enclosed vesicles (Pegtel and Gould, 2019), which were initially discovered in sheep reticulocytes in 1983 and then were named “exosomes” by Johnstone in 1987 (Pan and Johnstone, 1983; Johnstone et al., 1987). Several studies have indicated that the inward budding of the early endosomal membrane forms early endosomes and then matures into late endosomes which are often packed with small intraluminal vesicles (ILVs) and evolve into multivesicular bodies (MVBs) (Ohayon et al., 2021). After a series of intracellular transport, the degradation of vesicles occurs when some MVBs fuse with lysosomes. While other MVBs release ILVs such as exosomes when fusing with the plasma membrane (Kowal et al., 2014; Villarroya-Beltri et al., 2016). These exosomes can be detected in diverse bodily fluids, such as breast milk, saliva, blood, urine (Pisitkun et al., 2004; Caby et al., 2005; Admyre et al., 2007; Palanisamy et al., 2010). Simultaneously, numerous studies suggest that a broad range of cell types, including cardiomyocytes, immune cells, progenitor cells, fibroblasts, and stem cells can generate exosomes (Zitvogel et al., 1998; Doyle and Wang, 2019).

MVs were first described by Peter Wolf in 1967 and are generated by the outward budding of plasma membrane (Wolf, 1967), and they are also the result of dynamic interaction between redistribution of phosphatidylserine (PS) and the remodeling of cytoskeletal proteins (Zwaal and Schroit, 1997; Leventis and Grinstein, 2010); proteins and phospholipids are distributed very heterogeneously and form microdomains within the membrane. Aminophospholipid translocases tightly control this asymmetric distribution of PS on plasma membranes. Moreover, flippase is a translocase that translocates PS from the outer leaflet to the inner leaflet of the membrane, while floppase transfers phospholipids from the inner to the outer leaflet of the plasma membrane (Zwaal and Schroit, 1997; Hugel et al., 2005). Immediately afterwards, actin-



myosin interactions promote cytoskeleton contraction, aid cell membranes' budding, and ultimately form MVs (McConnell et al., 2009; Muralidharan-Chari et al., 2009). The number of these generated MVs relies on the physiological state and microenvironment of the donor cells while consumed MVs depends on the physiological state and microenvironment of the recipient cells (Zaborowski et al., 2015).

These EVs can enter the recipient cells *via* fusing with cellular membranes; on the other hand, it also enters the recipient cells *via* different endocytic pathways, including phagocytosis clathrin-mediated endocytosis, caveolae, or lipid raft-mediated endocytosis (Feng et al., 2010; Tian et al., 2014; Costa Verdera et al., 2017; Hou and Chen, 2021). Moreover, classical intercellular adhesion molecules also mediate the binding of EVs to the target cells (Zhao et al., 2015); specifically, on the one hand, TIM family members (TIM1, TIM4) PS transmembrane receptors are used by target cells for exosomes entry (Miyashita et al., 2007), on the other hand, exosomes derived from B cells containing a 2,3-linked dialkylated glycoprotein can be captured by lymph node and spleen CD169⁺ macrophages (Saunderson et al., 2014). At the same time, macrophages uptake exosome *via* C-type lectins expressed in macrophages and galectin-5 exposed to exosome (Barres et al.,

2010). Meanwhile, multiple mechanisms have been demonstrated to be associated with regulating the biogenesis of EVs, especially the endosomal sorting complex required for transport (ESCRT) or non-ESCRT mechanisms (Assil et al., 2015). ESCRT consists of four core subunits (ESCRT-0; ESCRT-I; ESCRT-II; ESCRT-III), where ESCRT-0 mediates substrate recognition and sorting, ESCRT-I and ESCRT-II are critical for mediating endosomal membrane inward budding, and ESCRT-III is responsible for shearing the neck of the budding body, thereby orchestrating the formation of an inward budding vesicle of MVBs (Hurley and Hanson, 2010; Hanson and Cashikar, 2012; Tarasov et al., 2021). Considering the biological complexity of EVs and is susceptible to the environment, there are yet no established criteria to distinguish different types of EVs, which limits the development of related studies (Amosse et al., 2017) (Figure 1).

4 Composition and physiological function of extracellular vesicles

Initially, EVs were considered waste products released by cells. Later studies, however, demonstrated that EVs mediate the

transmission of intercellular molecular biological information and play a crucial role in intercellular communication (Hessvik and Llorente, 2018; Harmati et al., 2021). Besides, EVs can transport bioactive cargoes, including RNA, DNA, proteins, and lipids (Chen F et al., 2020). Indeed, it both retains many of the biological properties of their parental cells and regulates recipient cells' physiological or pathological processes (Xing et al., 2021). Of the three types of EVs, exosomes are derived from MVBs, which budding inward from a raft-like domain and, therefore, have a slightly different lipid content than the cell membrane. Exosomes are rich in cholesterol, sphingomyelin, glycerophosphocholine, and phosphatidylcholine (Laulagnier et al., 2004; Raposo and Stoorvogel, 2013; Janas et al., 2015), and membrane surface markers such as integrins, Flotillin 1, CD63, CD81, Alix, TSG101, and cell adhesion molecules (CAMs) (Lotvall et al., 2014). Due to its small size, protective lipid bilayer, and surface receptors, exosome can mediate long-distance, inter-tissue systemic crosstalk (Mathieu et al., 2019; Pelissier Vatter et al., 2021).

The lipid composition and markers of MVs depend mainly on the composition of their cell membrane and also show particular antigens concerning the membrane composition of the original cell. For example, T cell MVs carry CD4 or T cell receptors (Choudhuri et al., 2014). Monocyte-derived MVs show CD14, while CD31, CD34, CD51, CD62E, CD144 and CD146 expressed on endothelium-derived MVs (Koga et al., 2005). Microvesicular cholesterol and hyperphosphatidylserine expose a lipid composition different from that of exosomes. Addition, the shape and density of MVs are irregular as well as the surface does not have any tetrapeptide (Muralidharan-Chari et al., 2010; Choudhuri et al., 2014). These vesicles consist of a lipid bilayer and have mRNAs, miRNAs, biologically active lipids, metabolites, and protein in which surface phospholipid bilayer (Ratajczak and Ratajczak, 2020), it also expresses the number of transmembrane proteins, such as integrins and selectins as in the parental cell membrane (Bobrie et al., 2011; Raposo and Stoorvogel, 2013). However, MVs contain a large number of cytoplasmic and plasma membrane proteins, such as cytoskeletal proteins, heat shock proteins, and protein tetrapeptides that accumulate on the surface of the plasma membrane, which is 100-fold more concentrated in MVs than in cell lysates (Escola et al., 1998; Zoller, 2009; Di Vizio et al., 2012; Morello et al., 2013), and contribute significantly to intercellular communication (Cocucci et al., 2009). On the other hand, the function of MVs depends on the cell types from which they originate (Muralidharan-Chari et al., 2010). For example, immune cell-derived MVs are involved in inflammatory responses, while endothelial cell-derived MVs are related to blood vessels (Morel et al., 2004). In addition to this, the procoagulant properties of MVs were reported for the first time in 1946 by Chargaff and West and later confirmed by Wolf in 1967, who described them as "platelet dust" (Wolf, 1967; Morel et al., 2004).

In recent years, there have been concerns regarding the relationship between EVs and diseases, with numerous studies focusing on cancer (Prieto-Vila et al., 2021), Parkinson's (D'Anca et al., 2019), infectious diseases (Rodrigues et al., 2018), kidney diseases (Thongboonkerd, 2019), and cardiovascular diseases, resulting in substantial influential outcomes. A great deal of research in cardiovascular disease suggests that EVs play an essential role in cardiac pathophysiology (Njock et al., 2015), and

EVs of diverse origins are necessary for maintaining cardiac function and homeostasis *via* influencing and interacting with each other (Yu and Wang, 2019). These subgroups exhibit a wide range of functions, making them an important source of potential biomarkers for early diagnosis, therapeutic drug delivery systems, or vaccine production systems (Bordanaba-Florit et al., 2021).

5 Mechanism of extracellular vesicles involved in cardiac remodeling after myocardial infarction

Numerous studies have shown that EVs have similar biological properties to parental cells. Interestingly, however, multiple cells produce EVs after MI, including cardiomyocytes, immune cells, ECs, progenitor cells, fibroblasts, and stem cells (Fujita et al., 2019; Berezin and Berezin, 2020). The presence of pro-inflammatory M1 macrophages (M1-MØ) and reparative M2 macrophages (M2-MØ) as well as N1 (Ly6G⁺CD206⁻) and N2 (Ly6G⁺CD206⁺) neutrophil phenotypes in the infarcted heart mean immune cells exhibit functions and phenotypic heterogeneity (Ma et al., 2016; Peet et al., 2020). N1 neutrophils are pro-inflammatory, with high expression of pro-inflammatory markers, such as TNF- α , IL-1 β , macrophage inflammatory protein 1 α (CCL3), and interleukin 12a (IL-12a), while anti-inflammatory Cd206 and interleukin 10 (IL-10) are highly expressed in N2 neutrophils. Similarly, monocytes differentiate into pro-inflammatory and anti-inflammatory. EVs of different origins and subtypes also have complex and diverse roles similar to their parental cells and play a dual role in post-infarction cardiac remodeling. These EVs maintain a dynamic balance between pro-and anti-inflammatory, pro-and anti-fibrotic. Thus, it is significant to investigate the involvement of EVs in post-infarction cardiac remodeling, providing new avenues to open up targeted therapeutic strategies.

5.1 Mechanism of extracellular vesicles associated with the initial inflammatory response following myocardial infarction

5.1.1 Mechanism of extracellular vesicles associated with the pro-inflammatory response after myocardial infarction

After MI, the I/R injury contributes to the increased release of various EVs from the heart, which on the one hand, increases the expression of pro-inflammatory cytokines by promoting M1-type polarization of macrophages, on the other hand, these EVs generated by I/R injury stimulate the inflammatory response *via* transferring miR-155-5p to macrophages activating the JAK2/STAT1 pathway (Ge et al., 2021). Furthermore, a large number of monocytes infiltrate the infarct zone and differentiate into pro-inflammatory macrophages, which generated exosomes containing pro-inflammatory miRNAs. When transported into CFs, these exosomes promote significant expression of inflammatory cytokines, including TNF- α , CCL2, IL-1 β , and IL-6 in CFs, thereby exacerbating the inflammatory response in the infarct area (Wang et al., 2017). On the other hand, M1 macrophage-

secreted exosomes can also transfer specific miR-155 to ECs, preventing migration and proliferation, affecting angiogenesis and repair, which in turn exert a pro-inflammatory effect (Liu Y et al., 2020).

Beyond that point, as one of the most critical members of the immune cell family, neutrophils play an essential role in this phase. It is well known that neutrophils are professional phagocytes that help to clear necrotic cells. However, recent studies have shown that excessive neutrophil accumulation or delayed clearance of neutrophils is detrimental and can act by secreting EVs (van der Pol et al., 2016). Depending on the production mechanism, neutrophil-derived EVs can be classified into two subtypes: neutrophil-derived MVs (NDMV) and neutrophil-derived trails (NDTRs). Pro-inflammatory miRNAs are highly expressed in the NDTRs, which are found in tissues where neutrophils migrate while inducing pro-inflammatory macrophage polarization and exacerbating the inflammatory response and cardiac injury (Ma, 2021; Youn et al., 2021). On the other hand, NDMVs can evoke an acute inflammatory response by stimulating ECs to produce inflammatory mediators, including MCP-1 and IL-6 and tissue factor as well as increase microvascular endothelial permeability (Mesri and Altieri, 1999; Ajikumar et al., 2019). Numerous factors influence the increase of neutrophils. Generally, myocardial injury mobilizes neutrophils rapidly from the spleen to the peripheral blood, where they engage in transcriptional activation prior to reaching the damaged area. AMI triggers the transient release of cardiomyocyte- and endothelium-derived EVs. Ly6C⁺ monocytes infiltrating the infarcted heart are stimulated to release chemokines and inflammatory cytokines by these vesicles, exacerbating the local inflammatory response (Loyer et al., 2018). Further studies demonstrated that endothelial cell-derived EVs mediated neutrophil mobilization from the spleen *via* EC-EV-VCAM-1 after AMI and induced transcriptional activation of blood neutrophils to facilitate miRNA-126-mRNA targeting (Akbar et al., 2022). At the same time, ABs are produced when a substantial number of cells appear apoptotic in infarct zones. However, these ABs do not contribute to the release of intracellular contents and trigger an inflammatory response due to their intact membrane structure, while a large number of necrotic cell contents are secreted into the extracellular area to trigger a dangerous related pattern and trigger a severe inflammatory reaction (Anversa et al., 1998).

Besides this, the current clinical treatment of MI is mainly through percutaneous coronary intervention (PCI). Available studies demonstrate that post-PCI vascular inflammation involves complex interactions between multiple cell types, these cells release proinflammatory cytokines while recruiting monocytes and neutrophils for transendothelial migration to surrounding tissues (Tucker et al., 2021), and EVs, as an important medium of intercellular communication, may play an important role in this process (S et al., 2013). On the other hand, Neutrophils mediate platelet aggregation and thrombus formation, while activating endothelial cells, macrophages and T lymphocytes to release inflammatory cytokines and promote an inflammatory cascade response (Vaidya et al., 2021). However, whether these immune cells release EVs in the process and whether these EVs play a role remains to be explored. Given the current context, future research directions regarding EVs in the regulation of microvascular

inflammation after PCI may focus on several directions. Firstly, EVs can be used as potential biomarkers to assess the degree of vascular inflammation; secondly, they may be critical mediators of intercellular communication, broadening the understanding of pathological mechanisms associated with the microvascular inflammation after PCI; thirdly, EVs can be used as drug carriers or targets to alleviate microvascular inflammation after PCI.

5.1.2 Mechanism of extracellular vesicles associated with the anti-inflammatory response following myocardial infarction

Macrophages are functionally and subtypically polarized into the classically activated M1 phenotype and the alternatively activated M2 phenotype. Despite its simplicity, this classification is still more accepted at this stage. M2 phenotype macrophages can mediate inflammation resolution by secreting anti-inflammatory mediators during days 3–5 following MI. Furthermore, it has been shown that the co-culture of the MSCs and macrophages can contribute to sustained high expression of CD206 and IL-10 and low expression of interleukin 12 (IL-12) and TNF- α on the surface of macrophages, resulting in a shift of macrophages towards the M2 phenotype (Chiossone et al., 2016). It has also been shown that mesenchymal stroma-derived exosomes promote macrophage polarization toward the M2 phenotype, which may attenuate the inflammatory cascade response and enhance subsequent repair activity, thereby limiting the infarct size. The mechanism is primarily associated with a large number of miR-182 transported in these exosomes, which regulate the phenotypic transition of macrophages *via* the TLR4/NF- κ B pathway (Zhao J. et al., 2019). These studies suggest that exosomes of different cellular origins that interact with macrophages may be one of the mechanisms by which the myocardium is protected.

NDMV is involved in the anti-inflammatory response as well as the pro-inflammatory response. These EVs have been demonstrated to have a significant anti-inflammatory effect on interacting cells, mainly by reducing the production of activated cytokines, including IL-1 β , TNF- α , IL-6, IL-10, and IL-12 (Byrne and Reen, 2002; Eken et al., 2008; Ren et al., 2008; Eken et al., 2010; Alvarez-Jimenez et al., 2018). Apart from this, neutrophils exert their anti-inflammatory action by secreting EVs, such as NDMVs containing anti-inflammatory miRNAs in foci of inflammation (Youn et al., 2021). At the same time, the administration of neutrophil-derived EVs carrying membrane-linked protein A1 (AnxA1) inhibited inflammation. Overexpression of AnxA1 activated the STAT3 signaling pathway to inhibit neutrophil infiltration during myocardial I/R injury (Dalli et al., 2008; Zhao C. et al., 2019). On the other hand, it has been shown that leukocyte-derived MVs (LMVs) impede the leukocyte inflammatory responses to lipopolysaccharide and stimulate anti-inflammatory cytokine transforming growth factor β 1 (TGF- β 1) secretion (Gasser and Schifferli, 2004). Specifically, membrane-linked protein 1 (an anti-inflammatory protein) expressed on the surface of these particles exerts this effect (Dalli et al., 2008). *In vitro*, the particles are taken up by B cells and monocytes, thereby modulating their activation to an anti-inflammatory phenotype. Such phenomenon mediates the immune cells' anti-inflammatory response in the early phase of acute infarction (Koppler et al., 2006).

Regulatory T (Treg) cells from the thymus or peripheral lymphoid organs are a subtype of T-cells (Richards et al., 2015). Inflammatory cell infiltration and macrophage polarization can be improved through paracrine action and direct contact. It inhibits cardiomyocytic apoptosis and is considered a potential target for reducing the inflammatory response to myocardial I/R injury. Recent studies have indicated that miRNA-181a is associated with immune regulation in cardiovascular disease. Furthermore, Wei et al. showed that miRNA-181a overexpression significantly suppressed inflammatory responses and increased Treg cell ratios by targeting the critical inflammatory transcription factor c-Fos. Additionally, miRNA-181a also attenuated the activation of dendritic cells (DCs) stimulated by oxidized low-density lipoprotein (ox-LDL). It is evident that miRNA-181a delivered by MSC-derived exosomes (MSC-Exos) combines the immunosuppressive effect of miRNA-181a with the cellular targeting ability of MSC-Exos and ultimately exerts a more substantial therapeutic effect on myocardial I/R injury (Wei et al., 2019).

Bone marrow mesenchymal stem cell (BMMSC) transplantation is considered potential therapeutic approach for MI; however, the body's immune response can contribute to significant early death of transplant cells (Karantalis et al., 2014). Increasing evidence points specifically to the BMMSC-derived exosomes have immunomodulatory, anti-inflammatory effects and can be used as an alternative to stem cell transplantation (Moghaddam et al., 2019). The NF- κ B pathway acts as a coordinator in inflammation and inhibiting the NF- κ B pathway prevents ventricular remodeling and cardiac rupture in mice with acute myocardial infarction (Pramanik et al., 2018). EVs of BMMSCs can regulate the inflammatory microenvironment *via* regulating the BCL6/MD2/NF- κ B signaling pathway, thereby delivering miR-302d-3p and attenuating the inflammatory response during cardiac remodeling after AMI (Liu et al., 2022). At the same time, it has also been shown that FNDC5 pretreated BMMSCs can secrete more exosomes that reduce the secretion of pro-inflammatory cytokines by macrophages while increasing the secretion of anti-inflammatory cytokines. MSCs-Exo and FNDC5-MSCs-Exo lessen the infiltration of inflammatory cells in ischemic heart tissue and improve the secretion of pro-inflammatory cytokines, thereby improving the inflammatory response in the heart early after myocardial infarction (Ning et al., 2021).

In addition, adipose mesenchymal stem cell-derived exosomes (ADMSC-Exos) exert anti-inflammatory effects by promoting macrophage M2 polarization and activating the S1P/SK1/S1PR1 signaling pathway to improve the inflammatory response in MI (Deng et al., 2019). Adipose stromal cells (ADSCs) can reduce MI severity (Kim et al., 2017), and ADSCs derived exosomes overexpression miR-126 have previously been shown to reduce inflammatory cytokines secreted by damaged cardiomyocytes (Luo et al., 2017). Furthermore, exosomes from miR-93-5p overexpressing ADSCs further target TLR4 to downregulate the expression of inflammatory cytokines such as IL-6, IL-1 β , and TNF- α in hypoxia-treated cardiomyocytes, thereby reducing autophagy-related proteins (LC3II and Atg7) expression and inflammation-related proteins (TLR4 and NF- κ B p65) in cardiac tissue and ultimately alleviated inflammatory response (Liu D et al., 2018; Pan W et al., 2019).

At the same time, further work in this field of research contributes to paramount observations or considerations to pre-clinical research using tissue engineering or matrices carrying multifunctional MSC-EVs as an alternative therapeutic approach with associated anti-inflammatory or fibrotic benefits. EVs from porcine cardiac adipose tissue-derived MSC (cATMSC) combined with biocompatible cardiac scaffolds are capable of effective delivery in post-infarct myocardial tissue, decreasing expression of inflammatory TNF- α , increasing expression of IL-1ra, blocking IL-1 α / β inflammatory effects and inducing anti-inflammatory IL-10 expression, which in turn attenuates the local inflammatory response in the myocardium. Meanwhile, the reduction of inflammatory mediators modulates the expression and activity of TGF- β , metalloproteinases, and other pro-fibrotic mediators, thereby reducing collagen synthesis and deposition and myocardial vascularization and fibrosis (Monguio-Tortajada et al., 2021; Monguio-Tortajada et al., 2022). Yet producing effective formulations of EVs and meeting the requirements for standardized clinical-grade biomanufacturing and regulatory, large-scale, GMP-compliant issues for EV production and clinical application remain a huge challenge (Courageux et al., 2022; Soler-Botija et al., 2022).

5.2 Mechanism of extracellular vesicles involved in scar repair after myocardial infarction

5.2.1 Mechanism of extracellular vesicles involved in promoting scar repair

CFs are the primary effector cells in the scar repair phase of myocardial fibrosis, which transdifferentiate to MFBs in response to different stimuli, secrete collagen, and are the primary source of ECM. The scar repair phase involves several processes such as pro-angiogenesis and pro-differentiation of CFs into MFBs. CircUbe3a from M2 macrophage-derived exosomes promotes the proliferation or migration of CFs, and transdifferentiation *via* the miR-138-5p/RhoC axis, thereby mediating post-infarction myocardial fibrotic repair (Wang Y. et al., 2021). On the other hand, EVs generated from cardiosphere-derived cells (CDC-EVs) can polarize M1-M ϕ to a proangiogenic phenotype dependent on upward regulation of arginase to contribute to scar repair (Mentkowski et al., 2020). Moreover, several studies suggested that the expansion and activation of CD4⁺ T-cells in the heart associated with post-infarction remodeling. Exosomes generated from activated CD4⁺ T-cells carrying miR-142-3p, which mediates local MFBs activation through the miR-142-3p-WNT signaling cascade, secreting TGF- β , and regulating the APC-GSK- β -linked protein signaling cascade to confer a profibrotic effect. Aggravates post-ischemic myocardial fibrosis (Cai et al., 2020). Though EVs affect the post-infarction microenvironment by transporting miRNAs, cardiovascular risk factors, including sex, age, genetics, smoking, overweight, and obesity, affect apoptosis and activation of the mother cells of EVs, which in turn affect the physicochemical properties of these vesicles. Thus, most miRNAs are transported by cell-free and EV-free.

Cardiomyocyte-derived exosomes facilitate the formation of myocardial fibrosis *via* cardiomyocyte-fibroblast interactions.

Previous study demonstrated that cardiomyocyte-derived exosomes enriched with miR-208a can promote the proliferation and differentiation of fibroblast toward MFBs (Yang et al., 2018). The molecular analysis then showed that miR-208a blocked the expression of Dyrk (bispecific tyrosine phosphorylation kinase), thereby upregulating fibrotic gene expression *via* the nuclear translocation of NFAT. Additionally, miR-217 was enriched in exosomes derived from cardiomyocyte and enhanced fibroblast activity by targeting the PTEN signaling cascade under pressure overload (Nie et al., 2018). At the same time, WNT3a in exosomes containing WNT protein can effectively trigger WNT/ β -catenin signaling. In the presence of TGF- β , WNT3a can effectively enhance the pro-fibrotic response of human CFs (Dzialo et al., 2019). In addition, human umbilical cord mesenchymal stem cell-derived exosomes (hUCMSC-Exos) can promote CFs into FMBs and secrete collagen into inflammatory environment, thereby promoting cardiac fibrotic scar repair (Shi et al., 2019).

During the fibrotic repair phase of post-myocardial infarction, the precursors of ECs such as late endothelial progenitor cells (EPCs) can repair the heart through paracrine mechanisms. Several studies have demonstrated that human endothelial histocyte-derived exosomes enhance the proliferation and angiogenesis of CFs *in vitro* (Ke et al., 2017). In addition, cardiac mesenchymal stem cells (C-MSCs), a novel subpopulation of MSCs derived from cardiac tissue, have been shown to play an irreplaceable role in cardiac regeneration. Notch signaling is thought to contribute to cardiac repair after myocardial injury. EVs derived from Notch1 overexpressed C-MSCs are essential in promoting angiogenesis and cardiac fibroblast proliferation, preventing cell death, and facilitating myocardial fibrosis repair (Xuan et al., 2020).

Furthermore, in a MI model, it has been demonstrated that ABs released by transplanted MSCs can enhance angiogenesis and improve recovery of cardiac function by regulating macroautophagy/autophagy in recipient ECs. BMMSCs undergo extensive apoptosis immediately after transplantation and release ABs phagocytosed by recipient ECs. Then, these ABs activate lysosomal function and promote the expression of TFEB (transcription factor EB), the master gene for lysosomal biogenesis and autophagy in ECs. Notably, TFEB is involved in the expression of autophagy-related genes in ECs and boosts angiogenesis and recovery of cardiac function after MI (Liu H et al., 2020). It has been shown that myocardial endocytic target and silence CDIP1 (cell death involved p53 target 1) gene *via* exosomal miRNA-21-5p, thereby downregulating activated caspase-3, which in turn inhibits apoptosis of ECs under ischemic and hypoxic conditions and facilitates post-MI angiogenesis and regeneration, which in turn involved in post-infarction scar repair (Liao et al., 2021).

