MASTERARBEIT | MASTER'S THESIS

Titel der Masterarbeit | Title of the Master's Thesis

Do SPARC and Desmin communicate via JNKs in cardiovascular progentior cells and does this contribute to the regulation of cardiomyogenesis?

Verfasst von | submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2023 / Vienna, 2023

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 834
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Biologie – Molecular Biology
Betreut von / Supervisor:	AO. Univ.-Prof. Mag. Dr. Georg Weitzer

Acknowledgements

I would like to take this opportunity to express my sincerest gratitude to the people who have helped me throughout the journey of completing this thesis.

I want to express my gratitude to my group leader, Georg, for his unwavering support and guidance throughout the entire process of this research project. He always took the time to listen to my ideas and encouraged me to pursue my project with dedication and confidence, even when things were not going as planned.

I am also deeply grateful to my colleagues, Claudia and Theresa, who make the lab a welcoming and inclusive place to work. Their technical expertise and friendly assistance were invaluable to me, and I couldn't have achieved this accomplishment without their help. I am so grateful to have found not just coworkers but friends in them.

I want to thank my co-Master students, Elisabeth, Florian, and Franziska, for their companionship and support during this journey. The good laughs and fun times we shared together made the experience all the more enjoyable.

Lastly, I would like to express my deepest gratitude to my family, who have been my rock throughout this journey. Their unwavering emotional and financial support has been invaluable, and I am deeply grateful for the freedom they have given me to pursue my dreams. Without their encouragement, this accomplishment would not have been possible. I would also like to give special thanks to my sister and my partner, who have always been a source of motivation and support and have played an integral role in my success.

The love and support of my family and friends have been an invaluable asset to me during this time.

I am truly blessed to have such wonderful people in my life, and I could not have achieved this milestone without their guidance, encouragement, and love.

Thank you from the bottom of my heart.

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Abbreviations

CB	cardiac bodies
CD-CVPC	cardiosphere-derived cardiovascular progenitor cell
BMP	bone morphogenic protein
Co-IP	co-immunoprecipitation
CoPP	cobalt protoporphyrin
CPC	cardiac progenitor cell
CS	cardiac sphere
CVPC	cardiovascular progenitor cell
ESC	embryonic stem cell
JNK	c-Jun N-terminal kinase
LIF	leukemia inhibitory factor
MAPK	mitogen activated protein kinase
qPCR	quantitative polymerase chain reaction
SC	stem cell
SPARC	secreted protein acidic and rich in cystein
WB	western blot

Abstract

Cardiovascular disease is the leading cause of death worldwide. While the heart was previously thought to be a purely postmitotic organ, it is now recognized that the heart harbors diverse populations of cardiovascular progenitor cells. Despite the strong scientific interest in stem cells, knowledge about the regulation of cardiomyogenesis in CVPCs is limited. There is evidence that the intermediate filament protein Desmin and the matricellular protein SPARC co-regulate cardiomyogenesis, but how they communicate and interact is unknown. Preliminary research has shed light on the JNK signaling pathway as a possible interface of these two proteins. This study examined the effects of intracellular SPARC levels on JNK phosphorylation using quantitative Western blot analysis. The physical interaction between Desmin, SPARC, and players of JNK signaling was elucidated by co-immunoprecipitation. To investigate the effects of inhibition of the JNK pathway on cardiomyogenesis, Cardiac Bodies were generated and treated with the JNK inhibitor SP600125. These experiments demonstrated that in SPARC-overexpressing cells, JNK phosphorylation is increased. Furthermore, Desmin was shown to physically interact with JNK2 and pJNK, and this binding was resolved in the presence of SP600125. Inhibition of JNK signaling also has a slowing effect on cardiomyogenesis in Cardiac Bodies. In summary, this provides evidence that SPARC and Desmin are able to influence JNK signaling and, vice versa, JNK signaling influences SPARC gene expression and protein levels, suggesting the JNK pathway as a promising interface for the communication of SPARC and Desmin in the regulation of cardiomyogenesis.

1 Introduction

1.1 The adult heart harbors cells with regenerative potential

The heart as the first organ originating from the mesoderm and developing during embryogenesis, ensures by its function the supply of all organs in the body with oxygen and nutrients and is thus essential for life.

The process of heart development is very tightly regulated. Through continuous, temporally, and spatially controlled remodeling processes, the early mesoderm is finally transformed into the four-chamber organ. The heart is formed by the differentiation of embryonic stem cells into cardiovascular progenitor cells (CVPCs), which gradually give rise to terminally differentiated cells that form the adult heart (Christoffels et al., 2000).

Not exclusively defects in heart formation leading to congenital heart defects pose life-threatening risks, but also diseases of the cardiovascular system, caused by behavioral risk factors such as an unhealthy diet, lack of exercise or smoking. As a result, cardiovascular diseases are among the most the predominant cause of death worldwide (WHO).

For a long time, the scientific community assumed that the heart was a purely postmitotic organ. However, Beltrami et al. (2001) described the discovery of a subpopulation of myocytes in the adult heart that are not terminally differentiated. This cell population was defined as Cardiovascular Progenitor Cells (CVPCs). Stem cell antigen-expressing CVPCs have been identified in the adult heart of various mammals, but it is controversial where these cardiac progenitor cells originate (Wu et al., 2008). One theory is that they arise during embryonic development and reside in a niche in the heart as a reserve. Another possibility is that they do not originate in the heart, but undifferentiated cells from other organs migrate into the heart and only there acquire properties of CVPCs (Bianconi et al., 2018).

CVPCs are cell lineages that are self-renewing in response to leukemia inhibitory factor (LIF). They show concomitant expression of both the stemness transcription factors Oct4, Sox2, and Nanog as well as the early myocardial transcription factors Nkx2.5, GATA4, and Isl-1. Upon LIF withdrawal CVPCs differentiate exclusively into functional cardiomyocytes, endothelial cells, and smooth muscle cells, suggesting that these cells are mesodermal intermediates (Hoebaus et al., 2013). Although these cells with

regenerative potential are found in the adult heart, they are unable to adequately repair injury to the heart or counteract chronic degeneration. Injuries lead to scarring and persistent damage, and new cardiac muscle cells cannot be regenerated efficiently (Taubenschmid and Weitzer, 2012).