5.2.2 Mechanism of extracellular vesicles involved in the anti-scar repair

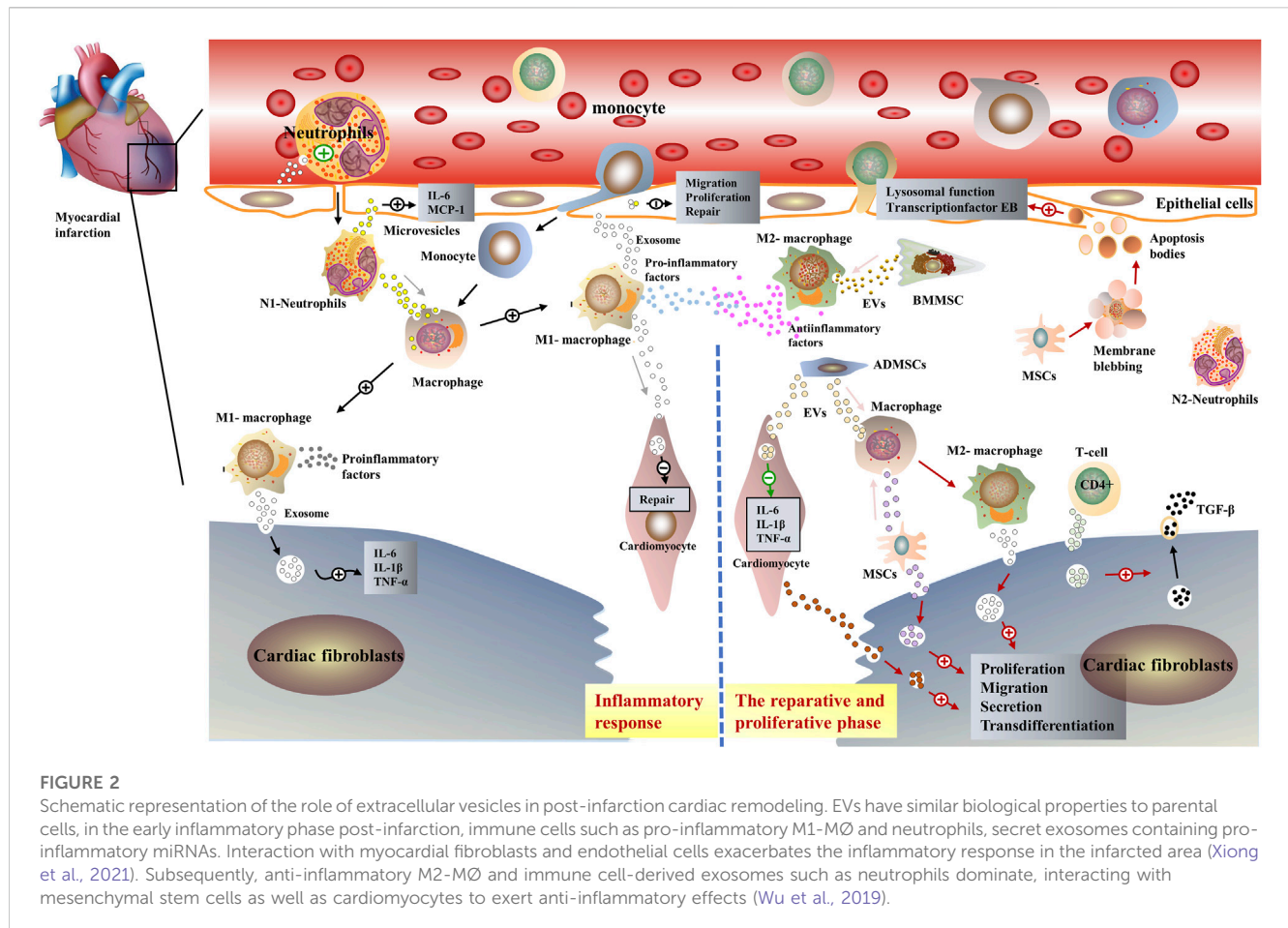
Scar repair is essential for maintaining cardiac structure in the early stage of MI, which involves angiogenesis and MFBs activation. Fibrotic scar repair is a double-edged sword, inadequate repair can contribute to cardiac rupture, and excessive and sustained fibrotic repair can lead to ventricular systolic-diastolic dysfunction and ultimately left heart failure (Ma et al., 2013; Ma et al., 2017).

Harmonizing the dynamic balance of pro-and anti-fibrosis is crucial for post-infarction cardiac remodeling.

BMMSC-derived M1 macrophage exosomes inhibit angiogenesis and myocardial regeneration following MI by activating the MALAT1/miR-25-3p/CDC42 signaling pathway as well as MEK/ERK axis, thereby impeding scar repair (Chen et al., 2021). These EV-loaded miRNAs are significant for the pathological process of fibrosis in addition to their involvement in the early anti-inflammatory response to cardiac remodeling. Previous studies have indicated that MSCs derived EVs containing miR-212-5p may ameliorate myocardial fibrosis after MI by inhibiting the NLR5/VEGF/TGF- β 1/SMAD signaling pathway, as evidenced by reducing the expression of TGF- β 1, collagen type I (Col I), and α -smooth muscle actin (α -SMA) (Wu et al., 2022). At the same time, MSC-derived extracellular vesicles (MSC-EVs) were also able to inhibit hypoxia-mediated cardiac fibroblast activation, in turn, exerting antifibrotic effects (Chen et al., 2014), secondly, these vesicles could be internalized by cardiac stem cells. Treatment of CSCs with MSC-Exo in an AMI model enhanced cell proliferation implantation and capillary density and reduced fibrotic area (Zhang et al., 2016). On the other hand, miR-199a-3p in exosomes released by BMMSCs targeted to the region of cardiac injury inhibited mTOR activation in myocardial tissue and subsequently activated induction of autophagy. Excessive autophagy eliminated the fibrotic fraction in the heart and attenuated tissue damage (Fan C et al., 2022). Concurrently, these exosomes can promote premature senescence of MFBs *in vitro* and enhance microvascular regeneration under stress, and ultimately improve cardiac function *via* modulating platelet-derived growth factor receptor- β (PDGFR- β) (Chen J et al., 2020; Wang et al., 2021a). Adipose-derived mesenchymal stem cells secreted miR-671-containing exosomes that attenuated myocardial fibrosis by inhibiting the TGFBR2/Smad2 signaling pathway (Wang et al., 2021b).

Cardiovascular progenitor cells (CVPCs) derived from human pluripotent stem cells are a promising source of myocardial repair with EVs mediating intercellular communication. Intramyocardial injection of HCVPC EVs into AMI mice on day 28 after MI significantly improved cardiac function and attenuated fibrosis while improving vascularization and cardiomyocyte survival in the marginal zone (Wu et al., 2020). In addition, it has been shown that ISX-9 is a small molecule with pro-survival, antioxidant, and regenerative properties, and that induction of CPCs by ISX-9 is an attractive cell-based cardiac regeneration therapy, and that these EVs contain a unique set of bioactive miRNAs, among which miR-373 has anti-fibrotic substantial effects. These new discoveries have important implications for preventing post-myocardial infarction remodeling (Xuan et al., 2019).

In preclinical studies, EVs derived from Cardiosphere-derived cells (CDCs) facilitated cardiogenesis and angiogenesis, ameliorated fibrosis, modulated immune responses, and generally improved cardiac function (Marban, 2018). MSCs mediate their disease-modifying biological activity by secreting paracrine factors, including EVs. Furthermore, like CDC-EVs, MSC-EVs contain a plethora of RNA species, like miRNAs. For instance, MSC-EVs from bone marrow-derived MSCs are enriched in miR-22 and have anti-fibrotic and anti-apoptotic properties in a mouse model of AMI



(Feng et al., 2014). This demonstrates a significant role in regulating fibrotic repair after infarction by intervening in extracellular vesicle-loading cargos (Figure 2).

6 Therapeutic strategies for targeting extracellular vesicles in regulating cardiac remodeling after myocardial infarction

6.1 Engineered extracellular vesicle-based therapeutics

Although native EVs make great achievements in preclinical research, realizing the precisely controllable release of these cargos for the target regions remains a challenge (Fan Z et al., 2022). Accordingly, to enhance the ability of EVs to load cargos, improve the controllability and precision of vesicular cargos released in the infarct region, further attenuate inflammation and maintain the homeostasis of fibrotic scar repair following MI, researchers distill experiences and inspirations from nano materials-based drug delivery research and engineer modification of extracellular vesicles, including bioengineering, chemical engineering, and physical engineering (Man et al., 2020; Nasiri Kenari et al., 2020; Wu et al., 2021). Targeted manipulation of the extracellular vesicles'

genes and proteins is an essential part of bioengineering, which can direct EVs to a specific tissue or organ and increase their utilization. For instance, EVs derived from cardiosphere-derived stem cells are labeled by infarcted heart targeting peptide, so-called "cardiac-homing peptide", the vesicles recruit to the infarct area and improve cardiac function by attenuating cardiac fibrosis, inducing angiogenesis, and promoting cardiomyocyte proliferation (Vandergriff et al., 2018). Meanwhile, there are also several studies demonstrated that HEK 293 cells transfecting with vectors encoding CTP-Lamp2b can derive exosomes with cardiac-targeting peptide (CTP)-Lamp2b on their membrane (CTP-Exo) (Kim et al., 2018), these vesicles can transport curcumin directly to the heart and improve cardiac function by regulating the PTEN/Akt/Bax signaling pathway associated with cardiomyocyte apoptosis via upregulating miR-144-3p (Kang et al., 2021). Furthermore, MSCs-derived exosomes modified with ischemic myocardium-targeting peptide CSTSMLKAC (IMTP) can specifically target ischemic myocardium, attenuating inflammation and apoptosis, promoting angiogenesis, and restoring cardiac function (Wang et al., 2018). On the other hand, Tian et al. found that exosomes (Hypo-Exo) derived from bone marrow mesenchymal stem cells (BMMSCs) under hypoxic conditions have excellent protections against ischemic diseases. The combination of Hypo-Exo with "CSTSMLKAC" peptide, an ischemic myocardial targeting peptide, significantly reduced

TABLE 1 Specific application and mechanism of miRNAs carried by EVs in post-infarction cardiac remodeling.

Stages	Species	Origin	Experimental model	Mechanism	References
Pro-inflammation	miR-155	M1 MØ - Exos	C57BL/6 J male mice	TNF- α ↑	Wang et al., 2017; Liu S et al. (2020)
			MI model	IL-1 β ↑	
				CCL2↑	
	miR-328-3p	CMs-Exos	BALB/C nude mice	Activate Caspase facilitate apoptosis	Huang P et al. (2021)
			MI model		
	miR-146a	ADSCs-Exos	SD male rat	EGR1↓	Pan W et al. (2019)
			MI model		
	miR-375	MØ -Exos	C57BL/6J male mice	Stimulate M1 MØ polarization	Garikipati et al. (2017)
			MI model		
Anti-inflammatory	miR-25-3p	MSC-Exos	BALB/c male mice	Disinhibit the expression of SOCS3	Peng et al. (2020)
			I/R injury model		
	miR-126	ADSC- Exos	SD male rat	IL-1 β ↓	Luo et al. (2017)
			MI model	IL-6↓	
				TNF- α ↓	
	miR-19a	hUCMSC-Exos	SD male rat	IL-1 β ↓	Huang P et al. (2020)
			AMI model	IL-18↓	
				JNK3/caspase-3↓	
	miR-181a	MSC-Exos	C57BL/6 male mice	Increase Tregs polarization	Wei et al. (2019)
			I/R injury		
	miR-181b	CDCs-Exos	WKY female rat	NF- κ B ↓	de Couto et al. (2017)
			I/R injury	Polarize MØ to an M2 phenotype	
	miR-23a-3p	hUCMSC-Exos	C57BL/6J male mice	Inhibit ferroptosis	Song et al. (2021)
			AMI		
	miR-22	MSC-Exos	C57BL/6J male mice	Reduce apoptosis	Feng et al. (2014)
			MI model		
	miR-182	MSC-Exos	C57BL/6 mice	Polarize MØ to an M2 phenotype	Zhao et al. (2019b)
			I/R injury		
	miR-93-5p	ADSC-Exos	SD male rat	TLR4/NF- κ B ↓	Liu J et al. (2018)
			AMI model		
	miR-129-5p	BMSCs-Exos	C57BL/6J male mice	Inflammatory cytokines↓	Wang et al. (2022)
			MI model		
	miR-24-3p	UMSC-Exos	—	NF- κ B ↓	Zhu et al. (2022)
				Polarize MØ to an M2 phenotype	
	miR-200b-3p	MSCs-EVs	C57BL/6J male mice	BCL2L1↓	Wan et al. (2022)
			MI model		
	miR-223	hUCMSCs-EVs	SD male rat	TNF- α ↓	Yang et al. (2022)
			MI model	IL-6↓	
				IL-1 β ↓	
	miR-1271-5p	M2 MØ -Exos	C57BL/6J male mice	SOX6↓	Long et al. (2021)

(Continued on following page)

TABLE 1 (Continued) Specific application and mechanism of miRNAs carried by EVs in post-infarction cardiac remodeling.

Stages	Species	Origin	Experimental model	Mechanism	References
	miR-302d-3p	MSC-EVs	AMI model		Liu et al. (2022)
			C57BL/6J male mice	TNF- α ↓	
			AMI model	IL-6↓	
Pro-fibrosis	miR-142-3p	CD4 ⁺ T cell	C57BL/6J male mice MI model	Activate myofibroblast	Cai et al. (2020)
		Exosome			
	miR-218-5p	EPC-Exos	SD rat	Promote CFs proliferation	Ke et al. (2021)
			MI model		
	miR-363-3p	EPC-Exos	SD rat	Promote CFs proliferation	Ke et al. (2021)
			MI model		
	miR-494-3p	DC-Exos	C57BL/6J male mice MI model	Promote angiogenesis	Liu Y et al. (2021)
	miR-146a	ADSC-Exos	SD male rat	EGR1↓	Pan J et al. (2019)
			MI model		
	miR-92a	CMS-Exos	C57BL/6J male mice MI model	α -SMA↑ periostin↑	Wang X et al. (2020)
	miR-142-3p	CD4 ⁺ T-cellT-cell	C57BL/6J male mice MI model	β -catenin↑	Cai et al. (2020)
		Exosome		Col1a1↑	
				Col3a1↑	
				α -SMA↑	
Anti-fibrosis	miR-155	M1 MØ - Exos	C57BL/6J male mice MI model	Inhibit the proliferation of CFs	Wang et al. (2017)
	miR-126	ADSC-Exos	SD male rat	Inhibit the tissue fibrosis	Luo et al. (2017)
			MI model		
	miR-22	MSC-Exos	C57BL/6J male mice	Reduce fibrotic area	Feng et al. (2014)
			MI model		
	miR-1246	EPC-Exos	SD male rat	Change of Fibroblasts to Endothelial Cells	Huang Y et al. (2021)
			MI model		
	miR-1290	EPC-Exos	SD male rat	Changes of Fibroblasts to Endothelial Cells	Huang J et al. (2021)
			MI model		
	miR-19a/19b	BMMSC-Exos	SD male rat	Reduce fibrotic area	Wang S et al. (2020)
			MI model		
	miR-29b-3p	BMSCs-Exos	SD male rat	Inhibit the proliferation, migration, and differentiation of CFs	Zheng et al. (2022)
			MI model		
	miR-106a-363	iCMs-EVs	SCID mice	Repressing Notch3	Jung et al. (2021)
			MI model		
	miR-4732-3p	MSC-EVs	Nude rats	Inhibit MFBs differentiation and the production of extracellular matrix	Sanchez-Sanchez et al. (2021)
			MI model		
	miR-212-5p	MSC- EVs	C57BL/6J male mice	COL I↓	Wu et al. (2022)
			MI model	α -SMA↓	
	miR-223	hUCMSCs-EVs	SD male rat	COL I↓	Yang et al. (2022)

(Continued on following page)

TABLE 1 (Continued) Specific application and mechanism of miRNAs carried by EVs in post-infarction cardiac remodeling.

Stages	Species	Origin	Experimental model	Mechanism	References
			MI model	COI III↓	
	miR-302d-3p	MSC- EVs	C57BL/6J male mice	Inhibit MFBs differentiation	Liu et al. (2022)
			AMI model		

apoptosis after MI *via* targeting Exo in ischemic heart regions, suggesting that IMT-Exo could be a novel drug carrier to enhance the specificity of drug delivery in ischemic diseases (Zhu et al., 2018). These findings demonstrated that the delivery of various cargoes in cardiac-targeted peptide-modified exosomes may be a promising strategy for MI treatment.

Alginate hydrogel possesses favorable biocompatibility and has been widely applied in tissue engineering and cellular engineering (Ruvinov and Cohen, 2016). Researchers have constructed an injectable conductive hydrogel to bind exosomes derived from human umbilical cord mesenchymal stem cells and injecting the hydrogel into injured rat hearts effectively prolongs the retention time of exosomes in the ischemic myocardium (Zou et al., 2021). Dendritic cell-derived exosomes (DEX) can activate Treg cells (Sakaguchi, 2004), and contribute to an early shift of macrophage subsets from an inflammatory M1 phenotype towards a reparative M2 phenotype after MI, thereby improving the immune microenvironment in the infarct area and offering the possibility of improving post-infarcted cardiac function. However, they have a short retention time and the therapeutic effects are transient. Accordingly, Researchers have developed a new drug delivery system that contains a sodium alginate hydrogel that continuously releases DEX, an approach that significantly increases therapeutic efficacy (Zhang et al., 2021). In addition, some investigators have also found that by combining the advantages of natural EVs and novel nanomaterials, engineered vesicles with biofilm/synthetic material chimeras were successfully constructed to enhance further the targeting and loading of natural vesicles with therapeutic molecules. Jin et al. found that engineered vesicles can effectively load microRNA-21 or curcumin for targeted delivery to achieve precise release of therapeutic molecules in macrophages, modulate the phenotypic transformation of macrophages, and effectively control inflammation levels (Andaloussi et al., 2013) (Table 1).

6.2 Drug pretreatment of extracellular vesicles to enhance its therapeutic effect

In addition to the engineered modification of EVs, several types of research focus on the drug regulation of EVs and make several significant findings.

Numerous experimental and clinical studies demonstrate that myocardium is protected from ischemic left ventricular remodeling by long-term antagonism of the purinergic GPCR P2Y₁₂ (Roubille et al., 2012; Nanhwan et al., 2014; Vilahur et al., 2016; Bansilal et al., 2018). Ticagrelor is a selective and reversible P2Y₁₂ receptor antagonist that induces exosome release from CPCs, resulting in

the release of anti-apoptotic HSP70. Simultaneous continuous pretreatment of cardiomyocytes with HCPC derived exosomes exposed to low-dose of ticagrelor attenuates hypoxia-induced apoptosis *via* acute phosphorylation and activation of ERK42/44, resulting in myocardial protection effect (Casieri et al., 2020). This research has clinical implications for enhancing the endogenous exosomal anti-hypoxic response and prevent the heart from ischemic injury by developing new non-invasive pharmacological approaches. In addition, atorvastatin pretreatment has been demonstrated to enhance the function of BMSC-derived exosomes in angiogenesis, cardiomyocyte protection, and long non-coding RNA H19 (lncRNA H19) is a mediator of regulating miR-675 expression and promoting atorvastatin pretreatment MSC-Exo (MSC^{ATV}-Exo) effect in angiogenesis. Importantly, lncRNA H19 and its downstream signaling pathways mediate the cardioprotective effects of MSC^{ATV}-Exo (Huang L et al., 2020). Other studies have shown that sulpiride/valsartan can improve cardiac function and ameliorate myocardial fibrosis by downregulating miR-181a in exosomes in a rodent chronic MI model (Vaskova et al., 2020).

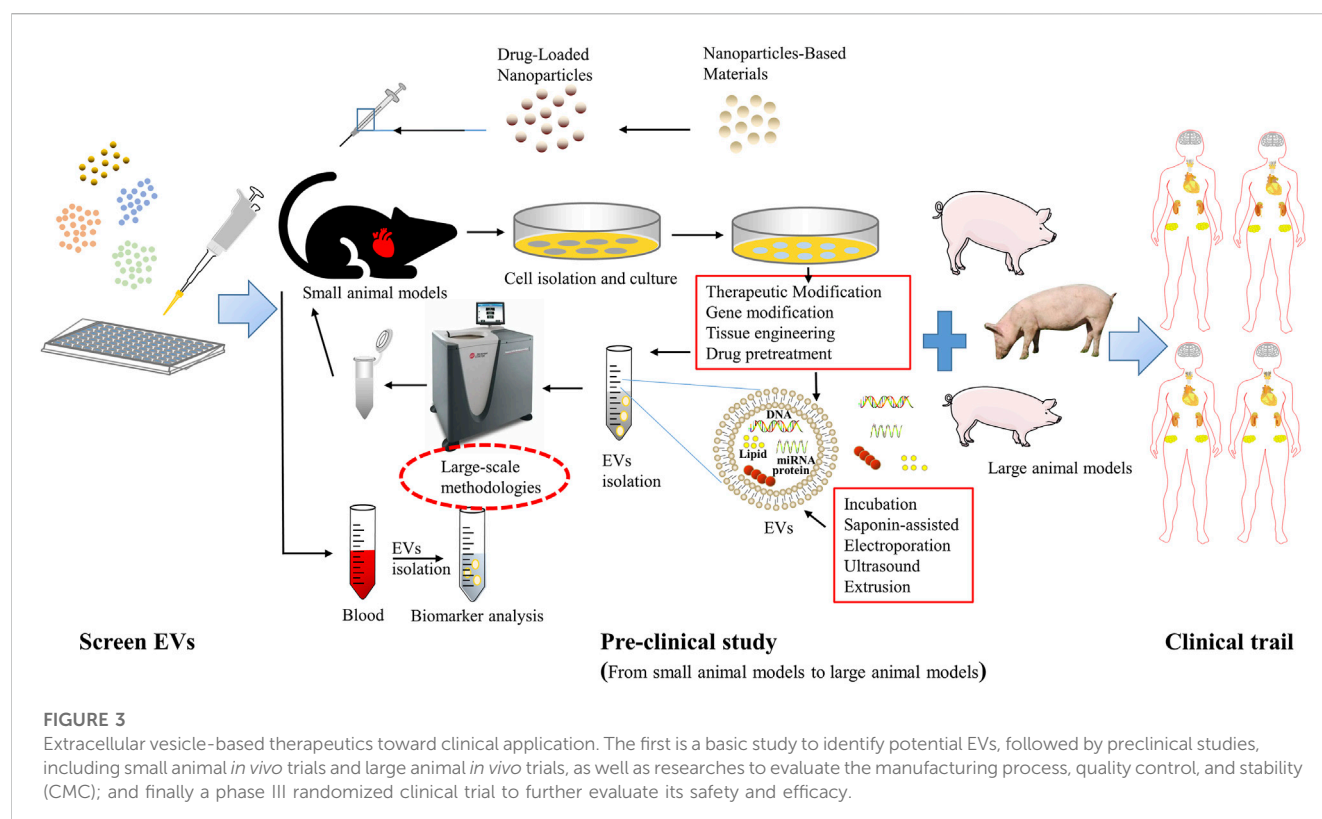
TCM has been practiced and developed for thousands of years. In recent decades, considerable progress and achievements have been made in studying the mechanism of TCM using modern molecular biology techniques (Zhou et al., 2016). One of the hotspots is the research on the targeting and regulation of EVs using TCM, such as the regulation of stem cells and immune cells. Stem cell transplantation is a promising therapeutic alternative to facilitate myocardial repair following MI, however, its clinical application is limited by the low preservation and survival rates of implanted cells (Feyen et al., 2016). In recent years, new strategies have been developed, including combined cell therapy with BMSC exosomes, which have been demonstrated that such strategies have anti-apoptotic, anti-inflammatory, and pro-angiogenic effects. Transplantation of exogenous vesicles into the ischemic heart within 30 min of MI significantly modulates the ischemic environment, including decreasing inflammatory IL-6 and TNF- α , enhancing SDF-1 expression and MSC survival. Additionally, several studies have demonstrated that combined pretreatment with hypoxia and the herbal compound Tongxinluo can effectively achieve better performance in facilitating cardiac repair through increasing CXCR4 expression, which provides new insights into stem cell therapy for cardiac rehabilitation (Xiong et al., 2022). Furthermore, Ruan et al. suggested that Suxiao Jiuxin pills modulate myocardial MSC-Exos, causing structural genetic chromatin remodeling in recipient cardiomyocytes and accelerating cardiomyocyte propagation (Ruan et al., 2018). Accordingly, researchers rolled out several studies to enhance EVs targeting, thus making them better delivery vehicles (Veerman et al., 2019).

7 Clinical applications and regulation of extracellular vesicles

Clinical applications of EVs are novel therapeutic modalities, including vaccines, diagnostic criteria, and drug delivery. The idea of using EVs as anti-tumor vaccines originated in the last century. Basic studies suggested that DEX can promote T-cell dependent antitumor effect, and phase I clinical trials (to vaccinate peptide-pulses DEX for patients with cancer) demonstrated that the feasibility and safety of inoculation of DEX (Escudier et al., 2005; Morse et al., 2005). Immediately after, Sophie et al. developed a second-generation DEX with enhanced immunostimulatory properties and produced large-scale IFN- γ -DEX vaccines while conducting phase II clinical trials (NCT01159288) under the guidance of Institut Gustave Roussy in France, in which DEX was administered to patients with non-small cell lung cancer with the aim of increasing progression-free survival (Viaud et al., 2011; Lener et al., 2015). In respiratory diseases, preclinical studies have established that MSC-Exos can ameliorate most of the pathological changes caused by lung infections. And a clinical study (NCT04602104) for treating acute respiratory distress syndrome (ARDS) with MSC-Exos Nebulizer is currently recruiting patients, this study explores a new approach to treat ARDS and assesses its safety by administering human mesenchymal stem cell exosomes (hMSC-Exos) aerosol inhalation to patients. It has also been shown that ExoFlo™, derived from allogeneic bone marrow mesenchymal stem cells, can reconstitute immunity, downregulate cytokine storm and restore oxygenation in patients with severe COVID-19,

demonstrating the promise of ExoFlo for the treatment of COVID-19 (Sengupta et al., 2020). In renal disease, intra-arterial and intravenous administration of MSC-Exos to patients with grade III-IV chronic kidney disease in phase II/III clinical trials was shown to ameliorate inflammation and renal function. No adverse events associated with MSC-Exos administration were observed in subjects during the 1-year follow-up period (Nassar et al., 2016; Guo et al., 2020). In cardiovascular system diseases, a phase II randomized clinical trial of intravenous ischemia-tolerant MSCs (itMSCs) in patients with non-ischemic cardiomyopathy showed that single-dose of intravenous itMSCs was safe and well tolerated compared with controls, increasing the patient's 6-min walk distance while eliciting systemic immunomodulatory effects associated with improved LVEF (Butler et al., 2017). Examples of these clinical trials showed the great therapeutic potential of EVs.

The complex properties of EVs make their specification and characterization difficult. Thus, in addition to controlling the final product by specification and characterization, the quality of EVs must be ensured by quality control of the raw material and regulation of the manufacturing process (Tsuchiya et al., 2022). EV manufacturing must be performed in accordance with Good Practice (GxP) regulations, which are a collection of quality guidelines and regulations designed to ensure that biological/medical products are safe, meet their intended use and follow quality processes in manufacturing, control, storage and distribution (Good Manufacturing, Good Laboratory, Good Distribution, Good Clinical, Good Scientific Practice) (Soekmadji et al., 2020). The biological activity of EV used as a therapeutic agent must be tested in a qualified bioassay, called a



“potency assay” (Lener et al., 2015). EVs are subject to detailed guidelines for the clinical application and manufacture of novel biomedical products in the EU. In Australia, the government’s Therapeutic Goods Administration (TGA) office provides rules and guidelines related to the manufacture and use of therapeutic agents often adopted in EU rules. In the United States, EV-based therapeutics for human use are regulated by the Center for Biologics Evaluation and Research (CBER) within the Food and Drug Administration (FDA) (Lener et al., 2015). (Figure 3).

8 Conclusion

Post-infarction cardiac remodeling is a complex and diverse pathological process consisting of two main phases: inflammatory response and fibrotic scar repair. Sudden and substantial cardiomyocyte death triggers a vigorous inflammatory response and subsequently activated MFBs, which secrete collagen, leading to myocardial fibrosis and consequent scar formation. Moderate myocardial fibrosis plays a critical protective role, maintaining the structural integrity of the chambers and preventing cardiac rupture, while persistent myocardial fibrosis can lead to cardiac stiffness and diastolic dysfunction, eventually causing HF. The search for new measures and approaches that can attenuate the post-infarction inflammatory response as well as maintain the dynamic balance of fibrosis repair is particularly critical. EVs of different cellular origins play a dual role in the early post-infarction inflammatory response and the subsequent repair of fibrotic scars. During the early inflammatory phase, these vesicles are involved in both pro-inflammatory and anti-inflammatory responses, and therefore, by achieving effective control of the release of pro-inflammatory vesicles and the delivery of their cargoes is of great importance to contain the early adverse persistent inflammatory response after infarction. At the same time, these vesicles play a dual role of pro-fibrotic and anti-fibrotic effects in the subsequent fibrotic scar repair phase, a property that also shows the importance of maintaining the dynamic balance of post-infarction fibrotic repair by modulating EVs. Currently, research on targeted regulation of EVs in cardiovascular diseases has focused on drug pretreatment as well as engineered modifications.

Research associated with the intercellular delivery of functional nucleic acids and proteins by EVs has demonstrated their advantages as cargo carriers, such as low immunogenicity, high stability and biocompatibility, long cycle life, and ability to cross the blood-brain barrier. These properties make EVs an important endogenous carrier for delivering therapeutic drugs, however, the current research on extracellular vesicle drug delivery is mainly focused on cancer and neurological related diseases, while preclinical studies on cardiovascular diseases are rare. The reasons for this are limited by the isolation of endogenous vesicles, storage, mass production techniques, low efficiency of targeted drug delivery and cellular uptake, on the one hand, and the unknown biological distribution of drug-loaded vesicles in the individuals treated with them, on the other hand, which may lead to difficult challenges in managing the use of exosomes in standardized methods

related to their isolation, quantification and outcome analysis. Currently, EVs were isolated by Polymer precipitation, Differential ultracentrifugation, Field-flow fractionation, and Gradient density ultracentrifugation. These methods always lacking in yield and purity. Distinct purification methods of EVs exist in relation to the physical or molecular characteristics of isolated EVs preparations and affect the results of downstream analysis. The best separation strategy for EVs is by applying different methods of isolation in order to achieve a desirable recovery and purity and report in detail about standardized approaches for isolation, this research is particularly important, especially in the context of promising clinical applications of EVs (Clos-Sansalvador et al., 2022). Second, drug loading cannot disrupt the membrane structure and content of EVs, and finding effective ways to load therapeutic drugs into these EVs remains a great challenge. Although, the use of EVs as drug delivery carriers is still in the developmental stage. However, with a deeper understanding of the physiological properties of EVs, improved isolation and drug delivery techniques for EVs, the understanding and conclusion of exosome-loaded drugs in other diseases (cancer, hepatitis, Parkinson’s disease, etc.) will help develop or expand future therapeutic approaches for post-infarction cardiac remodeling, mitigate the inflammatory response during post-infarction cardiac remodeling, precisely regulate the dynamic balance between pro- and anti-fibrosis, and ultimately the transition from basic research to clinical applications.

Author contributions

PC: Writing original draft and designing the figures. XW and QL: Revising the manuscript and visualization. TY: Revising the manuscript and providing good ideas. HZ and HQ: Conceiving and designing this review.

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Glossary

AMI acute myocardial infarction

EVs Extracellular vesicles

HF heart failure

MI myocardial infarction

I/R ischemia-reperfusion

DAMPs damage-associated molecular patterns

CFs cardiac fibroblasts

MSCs mesenchymal stem cells

CPCs cardiac progenitor cells

MVs microvesicles

ABs apoptotic bodies

RIPK3 receptor-interacting protein kinase three

RIPK1 receptor-interacting protein kinase 1

PRRs pattern recognition receptors

TNF tumor necrosis factor

IL-1 β interleukin 1 β

IL-6 interleukin 6

MCP-1 monocyte chemotactic protein-1

ATP adenosine triphosphate

NF- κ B nuclear factor κ B

ECs endothelial cells

PSCs progenitor stem cells

MFs myofibroblasts

TGF- β transforming growth factor β

ECM extracellular matrix

ILVs intraluminal vesicles

MVBs multivesicular bodies

PS phosphatidylserine

ESCRT endosomal sorting complex required for transport

CAMs cell adhesion molecules

CCL3 macrophage inflammatory protein 1 α

IL-1b interleukin 1b

IL-12a interleukin 12a

TNF- α tumor necrosis factor

IL-10 interleukin 10

CCL-2 chemokine (C-C motif) ligand 2

NDMVs neutrophil-derived MVs

NDTRs neutrophil-derived trails

AnxA1 membrane-linked protein A1

LMVs leukocyte-derived MVs

TGF- β 1 transforming growth factor β 1

DCs dendritic cells

ox-LDL oxidized low-density lipoprotein

BMMSC Bone marrow mesenchymal stem cell

ADSCs adipose stromal cells

Dyrk bispecific tyrosine phosphorylation kinase

MSC-Exos MSC-derived exosomes

hUCMSC-Exos human umbilical cord mesenchymal stem cell-derived exosomes

EPCs endothelial progenitor cells

CD31 cluster of differentiation 31

TCM traditional chinese medicine

C-MSCs cardiac mesenchymal stem cells

TFEB transcription factor EB

CDIP1 cell death involved p53 target 1

α -SMA α -smooth muscle actin

Col I collagen type I

MSC-EVs MSC-derived extracellular vesicles

PDGFR- β platelet-derived growth factor receptor- β

HPSC human pluripotent stem cell

CVPCs cardiovascular progenitor cells

CHP cardiac homing peptide

CDCs cardiosphere-derived cells

PCs progenitor cells

iCMs human iPSC-derived cardiomyocytes

IMTP ischemic myocardium-targeting peptide

AMSC-Exos adipose mesenchymal stem cell-derived exosomes

EGR1 growth response factor 1

M1-M $\emptyset$ M1 macrophages

M2-M $\emptyset$ M2 macrophages

ADSCs-Exos exosomes secreted by adipose-derived stem cells

CDCs-Exos exosomes secreted by Cardiosphere-derived cells

EPC-Exos exosomes secreted by endothelial progenitor cells

BMMSC-Exos exosomes derived from bone marrow mesenchymal stem cells

DC-Exos dendritic cells derived exosomes

CMs-Exos cardiomyocytes-derived exosomes

UMSC-Exos umbilical cord MSC-derived exosomes

DEX dendritic cell-derived exosomes

ARDS acute respiratory distress syndrome

hMSC-Exos human mesenchymal stem cell exosomes

itMSCs ischemia-tolerant MSCs



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Ginaton reduces M1-polarized macrophages in hypertensive cardiac remodeling via NF- κ B signaling

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Introduction: Macrophages play a critical role in cardiac remodeling, and dysregulated macrophage polarization between the proinflammatory M1 and anti-inflammatory M2 phenotypes promotes excessive inflammation and cardiac damage. Ginaton is a natural extract extracted from *Ginkgo biloba*. Because of its anti-inflammatory properties, it has long been used to treat a variety of diseases. However, the role of Ginaton in modulating the diverse macrophage functional phenotypes brought on by Ang II-induced hypertension and cardiac remodeling is unknown.

Methods: In the present study, we fed C57BL/6J mice in the age of eight weeks with Ginaton (300 mg/kg/day) or PBS control, and then injected Ang II (1000 ng/kg/min) or saline for 14 days to investigate the specific efficacy of Ginaton. Systolic blood pressure was recorded, cardiac function was detected by echocardiography, and pathological changes in cardiac tissue were assessed by histological staining. Different functional phenotypes of the macrophages were assessed by immunostaining. The mRNA expression of genes was assessed by qPCR analysis. Protein levels were detected by immunoblotting.

Results: Our results showed that Ang II infusion significantly enhanced the activation and infiltration of macrophages with hypertension, cardiac insufficiency, myocardial hypertrophy, fibrosis and M1 phenotype macrophages compared with the saline group. Instead, Ginaton attenuated these effects. In addition, *in vitro* experiments showed that Ginaton inhibited Ang II-induced activation, adhesion and migration of M1 phenotype macrophages.

Conclusion: Our study showed that Ginaton treatment inhibits Ang II-induced M1 phenotype macrophage activation, macrophage adhesion, and migration, as well as the inflammatory response leading to impaired and dysfunctional hypertension and cardiac remodeling. Ginaton may be a powerful treatment for heart disease.

KEYWORDS

ginaton, inflammation, hypertension, cardiac remodeling, macrophage polarization

Introduction

Cardiovascular disease is a major public health concern worldwide (Benjamin et al., 2019). Hypertension is the leading cause of cardiovascular disease, affecting approximately 1 billion people globally (Mouton et al., 2020). The prevalence of hypertension is increasing by more than 10% every year (Houston et al., 2011). Recent studies have shown that chronic high blood pressure can lead to cardiac hypertrophy, inflammation, fibrosis, and, ultimately, heart failure (Peet et al., 2020). Therefore, therapeutic strategies aimed at alleviating hypertension and preventing myocardial hypertrophy, inflammation, and fibrosis may help prevent hypertension-induced cardiac insufficiency or heart failure (Berthiaume et al., 2012; Dodd et al., 2012; Cavallera et al., 2014). During the development of hypertension, chronic pressure overload is closely associated with myocardial hypertrophy and inflammation, increasing the risk of heart failure and sudden death (DeBerge et al., 2019). Angiotensin II (Ang II) is a bioactive octapeptide derived from the renin-angiotensin-aldosterone system. Elevated levels of Ang II and its receptor type 1 angiotensinogen (AT1) promote cardiac remodeling and play an important role in the occurrence and development of hypertension (Yin et al., 2022). In addition to the known hemodynamic effects, Ang II also mediates cardiac remodeling through multiple signaling pathways, including protein kinase B/mammalian target of rapamycin (AKT/mTOR), mitogen-activated protein kinase (MAPK), nuclear factor- κ B subunit (NF- κ B), and transforming growth factor- β /mothers against decapentaplegic homolog (TGF- β /Smad) (Jin et al., 2015; Samak et al., 2016; Trial, et al., 2017; Mouton et al., 2018). Ang II stimulates hypertrophic remodeling by promoting proinflammatory cell infiltration into cardiac tissue. Recent studies have shown that Ang II stimulation can increase the adhesion of macrophages/monocytes to endothelial cells and subsequently migrate to cardiac tissue through endothelial cells, generate proinflammatory cytokines, activate multiple signaling pathways, and lead to cardiac remodeling (Peet et al., 2020). However, the roles of different functional phenotypes of macrophages in Ang II-induced hypertensive myocardial remodeling are unclear.

Macrophage monocytes are innate immune system pleiotropic cells that are critical for the initial inflammatory response and subsequent wound healing following injury in many tissues, including the heart (Labonte et al., 2014; Liu et al., 2014). Macrophages also play a central role in inflammation and host defense (Sica et al., 2012). Macrophages are monocyte-derived cells derived from proinflammatory (M1) and repair (M2) macrophages (Sica et al., 2007; Biswas et al., 2010). M1 phenotypic macrophages express many proinflammatory mediators, including tumor necrosis factor α (TNF- α), IL-1, IL-6, reactive nitrogen, and oxygen intermediates with potent microbicidal and tumoricidal activities, while M2 phenotypic expression molecules include resistin-like α (also known as FIZZ1), arginase 1 (Arg1), chitinase 3-like 3 (also known as Ym 1), IL-10, and Mrc1 (also known as CD206) (Gordon et al., 2010). These molecules are thought to be involved in parasite infection, tissue remodeling, and tumor progression (Mantovani et al., 2002). Recent studies have reported increased levels of proinflammatory cytokines, such as IL-6, secreted by M1 phenotypic macrophages in patients with unstable angina

and myocardial infarction; moreover, high levels predict adverse outcomes (Hanna et al., 2020). *In vitro* studies have found that M1 phenotype macrophages can also induce smooth muscle cell proliferation and release vasoactive molecules, including NO, endothelin, and eicosanoids, which are important consequences of lipoprotein oxidation and cytotoxicity (Khallou-Laschet et al., 2010; Tsimikas et al., 2011; Ndisang et al., 2014). Early arteriosclerotic plaques are infiltrated by M2 phenotype macrophages; however, with plaque progression, M1 phenotype macrophages gradually increase and dominate (Khallou-Laschet et al., 2010). The present study investigated the role of different phenotypes of macrophages in Ang II-induced hypertensive myocardial remodeling to provide new opportunities for the treatment of cardiovascular diseases.

Ginaton is a natural product extracted from *Ginkgo biloba* that has been used to treat cardiovascular and cerebrovascular diseases for decades (Li et al., 2020). Ginaton has well-known anti-inflammatory, antioxidant, and antiapoptotic effects (Gevrek et al., 2018). Previous studies demonstrated that Ginaton can downregulate the TGF- β signaling pathway and effectively improve renal interstitial fibrosis and the inflammatory response (Liang et al., 2021). However, the role of Ginaton in mediating Ang II-induced cardiac remodeling remains unclear.

Herein, we investigated the role of macrophages of different phenotypes in Ang II-induced myocardial remodeling in hypertension and the potential protective effect of Ginaton on Ang II-induced cardiac remodeling, providing a new therapeutic target for cardiovascular diseases.

Materials and methods

Animal study

In this study, 8-week-old C57BL/6 mice purchased from Jackson Laboratory (Sacramento, CA) were treated as wild-type (WT). We treated the mice with Ang II (1,000 ng/kg/min) or saline for 14 days. The hypertensive myocardial remodeling models and controls were established using osmotic micropumps (Alzet Model 1002, DURECT, Cupertino, CA). Ginaton injection was purchased from Chi Sheng Chemical Cooperation (Taiwan, China). To investigate the potential protective effects of Ginaton, we administered Ginaton (300 mg/kg/day) to the mice intragastrically the day before surgery, at a dose according to that reported previously (Sun et al., 2015). Phosphate-buffered saline (PBS) treatment was used as a control. All animal experiments were carried out in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, which were approved by the Dalian Medical University Animal Committee.

Measurement of systolic blood pressure and cardiac function

We used a tail-cuff device (BP-98, Softron, Japan) to record systolic blood pressure (SBP) and heart rate in all mice. We placed the mice on a retainer, completely exposing the tail, and then placed the tail-cuff device to measure their SBP and heart rate. Cardiac function was

measured in each mouse using a 30 MHz probe (Vevo 1100 system, VisualSonics, Toronto, Canada). By measuring the echocardiography of each mouse, we recorded the end-diastolic and end-systolic left ventricular anterior wall (LVAW) thickness, left ventricular posterior wall (LVPW) thickness, ventricular inner diameter (ID), LV ejection fraction (EF%), and LV fractional shortening (FS%).

Histopathology and immunofluorescence

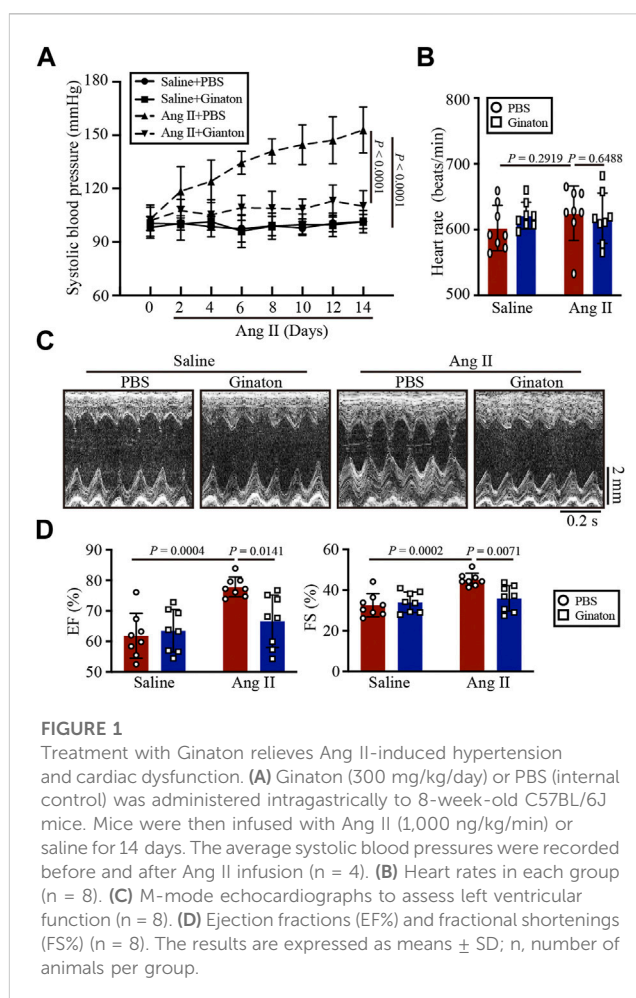
Heart tissue was fixed in 4% POM for >24 h. Part of the heart tissue was paraffin-embedded, while the other part was OCT-embedded. Masson's trichrome and hematoxylin and eosin (H&E) staining were performed on paraffin sections (4 μ m). The OCT sections (8 μ m) were treated with wheat germ agglutinin (WGA). Cardiac sections were stained with anti- α -SMA (ab124964, Abcam) by immunohistochemistry at 4°C overnight. The next day, after washing with PBS, the sections were incubated with the secondary antibody and DAB substrate. The color reaction was stopped with ddH₂O, and the sections were incubated with hematoxylin. Frozen sections or cells were fixed with 4% POM for 15 min at room temperature and then incubated with anti-CD68 (ab283654, Abcam), anti-CD206 (ab300621, Abcam), or anti-iNOS (ab283655, Abcam) at 4°C overnight. The next day, after washing with PBS, the sections were incubated with fluorescently labeled antibodies at room temperature for 30 min and then with DAPI at room temperature for 3 min. Pictures of the sections were taken at $\times 100/\times 200$ magnification on a fluorescence microscope (Olympus, BX53, Japan).

RNA isolation and quantitative real-time PCR

Total RNA was extracted from fresh heart tissue or cells. cDNA (1 μ g) was obtained using PrimeScript RT reagent (Takara, Japan). Quantitative real-time PCR was performed with an SYBR Green Premix Pro Taq HS qPCR Kit (AG11701). All mRNA levels were normalized to GAPDH by $\Delta\Delta$ Ct analysis.

Western blot analysis

Fresh heart tissue was added to RIPA lysis buffer to obtain total protein. Protein (25 μ g) was subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked with skim milk for 30 min at room temperature and then incubated with primary antibodies at 4°C overnight. The next day, after washing with TBST, the membranes were incubated with conjugated secondary antibodies (1:3000, CST) at room temperature for 1 h, and the bands were visualized by chemiluminescence. The main antibodies used in this study were: anti-TGF- β (ab215715, Abcam), anti-p-Smad2 (ab280888, Abcam), anti-Smad2 (ab33875, Abcam), anti-p-AKT (ab38449, Abcam), anti-AKT (orb11276, Biorbyt), anti-p-ERK1/2 (ab214036, Abcam), anti-ERK1/2 (ab184699, Abcam), anti-p-IKK α (ab138426, Abcam), anti-IKK α (ab32041, Abcam), anti-p-P65 (ab76302, Abcam), anti-P65 (ab16502, Abcam), and anti-GAPDH (ab8245, Abcam).



Cell adhesion and migration analysis

Macrophages were obtained from the tibias and femurs of WT mice and cultured in 1640 medium (Meilunbio, MA0215). Human umbilical vein endothelial cells (HUVECs) were cultured in ECM. Proclones from macrophages to adhere HUVECs were distributed as previously described (Yin et al., 2022). We pretreated HUVECs with Ginaton (100 μ g/ml) or PBS for 4 h and then with Ang II (100 nM) or saline for 24 h. Macrophages were labeled with PKH-26 fluorescent dye and added to HUVECs at a ratio of 10:1. After incubation for 1 h, the non-adherent cells were washed with PBS, while the adherent cells were photographed by microscopy.

HUVECs were cultured and treated as described in the adhesion experiments. Macrophages (5×10^4) were added to the upper chamber of a 24-well Transwell plate (8 μ m pore, Coning), and HUVEC-conditioned media were added to the lower chamber of the plate. After 24 h of incubation, the migrated cells were fixed, stained with DAPI, and observed by microscopy.

Statistics

Data are presented as means $\pm$ SD. Statistical analysis was performed using GraphPad Prism software. For statistical comparison, a one-way ANOVA was used, followed by Dunnett's multiple comparison tests.

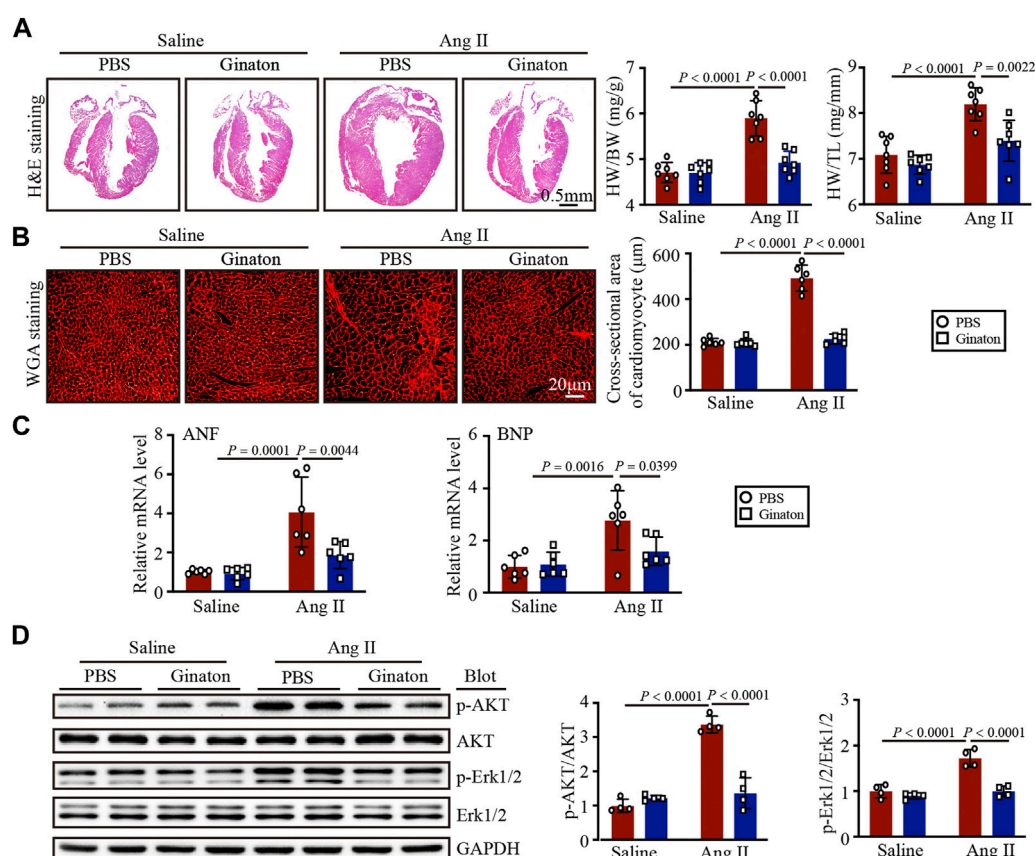


FIGURE 2

Ginaton administration rescues Ang II-induced cardiac hypertrophy. Ginaton (300 mg/kg/day) or PBS (internal control) was administered intragastrically to 8-week-old C57BL/6 J mice. Mice were then infused with Ang II (1,000 ng/kg/min) or saline for 14 days. **(A)** H&E staining of heart tissues in each group (left, $n = 4$, scale bar = 0.5 mm) and the ratios of heart weight to body weight (HW/BW) and HW to tibial length (HW/TL) (right, $n = 8$). **(B)** Heart sections stained with wheat germ agglutinin (WGA) (left, $n = 6$, scale bar = 20 μm) and quantification of myocyte cross-sectional areas (right, $n = 6$). **(C)** qPCR analyses of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) mRNA levels ($n = 6$). **(D)** Western blot analyses of the expression of phosphorylated (p)-AKT, AKT, p-ERK1/2, ERK1/2, and GAPDH proteins in heart sections (left, $n = 4$) and the relative protein levels of each (right, $n = 4$). The results are expressed as means $\pm$ SD; n , number of animals per group.

with the control group. Student's unpaired *t*-tests were used to compare the two groups. $p < 0.05$ was considered statistically significant.

Results

Ginaton treatment alleviates hypertension and cardiac dysfunction caused by Ang II

As shown in Figure 1A, WT mice were treated with Ginaton (300 mg/kg) and Ang II (1,000 ng/kg/min) for 14 days. PBS was used as the control. After 14 days of Ang II infusion, the SBP in the Ginaton-treated group was significantly lower than that in the PBS-treated group. However, after Ang II infusion, heart rates were similar in the Ginaton and PBS treatment groups (Figure 1B). Echocardiography showed enhanced cardiac function 14 days after Ang II infusion, with increased EF% and FS% in the

PBS-treated group, while treatment with Ginaton effectively mitigated this response (Figures 1C, D, Supplementary Table S1).