The therapeutic approach of drugs and devices may initially bring about a temporary improvement in cardiac function, but cannot replace the lost myocardium (Xin et al., 2013). The discovery of cardiac stem cells has provided a new potential alternative therapy for heart disease. However, for a future use of efficient stem cell-based therapies, a precise understanding of the molecular regulatory network of cardiogenesis, which controls the proliferation and differentiation of the cells is of importance.

1.1.1 Populations and properties of CVPCs

Several different populations of cardiac progentior cells (CPCs) have been identified that reside in the myocardium. These populations are distinguished by the specific surface markers they express.

One population of CVPCs was identified by Quaini et al. (2002) as expressing the surface receptor c-Kit, which is specific for somatic stem cells. Further studies found c-Kit positive CVPCs, which are also able to differentiate into adult cardiac cells and contribute to cardiac hypertrophy in patients with aortic stenosis. This population of cardiac stem cells expresses different stem cell-specific transcription factors, such as Nkx2.5, MEF2C, GATA4, and GATA5. In vivo, c-Kit expressing CVPCs are capable of differentiating into adult cardiovascular cells upon cardiac injury and improving cardiac function (Beltrami et al., 2003, Urbanek et al., 2003).

Another surface receptor expressed by different CVPC populations is Sca-1. Sca-1 is specific for somatic stem cells in mice and indispensable for the differentiation of cardiac stem cells into adult cardiac cells. CVPCs positive for Sca-1 have been shown to improve myocardial function after ischemic attack and contribute to positive remodeling of the murine heart (Huang et al., 2011, Wang et al., 2006).

The CS population refers to a heterogeneous population of CVPCs that are able to adhere to each other in cell suspension and form cell conglomerates called cardiac spheres (CS). These CS-derived CVPCs (CD-CVPCs) are characterized by

expressing stem cell specific surface receptors such as Sca-1 and c-Kit, as well as being CD34 and CD31 positive (Messina et al., 2004).

The term side population (SP) is used to describe a population of CVPCs that is able to eject the Hoechst dye that binds to DNA using the ABCG2 transporter. Oh et al. (2003) first identified the SP cells, which they isolated from mouse myocardium. Further studies described and characterized this population of CVPCs. SP CVPCs are capable of differentiating into mature cardiomyocytes in both mice and humans. SP cells expressing the surface receptors c-Kit and Sca-1 were identified, as well as other stem cell-specific transcription factors, such as Nkx2.5, MEF2C, and GATA4. These SP cells are also able to migrate to critical sites in injured hearts and differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells (Oyama et al., 2007).

However, knowledge of how these distinct populations of CVPCs are recruited and act in cardiovascular injury is limited. It is thought that there are two mechanisms by which CVPCs support injured tissue. One is through direct differentiation into or fusion with adult cardiac cells, and the other is through the delivery of paracrine factors that promote regeneration of injured tissue. Although several studies found evidence that CVPCs regenerate tissue through direct differentiation and fusion with already resident cells (Matsuura et al., 2004, Oh et al., 2003), other studies have shown that injected CPCs do not differentiate into adult cardiomyocytes, but beneficial effects occur through paracrine influence (Tang et al., 2016).

1.1.2 The Cardiac Stem Cell Niche

In order to self-renew and maintain stem cell status, stem cells require certain conditions, including contacts with other cells, as well as growth signals that they receive in both an autocrine and paracrine manner. This particular microenvironment, also known as a niche, harbors stem cells until they are stimulated to differentiate by stimuli.

In the heart, such niche-like structures exist in the myocardium, but this specific niche has not yet been precisely defined in terms of the cell types it harbors to maintain stem cell status. These niches are found primarily in the subepicardium and atrium of the heart. These niche-like structures mainly harbor fibroblasts. Fibroblasts are thought to contribute to stem cell self-renewal status, as some have been identified to express N-

cadherins and α4β1-integrins. Furthermore, fibroblasts secrete substances that stem cells need for self-renewal, such as Leukemia Inhibitory Factor (LIF) (Kuhn and Wu, 2010).

Some CVPCs are able to attach to each other and form cardio spheres, creating an environment for themselves that resembles the niche (Li et al., 2010).

Niche-like conditions are created in vitro for the CVPCs isolated from the adult mouse heart used in our studies. The CVPCs are maintained on a bed of LIF-secreting fibroblasts, which also serve as a base for attachment. If these feeder-cells are removed, the CVPCs begin to differentiate (Hoebaus et al., 2013).

1.1.3 Stem Cell Therapy Approaches for Heart Disease and Injury

Before new stem cell-based therapies can be developed for the injured heart, it is essential to understand the processes by which SCs contribute to the regeneration of damaged tissue.

One of the first approaches was to use stem cells harvested from bone marrow. It was assumed that these stem cells would take on properties of CPCs in the heart and differentiate into cardiomyocytes, a process known as transdifferentiation. Evidence was found that this is indeed the case, but it happens extremely rarely and in vanishingly small numbers, making it unsuitable as a therapeutic intervention (van Berlo et al., 2014).

The current scientific consensus agrees that the main influence of stem cells on the regeneration of damaged cardiac tissue occurs via paracrine factors. It has been shown many times that stem cells secrete increased growth factors, such as EGF, VEGF, and SDF-1, as well as signals for regeneration via relevant biologically active factors, such as TGF-β. These factors not only affect the immediate environment in the heart, but also enter the bloodstream and have systemic effects. These factors then act beneficially at multiple levels, such as recruiting resident progenitor cells, neovascularization, and reducing apoptosis of residing cardiomyocytes (Mirotsou et al., 2011).

Neovascularization of infarct tissue is an important part of regeneration because of the need to deliver oxygen and nutrients to injured areas. Stem cells play an important role in the formation of new blood vessels by secreting growth factors, such as VEGF, EGF,

and IGF-1, which stimulate vascularization. Specifically, this was shown in a study by Chimenti et al. (2010) for cardiosphere-derived CVPCs (CD-CVPCs). It was shown that an important player in the transmission of this paracrine signal from cardiac stem cells (CSCs) is the TGF-β co-receptor endoglin. SDF-1 is also a relevant growth factor leading to the formation of new blood vessels. Cheng et al. (2014) showed in a study that CD-CVPCs isolated from patients with advanced heart failure secrete significantly more SDF-1 than CD-CVPCs from the healthy heart. When these CD-CVPCs from patients with heart failure are injected into injured tissues, they increase endothelial cell division to a greater extent than CD-CVPCs from healthy hearts.