Ginaton treatment mitigates the Ang II-induced cardiac hypertrophic response

Next, we determined the role of Ginaton in Ang II-induced cardiac hypertrophy. After 14 days of Ang II infusion, PBS-treated mice exhibited the characteristics of myocardial hypertrophy, including increased left ventricular thickness, heart weight/body weight (HW/BW), heart weight/tibial length (HW/TL), and increased cardiomyocyte cross-sectional area; however, these effects were mitigated by Ginaton (Figures 2A, B). Ginaton also reduced the expression of hypertrophy markers (ANF and BNP) after Ang II infusion (Figure 2C). The primer sequences are listed in Supplementary Table S2. As shown in Figure 2D, Ginaton downregulated the Ang II-induced activation of AKT and

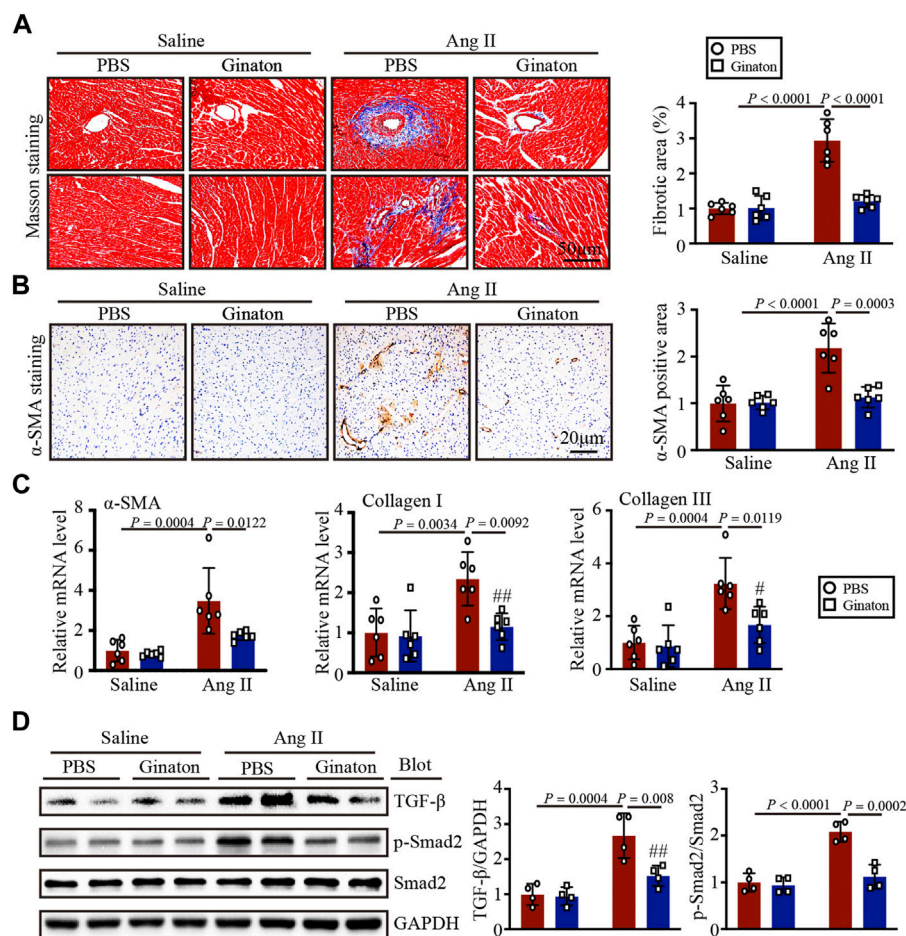


FIGURE 3

Ginaton reduces fibrosis and collagen deposition in Ang II-infused mice. Ginaton (300 mg/kg/day) or PBS (internal control) was administered intragastrically to 8-week-old C57BL/6J mice. Mice were then infused with Ang II (1,000 ng/kg/min) or saline for 14 days. **(A)** Masson's trichrome staining of cardiac sections (left, $n = 6$, scale bar = 50 μm) and quantification of the areas of collagen deposition (right, $n = 6$). **(B)** Immunohistochemical staining of heart tissues with antibody against α -smooth muscle actin (α -SMA) (left, $n = 6$, scale bar = 20 μm); quantification of α -SMA⁺ areas (right, $n = 6$). **(C)** qPCR analyses of α -SMA, collagen I, and collagen III mRNA levels ($n = 6$). **(D)** Western blot analyses of the expression of TGF- β , p-Smad2, Smad2, and GAPDH proteins in heart sections (left, $n = 4$) and the relative protein levels of each (right, $n = 4$). The results are expressed as means $\pm$ SD; n , number of animals per group.

ERK1/2 expression. Therefore, Ginaton attenuated the Ang II-induced cardiac hypertrophy response.

Ginaton treatment reduces Ang II-induced cardiac fibrosis

Masson's trichrome staining to investigate the role of Ginaton in Ang II-induced cardiac fibrosis showed that Ginaton treatment significantly reduced collagen deposition in the Ang II-infused heart compared to the PBS group (Figure 3A). Similarly, the number of α -SMA-positive myofibroblasts and the expression levels of α -SMA, collagen I, and collagen III mRNA were downregulated in Ginaton-treated mice compared to the PBS group (Figures 3B, C). As shown in Figure 3D, Ang II infusion induced increased TGF- β and Smad2 protein expression, while Ginaton treatment alleviated this response. These results suggested that Ginaton participates in Ang II-induced myocardial fibrosis through the TGF- β -Smad2 pathway.

Ang II infusion induces M1 but not M2 phenotype macrophage activation, while Ginaton treatment downregulates the Ang II-induced inflammatory response

To investigate the expression of different macrophage phenotypes in Ang II-infused hearts and the mechanism by which Ginaton improved Ang II-induced cardiac remodeling, we co-stained with the CD68 macrophage biomarker and the iNOS M1 phenotype macrophage biomarker. Immunofluorescent staining revealed that Ang II infusion induced high expression of iNOS⁺ M1 phenotype macrophages and that Ginaton reduced this Ang II-induced increase (Figure 4A). Furthermore, co-staining for the CD68 macrophage biomarker and the CD206 M2 phenotype macrophage biomarker showed no significant difference between Ang II infusion and Ginaton treatment on CD206⁺ M2 phenotype macrophages (Figure 4B). Similarly, Ang II

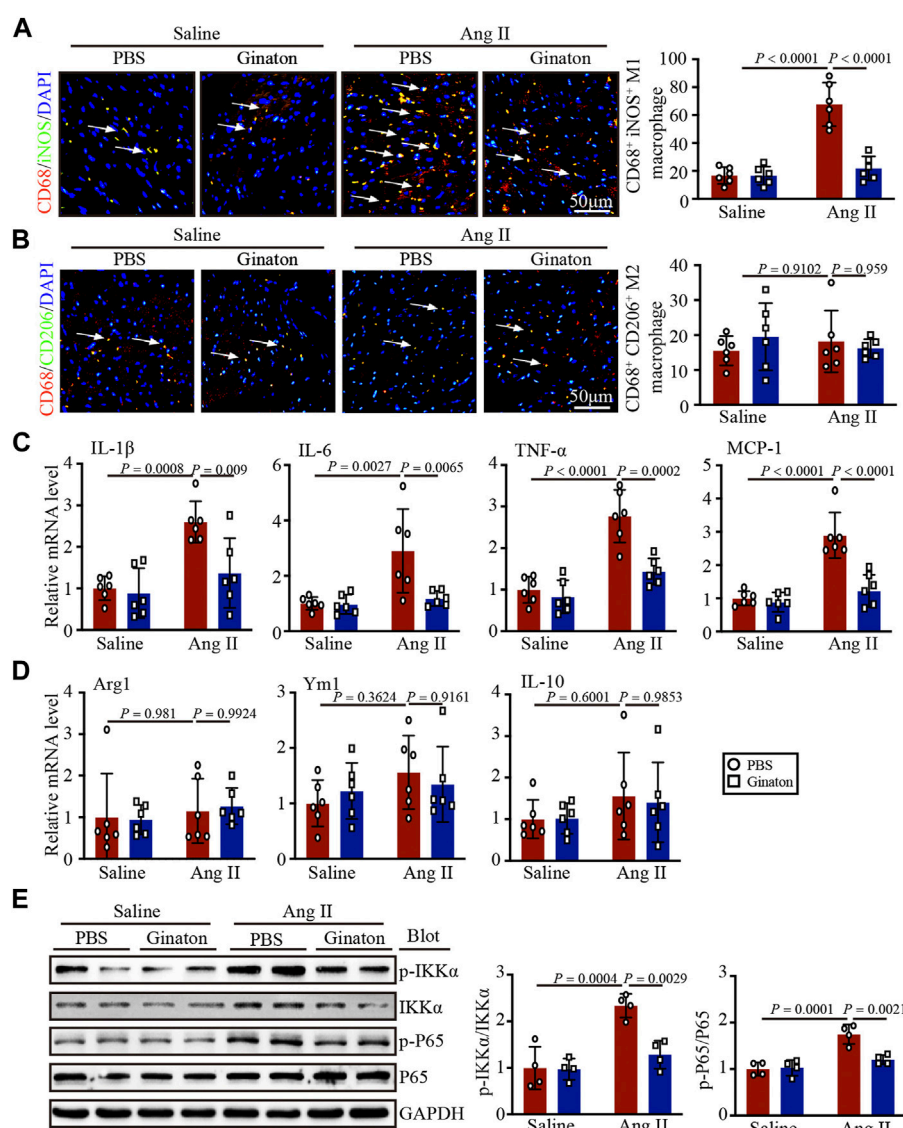


FIGURE 4

Ginaton decreases Ang II-induced M1 phenotype macrophage activation and inflammation through the NF- κ B signaling pathway. Ginaton (300 mg/kg/day) or PBS (internal control) was administered intragastrically to 8-week-old C57BL/6J mice. Mice were then infused with Ang II (1,000 ng/kg/min) or saline for 14 days. **(A)** Immunofluorescence staining in cardiac sections with CD68 and iNOS antibodies (left, $n = 6$, scale bar = 50 μ m) and quantification of CD68⁺ and iNOS⁺ macrophages in heart sections (right, $n = 6$). **(B)** Immunofluorescence staining of cardiac sections with CD68 and CD206 antibodies (left, $n = 6$, scale bar = 50 μ m) and quantification of CD68⁺ and CD206⁺ macrophages in heart sections (right, $n = 6$). **(C)** qPCR analyses of IL-1 β , IL-6, TNF- α , and MCP-1 mRNA levels ($n = 6$). **(D)** qPCR analyses of Arg1, Ym1, and IL-10 mRNA levels ($n = 6$). **(E)** Western blot analyses of p-IKK α , IKK α , p-P65, P65, and GAPDH expression in heart sections (left, $n = 4$) and the relative protein levels of each (right, $n = 4$).

infusion increased the mRNA levels of molecules associated with M1 phenotypic expression, including IL-1 β , IL-6, TNF- α , and MCP-1, while Ginaton rescued this Ang II-induced inflammatory reaction. The levels of M2 phenotypic expression molecules, including Arg1, Ym1, and IL-10 mRNA levels, did not differ between the Ang II infusion and Ginaton treatment groups (Figures 4C, D). Moreover, IKK α and p65 phosphorylation were reduced in the Ginaton-treated group compared to the PBS-treated group (Figure 4E). These results suggested that Ginaton relieved Ang II-induced cardiac inflammation *via* the accumulation of M1 phenotype macrophages in the heart.

Ginaton treatment reduces Ang II-induced M1 macrophage activation *in vitro*

To confirm the effect of Ginaton on the activation of M1 phenotype macrophages induced by Ang II, we pretreated macrophages with Ginaton (100 μ g/ml) or PBS for 4 h and with Ang II (100 nM) or saline for 24 h. We then co-stained the cells with the macrophage biomarker CD68 and the M1 phenotype macrophage biomarker iNOS. Immunofluorescence staining showed that Ang II treatment induced high expression of iNOS⁺ M1 phenotype macrophages and that Ginaton reduced

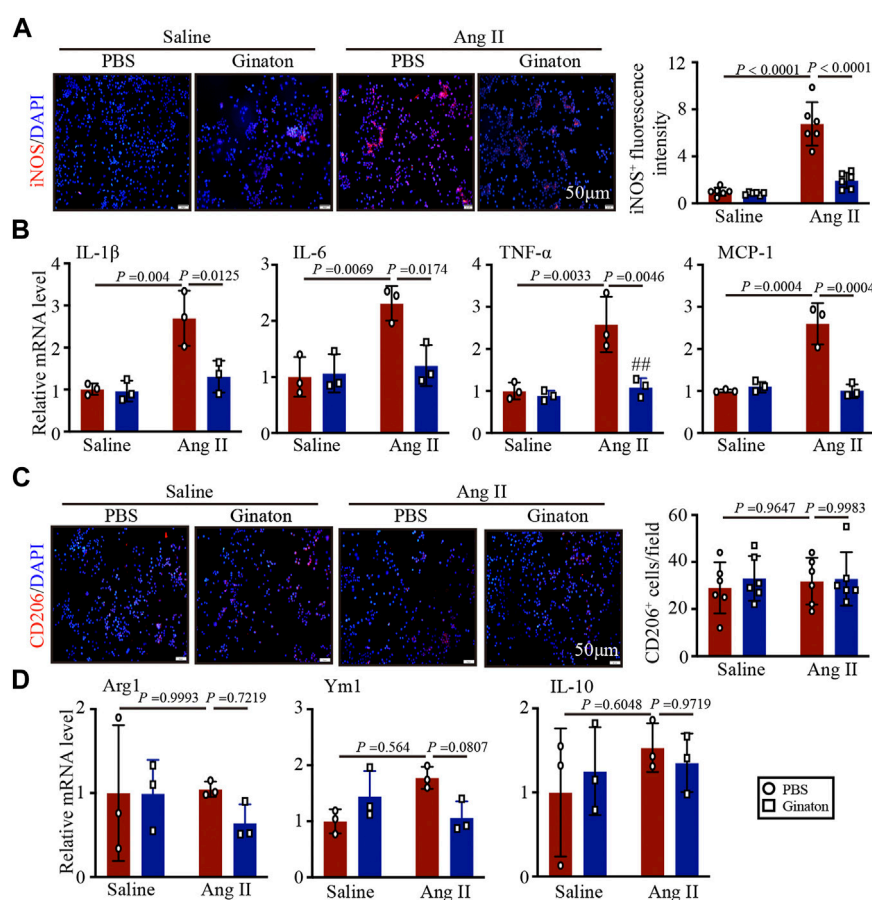


FIGURE 5

Application of Ginaton rescues the expression of M1 phenotype macrophages. Macrophages were treated with Ginaton (100 μg/ml) or PBS for 4 h and then with Ang II (100 ng/ml) or saline for 24 h. **(A)** Immunofluorescence staining in macrophages with CD68 and iNOS antibodies (left, n = 3, scale bar = 50 μm) and quantification of iNOS⁺ intensity (left, n = 3). **(B)** qPCR analyses of IL-1β, IL-6, TNF-α, and MCP-1 mRNA levels (n = 6). **(C)** Immunofluorescence staining of macrophages with CD68 and CD206 antibodies (left, n = 3, scale bar = 50 μm) and quantification of CD206⁺ intensity (left, n = 3). **(D)** qPCR analyses of Arg1, Ym1, and IL-10 mRNA levels (n = 6). The results are expressed as means ± SD; n, number of animals per group.

the increase in iNOS⁺ M1 phenotype macrophages induced by Ang II *in vitro* (Figure 5A). Moreover, Ginaton reduced Ang II-induced mRNA expression of the M1 phenotype molecules IL-1β, IL-6, TNF-α, and MCP-1 (Figure 5B). Neither immunofluorescence staining nor qPCR results showed differences in M2 phenotype macrophages between the Ginaton and Ang II treatment groups (Figures 5C, D).

Ginaton treatment inhibits macrophage adhesion and migration *in vitro*

Coculture experiments of macrophages with HUVECs and Transwell assays showed that Ang II treatment increased macrophage adhesion and migration to HUVECs, an effect that was inhibited by Ginaton (Figures 6A, B). The results showed that Ginaton could reduce the adhesion and migration of macrophages to HUVECs.

Discussion

The results of the present study demonstrated the protective effect of Ginaton on Ang II-induced cardiac remodeling in mice. After 14 days of Ang II infusion, the mice showed significantly elevated SBP, upregulated expression of M1 phenotype macrophages, monocyte/macrophage adherence to vascular endothelial cells, and subsequent migration to the heart tissue across the endothelium, producing proinflammatory cytokines, whose activation led to multiple signaling pathways of hypertensive cardiac remodeling. In contrast, Ginaton alleviated Ang II-induced hypertension, the inflammatory response, and monocyte/macrophage adhesion and migration, which might provide a new perspective for the treatment of hypertension (Figure 7).

Hypertension is a major risk factor for heart failure and is characterized by chronic low-grade inflammation, which leads to poor cardiac remodeling (Mikolajczyk et al., 2021). Although macrophages play a critical role in cardiac remodeling,

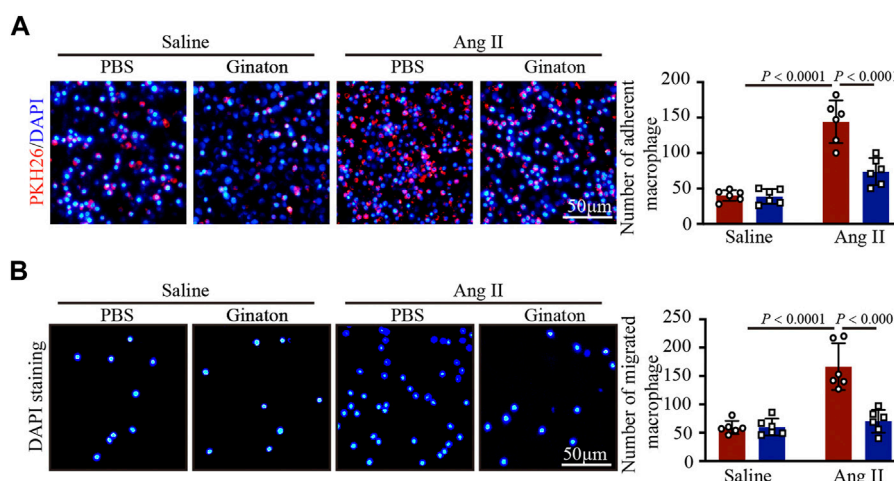


FIGURE 6

Ginaton reduces the increase of Ang II-induced macrophage adhesion and migration. **(A)** Human umbilical vein endothelial cells (HUVECs) were pretreated with Ginaton (100 μ g/ml) or PBS for 4 h, and then with saline or Ang II (100 nM) for 24 h. PKH-26 staining of macrophages (left, $n = 6$, scale bar = 100 μ m) and analysis of adherent cells (right, $n = 3$). **(B)** Macrophages (5×10^4) were added to the upper chamber on Transwell plate, and HUVEC-conditioned medium was added to the lower chamber of the plate. DAPI staining of macrophages (left, $n = 6$, scale bar = 100 μ m) and analysis of mitigated cells (right, $n = 3$). The results are expressed as mean $\pm$ SD; n , number of animals per group.

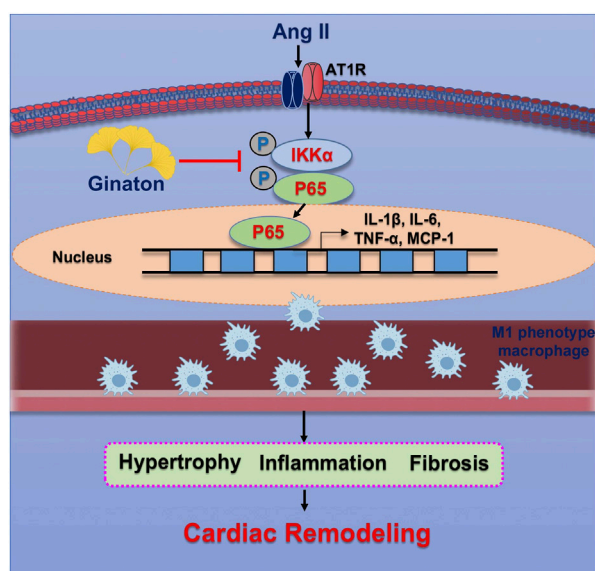


FIGURE 7

Operational representation of the method through which Ginaton controls cardiac remodeling caused by Ang II. By encouraging M1 phenotype macrophages to adhere to the endothelium and infiltrate the heart, Ang II infusion increases NF- κ B activation, which in turn causes cardiomyocyte hypertrophy, fibrosis, and subsequent cardiac remodeling. Ginaton administration successfully reverses these effects.

dysregulated macrophage polarization between the proinflammatory M1 and anti-inflammatory M2 phenotypes promotes excessive inflammation and cardiac damage (Gullestad et al., 2012; Varol et al., 2015). M1 phenotype macrophages express many proinflammatory mediators, including TNF- α , IL-1, IL-6, and

iNOS, while M2 phenotype macrophages express molecules including Arg1, IL-10, Ym1, and CD206 (Lavine et al., 2014; Kologrivova et al., 2021). Many key transcription factors are involved in macrophage polarization, such as signal transducers and activators of transcription (STATs), interferon regulatory factors (IRFs), nuclear factors (NF- κ B), activator protein (AP) 1, and peroxisome proliferator-activated receptor (PPAR)- γ (Ohmori et al., 1997; Bouhrel et al., 2007; Odegaard et al., 2007; Satoh et al., 2010; Krausgruber et al., 2011; Lawrence et al., 2011; Oeckinghaus et al., 2011; Schonthaler et al., 2011). NF- κ B is a family of transcription factors involved in many biological processes, including the immune response, inflammation, cell growth, survival, and development. NF- κ B protein is usually expressed by a series of inhibitors, including κ Ba immobilized in the cytoplasm. The typical NF- κ B activation pathway depends on I κ Bs, particularly I κ Ba-inducible degradation (Mitchell et al., 2016). The degradation of I κ Ba is mediated through its phosphorylation by I κ B kinase α (IKK α) (Behar et al., 2013). The present study focused on macrophage phenotypes and their impacts on the outcome of hypertensive cardiac remodeling. We found that following infusion with Ang II, macrophages tended to switch to an M1 phenotype and expressed higher levels of iNOS and more pro-inflammatory cytokines such as IL-1 β , IL-6, TNF- α , and MCP-1. Ang II infusion also increased p-IKK α and P-p65 expression. In further investigations, we examined the expression of CD206⁺ M2 phenotype macrophages and proinflammatory cytokines such as Arg1, Ym1, and IL-10. We found that Ang II infusion did not significantly increase the expression of M2 phenotype macrophages. These results suggested that Ang II infusion activated the proinflammatory response in the heart and that the activation of macrophage adhesion and migration was a major cause of cardiac remodeling.

Ginaton, a natural product extracted from *Ginkgo biloba* leaves, has been widely used worldwide and has therapeutic effects on a

variety of diseases (Tian et al., 2017). Recent studies have found that treatment with Ginaton alleviated renal interstitial fibrosis through the TGF- β pathway and that Ginaton also reduced TNF- α , IL-1 β , and 5-hydroxytryptamine levels in the hippocampus of mice with heart failure (Zhang et al., 2019). In the present study, after treatment with Ginaton, Ang II-induced hypertrophy and fibrosis were both alleviated. Moreover, we detected the expression of macrophages with different phenotypes in Ang II-infused hearts. The results showed markedly decreased levels of M1 phenotype macrophages and proinflammatory cytokines after Ginaton treatment, with no obvious change in M2 phenotype macrophages or proinflammatory cytokines. Ginaton also reduced the Ang II-induced activation of macrophage adhesion and migration. These results indicate that Ginaton may offer an approach for the treatment of hypertensive cardiac remodeling.

In conclusion, our results showed that Ginaton treatment inhibited Ang II-induced M1 phenotype macrophage activation, adhesion and migration, and the inflammatory response, leading to impaired and dysfunctional hypertension and cardiac remodeling. Therefore, Ginaton may be a powerful treatment for heart disease.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

Ethics statement

The animal experiments were performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, and the study protocols were approved by the Animal Committee of Dalian Medical University.

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Author contributions

JW and EC performed the histological staining and the *in vitro* studies. JW performed molecular biology experiments. XA and JW wrote, revised, and gave their approval for the final version of the manuscript to be published. The final manuscript was read and approved by all authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2023.1104871/full#supplementary-material>

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Atrioventricular node dysfunction in pressure overload-induced heart failure—Involvement of the immune system and transcriptomic remodelling

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Heart failure is associated with atrioventricular (AV) node dysfunction, and AV node dysfunction in the setting of heart failure is associated with an increased risk of mortality and heart failure hospitalisation. This study aims to understand the causes of AV node dysfunction in heart failure by studying changes in the whole nodal transcriptome. The mouse transverse aortic constriction model of pressure overload-induced heart failure was studied; functional changes were assessed using electrocardiography and echocardiography and the transcriptome of the AV node was quantified using RNAseq. Heart failure was associated with a significant increase in the PR interval, indicating a slowing of AV node conduction and AV node dysfunction, and significant changes in 3,077 transcripts (5.6% of the transcriptome). Many systems were affected: transcripts supporting AV node conduction were downregulated and there were changes in transcripts identified by GWAS as determinants of the PR interval. In addition, there was evidence of remodelling of the sarcomere, a shift from fatty acid to glucose metabolism, remodelling of the extracellular matrix, and remodelling of the transcription and translation machinery. There was evidence of the causes of this widespread remodelling of the AV node: evidence of dysregulation of multiple intracellular signalling pathways, dysregulation of 109 protein kinases and 148 transcription factors, and an immune response with a proliferation of neutrophils, monocytes, macrophages and B lymphocytes and a dysregulation of 40 cytokines. In conclusion, inflammation and a widespread transcriptional remodelling of the AV node underlies AV node dysfunction in heart failure.

KEYWORDS

heart failure, atrioventricular node, heart block, RNA sequencing, transcriptome, ion channels, inflammation

Abbreviations: AV, atrioventricular; ECG, electrocardiogram; HF, heart failure; RNAseq, RNA-sequencing; TAC, transverse aortic constriction.

Introduction

Heart failure (HF) is associated with atrioventricular (AV) node dysfunction. First-degree AV block is present in 15%–51% of HF patients as compared to a prevalence of 2.1%–4% in the general population (Aro et al., 2014; Nikolaidou et al., 2016). First-degree AV block in HF is associated with an increased risk of mortality and HF hospitalisation, and optimisation of AV delay in patients by means of cardiac resynchronisation therapy is an important treatment (Nikolaidou et al., 2016). AV node dysfunction has also been widely observed in animal models of HF (e.g., Nikolaidou et al., 2015). The aim of this study was to investigate the causes of the AV node dysfunction by studying all changes occurring in the AV node in HF. For this, we used RNAseq to study the transcriptome of the AV node. The aetiology of HF in patients is likely to be mixed and complicated (Roger, 2021). Nevertheless, hypertension is the commonest cause of HF (Bui et al., 2011) and, although it is not a model of hypertension *per se* (Riehle and Bauersachs, 2019), we studied a mouse model of pressure overload-induced HF: the transverse aortic constriction model (Richards et al., 2019). Widespread changes were observed (in 3,077 transcripts), showing that AV node dysfunction is the result of the failure of multiple cellular systems.

Materials and methods

Male eight-week-old C57Bl/6N mice underwent transverse aortic constriction (TAC) or sham operations. Mice were terminated 8 weeks post-surgery or when HF symptoms developed. In the conscious mouse, the heart rate was measured weekly following surgery using an ECGenie. Prior to termination, in anaesthetised mice, echocardiography was carried out to determine heart function, and the electrocardiogram (ECG) was recorded to determine the PR interval, etc. AV node biopsies were collected from the triangle of Koch bordered by the coronary sinus, tendon of Todaro and tricuspid valve annulus (Li et al., 2008); the base, height and area of the triangle was ~ 0.5 mm, ~ 0.75 mm and 0.19 mm², respectively. AV node biopsies taken from three mice were pooled and three such replicates were obtained from HF (TAC) and control (sham-operated) mice. RNA was extracted and sequenced (RNAseq) to yield the expression level of 55,385 transcripts. Statistical analysis was carried out to determine significant differences in transcript expression, and gene ontology and pathway analysis was carried out to understand the functional significance of the data. All experimental procedures were approved by the University of Manchester and were in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Further details of the methods used are given in the [Supplementary Materials and Methods](#).

Results

TAC mouse model of pressure overload-induced HF and AV node dysfunction

Figure 1A shows that from day 36 following TAC surgery, some mice reached prescribed endpoints and had to be culled, whereas

none of the control sham-operated mice had to be culled. Figures 1B–D shows that by the end of the experiment, in the case of the mice subjected to TAC surgery, the body weight was significantly reduced, and the heart weight and heart weight:body weight ratio were significantly increased, indicative of HF. The relative loss of body weight (as compared to the body weight of the control mice) occurred progressively after the TAC surgery ([Supplementary Figure S1A](#)). Following TAC surgery, the mice developed congestive HF—the lung weight:body weight ratio was significantly increased in the mice subjected to TAC surgery by the end of the experiment (Figure 1E). Echocardiography at the end of the experiment confirmed HF: fractional shortening and ejection fraction were significantly reduced and left ventricular mass and end diastolic and systolic diameters were significantly increased ([Supplementary Figure S1B–F](#)). HF was accompanied by electrophysiological changes: the ECG was recorded in the anaesthetised mouse at the end of the experiment, and in the HF mice there was a significant increase in the PR interval indicative of AV node dysfunction (a slowing of AV node conduction) (Figure 1G). There was also a significant decrease in the heart rate and significant increases in the QRS duration and uncorrected and corrected QT intervals in the HF mice as expected (Figures 1F,H–J). See the [Supplementary Material](#) for further discussion of the data as well as justification of the mouse TAC model as a model of HF. From this point onwards, pressure overload-induced HF will be simply referred to as HF.

Transcriptomic remodelling of the AV node

The transcriptome of the AV node was determined using RNAseq, and to have sufficient material, AV node tissue isolated from three mice was pooled. Three biological replicates (each a pool from three mice) were isolated from each cohort of mice (the study therefore involved nine HF and nine control mice). 55,385 transcripts were detected and their abundance quantified. Principal component analysis (PCA) for each of the three technical replicates in the transcriptomics dataset showed clear distinction of the samples into two groups corresponding to the HF and control cohorts of mice (Figure 2A). 64% of the variation between samples (PC1) was attributed to HF, whereas 19% of the variation (PC2) was attributed to variation between technical replicates (Figure 2A). Figure 2B shows a volcano plot of the Benjamini-Hochberg-adjusted *p*-value against the fold change for all transcripts. The dashed line corresponds to *p* = 0.05, the threshold for significance. There were significant changes in the abundance of 3,077 transcripts (5.6% of the total). Unsupervised hierarchical clustering of the transcripts changing significantly in HF resulted in a grouping of the HF and control cohorts of mice into two distinct clusters (Figure 2C). 1,337 transcripts were significantly downregulated and 1,740 transcripts including well-known HF markers were significantly upregulated in HF (Figures 2B,C; [Supplementary Figure S2](#)). A gene ontology analysis was carried out of the transcripts significantly downregulated or upregulated in HF using topGO and this showed that HF had widespread effects on the cellular systems of the AV node. In Figures 2D,E the most significant of these pathways is shown (*p* < 0.01). The set of transcripts downregulated in HF is enriched for transcripts

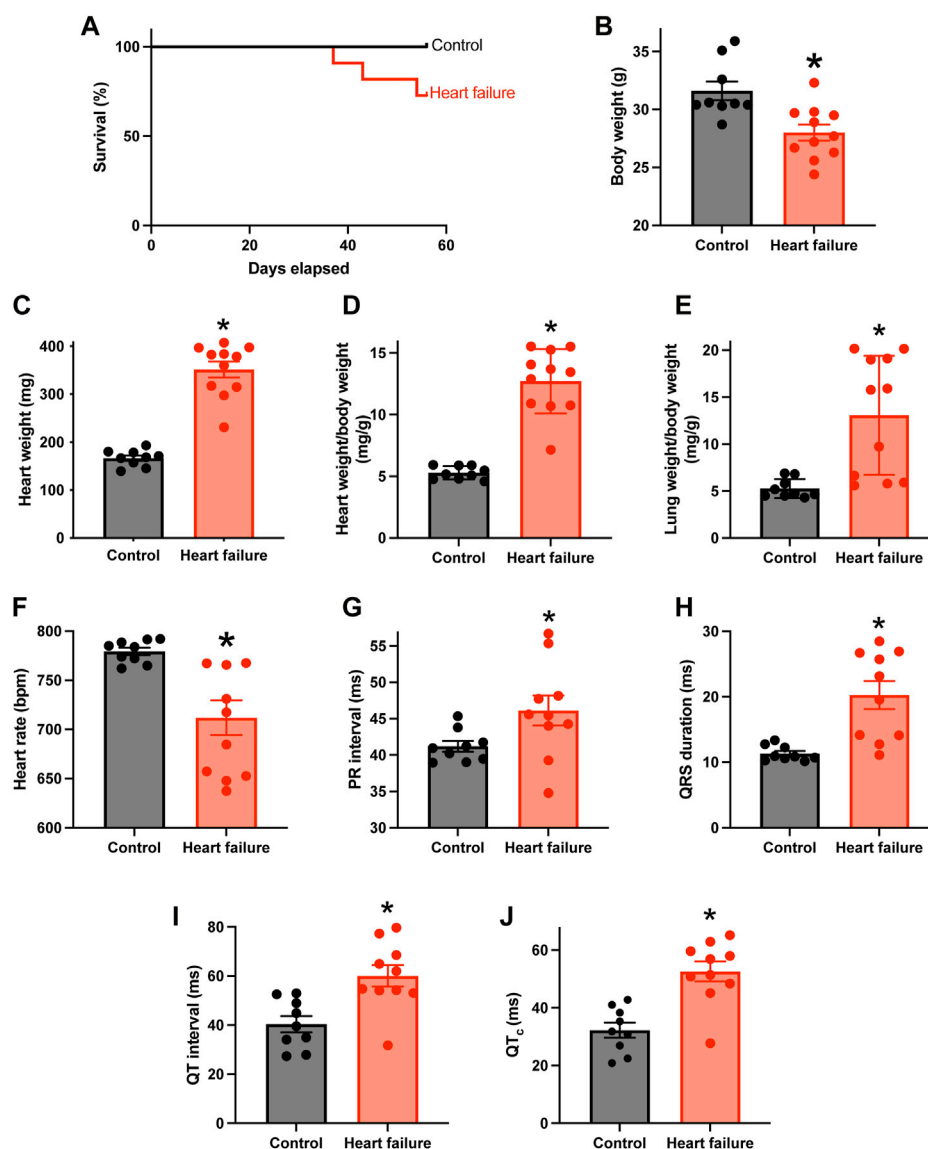


FIGURE 1

HF model with AV node dysfunction. (A), percentage survival of control ($n = 9$; subject to sham surgery) and HF ($n = 11$; subject to TAC surgery) mice in the days following surgery. (B–F), body weight (B), heart weight (C), heart weight:body weight ratio (D), lung weight:body weight ratio (E) and *in vivo* heart rate (F); measured using the ECGenie) of the two cohorts of mice ($n = 9$ and 11) at the end of the experiment. (G–J), PR interval (G), QRS duration (H), QT interval (I) and corrected QT interval (J) of the two cohorts of mice ($n = 9$ and 10) measured in the anaesthetised animals at the end of the experiment. In B–J, means $\pm$ SEM as well as individual data points shown. $*p < 0.05$.

related to “excitation and ion transport” for example, (Figure 2D), whereas the set of transcripts upregulated in HF is enriched for transcripts related to “intracellular Ca^{2+} ,” “contraction,” “metabolism,” “apoptosis,” “signalling,” “extracellular matrix” and the “immune system” for example, (Figure 2E). The effects of HF on some of these systems is now considered in detail.

Downregulation of transcripts in the AV node expected to impact conduction

There was a remodelling of ion channel transcripts in the AV node in HF, some of which are expected to impact AV node

conduction. Whereas *Scn5a* responsible for the principal Na^+ channel in the heart, Nav1.5, was unaffected (Figure 3A), other less abundant Na^+ channel transcripts were significantly downregulated (Figure 3B). *Scn10a* (responsible for Nav1.8) was scarce compared to *Scn5a*, but it was significantly downregulated (Figure 3B) and this may be important: GWAS studies have implicated *Scn10a* as a modulator of cardiac conduction (Park and Fishman, 2014) including the PR interval (Chambers et al., 2010; Holm et al., 2010; Pfeufer et al., 2010), and mice treated with a selective inhibitor of Nav1.8 channels show a marked prolongation of PR and QRS intervals (Park and Fishman, 2014). The mechanism of action of *Scn10a* is unclear (Park and Fishman, 2014). Three key Ca^{2+} channel transcripts were significantly downregulated: *Cacna1d*, *Cacna1g* and

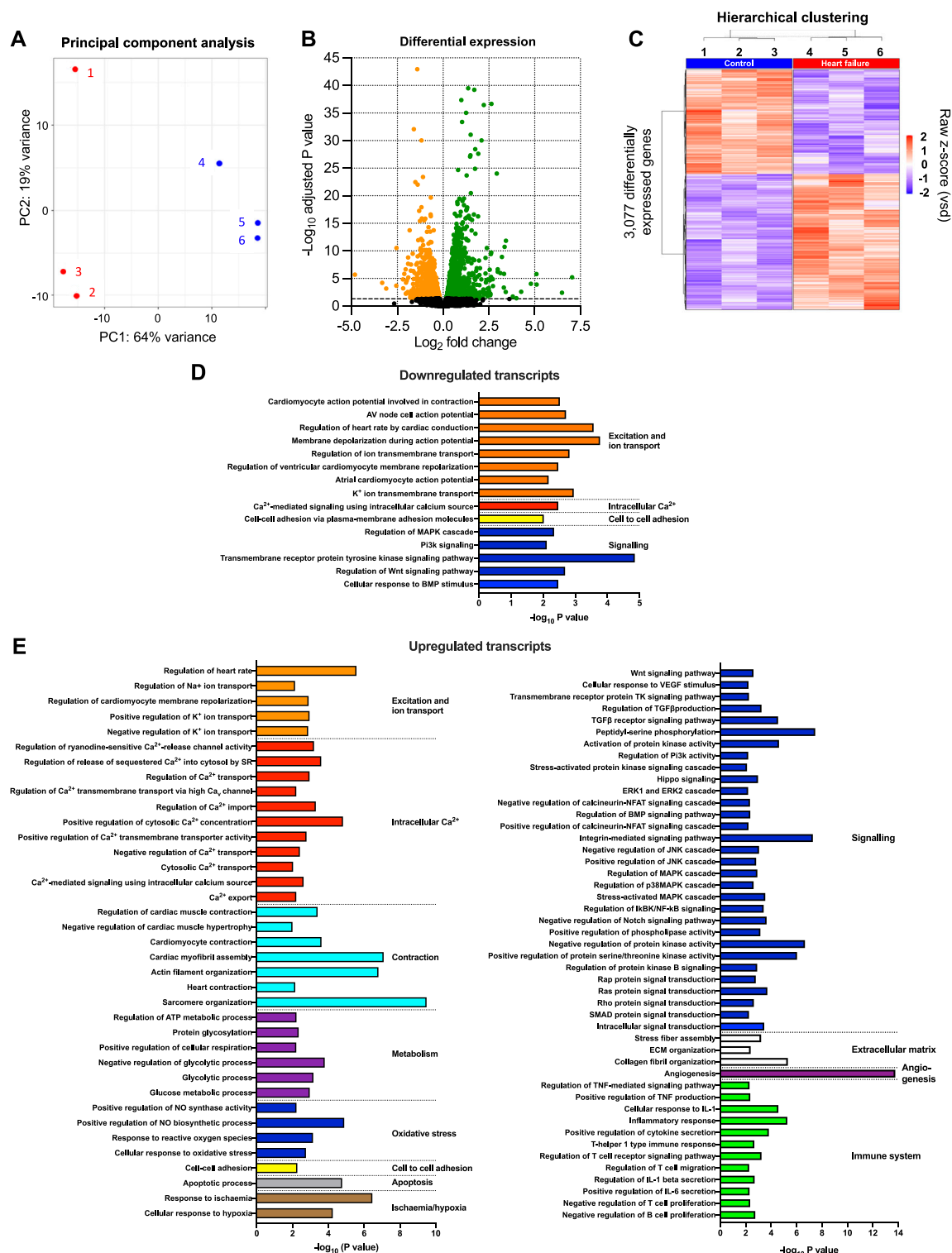


FIGURE 2

Transcriptomic remodelling of the AV node in HF. (A), principal component analysis (PCA) of all six replicates based on the 500 most highly expressed transcripts. The x-axis shows the PCA 1 score, which represents the maximum variance direction in the data set. This is principal component 1 (PC1) and it accounts for 64% of the variance in the samples and can be attributed to the variance between the control and HF cohorts. The y-axis shows the PCA 2 score, which represents the second highest variance direction in the data set. This is principal component 2 (PC2) and it accounts for 19% of the variance in the samples and can be attributed to the variance among all samples. The red symbols (1, 2 and 3) represent the three HF technical replicates and the blue symbols (4, 5 and 6) represent the three control technical replicates. Each technical replicate contained three pooled AV node biopsies. (B), volcano plot showing $-\log_{10}$ adjusted p -value plotted against $\log_2$ fold change for each transcript measured. Dashed line corresponds to adjusted $p = 0.05$. The red and blue symbols show the 3,077 transcripts significantly upregulated (red symbols; 1,740 transcripts) or downregulated (blue symbols; 1,337 transcripts). (C), hierarchical clustering of the 3,077 differentially expressed transcripts (adjusted $p < 0.05$) performed using Pearson's correlation distance and ward, D2 agglomeration method. Data for the six samples shown. Expression (raw z-score) goes from high (red) to low (blue). (D and E), functional enrichment analysis performed using the topGO R package on downregulated (D) and upregulated (E) differentially expressed genes in the HF samples compared to the control samples. Significantly enriched biological process GO terms related to cardiac function are presented.

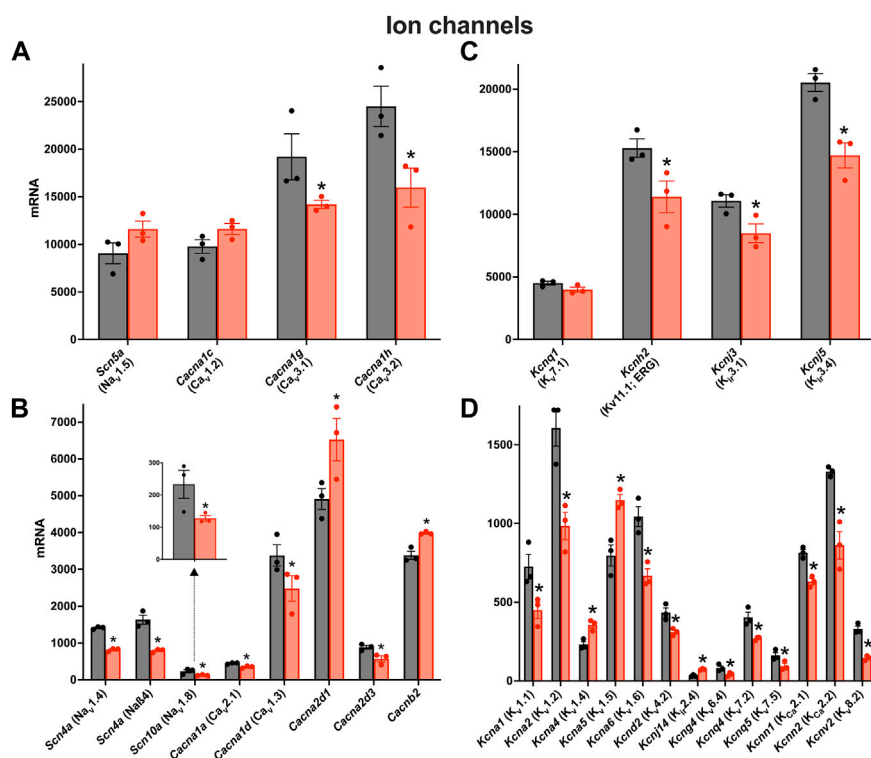


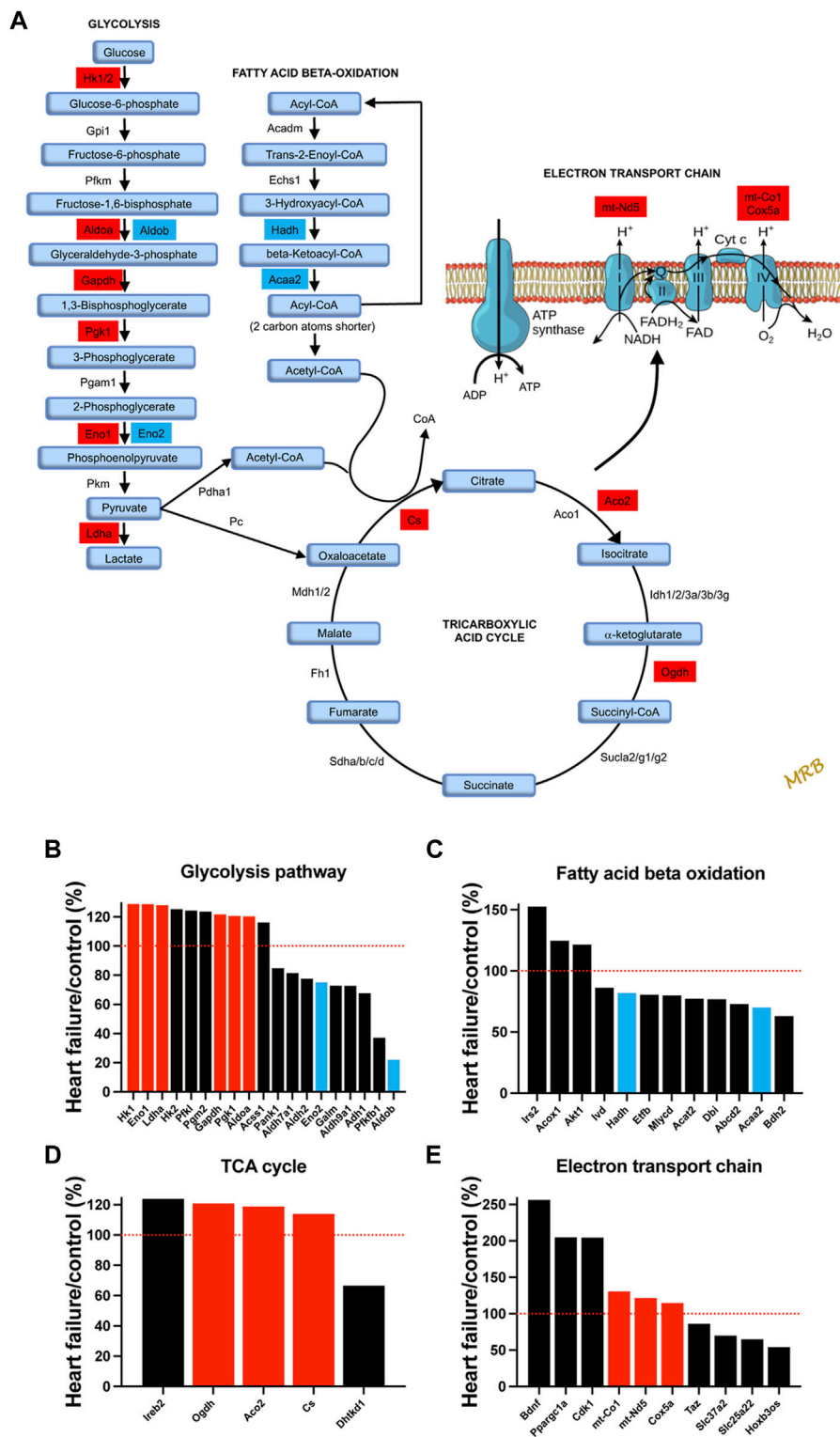
FIGURE 3

Downregulation of many key ion channel transcripts in the AV node in HF. (A–D), expression of transcripts for Na⁺ and Ca²⁺ channels (A and B) and K⁺ channels (C and D) in control (black bars) and HF (red bars). Highly expressed ion channel transcripts are shown in the upper panels (A and C) and more poorly expressed ion channel transcripts are shown in the lower panels (B and D). The data for *Scn10a* are also shown in a zoomed panel. Gene names and more common names are shown. Means $\pm$ SEM as well as individual data points shown **p* < 0.05.

Cacna1h, responsible for the L- and T-type Ca²⁺ channels, Cav1.3, Cav3.1 and Cav3.2 (Figures 3A,B). This is likely to be important, because in mice, loss-of-function of Cav1.3 and Cav3.1 impairs AV conduction and in humans AV block has been attributed to loss-of-function of Cav1.3 and Cav3.1 (Mesirca et al., 2015). The Ca²⁺ channel accessory subunit, *Cacna2d3*, was significantly downregulated (Figure 3B) and it too has been linked to cardiac conduction (Auxerre-Planté et al., 2019). However, *Cacna2d1* was significantly upregulated (Figure 3B) and this is expected to increase the L-type Ca²⁺ current (Templin et al., 2011). Three key abundant K⁺ channel transcripts were significantly downregulated, *Kcnh2*, *Kcnj3* and *Kcnj5* (Figure 3C); *Kcnh2* is responsible for the ERG (Kv11.1) channel, and *Kcnj3* and *Kcnj5* are responsible for Kir3.1 and Kir3.4, which together make up the ACh-activated K⁺ channel. Regan et al. (Regan et al., 2005) observed that selective pharmacological block of ERG (responsible for the K⁺ current, *I_{K,r}*) and KvLQT1 (responsible for the K⁺ current, *I_{K,s}*) both cause a prolonged AH interval and AV node effective refractory period in rats. Although *Kcnh2* responsible for ERG was downregulated in HF, *Kcnq1* responsible for KvLQT1 was not (Figure 3C). Regardless, this demonstrates that K⁺ channels are able to affect AV node conduction and refractoriness. Mesirca et al. (Mesirca et al., 2016) reported that ablation of the ACh-activated K⁺ current (carried by Kir3.1 and Kir3.4) relieves the AV block in Cav1.3 knockout mice. The downregulation of *Kcnj3* and *Kcnj5* responsible for Kir3.1 and Kir3.4 in HF (Figure 3C) could, therefore, be a compensatory mechanism, although it is also

expected to reduce the response of the AV node to vagal stimulation. There were many significant changes of the less abundant K⁺ channel transcripts (Figure 3D). *Kcna1* and *Kcna2* (responsible for Kv1.1 and Kv1.2) were both significantly downregulated in HF (Figure 3D). In the mouse, systemic knockout of *Kcna1* (Kv1.1) results in AV block—although this was attributed to the autonomic nervous system, it could be a direct action on the AV node (Glasscock et al., 2010; Glasscock, 2019). Transgenic mice expressing high levels of activated RhoA (a GTPase implicated in cardiac hypertrophy) develop lethal ventricular failure (Sah et al., 1999). The mice develop AV block and Sah et al. (1999) hypothesised that this was the result of RhoA physically associating with and suppressing the activity of Kv1.2 (Cachero et al., 1998). Interestingly, *RhoA* was significantly upregulated in the AV node in HF in the present study (data not shown). Transcripts for four Ca²⁺-activated K⁺ channels were present and *Kcnn2* (responsible for K_{Ca}2.2 or SK2) was the most abundant (Figure 3D). *Kcnn2* and *Kcnn1* (responsible for K_{Ca}2.1 or SK1) were significantly downregulated in HF (Figure 3D) and this is likely to be functionally important. In the mouse, deletion of one copy of the *Kcnn2* gene results in AV node dysfunction—a prolongation of the AV node action potential, a slowing of AV node spontaneous activity and an increase in the PR interval—and deletion of both copies results in complete AV block (Zhang et al., 2008).

It is possible that the changes in ion channel expression are dependent on the severity of HF. To explore this, for each of the six pooled samples measured, the expression of transcripts for key ion



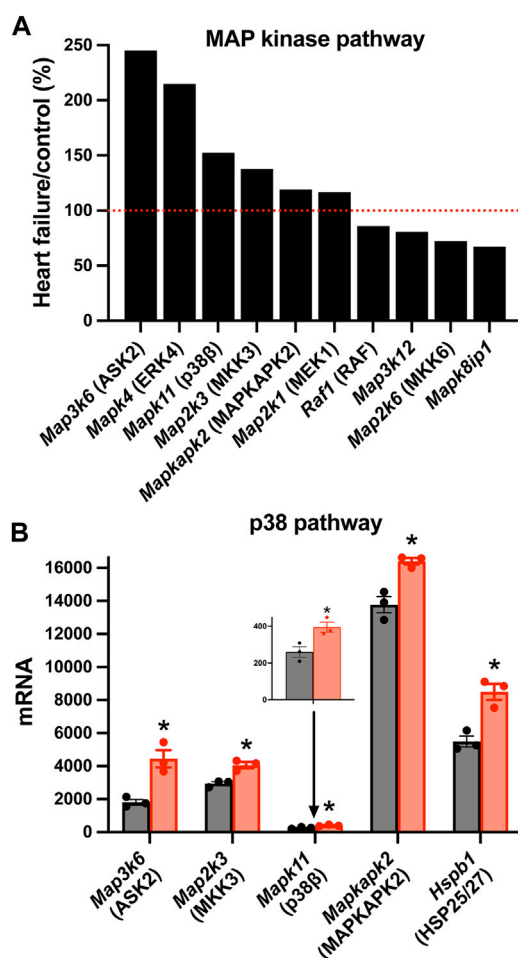


FIGURE 5

Changes in MAP kinase pathway transcripts in the AV node in HF. (A), significant changes in the expression of MAP kinase pathway transcripts. Expression in HF mice is shown as a percentage of that in control mice. Red dotted line corresponds to 100%. (B), mean (+SEM) expression (as well as individual data points) of p38 pathway transcripts in control (black bars) and HF (red bars) mice. The data for *Mapk11* are also shown in a zoomed-in panel. * $p < 0.05$.

channel subunits was plotted against *Myh7*, a well-known HF marker, and in all cases with one exception there was a significant correlation (Supplementary Figure S3). However, they were not correlated with *Nppa* and *Nppb*, two other HF markers.