As a result of myocardial infarction, a process known as cardiac remodeling commonly occurs. In this process, the heart undergoes various changes at the cellular and molecular levels that result in impairing the organization of cardiomyocytes and also disturbing the balance of the extracellular matrix, thereby affecting the heart's function. There are several indications that the application of various stem cell-based interventions positively influences the course of this adverse cardiac remodeling. For example, injection of CPCs prevents excessive deposition of collagen in infarct tissue and strengthens the left ventricle (Savi et al., 2016). A phase 2 clinical trial also demonstrated that CD-CVPCs have a significant positive effect on the size, mass, and volume of scar tissue after myocardial infarction (Ishigami et al., 2017).

While these aforementioned studies sound promising regarding the use of CPCs for cardiac regeneration, the data on human clinical trials is inconsistent. While several meta-analyses found a small but significant improvement with the application of stem cells (Cong et al., 2015, Liu et al., 2014), these have been disproved in a study that examined the individual outcomes of stem cell treated patients (Gyöngyösi et al., 2015). This shows that there is an urgent need to find new methods to exploit the beneficial effects of stem cells on cardiac regeneration.

One promising method to augment the therapeutic effects of CPCs is pharmacological preconditioning. This involves treating isolated stem cells with a compound designed to enhance their healing abilities. Indeed, stem cells previously treated with oxytocin secreted significantly more beneficial cytokines, resulting in improved survival of co-cultured cardiomyocytes. In another study, CSCs were treated with cobalt protoporphyrin (CoPP), which induces heme oxygenase 1. The treated CSCs not only showed significantly increased resistance to apoptosis but also secreted substantially

more growth factors and cytokines (Cai et al., 2012). Various other studies demonstrated that in vitro preconditioning of stem cells with growth factors or environmental factors also enhanced the therapeutic effect of injected CSCs (Hosoyama et al., 2015, Yu et al., 2013).

Despite such novel approaches, there is still an urgent need to better understand the cellular processes through which CPCs act and contribute to the regeneration of injured tissue. With a more comprehensive knowledge of how CPCs and their differentiation are regulated, methods can also be developed to make them more effective healing agents.

1.2 Regulation of cardiomyogenesis in CVPCs

1.2.1 Transcription factor network

The exact differentiation pathway from stem cell to adult cardiac cell remains unresolved at the level of the exact transcription factor network, but there are clues as to how the network is constructed. An embryonic stem cell expresses the stem cell transcription factors Oct4 and Sox2, and these contribute to maintaining stem cell status by upregulating Nanog. However, there is also evidence that Oct4 and Sox2 activate the *Brachyury* gene, which is one of the earliest expressed genes when a stem cell differentiates into an adult cardiac cell (Thomson et al., 2011). Thus, it can be assumed that regulatory communication already exists at this level, as Nanog and Brachyury also interact and regulate which cell retains stem cell status and which cell differentiates (Sarkar et al., 2012). At the next level of regulation, Brachyury binds to and activates the promoter of *MESP1*. MESP1 is again one of the proteins that is expressed earliest in cardiovascular lineage progenitor cells (David et al., 2011). MESP1 is itself a transcription factor that when knocked out leads to severe defects in the heart. *Mesp1* can also be directly activated by Oct4, as well as by Eomesodermin. Next, MESP1 then activates *DKK1*, which inhibits Wnt-Signaling and therefore promotes the differentiation of CVPCs into cardiomyocytes (David et al., 2008). MESP1, as a transcription factor, also triggers the expression of the next enormously important TF, namely Nkx2.5 (Bondue et al., 2008). Nkx2.5 subsequently activates the transcription factors GATA4 and MEF2C. GATA4 and Nkx2.5 physically bind to each other and interaction with each other triggers cardiomyogenesis.

Nkx2.5 and GATA4 together regulate other target genes to promote cardiomyogenesis. These include *MEF2C*, which is impaired upon knock-out of the Nkx2.5 gene (Tanaka et al., 1999). Furthermore, Nkx2.5 and GATA4 affect different signaling pathways. Upon knock-down of *Nkx2.5*, β-catenin is upregulated, leading to the activation of the Wnt-pathway and subsequently to the upregulation of *Brachyury* (Yamaguchi et al., 1999).

This network of transcription factors is described here in a very simplified way, as there are a large number of other players involved and the interconnections are very complex.

1.2.2 The IF-Protein Desmin

Intermediate filaments play an important role in intracellular and intercellular communication and are an important factor in healthy cardiac function. The type III intermediary filament protein Desmin is considered one of the earliest expressed proteins specific for cardiac muscle cells. Desmin is expressed in mesodermal cells destined for cardiac muscle, as well as in cardiac muscle side population stem cells (CSPCs) and muscle-specific satellite cells (Capetanaki et al., 2015). Desmin fulfills important roles in cardiac muscle contraction and is also involved in electrical signal transduction in the heart (Tsikitis et al., 2018).

One of the most important functions of Desmin is to provide physical structure to the cell and to establish connections between cell organelles. However, its function is not only to create a structural scaffold in the cell between the cytoskeleton, organelles, and the nucleus. There is also evidence that intermediate filaments act very dynamically in the cell and are involved in the regulation of important cellular processes. These regulated processes include homeostasis, cell differentiation, and various pathologies and cell aging (Hyder et al., 2011). When the Desmin gene is knocked out, no overt defects are initially seen during mouse embryogenesis, but adult mice later show severe cardiac defects (Mavroidis et al., 2015). Some human skeletal and cardiac muscle diseases have therefore been linked to various mutations in the Desmin gene.

Moreover, it has been shown that the absence of Desmin causes mitochondria to be impaired in both structure and function (Milner et al., 2000). However, a very compelling finding is that Desmin knock-out has severe consequences in the nucleus,

where the muscle-specific transcription factors MyoD and myogenin are diminishedly expressed in embryonic stem cells during differentiation (Weitzer et al., 1995).

Desmin interacts directly with a variety of proteins in cardiomyocytes, such as other intermediate filaments, structural proteins, signaling proteins, chaperones or proteases. This gives Desmin the ability to interfere with many different processes in the cell. Furthermore, Desmin can migrate into the nucleus and bind to transcription factors and activate the expression of genes (Hol and Capetanaki, 2017). Desmin is also able to bind directly to DNA and thus affect gene expression (Tolstonog et al., 2005).

Other effects of Desmin on cardiomyogenesis in CVPCs include increases in the expression levels of the transcription factor Nkx2.5 (Fuchs et al., 2016). Furthermore, a beneficial effect on the differentiation of primitive mesodermal cells into rhythmically contracting cardiomyocytes was detected (Hofner et al., 2007). These results provide evidence that Desmin is able to influence early cardiomyogenesis by regulating cardiac-specific genes.

1.2.3 The extracellular matrix protein SPARC

By definition, a matricellular protein associates with the extracellular matrix (ECM) but does not primarily contribute to the structure of the ECM, as does collagen or laminin (Bornstein, 2000). SPARC, which stands for secreted protein acidic and rich in cysteine, also called osteonectin or BM-40, is one of these matricellular proteins and presents itself as prototypically collagen-binding. Like any protein of the SPARC family, it exhibits a conserved EC domain with an E-F hand motif required for calcium binding (Bradshaw, 2012).

SPARC-KO mice show various deficiencies regarding the composition and assembly of the extracellular matrix, such as a collagen deficiency in the connective tissue of various organs (Bradshaw, 2009). In addition, SPARC has an important function in the regulation of matrix metalloproteinases, whose role is in the turnover and remodeling of the ECM. In this manner, the addition of exogenous SPARC can significantly increase the activity of MMPs (Tremble et al., 1993).

When it comes to cell survival, SPARC shows very contradictory functions. On the one hand, it has been shown that SPARC prolongs the survival of cells that have been

condemned to apoptosis by signals from neighboring cells (Portela et al., 2010). Similar results were found in melanoma, where downregulation of SPARC led to apoptosis of cancer cells (Fenouille et al., 2010). In contrast, there is evidence that SPARC promotes apoptosis in certain cancers such as breast cancer, pancreatic cancer, and colorectal cancer and is therefore used adjunctively with chemotherapies as it increases caspase 8 activity (Rahman et al., 2011). In this case, SPARC seems to exhibit a dichotomy regarding its function.

SPARC plays an important role during embryogenesis, where it is highly expressed in the parietal endoderm and the developing heart (Holland et al., 1987). Furthermore, in studies of cardiomyogenesis, it was observed that SPARC caused cardiomyocytes to differentiate more rapidly. In addition, SPARC contributes to increased gene expression of Nkx2.5 and BMP-2, factors typical of cardiac development (Stary et al., 2005).

SPARC has also been shown to be involved in the transmission of growth factor-signaling mediated by cell surface receptors and plays a role in their modulation. These regulated receptors include VEGF, bFGF, and TGF-beta. In pericytes, TGF-beta activity has been shown to be dependent on the binding of SPARC to the receptor endoglin (Rivera and Brekken, 2011).

It is proposed that SPARC regulates growth factor signaling pathways through a variety of cell-dependent mechanisms. It was demonstrated that this mainly occurs in the extracellular space and that the regulation of receptor activity involves interaction with the receptor itself or its ligands (Bradshaw, 2012).

1.3 The role of JNKs

JNK (c-Jun N-terminal kinase) belongs to the MAPKs (mitogen-activated protein kinases), which are responsible for the regulation of important processes in the cell, such as proliferation, apoptosis, and differentiation. JNK is encoded by three different genes (*JNK1, JNK2, JNK3*), each of which is still subject to different splicing (Dreskin et al., 2001). JNK1 and JNK2 are present in most tissues, whereas JNK3 is mainly expressed in the nervous system and testis (Kumagae et al., 1999). Different splice sites from the three JNK genes result in a total of 16 different isoforms of the kinase, which primarily phosphorylates serine-proline (SP) and threonine-proline (Noessner et

al.) consensus sites. JNK1 and JNK2 have two alternative splice sites, the first of which defines whether the alpha or beta form of the protein is presented and the second of which determines the molecular weight of either 46 or 54 kDa (Gupta et al., 1996).

Evidence suggests that JNKs associate with upstream activating proteins and phosphorylation targets via molecular scaffolds. These scaffolds contribute to the speed and efficiency of signal transduction from the cell membrane to the nucleus and provide another regulatory target of phosphatases (Whitmarsh et al., 1998).

1.3.1 The JNK Signaling Pathway and its crosstalk

Activation of JNKs occurs in response to stimuli such as cellular stress, growth factors or cytokines. Looking at the main signaling pathway, these influences lead to the activation of Rho-GTPases in the cell membrane and result in the activation of MAPKK proteins, which are localized close to the cell membrane (Chang and Karin, 2001). This activates MKK7, which then phosphorylates various JNKs at specific threonines and tyrosines within a conserved tripeptide, thereby activating them. Activated JNK is subsequently itself capable of phosphorylating its substrates at threonine and serine residues (Kumar et al., 2015). One of the JNK phosphorylation targets is AP-1, which stands for transcription factor activator protein-1. AP-1 is a protein complex composed of Jun-proteins and their dimerization partners, the Fos-proteins, and its main activity is the activation of various transcription factors such as p53, Elk1, and c-Myc, as well as activating Bcl-2 in mitochondria, which regulates apoptosis. Through these transcription factors, important processes such as cell proliferation, apoptosis, differentiation, and cell survival are regulated (Shaulian and Karin, 2002) (Figure 1).