Changes of further ion channels and transporters are considered in the (Supplementary Figures S4–S7).

Evidence of a shift from fatty acid to glucose metabolism

It is well known that HF is associated with metabolic remodelling and energy insufficiency (Doenst et al., 2013; Wende et al., 2017). This insufficiency is thought to play an important role in the development of HF. Under normal conditions, the heart derives 50%–70% of the ATP needed from fatty acids with glucose

contributing less (Wende et al., 2017). However, under stress conditions, there can be a shift from fatty acid to glucose utilisation (Tran Diem and Wang Zhao, 2019). Glucose generates more ATP than fatty acid for each mole of oxygen (Shiojima and Walsh, 2006). For the first time, we show that a qualitatively similar metabolic remodelling occurs in the AV node. Transcripts which change significantly in HF and are associated with glycolysis, fatty acid beta-oxidation, the tricarboxylic cycle, and the electron transport chain are shown in Figure 4. Glucose entry into cardiac myocytes is facilitated by GLUT1–13 transporters (Kewalramani and Rodrigues, 2009), but there were no changes in the corresponding transcripts (*Slc23a1–13*; Supplementary Figure S8A). Transcripts for six key enzymes in glycolysis were significantly upregulated in HF (Figure 4B) and are highlighted red in the metabolic pathway (Figure 4A). In two cases there appeared to be a partial isoform switch: whereas *Aldoa* and *Eno1* were significantly upregulated in HF, *Aldob* and *Eno2* were significantly downregulated (Figures 4A,B). Fatty acid is transported into cardiac myocytes by FAT/CD36 (*Cd36*), FABPpm (*Got2*) and FATP1–6 (*Slc27a1–6*) transporters (Chabowski et al., 2008); *Cd36* was significantly upregulated and *Slc27a1* was significantly downregulated (Supplementary Figure S8B). Transcripts for two key enzymes in the fatty acid beta-oxidation pathway (*Hadh* and *Acaa2*) were significantly downregulated in HF (Figure 4C) and are highlighted blue in the metabolic pathway shown in Figure 4A. Although in the same set of transcripts shown in Figure 4C, *Irs2* and *Akt1* were upregulated in HF, AKT enhances glycolysis by promoting glucose uptake and may inhibit fatty acid oxidation (Shiojima and Walsh, 2006), and IRS similarly enhances glycolysis and inhibits fatty acid oxidation by acting via AKT (as well as AMP-activated protein kinase) (Long et al., 2011). *Ppargc1a* (PGC-1α) is a master regulator of mitochondrial functions (Austin et al., 2011) and *Ppargc1a* and also *Ppara* are involved in transcriptional regulation of fatty acid oxidation enzymes (Wende et al., 2017); both were upregulated (Supplementary Figure S9A). AMP-activated protein kinase is a central regulator of metabolism and it promotes both glycolysis and fatty acid oxidation (Kewalramani and Rodrigues, 2009), and there was an upregulation of three key AMP-activated protein kinase subunits (Supplementary Figure S9B). Transcripts for three key enzymes in the tricarboxylic acid cycle were significantly upregulated in HF (Figure 4D) and are again highlighted red in the metabolic pathway (Figure 4A). Finally, transcripts for three key enzymes in the electron transport chain were significantly upregulated in HF (Figure 4E) and are highlighted red in the metabolic pathway (Figure 4A); *mt-Nd5* is responsible for NADH dehydrogenase 5, part of complex I, and *mt-Co1* and *Cox5a* are responsible for cytochrome c oxidase subunits I and 5a of complex IV. In addition, *Ppargc1a* (the master regulator of mitochondrial functions) was significantly upregulated (Figure 4E) as already discussed and this has been shown to affect the electron transport chain (Austin et al., 2011).

Evidence of activation of intracellular signalling pathways and transcription factors

There was a significant upregulation of key transcripts in various intracellular signalling pathways and, as an example, the mitogen-

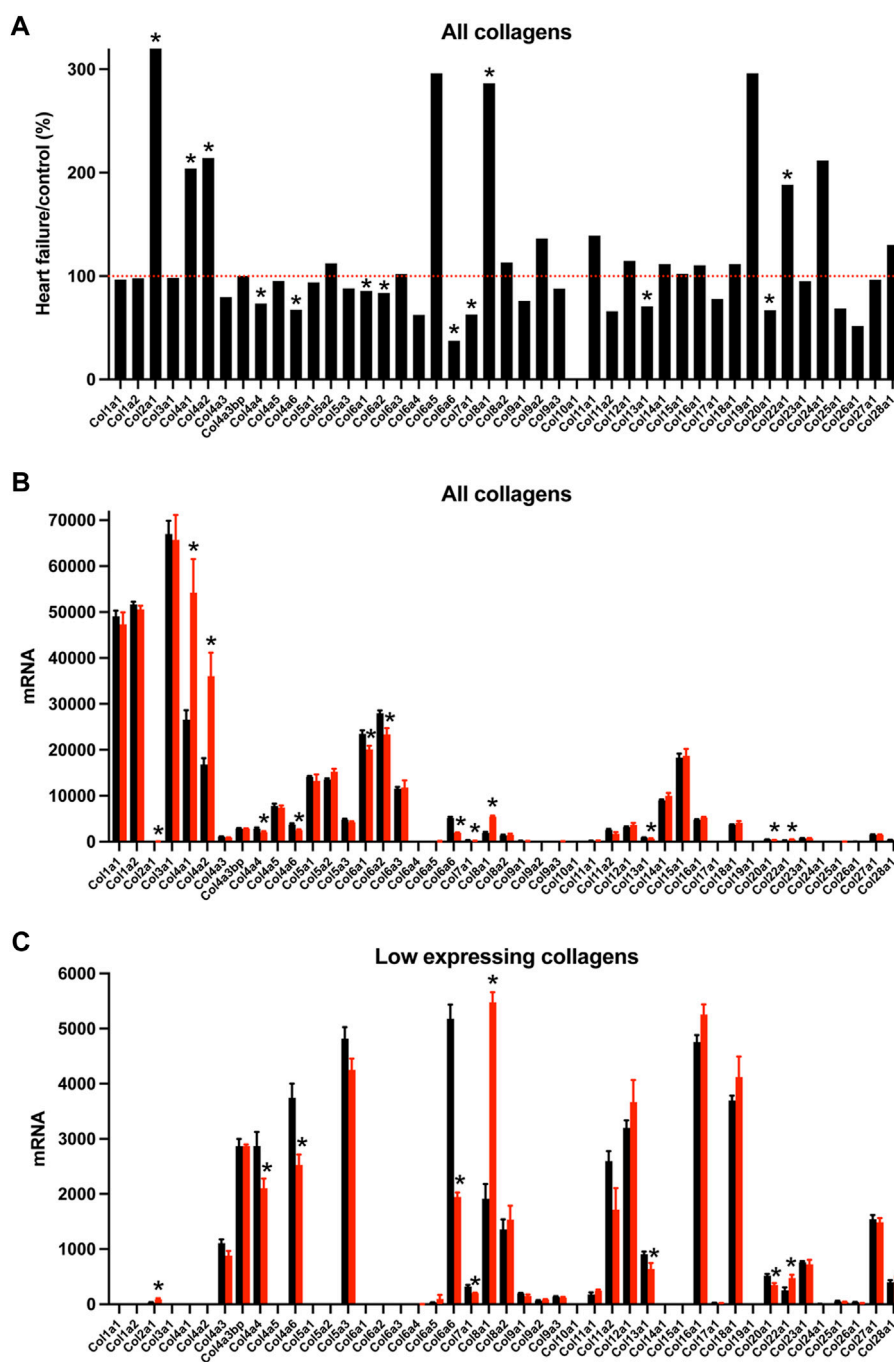


FIGURE 6

Transcriptomic remodelling of the extracellular matrix of the AV node in HF. (A), expression of all collagen transcripts in HF mice as a percentage of that in control mice. Red dotted line corresponds to 100%. (B and C), expression of highly expressed collagen transcripts (B) and more poorly expressed collagen transcripts (C) in control (black bars) and HF (red bars) mice. Only means + SEM shown (individual data points not shown for clarity). * $p < 0.05$.

activated protein (MAP) kinase pathway will be considered here. MAP kinases play an important signalling role in the heart (Rose et al., 2010). Various members of the family were significantly affected in the AV node in HF (Figure 5A). In particular, various members of the p38 signalling pathway (one arm of the MAP kinase signalling network) were upregulated (Figure 5B). MAP kinase

signalling is prototypically activated by three tiers of phosphorylation: a MAP kinase kinase phosphorylates and activates a MAP kinase, which in turn phosphorylates and activates a MAP kinase (mediator of biological responses) (Rose et al., 2010). In the p38 pathway, *Map3k6* was upregulated (Figure 5); *Map3k6* is responsible for ASK2, which together with

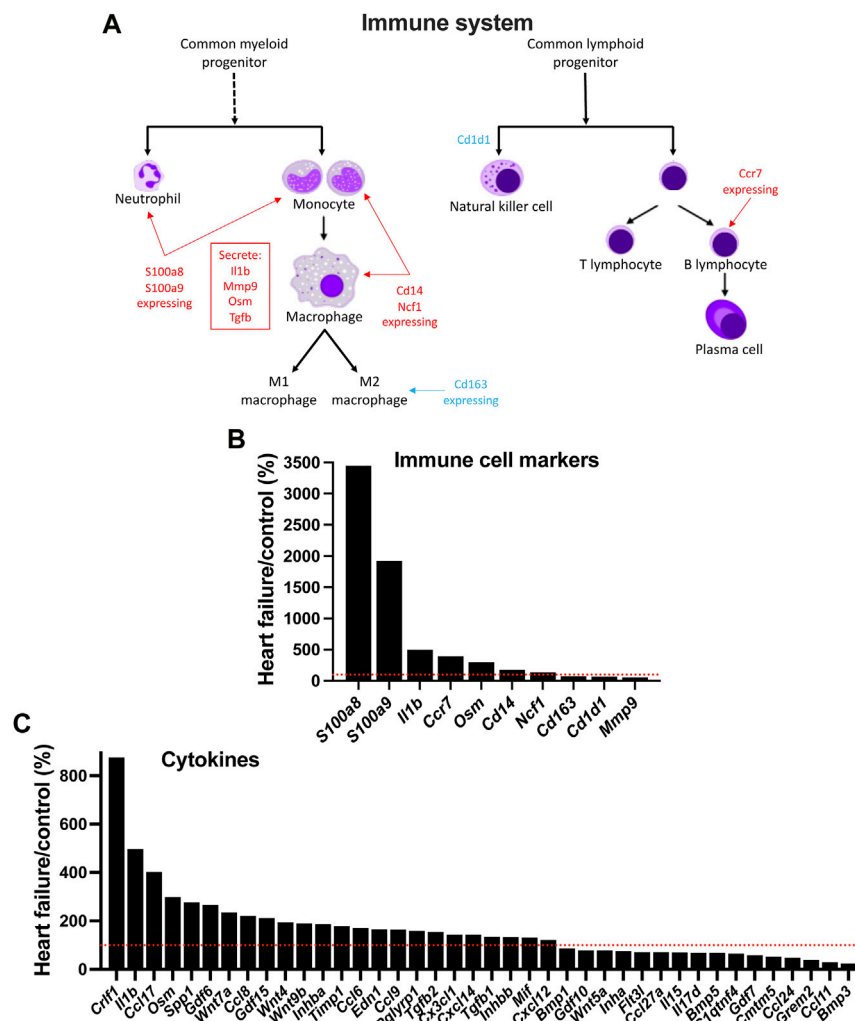


FIGURE 7

Evidence of infiltration of the AV node by immune cells in HF. (A), schematic diagram of the cells of the immune system. (B), expression of immune cell markers (transcripts) in HF mice as a percentage of that in control mice. (C), expression of cytokine transcripts in HF mice as a percentage of that in control mice. Red dotted lines correspond to 100%.

ASK1, acts as a MAP kinase kinase (Takeda et al., 2007). *Map3k6* has previously been reported to be upregulated in the heart in the mouse model of pressure overload-induced HF (Matkovich et al., 2012). In the p38 pathway, *Map2k3* was upregulated (Figure 5); *Map2k3* is responsible for MKK3 and acts as a MAP kinase. Finally, in the p38 pathway, *Mapk11* was upregulated (Figure 5); *Mapk11* is responsible for p38 β (one of four p38 isoforms present). p38 α/β phosphorylates the downstream kinase, MAPKAPK2, which phosphorylates the heat shock protein HSP25/27 and the transcripts for both (*Mapkapk2* and *Hspb1*) were significantly upregulated (Figure 5B). p21-activated kinases (PAKs) are a group of p38 activators and *Pak2* and *Pak3* were upregulated in HF (although *Pak6* was downregulated; data not shown) (Zarubin and Han, 2005). The p38 pathway is activated in HF and the resultant pathological remodelling can lead to cardiac arrhythmias (Romero-Becerra et al., 2020). Interestingly, p38 β regulates Hippo signalling (Huang et al., 2016). p38 inhibition improves heart function in pressure-overload right ventricular

hypertrophy (Kojonazarov et al., 2017). The activity of MAP kinases are tightly controlled by a family of dual-specificity MAP kinase phosphatases (DUSPs) and they have been shown to have a variety of physiological and pathological roles (Liu and Molkentin, 2016). 10 DUSP transcripts were significantly up or downregulated in HF (data not shown).

There was also a significant upregulation of key transcripts in the protein kinase A, Ca²⁺-calmodulin-dependent protein kinase II (CaMKII), Hippo, WNT, JAK-STAT, and NOTCH signalling pathways (Supplementary Figures S10–S13). Protein phosphorylation is an important signalling mechanism, and it is determined by a balance of protein kinases and phosphatases and there was a remodelling of both protein kinases and phosphatases at the transcript level (Supplementary Figures S14, S15). Transcript expression is primarily governed by transcription factors, and in the mouse Zhou et al. (2017) have identified 941 transcription factors. Of these, transcripts for 927 were identified in the AV node and 148 (16%) changed significantly in HF (Supplementary Figure S16). 81

TABLE 1 Transcripts which changed significantly in the AV node in HF and the corresponding genes have been identified by GWAS studies as being associated with the PR interval or naturally-occurring mutations in the corresponding genes are associated with AV block. Gene/transcript name, transcript abundance in HF as a percentage of that in control animals, adjusted *p*-value of the change in HF, and notes on the functions of the genes shown.

Gene	HF/ control (%)	Adjusted <i>p</i> -value	Function
GWAS studies			
<i>Camk2d</i>	116.3	0.028	Signalling molecule uniquely positioned to promote HF and arrhythmias; suggested to be causative of sinus node dysfunction Wu and Anderson (2014)
<i>Fat1</i>	121.6	0.043	FAT cadherins are evolutionarily conserved cell adhesion molecules, which play key roles in modulating tissue morphogenesis Helmbacher (2018)
<i>Fermt2</i>	121.3	0.0055	Important in integrin activation and cell-cell adhesion Yan et al. (2020)
<i>Fign</i>	62.0	2.04E-05	Unknown
<i>Id2</i>	79.4	0.033	Transcriptional regulator; a molecular pathway including ID2, TBX5 and NKX2-5 is required for cardiac conduction system development Moskowitz et al. (2007)
<i>Lrch1</i>	141.5	0.0015	Unknown
<i>Meis1</i>	82.7	0.014	Homeobox transcription factor essential for vertebrate heart development and normal physiology of adult heart Dupays et al. (2015)
<i>Myocd</i>	188.6	1.71E-10	Transcription factor; upregulated in cardiac tissue from patients with heart disease; upregulation causes left ventricular systolic dysfunction, and impairment of electrical activity and hypertrophy; downregulation attenuates cardiac muscle dysfunction and death Miano (2015)
<i>Tbx3</i>	69.3	0.0032	Transcription factor; involved in formation of AV node
<i>Tmem182</i>	120.8	0.0092	Unknown
<i>Scn10a</i>	54.5	0.0047	Modulator of AV node conduction Park and Fishman (2014)
Studies of naturally-occurring mutations			
<i>Lmna</i>	120.8	0.0022	Mutations known to be linked to high degree AV block Crasto et al. (2020)
<i>Prkag2</i>	163.7	5.13E-05	Gain-of-function mutations known to be linked to AV node dysfunction Gollob (2008)
<i>Trpm4</i>	66.0	8.86E-08	Loss-of function mutations known to be linked to AV block Bianchi et al. (2018)

(55%) were upregulated and 67 (45%) downregulated ([Supplementary Figure S16](#)).

Remodelling of extracellular matrix transcripts

Nodal dysfunction has frequently been attributed to fibrosis, i.e., a proliferation of the fibrillar collagens ([Suarez-Mier et al., 1995](#); [Bailey et al., 2007](#); [Swaminathan et al., 2011](#); [Csepe et al., 2015](#)), an important component of the extracellular matrix. The “Matrisome” from [Naba et al., \(2016\)](#) lists 1,110 transcripts in the mouse known to be associated with the extracellular matrix divided into four different categories. In the AV node in HF, many significant changes in Matrisome transcripts were observed (221 transcripts, i.e. 20% of the total) showing that there was likely a remodelling of the extracellular matrix. Collagens are the most abundant proteins in the extracellular matrix. Transcripts for two fibrillar collagens were present, types 1 and 3; they were the most abundant of the collagens, but they did not change in HF ([Figure 6](#)). Basement membranes are dense extracellular matrix structures, and previously we have shown that basement membrane surrounds each sinus node myocyte ([Linscheid et al., 2019](#)). Collagen type 4 is a major component of basement membranes ([Steffensen and Rasmussen, 2018](#)) and *Col4a1*

and *Col4a2*, the two most abundant collagen type 4 α -chain transcripts, were significantly upregulated in the AV node in HF ([Figure 6](#)). Collagen type 4 expression is known to be altered in various pathophysiological conditions ([Shamhart and Meszaros, 2010](#)). An increase in collagen type 4 may thicken the basement membrane of myocytes and impede extracellular communication with other cells ([de Castro Brás et al., 2014](#)). Collagen type 6 α -chain transcripts were significantly downregulated in the AV node in HF ([Figure 6](#)). Collagen type 6 carries out cytoprotective functions as well as mechanical roles ([Cescon et al., 2015](#)). In part by binding to collagen type 4 in the basement membrane, collagen type 6 links the myocyte to the extracellular matrix. In dilated cardiomyopathy in the human, [Gil-Cayuela et al., \(2016\)](#) reported there is an increase in the transcript (*Col8a1*) and protein for the non-fibrillar collagen eight $\alpha 1$ subunit, and there is a significant relationship between left ventricular mass and *Col8a1* expression. There was also a significant upregulation of *Col8a1* in the AV node in HF ([Figure 6](#)). [Gil-Cayuela et al., \(2016\)](#) hypothesise that overexpression of a number of non-fibrillar collagen genes may promote pathological remodelling by inducing apoptosis and hypertrophy of cardiomyocytes. The sum of all collagen transcripts was not significantly altered in HF (data not shown). Changes in other components of the Matrisome are considered in the Supplementary Results ([Supplementary Figures S17–S19](#)).

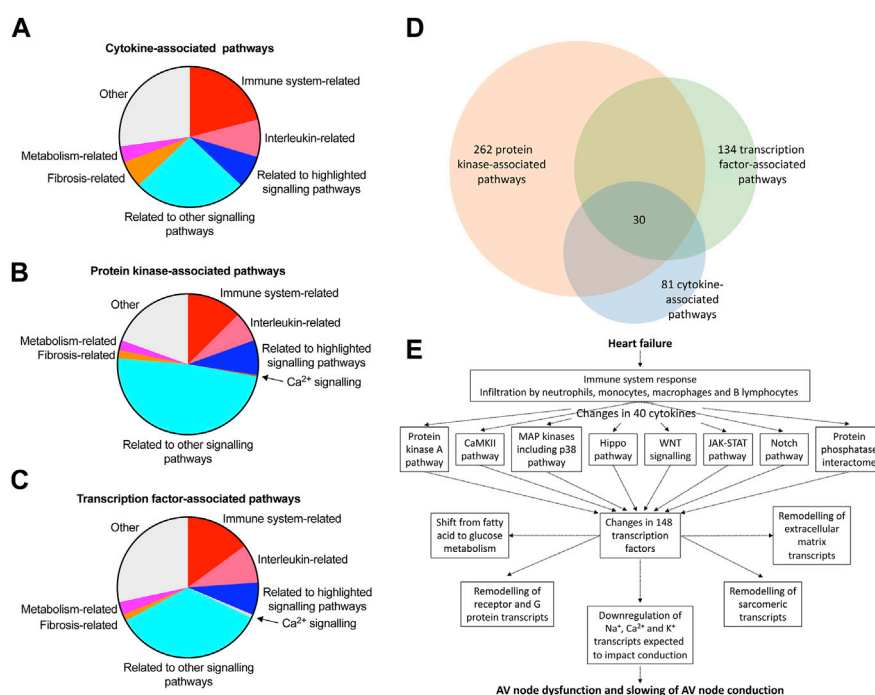


FIGURE 8

Pathways involved in AV node dysfunction in HF. (A–C), summary of canonical pathways (identified by Ingenuity Pathway Analysis) associated with the cytokines (A), protein kinases (B) and transcription factors (C) changing significantly in the AV node in HF. Details of the pathways are given in the Supplementary File, Canonical pathways. xlsx. 81, 262 and 134 pathways associated with the cytokines, protein kinases and transcription factors, respectively, were identified in total and the area of the pie chart for each group of pathways reflects the number of pathways in each group. (D), Venn diagram of the pathways associated with the cytokines, protein kinases and transcription factors; there is substantial overlap of the pathways and 30 pathways were common to the cytokines, protein kinases and transcription factors. (E), schematic diagram showing a potential chain of events in the AV node during HF leading to AV node dysfunction. Although the schema is based on the results of this study, it remains hypothetical. Although unidirectional arrows are drawn, they could be bidirectional.

Evidence of an immune response

In HF, although inflammation plays a key role in the development of ventricular dysfunction (Martini et al., 2019; Strassheim et al., 2019), it is not known whether it plays a similar role in AV node dysfunction. Using single cell RNAseq to measure the expression of transcript markers, Martini et al. (2019) showed that the myocardium is infiltrated by a variety of immune cell types in the mouse TAC model of HF. Figures 7A,B shows the expression of various immune cell marker transcripts in the AV node which changed significantly in HF. *S100a8* and *S100a9*, expressed in neutrophils and monocytes, were upregulated (Figures 7A,B). During inflammation, *S100A8/A9* is released and modulates the inflammatory response by stimulating leukocyte recruitment and inducing cytokine secretion (Wang et al., 2018). *Cd14* is expressed in monocytes, macrophages, dendritic cells and neutrophils (Marcos et al., 2010) and was upregulated in the AV node in HF (Figures 7A,B). *Ncf1* forms a subunit of NADPH oxidase, which is primarily active in phagocytes (including monocytes and macrophages) and plays an essential role in the immune system; *Ncf1* was upregulated in the AV node in HF (Figures

7A,B). These data suggest that neutrophils, monocytes and macrophages are infiltrating the AV node in HF. However, this may not be true of all macrophages, which exist as M1 cells (pro-inflammatory) or M2 cells (involved with resolution of inflammation and repair of damaged tissues). The data suggest a decline in the M2 cell type: the M2-like marker, *Cd163* (Martini et al., 2019), was downregulated and also the repair associated transcript, *Mmp9* (Martini et al., 2019), was downregulated (Figures 7A,B). *Cd1d1* is involved in natural killer T cell development (Sundararaj et al., 2018) and this too was downregulated (Figures 7A,B). However, phagocytes may not be the only immune cell type to be infiltrating the AV node in HF, because *Ccr7*, a marker of B lymphocytes (Mcheik et al., 2019), was also upregulated (Figures 7A,B). Cytokines are signalling molecules, largely secreted by cells of the immune system (predominantly macrophages and T helper cells), which mediate communication among immune and non-immune cells and are the means by which immune cells may impact the heart. Figure 7C shows that a large number of cytokine transcripts were significantly altered in the AV node in HF, some with known connections to HF; see Supplementary Figure S20 for further information.

HF-dependent changes in transcripts identified by GWAS as determinants of the PR interval or associated with hereditary AV node dysfunction

GWAS studies have identified 44 genes associated with an abnormal PR interval (van Setten et al., 2018). Of these, the transcripts associated with 11 genes changed significantly in the AV node in HF (Table 1) and therefore could be involved in the AV node dysfunction in HF. The function of some of the transcripts (*Fat1*, *Fermt2*, *Fign*, *Lrch1*, *Tmem182*) is either unknown or has an unknown relevance for the AV node; some transcripts are involved in heart development (*Meis1*, *Id2*, *Tbx3*); two transcripts are known to be involved in HF (*Camk2d*, *Myocd*); and one transcript (*Scn10a*) is known to modulate AV node conduction (Table 1). Three transcripts known to be associated with hereditary AV node dysfunction (*Lmna*, *Prkag2*, *Trpm4*; Table 1) changed significantly in the AV node in HF.

Other HF-dependent changes

There were many other HF-dependent changes and some of these are considered in the Supplementary Figures S22–S25.

Discussion

This study has shown that in a model of pressure overload (hypertension)-induced cardiac hypertrophy and HF there is a significant prolongation of the PR interval, evidence of AV node dysfunction as observed in HF patients (Nikolaidou et al., 2016). This was accompanied by significant changes in 5.6% of the transcriptome of the AV node: for example, in transcripts responsible for the PR interval, the sarcomere, fatty acid and glucose metabolism, the extracellular matrix, and the transcription and translation machinery. Importantly, there were also significant changes in transcripts involved in multiple signalling molecules and pathways, all of which have the potential to be involved in cardiac remodelling. Finally, there was evidence of an activation of the immune system and a proliferation or infiltration of macrophages and other immune cells in the AV node. The complexity and multiplicity of effects of HF on the AV node is a novel finding. There are likely to be many more effects on the AV node; the reader can peruse the list of all transcripts in the Supplementary Material (All transcripts. xlsx).

Potential immediate cause of AV dysfunction

In the AV node in HF there was a downregulation of key ion channel transcripts involved in AV node conduction: *Scn10a* responsible for Nav1.8; *Cacna1d* (Cav1.3) in part responsible for the L-type Ca^{2+} current, $I_{\text{Ca,L}}$; *Cacna1g* (Cav3.1) and *Cacna1h* (Cav3.2) responsible for the T-type Ca^{2+} current, $I_{\text{Ca,T}}$; and *Kcnh2* (ERG) responsible for the rapid delayed rectifier K^{+} current, $I_{\text{K,r}}$. These changes could be responsible for the slowing of AV node conduction and AV node dysfunction (Regan et al., 2005; Chambers et al., 2010; Holm et al., 2010; Pfeufer et al., 2010; Park and Fishman, 2014; Mesirca et al., 2015; Auxerre-Planté et al., 2019).