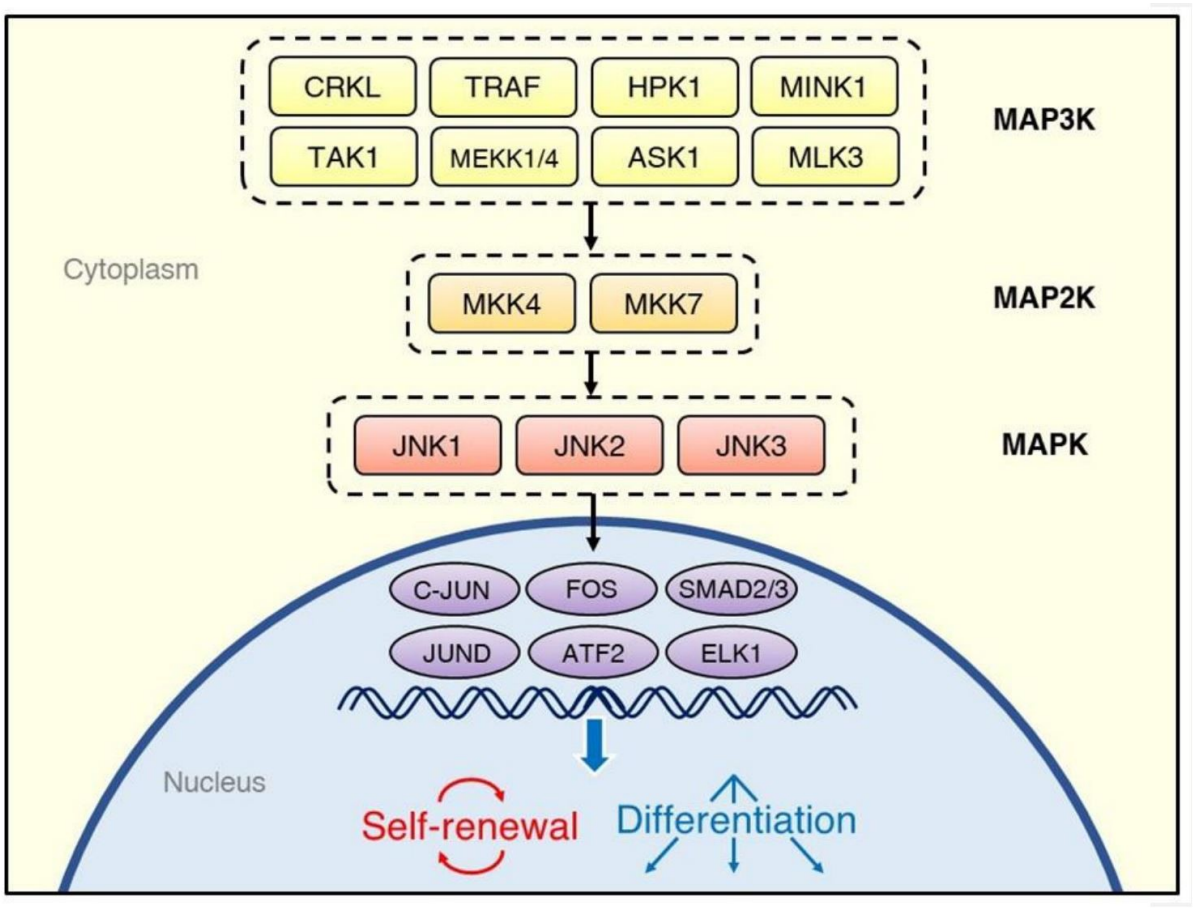

Figure 1 ***Activation cascade of the JNK pathway in stem cells.*** *Different stimuli from the environment activate the JNK signaling pathway in stem cells, which respond either by maintaining stem cell status or by differentiation. Figure taken and adapted from Semba et al. (2020).*

Scaffold proteins such as JIPs (JNK interacting protein), beta-arrestin 2, CrkII and filamin represent an important way to regulate the activity of JNK itself. These proteins act on JNK and its associated interacting partners at various locations in the cell and are key regulators in the activation of kinase activity and selection of phosphorylation targets (Haefliger et al., 2003).

Furthermore, regulation of MAPKs/JNKs has also been shown to occur through the TGF-beta/BMP pathway (Derynck and Zhang, 2003, Moustakas and Heldin, 2005). Communication between TGF-beta and the HER2/Neu/ErbB2 pathway has been established by several studies, which plays a role in the activation of the MAPK and PI3K/Akt pathways. This is particularly relevant in the regulation of mammary epithelial cells and breast cancer development (Seton-Rogers and Brugge, 2004, Siegel et al., 2003). In addition, JNK activation has been shown to have a suppressive effect on the overall expression of TGF-beta 1 (Ventura et al., 2004). Further studies suggest that

during osteoblast differentiation, activating signals from various BMPs are processed through JNK signaling pathways (Gallea et al., 2001, Guicheux et al., 2003) (Figure 2).

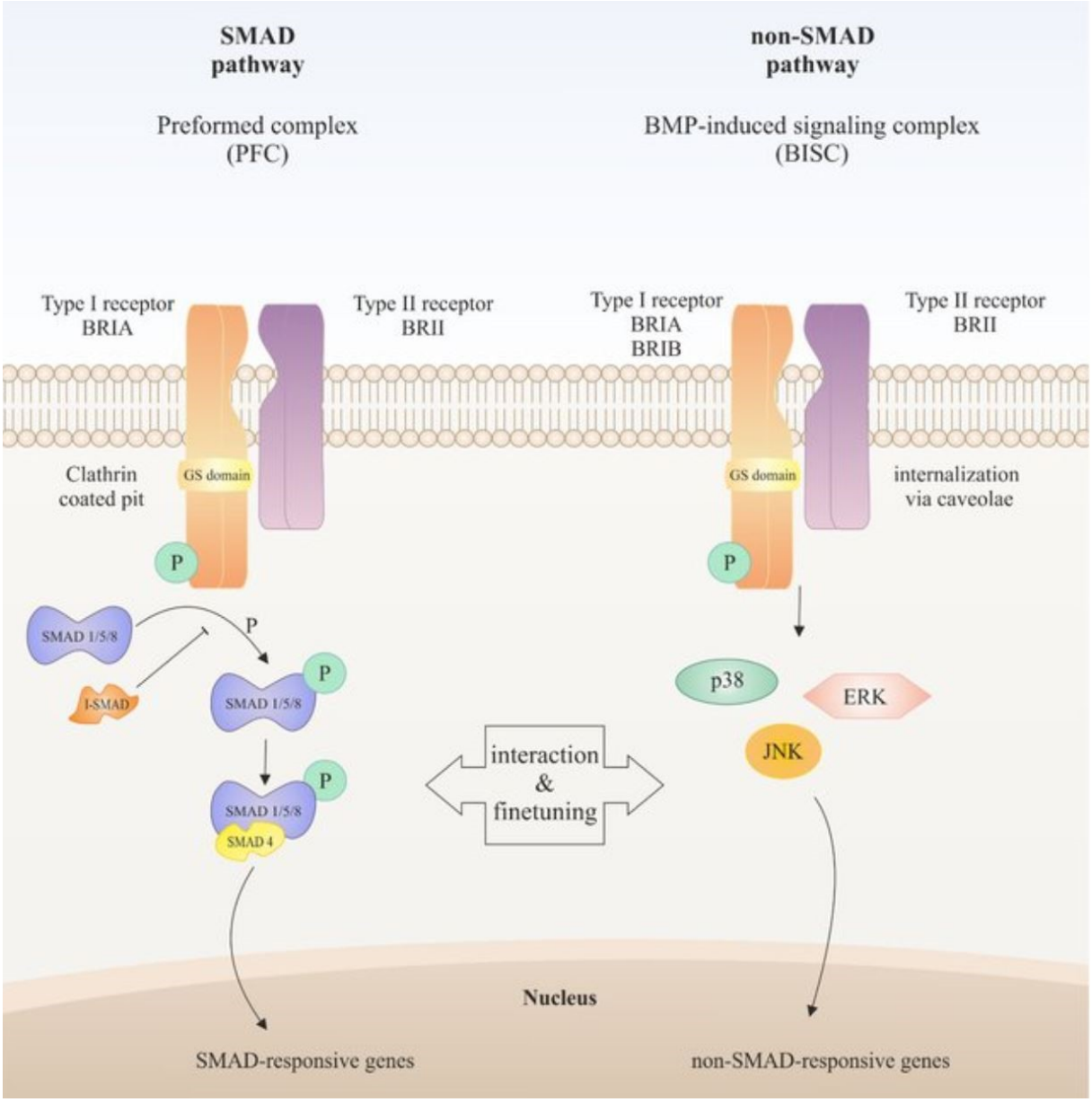

Figure 2 ***Schematic representation of BMP induced signaling pathway****. BMP can act either through the canonical SMAD pathway or non-canonically through the MAPK pathway and JNK. In the canonical pathway, BMP binds to the cognate receptor, whereupon the receptor dimerizes, becomes phosphorylated, and transmits the signal to Smad1/5/8. However, BMP can also act through alternative signaling pathways. Thus, the signal is transmitted through the bound receptor to p38, ERK or JNK, whereupon other target genes are activated. Figure taken and adapted from Siverino (2020).*

1.3.2 JNK Interaction with IF-Proteins and matricellular Proteins

Intermediate filaments have the ability to bind to the c-Jun N-terminal kinase and regulate its activity. Studies have shown that Desmin plays a role in the transmission of stress signals via JNK, as it stimulates JNK when the cell is exposed to stress (Palmisano et al., 2015). Physical interactions between Desmin and JNK2 have been confirmed experimentally. In bladder smooth muscle cell disease, Desmin and Vimentin are overexpressed, which activates JNK and subsequently decreases smooth muscle cell contractility (Javed et al., 2020). In contrast, sequestration of JNK by Keratin 8 results in inhibition of phosphorylation of c-Jun by JNK (He et al., 2002).

There are important interactions in the cell between the JNK-signaling pathway and matricellular proteins. Some genes encoding proteins of the ECM and ECM-associated proteins are regulated by TGF-beta through JNK. Fibronectin, a glycoprotein of the ECM, is increasingly expressed as a consequence of activation of the JNK pathway by TGF-beta (Hocevar et al., 1999). Another matricellular protein regulated by JNK and c-Jun is SPARC. It has been shown that in MCF7 cells, SPARC expression is enhanced by c-Jun phosphorylation (Rinehart-Kim et al., 2000). In another study, limbal epithelial stem cells proliferated at enhanced levels by addition of SPARC but differentiated less spontaneously, which was mediated via c-Jun and the MAPK pathway (Zhu et al., 2020).

1.3.3 JNK Signaling in Stem Cells

During the last decade, research has shown that the JNK signaling pathway plays an important role in the regulation of stem cells. If the JNK signaling pathway is inhibited during embryogenesis, this leads to defects in embryonic development (Hilberg et al., 1993). Despite the high research interest in stem cells, studies on the role of JNK signaling in stem cells have been limited to date. In ESCs, gene expression of key players of the JNK pathway was compared between differentiated and undifferentiated ESCs and downregulation of c-Jun, MAP4K1, and MAP3K7 was found in differentiated cells (Kim et al., 2006). This suggests that JNK in ESCs contributes to the maintenance of stem cell status. Further studies have found that the application of JNK inhibitors such as SP600125 in ESCs results in stimulating differentiation and downregulating Oct4 and Nanog gene expression (Brill et al., 2009). Another study by Xu and Davis

(2010) found that knock-out of JNK1 and JNK2 caused ESCs to proliferate faster, but progenitor cells were later unable to differentiate.

In contrast to the results for ESCs, JNK signaling appears to play a stimulatory role in differentiation in somatic progenitor cells. Indeed, in intestinal stem cells, activation of JNK1 resulted in stimulating differentiation, as well as proliferation (Sancho et al., 2009). Also in neural stem cells, it was shown that cells switch from Wnt signaling to JNK signaling during differentiation. Inhibition of JNK reduced neural stem cell differentiation (Bengoa-Vergniory et al., 2014). In addition, activation of the JNK pathway was shown to promote phosphorylation of STAT1/3, and thus the expression of genes important for NSC differentiation (Wei et al., 2014).

These results reveal opposing roles of JNK in embryonic stem cells and somatic progenitor cells, indicating that JNK has cell-specific functions. It is important to define this function for additional types of progenitor cells.

1.3.4 Inhibition of the JNK Signaling Pathway

As the importance of the JNK pathway has steadily increased in recent years and is relevant to a variety of cellular processes and pathologies, a large number of biologically active molecules have also been developed and explored for inhibition of JNK signaling. Thus, pyrimidines, pyridines, thiopene carboxamides, pyrazoles, isoquinolones, piperazines and various derivatives of all these compound classes serve as inhibitors of the JNK pathway and perform their function largely by preventing the binding of ATP to JNK and thus its phosphorylation and activation. Overall, these more than 100 described molecules exhibit varying effect concentrations, specificities, and off-target effects.

One inhibitor of JNK that has been applied in a large number of studies is the 1,9-pyrazoloanthrone analog SP600125 (Figure 3). SP600125 inhibits phosphorylation of JNK in a reversible manner by acting as an ATP competitor. In doing so, it inhibits JNK1 and JNK2 in vitro at an IC50 of 40 nM and JNK3 at an IC50 of 90 nM. However, when used in vivo, this concentration must be significantly exceeded to counteract with the increased supply of ATP.

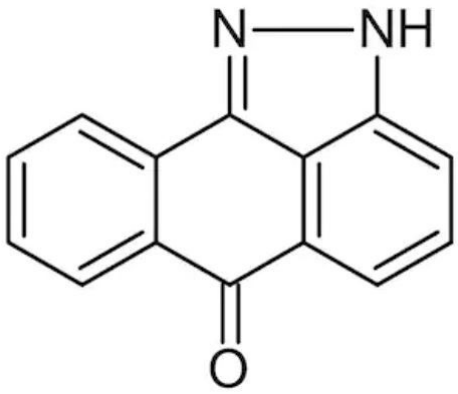

Figure 3 ***Molecular structure of the JNK inhibitor SP600125****. SP600125 is an ATP analog and acts by binding to the ATP binding site instead of ATP, thus preventing phosphorylation.*

It was also shown that application of SP600125 downregulated some genes involved in the inflammatory response, such as TNF-α, IL-2, and IFN-γ. Moreover, in cultures of CD4 cells, SP600125 prevented them from differentiating. Furthermore, SP600125 counteracts the activation of the TGFβ/BMP pathway, which was shown by preventing differentiation triggered by BMP-9 signaling in mesenchymal stem cells. SP600125 was also tested in embryonic stem cells and was shown to promote differentiation of ESCs.