Widespread transcriptomic remodelling of the AV node

Transcriptomic remodelling of the AV node in HF was not restricted to ion channels—there was a remodelling of other systems as well. There was a transcriptomic remodelling of receptors and G proteins, which potentially could impact AV node conduction (Supplementary Figure S22). Some of the transcriptomic remodelling in the AV node in HF is similar to that reported for the rest of the heart, for example, the changes in natriuretic peptide and myosin heavy chain transcripts (Supplementary Figure S2) (Dirkx et al., 2013; Man et al., 2018). The observed changes in metabolic transcripts are qualitatively similar to changes observed in whole heart studies: in a rat TAC model of HF, Bugger et al. (2009) reported that six proteins involved in fatty acid beta-oxidation were downregulated including HADH (downregulation of *Hadh* was observed in this study—Figure 4C), three involved in glycolysis were upregulated, two involved in the tricarboxylic acid cycle were upregulated including aconitase 2 (upregulation of *Aco2* was observed in this study—Figure 4D), and some proteins involved in the electron transport chain were upregulated and some downregulated including subunits of NADH dehydrogenase and cytochrome c oxidase. In the mouse TAC model of HF, a decreased abundance of proteins involved in fatty acid metabolism and increased abundance of proteins involved in glycolysis has been reported in the ventricles (Dai et al., 2012). HF is reported to result in fibrosis and this can be arrhythmogenic by interrupting the conduction of the action potential (Nguyen et al., 2017). However, there was no evidence of upregulation of transcripts for the fibrillar collagens, *Col1* and *Col3*, in the AV node in HF, although there was a widespread transcriptional remodelling of the extracellular matrix (Figure 6 and Supplementary S17–S19).

Potential causes of the adverse remodelling of the AV node

This study has shown a transcriptomic remodelling of the protein kinase A, CaMKII, p38-MAP kinase, Hippo, WNT, JAK-STAT and Notch signalling pathways as well as the protein phosphatase interactome in the AV node in HF; it has also shown evidence of an immune response; each of these has been shown in other studies to be involved in cardiac pathological remodelling in disease. This study has also shown significant changes in signalling molecules: 40 cytokines, 109 protein kinases and 148 transcription factors (Figure 7C, Supplementary S14 and S16). Ingenuity pathway analysis was used to identify the canonical pathways significantly associated with the cytokines, protein kinases and transcription factors and the results are given in a Supplementary file (Canonical pathways. xlsx) and summarised in Figures 8A–C; in each case, many molecules are associated with the immune system especially the interleukin pathway, many are associated with intracellular signalling pathways (both signalling pathways highlighted in this study and many other pathways), and some are associated with Ca^{2+} signalling, metabolism and fibrosis. Figure 8D shows a large amount of overlap between the three sets of associated canonical pathways and this increases confidence in the predictions. The highlighted

signalling pathways identified by the canonical pathway analysis include protein kinase A signalling, p38 MAPK signalling, PAK signalling, HIPPO signalling, WNT/ β -catenin signalling, JAK/Stat signalling, STAT3 pathway and Notch signalling—this increases the likelihood that these pathways are involved. The involvement of multiple signalling pathways in the AV node in HF is not surprising because there were widespread changes (involving >3,000 transcripts) in the AV node in HF. This raises reservations concerning therapeutic strategies targeting only one pathway.

The activation of the immune system has the potential to be the driver of all the other changes observed in the AV node. The function of the immune system is not only to protect the animal from invasion by foreign organisms. In the case of the heart, it is also concerned with repair of the heart after myocardial infarction (Kologrivova et al., 2021), the adverse remodelling of the working myocardium in heart disease (Strassheim et al., 2019; Murphy et al., 2020), and even ageing (Appel et al., 2021). For the first time, we show here evidence that in HF there is a proliferation of immune cells (neutrophils, monocytes, macrophages and B lymphocytes) in the AV node (Figure 7). One of the principal lines of evidence for a proliferation of neutrophils and monocytes were increases in *S100a8* and *S100a9* to 3,447% and 1,923% of control (Figure 7B). During inflammation, S100A8/A9 is actively released and exerts a critical role in modulating the inflammatory response and serves as a candidate biomarker for disease (Wang et al., 2018). S100A8/A9 plasma levels are significantly higher in chronic HF patients (Wang et al., 2018).

In the AV node in HF there was an increase in transcripts for proinflammatory cytokines (and proinflammatory cytokine-related molecules): *Il1b* (IL-1b), *Osm* (oncostatin M; cytokine of interleukin-6 family), *Tnfrsf1a* (TNF receptor 1), *Tnfrsf1b* (TNF receptor 2), *Tgfb1* (TGF β -1) and *Tgfb2* (TGF β -2) (Figure 7C). *Lgals3*, responsible for galectin-3, which can have cytokine-like regulatory actions in immune cells (Jeon et al., 2010), was also significantly upregulated in the AV node in HF (Supplementary Figure S20C). These upregulated cytokines and cytokine-related molecules have a known role in HF: *Il1b* is upregulated in HF and associated with worse prognosis and trials have been undertaken to evaluate the role of interleukine-1 blockade to reduce inflammation and ventricular remodelling in HF (Van Tassell et al., 2015; Szekely and Arbel, 2018). There is evidence that IL-1b downregulates $I_{Ca,L}$ and K^+ current and Ca^{2+} -handling proteins (Szekely and Arbel, 2018). Patients suffering chronic dilated cardiomyopathy show upregulation of oncostatin M and its receptor, O β (oncostatin M receptor; *Osmr*) (Kubin et al., 2011). Cardiac oncostatin M (*Osm*) induces dedifferentiation of cardiomyocytes and promotes progression of HF, and an antibody targeting O β or genetic inactivation of a single allele of *Osmr* reduces cardiomyocyte remodelling and dedifferentiation and improves cardiac performance and survival (Kubin et al., 2011; Pöling et al., 2013). TNF- α and its receptors have both pathogenic and cardioprotective roles in heart disease (Rolski and Blyszczuk, 2020). In the mouse TAC model of HF, there is a correlation between progressive hypertrophy and increasing myocardial levels of TNF- α and

TNF receptor 1 (Rolski and Blyszczuk, 2020). Increased concentrations of circulating TNF receptors 1 and 2 are associated with increased risks of cardiovascular events and mortality in patients with stable coronary heart disease (Carlsson et al., 2018). TGF- β is another cytokine. Myocardial TGF- β expression is upregulated in animal models of myocardial infarction and cardiac hypertrophy and in patients with dilated or hypertrophic cardiomyopathy, and it plays an important role in cardiac remodelling and fibrosis (Dobaczewski et al., 2011). Galectin-3 overexpression and secretion is associated with several diseases including HF, in which galectin-3 has been shown to be a useful biomarker (Gehlken et al., 2018; Suthahar et al., 2018). Galectin-3 induces pathologic remodelling of the heart, and is a culprit in the development of cardiac fibrosis in HF and electrical remodelling in atrial fibrillation (Takemoto et al., 2016; Gehlken et al., 2018). Proof that inflammation is the cause of AV node remodelling and dysfunction in HF is beyond the scope of this study. However, if it is the cause there may be a correlation between the two and with one exception there are significant correlations between the two well-known heart failure markers, *Nppa* and *Nppb* (responsible for ANP and BNP) and the macrophage marker, *Cd14*, the cytokine transcript, *Crlf1*, and the transcript for the cytokine-like galectin-3, *Lgals3* (Supplementary Figure S21).

In conclusion, the ultimate reason for the dysfunction of the AV node in HF is likely to be the downregulation of Na^+ , Ca^{2+} and K^+ channel transcripts, as a result of transcription factors—as well as micro-RNAs (Yanni et al., 2020). It is feasible that these changes are driven by the protein kinase A, CaMKII, p38-MAP kinase, Hippo, WNT, JAK-STAT and Notch signalling pathways as well as the proliferation of immune cells and upregulation of cytokine transcripts in the AV node in HF. Figure 8E presents a hypothetical scheme of the possible hierarchy of changes. In addition to this, the changes in transcripts known to be related to AV block listed in Table 1 have also to be considered.

Data availability statement

The raw data from this study are available in a repository: <https://www.ebi.ac.uk/biostudies/arrayexpress/arrayexpress/studies/E-MTAB-12384?accession=E-MTAB-12384>. Further data are available in the Supplementary Material.

Ethics statement

The animal study was reviewed and approved by Experiments carried out in the UK were in accordance with the UK Animals (Scientific Procedures) Act (1986) and UK Home Office project licence PPL PBA6A87CA.

Author contributions

MRB and HD obtained funding for the study and supervised the experiments. CW and MZ carried out the experiments. MRB and

CW with assistance and direction from AS and MS analysed the data. MRB and CW wrote the manuscript with contributions from others. CW, MZ, MS, MH, AS, HD, and MRB read and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2023.1083910/full#supplementary-material>

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Cannabinoid receptor agonist attenuates angiotensin II-induced enlargement and mitochondrial dysfunction in rat atrial cardiomyocytes

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Pathological remodeling of atrial tissue renders the atria more prone to arrhythmia upon arrival of electrical triggers. Activation of the renin-angiotensin system is an important factor that contributes to atrial remodeling, which may result in atrial hypertrophy and prolongation of P-wave duration. In addition, atrial cardiomyocytes are electrically coupled *via* gap junctions, and electrical remodeling of connexins may result in dysfunction of coordinated wave propagation within the atria. Currently, there is a lack of effective therapeutic strategies that target atrial remodeling. We previously proposed that cannabinoid receptors (CBR) may have cardioprotective qualities. CB13 is a dual cannabinoid receptor agonist that activates AMPK signaling in ventricular cardiomyocytes. We reported that CB13 attenuates tachypacing-induced shortening of atrial refractoriness and inhibition of AMPK signaling in the rat atria. Here, we evaluated the effects of CB13 on neonatal atrial rat cardiomyocytes (NRAM) stimulated by angiotensin II (AngII) in terms of atrial myocyte enlargement and mitochondrial function. CB13 inhibited AngII-induced enhancement of atrial myocyte surface area in an AMPK-dependent manner. CB13 also inhibited mitochondrial membrane potential deterioration in the same context. However, AngII and CB13 did not affect mitochondrial permeability transition pore opening. We further demonstrate that CB13 increased Cx43 compared to AngII-treated neonatal rat atrial myocytes. Overall, our results support the notion that CBR activation promotes atrial AMPK activation, and prevents myocyte enlargement (an indicator that suggests pathological hypertrophy), mitochondrial depolarization and Cx43 destabilization. Therefore, peripheral CBR activation should be further tested as a novel treatment strategy in the context of atrial remodeling.

KEYWORDS

atrial fibrillation (AF), cardiomyocytes (CMs), angiotensin II, cannabinoid (CB) receptor 1, cannabinoid receptor (CB(2)), cannabinoid

1 Introduction

Atrial remodeling can occur due to multiple factors including age, genetic predisposition, and underlying cardiovascular disease (Heijman et al., 2014). Cardiovascular comorbidities including hypertension, heart failure, and valvular disease contribute to the development of atrial remodeling (Chiang et al., 2012). These underlying cardiovascular risk factors result in atrial remodeling making tissue vulnerable to triggers, re-entry, and ectopic firing, thus initiating arrhythmic episodes (Heijman et al., 2014; Forrester et al., 2018). Current pharmacotherapies for some of the consequences of atrial remodeling (such as atrial fibrillation) focus on maintenance of heart rate and rhythm through ion channel blockade. However, they are only modestly effective and can paradoxically lead to the development of life-threatening arrhythmias as well as additional side effects. (Dobrev and Nattel, 2010). Patients with underlying cardiovascular diseases are prone to disease progression beyond atrial remodeling. Enhanced activation of the renin-angiotensin system (RAS) in these patients results in structural and electrical atrial remodeling that markedly increases AF substrate (Schotten et al., 2011). Enhanced levels of angiotensin II (AngII) leads to left atrial enlargement and prolongation of P-wave duration, indicative of structural and electrical atrial remodeling (Kim et al., 2017; Forrester et al., 2018; Jansen et al., 2020). Thus, AngII likely plays an important mechanistic role in the development and consequences of atrial remodeling.

In contrast, adenosine monophosphate-activated protein kinase (AMPK) activation during AF appears to improve atrial structural, contractile, and electrical properties by regulating atrial metabolism and adenosine triphosphate (ATP) expenditure (Kim and Young, 2015; Brown et al., 2021). AMPK is a signaling protein sensor that monitors cellular energy level and is present in all mammalian cells, including atrial and ventricular cardiomyocytes (Arad et al., 2007; Harada et al., 2012). The increased activation rate of atrial cardiomyocytes during AF increases metabolism and energy consumption, resulting in depletion of ATP (Mihm et al., 2001; Brandenburg et al., 2016). Compounds that activate AMPK may mitigate the metabolic demands resulting from AF (Harada et al., 2015; Kim and Young, 2015). In this regard, our group has found that cannabinoid receptor (CBR) ligands exert cardioprotective effects in ventricular hypertrophy models *in vitro* and *in vivo* presumably due to activation of AMPK (Lu et al., 2014; Lu et al., 2020). Recently, we also demonstrated that CB13, a dual CBR agonist with limited brain penetration, is an AMPK activator that prevents tachycardia-induced electrical remodeling and improves energy metabolism in the rat atria *ex vivo* (Lee et al., 2021). In addition to activating AMPK, CB13 appears to alter gap junction, Cx43, a signal conduction protein that connects cardiomyocytes and is important for synchronous atrial contraction (Qiu et al., 2016; Lee et al., 2021). More recently Cx43 has been demonstrated to be a potential downstream signaling mediator of AMPK activation (Qiu et al., 2016). In fact, in the heart, a key upstream kinase involved in regulating AMPK activity is the serine/threonine kinase, LKB1 (Sakamoto et al., 2006). LKB1 activates AMPK α by phosphorylating Thr172 (Hawley et al., 1996; Hong et al., 2003). Inhibiting AMPK by deleting LKB1 leads to atrial enlargement, fibrosis, and fibrillation as well as reduced AMPK and Cx43 (Kim et al., 2015; Ozcan et al., 2015). Thus, CB13, as an activator of

AMPK, warrants further study as a potential therapeutic to attenuate pathological remodeling of atrial cardiomyocytes.

In this study, we examined the effect of CB13 on AngII-induced atrial myocyte enlargement and mitochondrial dysfunction, as characteristics of AF, using neonatal rat atrial cardiomyocytes (NRAM). Neonatal rat cardiomyocytes are often used to study cardiac myocyte hypertrophy. They respond to pharmacological (Shubeita et al., 1990; Knowlton et al., 1991) and mechanical stimuli (Sadoshima et al., 1992) with hallmarks (Chien et al., 1991) of hypertrophy that mimic those *in vivo*: changes in cell size, myocyte architecture, protein synthesis, and expression of hypertrophic genes. Our findings demonstrate that CB13 has cardioprotective effects *via* AMPK activation, independently of LKB1, and prevents structural, metabolic and gap junction abnormalities in neonatal rat atrial myocytes.

2 Materials and methods

2.1 Materials

AngII was from Sigma Aldrich (St. Louis, MO). CB13 (1-Naphthalenyl[4-(pentyloxy)-1-naphthalenyl]methanone, compound C (dosmorphin, (6-[4-(2-Piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo [1,5-a]pyrimidine)), JC-1 (tetraethylbenzimidazolylcarbocyanine iodide) Mitochondrial Membrane Potential assay kit and Mitochondrial Permeability Transition Pore (mPTP) assay kit were from Cayman Chemical (Ann Arbor, MI). Primary antibodies from Cell Signaling Technology (Danvers, Massachusetts) for phospho-AMPK α (Thr172) (1:1000, cat. 2535), AMPK α (1:1000, cat. 2603), phospho-LKB1 (1:1000, cat. 3482), LKB1 (1:1000, cat. 3047) and Abcam (Toronto, Ontario) connexin 43 (1:7500, cat. ab11370), CB1R (1:500, cat. ab23703), and CB2R (1:5000, cat. ab45942) were used.

2.2 Neonatal rat atrial cardiomyocytes

This study was approved by the University of Manitoba Animal Care Committee and follows Canadian Council of Animal Care guidelines. Use of neonatal myocytes is an accepted *in vitro* model for hypertrophy (Bishopric et al., 1987; Bishopric and Kedes, 1991; Yamazaki et al., 1995; Rokosh et al., 1996; Wu et al., 1996; Liang et al., 1997; Adams et al., 1998; Liang and Gardner, 1998; Wang et al., 1998; Liang and Gardner, 1999; Montessuit and Thorburn, 1999; Zhu et al., 2000; Lim et al., 2001; Takemoto et al., 2001; Higuchi et al., 2003; Liang et al., 2003; Anderson et al., 2004). This protocol is adapted from our NRVM protocol (Wu et al., 1989; Lu et al., 2020). Atria were isolated from 1 to 3-day-old neonatal Sprague-Dawley rats and digested at 37°C with 0.1% trypsin and 0.002% DNase in Ca²⁺- and bicarbonate-free Hanks HEPES (CBFHH) buffer with agitation in repeating 5-min cycles. After each digestion cycle the digested cells were suspended in bovine calf serum (BCS) in 50 mL conical tubes on ice; once the 50 mL conical tube was full it was centrifuged at 60 rpm for 15 min to obtain the cell pellet. Cell pellets were filtered through a 100 μ m strainer. To distinguish non-cardiomyocytes from NRAM, cells were seeded (i.e., pre-plated)

on T75 tissue culture flasks for 75 min in Dulbecco's Modified Eagle Medium (DMEM) containing 10% cosmic calf serum (CCS) and 1% penicillin-streptomycin (P/S). Pre-plating allowed non-cardiomyocytes to adhere to the tissue culture flask, whereas most NRAM remained suspended in the culture medium. NRAM were collected from flasks and seeded in 0.1% gelatin-coated 6, 12, 24, and 48 well culture plates in 10% CCS DMEM for 24 h. Cells were serum-starved for 24 h prior to experiments/treatments in 0% CCS DMEM.

2.3 Treatments

NRAM were rendered quiescent by serum starvation and immediately used for experiments. NRAM were pre-treated for 1 h with CB13 (1 μ M) in the presence or absence of chemical inhibitors of AMPK (compound C; 1 μ M; 1 h) (Lu et al., 2020). Our selection of concentrations (and time points) is predicated on first, our previous dose response analyses which found that 1 μ M of CB agonists attenuated ventricular myocyte hypertrophy, and this was blocked by 0.1 μ M of CB antagonists (Lu et al., 2014), and second, reports that sub- or low μ M concentrations ablate other effects of endocannabinoids (Underdown et al., 2005; Romano and Lograno, 2006; Lam et al., 2007). In the absence of pharmacological treatment, NRAM were exposed to vehicle controls. Following the 1 h pretreatment, all treatments (compound C and CB13) remained in the culture media for the entirety of the experimental protocol. Atrial cardiomyocyte hypertrophy and mitochondrial dysfunction were stimulated in NRAM by AngII (10 μ M; 24 h) (Kim et al., 2017; Jansen et al., 2018; Chen et al., 2020). Incubation times are based on the results of previously reported time course experiments monitoring CB13 effects in ventricular myocytes (Lu et al., 2014).

2.4 Cell surface area measurements

NRAM were cultured in 12-well plates (1.5×10^6 cells/mL) and treated as required. NRAM were fixed with 4% paraformaldehyde, followed by permeabilization with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and blocked with PBS containing 2% bovine serum albumin (BSA), as previously described (Anderson et al., 2004). NRAM were incubated with mouse anti-rat sarcomeric α -actinin primary antibody overnight. NRAM were washed three times with PBS and incubated with Alexa Fluor[®] 488-conjugated secondary anti-mouse IgG1 antibody (ThermoFisher Scientific) for 1 h at room temperature. After washing with PBS, NRAM were viewed using an Olympus IX81 brightfield fluorescence microscope. Surface areas of individual cells were quantified using ImageJ software.

2.5 Mitochondrial membrane potential ($\Delta\Psi_m$) imaging

To assess relative changes in mitochondrial membrane potential ($\Delta\Psi_m$) in NRAM, the JC-1 Mitochondrial Membrane Potential assay kit was used, as per the manufacturer's protocol (Cayman Chemical), and as previously described (Lu et al., 2020). JC-1 is a

lipophilic fluorescent reagent; in healthy mitochondria with high $\Delta\Psi_m$, JC-1 concentrates as J-aggregates and emits red fluorescence. In mitochondria with reduced $\Delta\Psi_m$, JC-1 is monomeric and emits green fluorescence.

NRAM were cultured in 24-well plates (0.75×10^6 cells/well). Following treatments, NRAM were incubated with JC-1 for 30 min at 37°C. NRAM images were acquired using an Olympus IX81 brightfield fluorescence microscope. Samples were ex/em at 485/535 nm for monomer green fluorescence and 560/595 nm for aggregate red fluorescence. The ratio of aggregate to monomer (red:green) fluorescence was measured as an indicator of changes in $\Delta\Psi_m$ via corrected total cell fluorescence (CTCF) using ImageJ software, where; CTCF = integrated density – (area of cell x mean fluorescence of background) and compared as aggregate CTCF: monomer CTCF.

2.6 Mitochondrial permeability transition (mPT) imaging

Mitochondrial Permeability Transition (mPT) Pore assays were performed by measuring mPT pore (mPTP) opening status in NRAM using a commercially available kit (Cayman Chemical, Michigan, United States) according to manufacturer specifications, and as previously described (Lu et al., 2020). This mPTP assay kit utilizes a calcein: cobalt technique that is multiplexed with TMRE. Calcein: cobalt dual staining is a recognized method to assess the degree of mPTP opening. Fluorescence contrast between the mitochondrial puncta and cytosol was measured to quantify mPTP.

NRAM were cultured in 48-well plates (0.5×10^6 cells/well) and treated as required. NRAM were coloaded with calcein, cobalt and TMRE for 15 min, followed by a 5 min wash with PBS. The wavelengths for calcein ex/em 485/535 and TMRE ex/em 545/571, approximately. Fluorescence was measured using an Olympus IX81 inverted fluorescence microscope. Fluorescence contrast was determined as the difference in fluorescent calcein intensity between mitochondrial puncta and cytosol using ImageJ. For each replicate, images were captured for 5 cells, and fluorescent calcein intensity was measured in three distinct fields.

2.7 Western blotting

NRAM were seeded in 12-well plates (1.5×10^6 cells/well) and treated as required. NRAM lysates were prepared in radioimmune precipitation assay (RIPA) lysis buffer with phosphatase and protease inhibitor cocktails, as previously described (Lee et al., 2021). Protein concentration was determined by bicinchoninic (BCA) assay (Thermo Fisher Scientific, Massachusetts, USA). NRAM samples were loaded on Mini-PROTEAN TGX stain-free gels (Bio-Rad, California, USA) at 10 μ g/lane and transferred to PVDF membrane (Bio-Rad). Membranes were blocked and primary antibodies were incubated overnight at 4°C. Primary antibody dilutions are listed under *Materials*. Secondary antibodies were from Cell Signaling. As applicable, membranes were stripped and reprobed. Blots were imaged

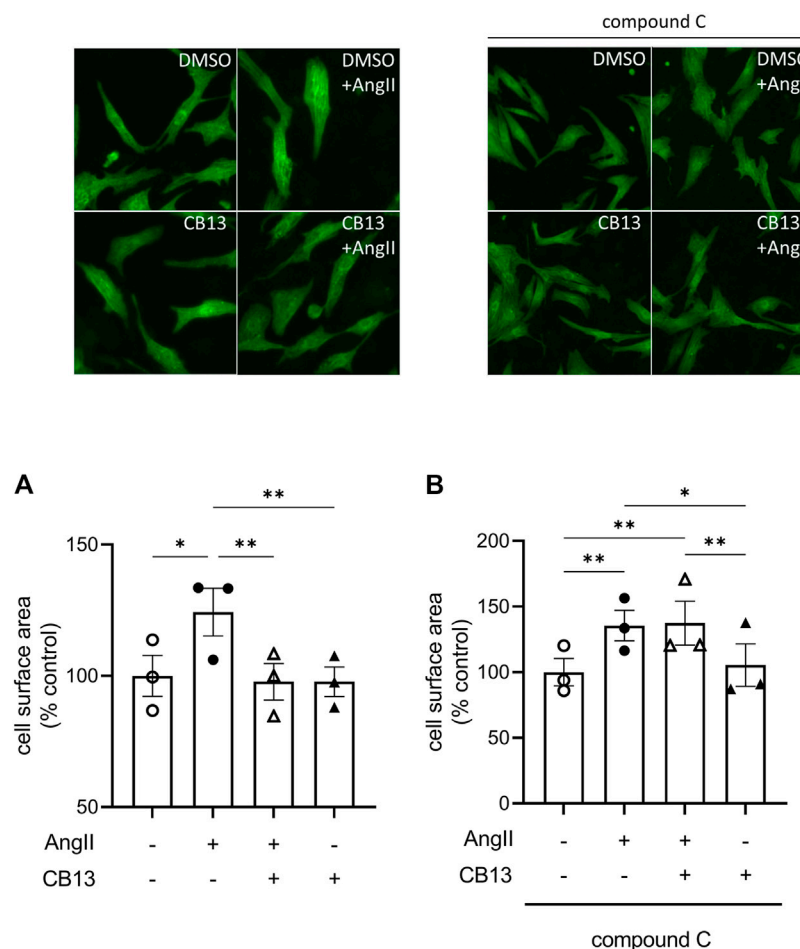


FIGURE 1

CB13 suppressed AngII-induced enhancement of NRAM cell surface area in an AMPK-dependent manner. **(A)** The ability of AngII to induce NRAM enlargement was abolished by CB13 treatment. **(B)** Treatment of NRAM with compound C, an AMPK inhibitor, prevented the ability of CB13 to suppress NRAM enlargement. Serum-deprived NRAM were pre-treated with CB13 (1 μ M, 1 h) in the presence or absence of compound C (1 μ M, 1 h), followed by treatment with AngII (10 μ M). All treatments remained in media for 24 h. Results are presented with representative fluorescent images. Data are presented as mean $\pm$ SEM. $n = 3$. $^{**}p < 0.01$.

with Bio-Rad ChemidocTM MP Imaging system and analyzed using Bio-Rad stain-free technology. Proteins of interest were normalized to total protein on the same stain-free membrane and quantified with Image Lab Software (Bio-Rad, Mississauga, Ontario). Each Western blot represents a single replicate (i.e., an independent experimental day). Within each replicate, NRAM were isolated from 60 rat pups, pooled, and plated. Protein from 3 wells was pooled to achieve sufficient protein amounts for Western blotting.