SP600125 has been successfully used to treat several diseases. The compound is able to positively influence the course of Alzheimer's disease and Huntington's disease, thus exhibiting neuroprotective properties (Braithwaite et al., 2010, Chen et al., 2010).

SP600125 is also used in the treatment of various types of cancer. It has been shown that the application of SP600125 in glioblastoma decreases the proliferation of cancer cells and promotes their differentiation (Matsuda et al., 2012). This effect has also been shown in other cancers, such as pancreatic cancer, where inhibition of JNK by SP600125 decreases the stem cell status of cancer cells (Okada et al., 2014).

A disadvantage in the application of such kinase inhibitors is the fact that in most cases other kinases are also inhibited, since hardly any inhibitors are really specific for their target. This must be considered when evaluating the results of applications of such inhibitors.

1.3.5 AP-1 Complex and Target Genes

Activator Protein 1 Complex (AP-1) is a product of dimerization of a variety of different transcription factors and one of the major substrates of JNK. It is generally accepted that AP-1 processes numerous extracellular signals and is a node for the differential activation of a variety of target genes. AP-1 allows cells to respond to various environmental stimuli and to adapt gene expression to them. Due to the importance of this complex, it is evident that disturbances in the activity of AP-1 are the cause of many pathologies and cancers (Shaulian and Karin, 2002).

When talking about AP-1, one is most likely to think of dimers of Jun and Fos family proteins. If AP-1 is seen in a broader context, dimers also include proteins of the ATF and MAF families. However, ATF and MAF play a role only in specific tissues, whereas Jun and Fos act globally (Eferl and Wagner, 2003).

The transcription factor Jun is able to form homodimers, whereas Fos cannot and is only found in heterodimers with Jun or other proteins of the AP-1 complex. What is interesting here is that Jun/Fos heterodimers have a stronger binding affinity for DNA and also result in stronger transcriptional activation than Jun/Jun homodimers (Halazonetis et al., 1988).

Depending on which dimers are formed, different consensus sequences in DNA are recognized and bound. For example, Jun/Jun and Jun/Fos have been shown to bind preferentially to the TRE element, which is also known as the AP-1 motif. A lower affinity is exhibited by these dimers to the CRE element. Although these two elements are the main binding sites for the canonical AP-1 dimers, they are still capable of binding countless other elements in DNA (Eferl and Wagner, 2003).

When talking about AP-1, it is important to mention that there is a paradox regarding its function, because although it is such an important complex and the subject of countless studies, we are still far from knowing the full extent of its target genes and understanding the ways in which it regulates its expression. At the beginning of research on AP-1, mainly chromatin immunoprecipitation and reporter gene assays were performed to elucidate its function, now we know that these methods have created a bias in the body of research. It was only with the increased use of -omics methods that it became known that AP-1 not only acts by binding directly to the promoter of target genes, but it also functions like a remote control for genes as it binds

to distant enhancer regions that are only moved in close proximity to promoter elements by wide-ranging chromatin interactions (Bejjani et al., 2019).

Of interest to our study is the fact that c-Jun in the AP-1 complex regulates the SPARC gene. So it was discovered by Rinehart-Kim et al. (2000) that in breast cancer cells SPARC is one of the major target genes upregulated by c-Jun. Another study in breast cancer cells confirmed the findings that SPARC upregulation is proportional to the upregulation of c-Jun (Zajchowski et al., 2001). Briggs et al. (2002), however, postulate a dichotomy in the relationship between SPARC and c-Jun, for whereas these studies found a directly proportional relationship between c-Jun activity and SPARC expression, conversely, in embryonic fibroblasts, c-Jun was shown to downregulate SPARC expression (Vial and Castellazzi, 2000).

1.4 Hypothesis and aim of the Study

As the above-described evidence shows, both the intermediate filament Desmin and the matricellular protein SPARC play important roles in cardiac development and differentiation of stem cells into adult cardiac cells. However, how these two proteins interact and jointly orchestrate cardiac cell development is not known. Since previous studies have shown that SPARC expression is regulated by JNK mediated phosphorylation of c-Jun and Desmin can communicate signals via JNK through its scaffolding and regulating function, we hypothesize that the JNK signaling pathway mediates communication between Desmin and SPARC. The aim of this master's thesis is to elucidate whether Desmin and SPARC communicate via the JNK pathway, as well as to determine what other factors are involved in regulation and to what extent this communication contributes to the regulation of cardiomyogenesis.

2 Materials and Methods

2.1 Cell Lines

Table 1 Overview of the used CVPC cell lines and their Genotype

Genotypes	Clones
Wild Typ - SPARC +/+, Desmin +/+	A5B8/1
SPARC +/-	K11S, K17S
SPARC +/+/ect	K4S, K10S
SPARC-mCherry	SmC K5-6
Desmin +/-	2Hd, 1Dd, 3Bd
Desmin +/+/ect	K4D, K12D
Desmin-mCherry	DmC(N28)
mCherry	mC-K8

2.2 Buffers, Stocks and Media

2.2.1 PBS (Phosphate Buffer Saline)

1:10 dilution of the 10x PBS:

80g NaCl, 2g KCl, 10.72g Na2HPO4x7H2O and 2g KH2PO4 is dissolved in 800ml MilliQ water and the pH is adjusted to 7.2 with saturated Na2HPO4x7H2O solution.

After filling up with MilliQ water to the 1l mark, the solution is sterile filtered and stored at room temperature.

2.2.2 Trypsin

3.5g NaCl, 0.5g D-glucose, 0.09g Na2HPO4x7H2O, 0.185g KCl, 0.12g KH2PO4, 0.2g

EDTA, 1,25g Trypsin, 1, 5g Tris Base.

Components are dissolved in 500ml MilliQ water and the pH is adjusted to 7.6 with concentrated HCl. The solution is sterile filtered and stored at -20°C.