2.8 Statistical analysis

Data are reported as mean $\pm$ SEM. Experimental data were analyzed using GraphPad Prism 9 software. Normality assumptions were tested using the Shapiro-Wilk test for normality. For normally distributed data, a one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test for multiple comparisons was performed. A p -value of ≤ 0.05 was considered significant.

3 Results

3.1 CB13 suppresses AngII-induced myocyte enlargement in an AMPK-dependent manner

In patients with cardiovascular diseases, enhanced activation of the RAS system gives rise to atrial remodeling and increased risk for AF (Jansen et al., 2020). Here, we first examined whether CB13 would reduce a hallmark of pathological hypertrophy (that is, increased cell surface area) in atrial cardiomyocytes (Figure 1). Indeed, AngII, increased NRAM surface area compared to control cells ($124.6 \pm 5.0\%$ vs. control; $p \leq 0.01$) (Figure 1A). However, CB13 markedly reduced the AngII-dependent NRAM enlargement ($97.9 \pm 1.5\%$ vs. $124.6 \pm 5.0\%$; $p \leq 0.01$) (Figure 1A) indicating that CB13 suppresses atrial myocyte enlargement induced by RAS system activation.

We previously demonstrated that CB13 inhibits hypertrophy in ET1-treated NRVM and that disruption of AMPK signaling

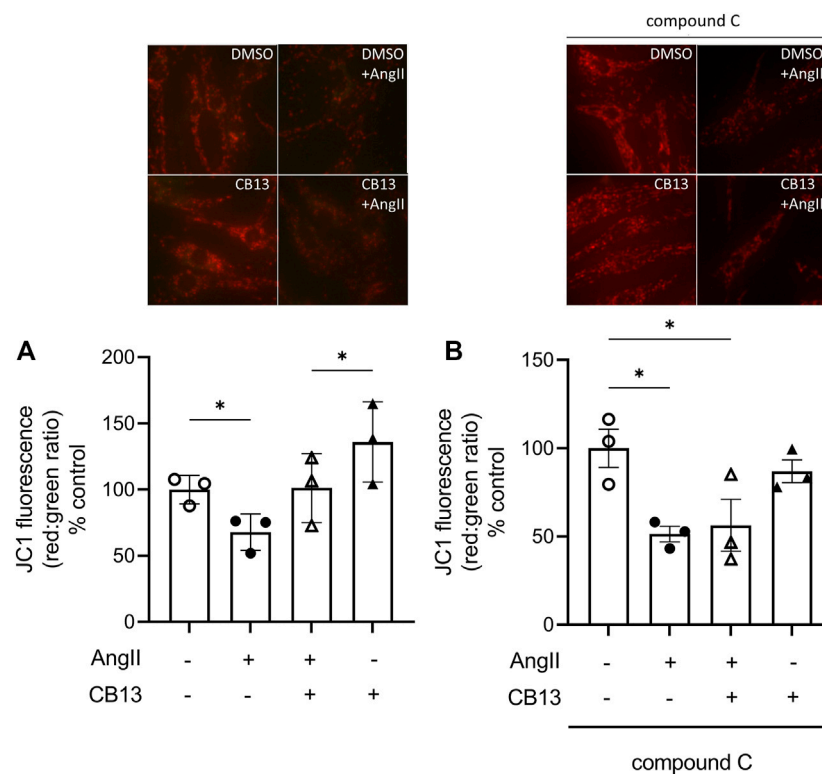


FIGURE 2

CB13 prevented AngII-induced mitochondrial membrane depolarization in an AMPK-dependent manner. The ratio of red aggregates to green monomers (red:green) reflects membrane depolarization. (A) CB13 treatment prevents AngII-induced mitochondrial membrane depolarization. AngII induces membrane depolarization, as indicated by a decrease in red:green, which is attenuated by CB13. (B) Compound C, an AMPK inhibitor, prevented the ability of CB13 to alter mitochondrial membrane depolarization. Rescue of red:green by CB13 was prevented by compound C treatment. Serum-deprived NRAM were pre-treated with CB13 (1 μ M, 1 h) in the presence or absence of compound C (1 μ M, 1 h), followed by treatment with AngII (10 μ M). All treatments remained in media for 24 h. Results are presented with representative fluorescent images. Data are presented as mean $\pm$ SEM. $n = 3$. * $p \leq 0.05$ and *** $p < 0.001$.

using a chemical inhibitor, compound C, abolished this effect (Lu et al., 2014). Thus, to determine whether CB13 suppresses enhancement of cell surface area in NRAM through an AMPK-dependent mechanism, we treated cells with compound C (Figure 1B). AngII increased NRAM surface area ($136.0\% \pm 3.5\%$ vs. control, $p < 0.01$). However, the ability of CB13 to prevent AngII-induced enlargement of NRAM was completely abolished by compound C ($137.0\% \pm 4.4\%$ vs. $136.0\% \pm 3.5\%$; ns) (Figure 1B). This suggests that CB13 prevents NRAM enlargement in an AMPK-dependent manner.

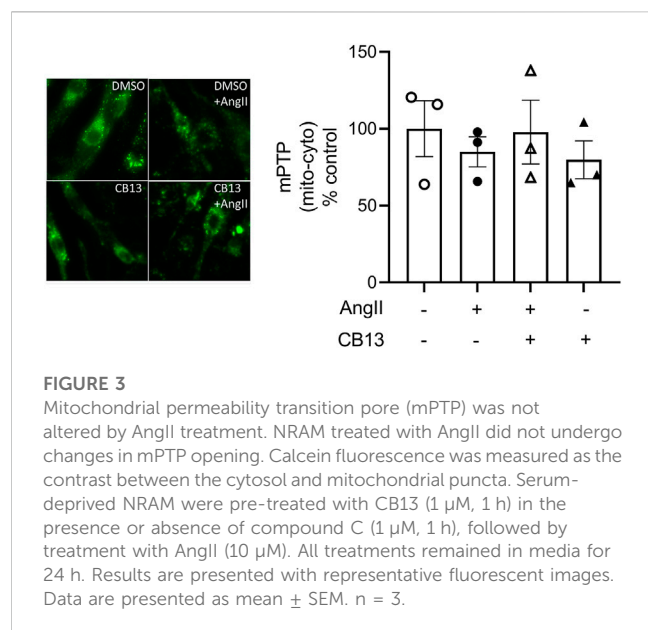
3.2 CB13 prevents AngII-induced mitochondrial membrane depolarization in an AMPK-dependent manner

Mitochondrial dysfunction underlies atrial metabolic remodeling (Harada et al., 2017; Brown et al., 2021). Therefore, mitochondrial membrane depolarization was measured as a parameter of metabolic remodeling. $\Delta\Psi_m$ is determined by the ratio between red J-aggregates to green monomers. The lipophilic and cationic dye, JC-1, selectively enters the mitochondria and changes from green to red as $\Delta\Psi_m$

increases. When cells are healthy, and the $\Delta\Psi_m$ is high, JC-1 forms red J-aggregates. When cells are apoptotic and unhealthy JC-1 remains in monomeric form and will form green monomers. AngII-induced a decline in red aggregate:green monomer ($67.3\% \pm 4.1\%$ vs. control; $p < 0.05$), indicating reduced $\Delta\Psi_m$ (Figure 2A). Mitochondrial membrane depolarization was prevented by CB13 in AngII-treated cells ($100.1\% \pm 9.4\%$ vs. $67.3\% \pm 4.1\%$; $p < 0.05$). When NRAM were pre-treated with compound C, CB13 no longer attenuated the decline in red:green (i.e., mitochondrial membrane depolarization) compared to AngII ($59.3\% \pm 13.8\%$ vs. $58.8\% \pm 8.7\%$; ns) (Figure 2B). Therefore, CB13 attenuated AngII-induced mitochondrial membrane depolarization in an AMPK-dependent manner.

3.3 Mitochondrial permeability transition pore (mPTP) is not altered by AngII

To measure mPTP atrial cardiomyocytes were coloaded with calcein, cobalt and TMRE and images were acquired. Fluorescence contrast between the mitochondrial puncta and cytosol was measured to quantify mPTP. NRAM treated with AngII did not exhibit alterations in mPTP opening, nor did CB13 influence mPTP either in the presence or absence of AngII (Figure 3).

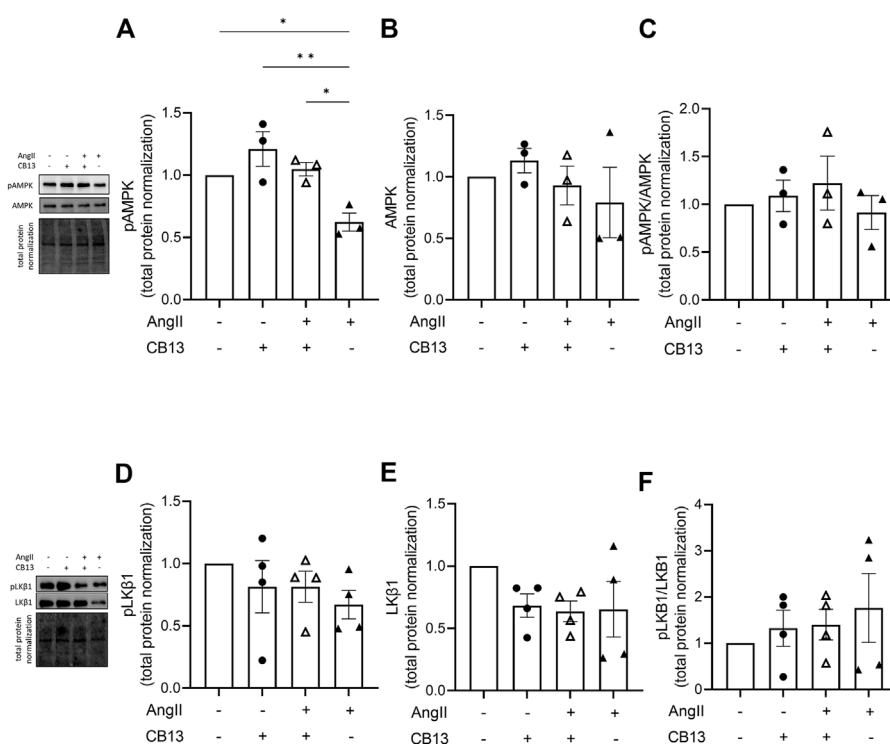


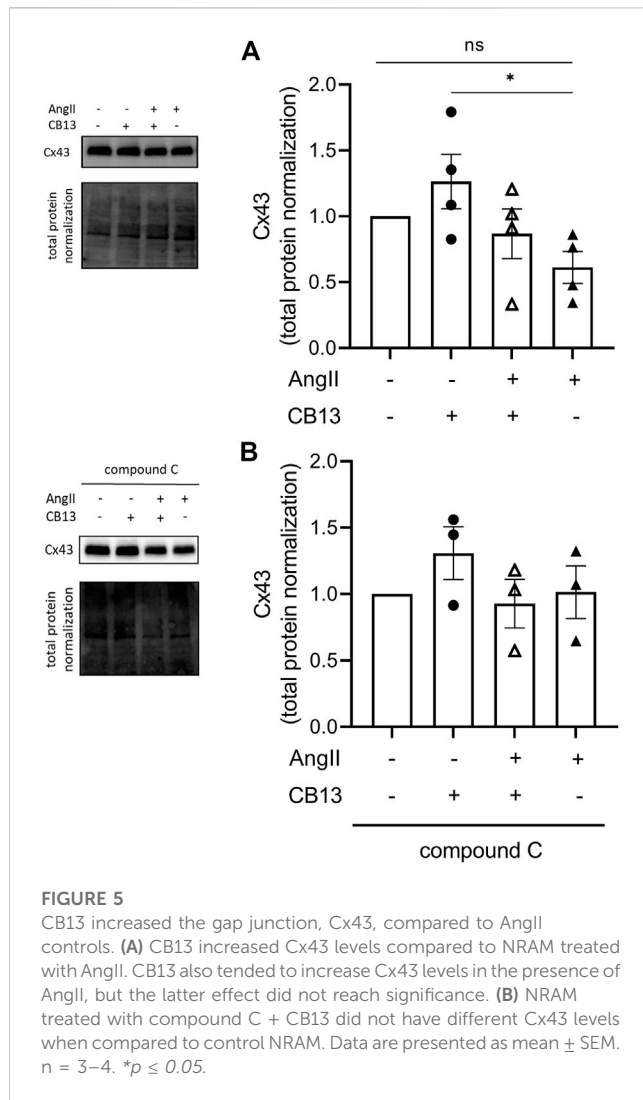
3.4 Biochemical effects of CB13 in atrial cardiomyocytes exposed to AngII

Biochemical analyses of relevant signaling mediators were performed using NRAM protein lysates. We began by

investigating phosphorylation of AMPK α at Thr172, an indicator of activation status. AngII treatment decreased phosphorylation of AMPK α (0.62 ± 0.07 vs. control; $p < 0.05$) (Figure 4A left). CB13 treatment abolished the inhibitory effects on AMPK α activation elicited by AngII (1.05 ± 0.05 vs. 0.62 ± 0.07 ; $p < 0.05$) (Figure 4A left). Neither AngII nor CB13 altered phosphorylation or total expression LKB1 (Figure 4B). Note also there were no changes in protein expression between native AMPK α nor native LKB1 regardless of treatment (Figures 4A, B, right). It should also be mentioned that while the proportion of pAMPK in the context of total AMPK does not change (panel C), this is in part because there is seemingly a trend, albeit statistically insignificant, toward reduced total AMPK. The overall finding remains the same. AMPK signaling is depressed in AngII-treated atrial myocytes.

The gap junction, connexin 43 (Cx43), is a major connexin in the heart that mediates cardiomyocyte electrical coupling; underexpression of this key protein is linked to AF (Ozcan et al., 2015). More recently, Cx43 has been associated as a downstream signaling mediator of AMPK (Ahmad Waza et al., 2012; Qiu et al., 2016). AngII tended to inhibit Cx43 expression compared to control and the addition of CB13 tended to restore the AngII-induced reduction of Cx43 levels although both trends did not reach statistical significance. Of note, compared to cells treated with AngII alone, the cells treated with CB13 alone had markedly elevated Cx43 levels and this difference was clearly significant





(Figure 5A). NRAM treated with compound C + CB13 did not have different Cx43 levels when compared to control NRAM (Figure 5B).

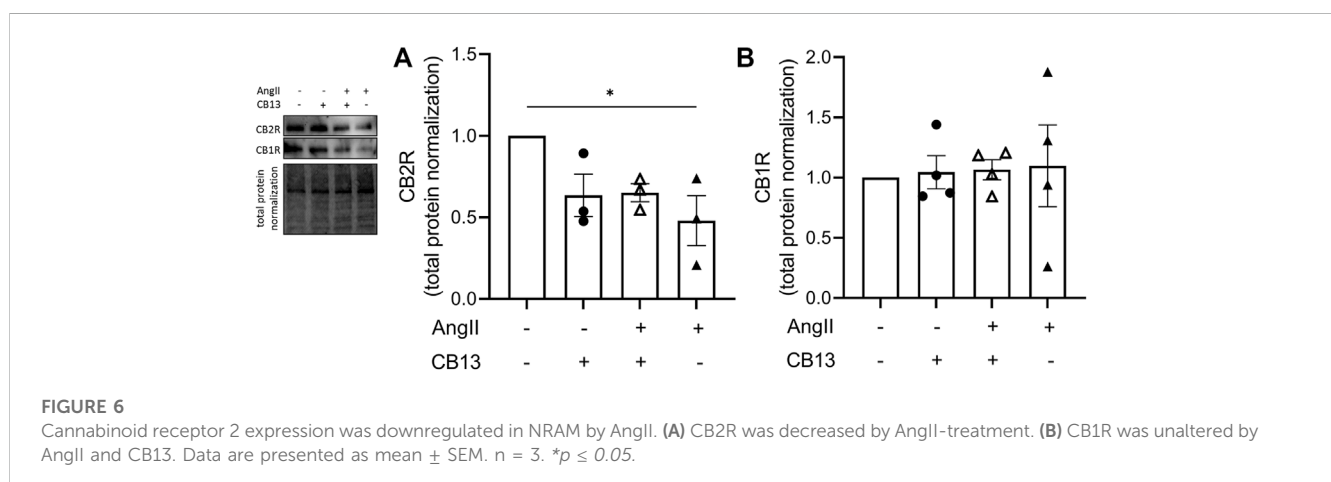
Lastly, we investigated the expression levels of cannabinoid receptor 2 (CB2R) and cannabinoid receptor 1 (CB1R). CB2R

was downregulated by AngII in NRAM (0.48 ± 0.15 vs. control; $p < 0.05$) (Figure 6A). In contrast, CB1R was unaltered by AngII and/or CB13 treatment (Figure 6B).

4 Discussion

Our findings demonstrate that the CBR ligand, CB13, attenuates atrial myocyte enlargement and mitochondrial dysfunction in NRAM through AMPK signaling, building upon previous evidence for beneficial effects of CBR ligands in stressed cardiomyocytes (Lu et al., 2014; Lu et al., 2020; Lee et al., 2021). In addition, our data may suggest that AMPK-dependent changes in Cx43 expression also contribute to the possible beneficial effect of CBR ligands. Overall, this study demonstrates three novel findings regarding the effects of CB13 in NRAM: 1) the anti-hypertrophic ability of CB13, previously detected in ventricular cardiomyocytes, likely extends to atrial cardiomyocytes (at least in terms of enhanced cell surface area) and is AMPK-dependent; 2) CB13 inhibits mitochondrial depolarization in a manner dependent on AMPK, but independent of mPTP; 3) AMPK activation (that is independent of LKB1 signaling) and Cx43 are effectors of cardioprotection against AngII-induced atrial stress.

RAS activation appears to play a key role in atrial remodeling and its consequences, particularly in patients with cardiovascular diseases (Allessie et al., 2001; Kim et al., 2017). Increased RAS activation leads to multiple alterations in the atria including hypertrophy and abnormal contractile function of the tissue (Allessie et al., 2001; Schotten et al., 2011). Our findings show that CB13 prevented the AngII-induced increase in cell surface area of NRAM. This finding extends upon our previous results of growth inhibition in NRVM and demonstrate the global nature of beneficial CBR activation in the heart (Lu et al., 2014). Furthermore, we demonstrated that AMPK inhibition, using compound C blocked the ability of CB13 to attenuate myocyte enlargement. This indicates that the molecular mechanism behind the beneficial effect of CBR activation is logically related to metabolic terms. Furthermore, this finding is consistent with the multiple recent reports regarding the possible beneficial effects of AMPK activation in the context of AF (Ikeda et al., 2009; Harada et al., 2015; Lenski et al., 2015; Brown et al., 2021).



Metabolic stress, leading to a reduction in ATP and an increase in ROS, is an important contributor to atrial remodeling (Brown et al., 2021). This is often exacerbated by remodeling-induced arrhythmia wherein the rapid atrial rate results in energy depletion and acute metabolic stress. This in turn activates AMPK as a compensatory mechanism (Harada et al., 2012; Harada et al., 2015). In fact, the progression from atrial remodeling to persistent arrhythmia has been linked to a decrease in AMPK activation (Brown et al., 2021), and this is improved by interventions that promote AMPK activation (Harada et al., 2015). Thus, reduced AMPK activation may be an underlying mechanism that permits the deleterious consequences of atrial remodeling. Our findings show activation of AMPK mediates the ability of CB13 to attenuate NRAM enlargement. Our group also recently demonstrated that CBR ligand activation prevented AMPK inhibition and atrial electrical remodeling following atrial tachypacing *ex vivo* (Lee et al., 2021). Overall, the current results, in conjunction with previous reports, support the notion that AMPK activation may be a viable treatment modality in the context of structural and electrical atrial remodeling. Given the apparent safety of CBR activation in hemodynamic terms (Lee et al., 2021), this treatment modality might be an attractive therapeutic option in this regard.

The mechanism by which CB13 activated AMPK is unclear. Although LKB1 activates AMPK α by phosphorylating Thr172 (Hawley et al., 1996; Hong et al., 2003), we found that CB13 failed to activate LKB1 in rat atria *ex vivo* (Lee et al., 2021). One possibility is that AMPK activation is instead achieved by suppression of the AMPK phosphatase, PPM1A. PPM1A reduced AMPK phosphorylation/activation in the presence of aldosterone (Tabony et al., 2011), which is a known hormonal stimulus of AF substrate.

An additional aspect of metabolic remodeling is mitochondrial dysfunction. $\Delta\Psi_m$ was reduced in AngII-treated NRAM, indicating a decline in proton motive force and an overall reduction in free energy available to generate ATP (Brown and O'Rourke, 2010). However, $\Delta\Psi_m$ was restored to control levels by CB13 in an AMPK-dependent manner. This contrasts with the AMPK-independent ability of CB13 to prevent reduced mitochondrial depolarization in NRVM (Lu et al., 2020). However, this finding was likely not due to complete lack of AMPK involvement but rather simultaneous loss of CBR/AMPK effect on the mitochondrial electron transport chain (Lu et al., 2020).

The role of mPT in cardiovascular injury is well-studied, as opening of mPTPs results in apoptosis and cell death in myocardial infarction and left ventricular dysfunction (Minners et al., 2000; Clarke et al., 2002; Hausenloy et al., 2002; Argaud et al., 2004; Hausenloy et al., 2004; Argaud et al., 2005; Oka et al., 2008). However, in the context of arrhythmia, the role of mPTP activity remains unclear (Brown and O'Rourke, 2010). While the collapse in $\Delta\Psi_m$ has been demonstrated in numerous studies and leads to arrhythmias (Romashko et al., 1998; Huser and Blatter, 1999; Zorov et al., 2000; Aon et al., 2003; Lu et al., 2020), the mechanistic basis is poorly understood (Brown and O'Rourke, 2010; Akar et al., 2005; Brown et al., 2008). AngII-induced atrial mitochondrial membrane depolarization is not linked to mPT, as opposed to the $\Delta\Psi_m$ -mPTP coupling in ET-1-treated NRVM. The contradictory involvement of mPTP in CB13-treated NRAM but not CB13-treated NRVM is

possibly explained by functional differences of ion complexes within the inner membrane that are involved in $\Delta\Psi_m$. For example, blocking the inner membrane ion channel, IMAC, was protective against arrhythmias by preventing $\Delta\Psi_m$ reduction, improving left ventricular developed pressure, and preventing action potential shortening (Billman et al., 1998; Aon et al., 2003; Brown et al., 2008). As noted above, we recently reported that CB13 treatment prevents atrial refractoriness shortening in an *ex vivo* rat model exposed to atrial tachypacing, suggesting prevention of action potential shortening by CB13 (Lee et al., 2021). It is plausible that CB13 counteracted the tachypacing-related remodeling by modulating $\Delta\Psi_m$ via IMAC. However, this possibility should be further confirmed in future studies. Overall, AngII induces mitochondrial dysfunction in NRAM, and utilizing therapeutics that target metabolic alterations and preserve $\Delta\Psi_m$ may prevent the development of arrhythmias.

The aforementioned structural remodeling is often accompanied by metabolic and electrical remodeling (Brown et al., 2021). In addition to AMPK activation, our data suggest that Cx43 may be a downstream effector of CB13. Cx43 is a major gap junction assembled from connexin proteins that are necessary for cell-to-cell electrical communication. Found in both ventricular and atrial myocardial tissue (Severs et al., 2008; Glukhov et al., 2012), Cx43 is downregulated during several atrial remodeling studies (Akar et al., 2007; Yan et al., 2013; Lee et al., 2021). Ozcan et al. also demonstrated that aspirin and metformin, both activators of AMPK, preserved Cx43 protein levels and atrial size in an LKB1 knockout mouse model (Ozcan et al., 2020). Our finding that CB13 preserves Cx43 levels offers insight into the potential effect of CB13 in terms of electrophysiological coupling between NRAM. Whether CBR/AMPK crosstalk is required for CB13 effects on Cx43 remain to be elucidated. Cx43 is reportedly a downstream signaling effector of AMPK activation, possibly through K_{ATP} channels (Ahmad Waza et al., 2012; Qiu et al., 2016). Our data show that in NRAM, AngII reduced phosphorylation of AMPK, CB13 rescued AMPK activation, and concomitantly prevented AngII-induced suppression of Cx43. We further demonstrate that CB13 increased Cx43 compared to AngII-treated NRAM, which is consistent with our previous report that CB13 rescued Cx43 in an acute tachypaced atrial model (Lee et al., 2021). This finding supports the notion that Cx43 expression levels are linked to AMPK activation status in a way that may be clinically relevant in the context of atrial remodeling. Moreover, the effect of CB13 on cellular localization of Cx43 is unknown, though there is some evidence that dexmedetomidine, an α_2 -agonist with antiarrhythmic properties, increased the distribution of Cx43 in the context of ischemic cardiomyopathy via AMPK activation. Whether CBR/AMPK signaling likewise affects Cx43 distribution is an interesting question that warrants further study, and therefore this was added to the discussion. Lastly, CB2R was downregulated in atrial cardiomyocytes by AngII. This may suggest that the inhibitory effect of AngII on CB2R is perhaps a route by which AngII prevents AMPK signaling.

In summary, our study demonstrates that CB13 prevents hallmarks of AngII-induced structural, metabolic, and possibly also electrical remodeling in a largely AMPK-dependent manner. Specifically, the CBR/AMPK axis prevents AngII-dependent NRAM enlargement, restores $\Delta\Psi_m$, and may address electrophysiological

aberrations by rescuing expression of Cx43. CBR-based interventions therefore warrant further study as a potential therapeutic approach to the clinical problem of deleterious atrial remodeling.

5 Limitations

Our study interrogated how CB13 mitigates aspects of atrial remodeling and hypertrophy such as atrial myocyte enlargement using neonatal cardiomyocytes *in vitro*. As neonatal cardiomyocytes are historically viewed as a suitable model for interrogating hypertrophic pathology, as patients with heart failure demonstrate reversion to cardiomyocyte neonatal phenotype during disease progression, our conclusions within this model are justified. However, in the future, this research direction would be strengthened by transitioning to iPSC-derived cardiac myocytes as experimental paradigm. Furthermore, this study is based upon previous findings in *ex vivo* Langendorff intact hanging hearts (Lee et al., 2021). An important next step will be to investigate the effects of CB13 *in vivo* using a novel tachypacing methodology (Lee et al., 2021; Murninkas et al., 2021).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by University of Manitoba.

Author contributions

DA: Contributed to the conception and design of the study, performed all experiments, analyzed all data, generated all figures,

prepared the first draft of the manuscript. YE: Conceived and designed the study and contributed to the completion of the manuscript. HA: Conceived and designed the study, guided the data analysis and design of the figures, and led the completion of the manuscript. All authors contributed to the preparation of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2023.1142583/full#supplementary-material>

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