Aliquots in use are stored at 4°C.

2.2.3 Gelatin-Solution

1:10 Dilution of the 1x stock solution with MilliQ

1x stock solution: 10g gelatin is dissolved in 1l MilliQ water and autoclaved.

Store the solution at room temperature.

2.2.4 Media for fibroblasts (M10Gi)

89% DMEM, 10% FBS: Fetal Bovine Serum (Gibco), 1% GPS (Glutamin-Penicillin-Streptomycin)

2.2.5 Media for CVPCs (M15Hy/Si)

83% DMEM, 15% FBS: Fetal Bovine Serum (HyClone/Sigma), 1% GPC (Glutamin, Penicillin-Streptomycin), 1% β-Mercaptoethanol

2.2.6 Protein Lysis Buffer

7 ml	H_2O
300 µl	NaCl (5M)
100 µl	NP-40
1 ml	Glycerol
200 µl	Tris (1M) pH 7.4
1.4 ml	H_2O

Protease Inhibitor (PI) Cocktail EDTA-free 100x and 0.5 M EDTA is added freshly.

2.3 Primer List

Table 2 Overview of the used Primers for qPCR

Gene	Sequence [5'-3']		amplicon [bp]
	FWD	**REV**	
Rpl32	TTGTTGCTCCCATAACCGATGT	GCCTCTGGTGAAGCCCAAG	177
Nkx2-5	CACTTTATTGACGTAGCCTG	AAAACATAAATACGGGTGGG	154
Gata4	GAAAACGGAAGCCCAAGAAC	GGGAGGGTCTCACCAGCA	67
Tgfb2	TGGCTTCACCACAAAGACAG	TCGCTTTTATTCGGGATGAT	104
Tgfb1	GATACGCCTGAGTGGCTGTC	TTTGGGGCTGATCCCGTTG	156
Klf4	CCAAAGAGGGGAAGAAGGTCG	GTGCCTGGTCAGTTCATCGG	198
SPARC	CTCAAAAATGTCCTGGTCAC	CTCATGGATCTTCTTCACAC	93
Desmin	ACACCTAAAGGATGAGATGG	GAGAAGGTCTGGATAGGAAG	147
JNK1	GAACAGGATTGAGTAGCGGC	TCATGATGGCAAGCAATTAGTC	139
JNK2	CTGGAGCCCAAGGAATTGT	GCGTTTGGTTCTGAAAAGGA	92

2.4 Antibodies

Table 3 Overview of the used antibodies

Primary Antibodies

Target	Cat #	Application	Obtained from
Vinculin	700062	WB	Thermo Fischer Scientific
SPARC	MA5-29821	WB	Thermo Fischer Scientific
TGF beta-1	MA5-15065	WB	Thermo Fischer Scientific
TG beta-2	710276	WB	Thermo Fischer Scientific
Smad2	MA5-15112	WB	Thermo Fischer Scientific
Phospho-Smad2	MA5-15122	WB	Thermo Fischer Scientific
Desmin	PA5-117909	WB	Thermo Fischer Scientific
JNK1	PA5-80795	WB	Thermo Fischer Scientific
Desmin	14-9747-82	WB	Thermo Fischer Scientific
SPARC	MA1-43027	WB	Thermo Fischer Scientific
mCherry	M11217	Co-IP	Thermo Fischer Scientific
BMP2	PA5-85956	WB	Thermo Fischer Scientific
c-Jun	MA515172	WB	Thermo Fischer Scientific
Smad1/5	PA580036	WB	Thermo Fischer Scientific
Phospho-Smad1/5	MA515124	WB	Thermo Fischer Scientific
JNK2	MA5-32191	WB	Thermo Fischer Scientific
pJNK1/2	44-682G	WB	Thermo Fischer Scientific

Secondary Antibodies

IRDye® 680RD Goat anti-Mouse IgG	LICOR
IRDye® 800CW Goat anti-Mouse IgG	LICOR
IRDye® 680RD Goat anti-Rabbit IgG	LICOR
IRDye® 800CW Goat anti-Rabbit IgG	LICOR

2.5 Fibroblasts

2.5.1 Fibroblast Cultivation

Stem cells require feeder cells for their growth. These feeder cells are cultivated by using the cell line SNL 76/7, which is derived from a STO (S=SIM (Sandos Inbred mice) TO=Thioguanine/Ouabain resistant) fibroblast cell line with additional LIF (Leukemia Inhibitory factor) genes. By secreting the stem cell "renewal factor" LIF, the cells remain undifferentiated. The cells are thawed and plated with M10Gi medium on 10cm plates.

2.5.2 Splitting of Fibroblasts

To maintain appropriate confluence of SNL 76/7, cells must be split at least once a week. To do this, the medium is removed by suction and the cells are washed with 1xPBS to remove media residues. Then trypsin is added, and the plate is incubated for 5 to 7 minutes at 37°C and 5% CO2 to release cell adhesion. Meanwhile, a new 10cm plate with approximately 10ml of M10Gi medium is prepared. After 6 minutes, trypsin is inhibited by adding M10Gi medium and then suspending (at least three times the amount of medium as trypsin). Depending on the desired cleavage ratio (based on the confluence of the cells), the appropriate volume of cell suspension is plated out on the prepared plate and stored at 37°C and 5% CO2.

2.5.3 Feeder Cells

When SNL 76/7 reach a confluence of about 70-80%, they are mitotically inactivated by adding Mitomycin C. The volume is therefore reduced to 4ml, 80µl of Mitomycin C is added and incubated for 4h at 37°C and 5% CO2 to inhibit proliferation. 24-well plates are covered with 500µl of 0.1% gelatin solution each. After 4h cells are washed twice with 5ml 1x PBS and then trypsinized to dissolve the cell adhesion. The reaction is stopped by the addition of M10Gi medium. After pooling and proper suspension, cells are counted using a cell counter. The counted cells are then diluted with M10Gi medium to achieve a density of 30*104 cells/ml. 500µl of the dilution solution is dropped onto the prepared 24-well plate and incubated at 37°C and 5% CO2 for 24h. Once a week the cells are fed with 1.5 to 2ml M10Gi medium.

2.6 Cardiovascular Progenitor Cells (CVPCs)

2.6.1 CVPC thawing and cultivation

Cardiovascular progenitor cells must be grown on a layer of feeder cells, which secrete LIF to maintain CVPCs in an undifferentiated state. The frozen cells are thawed, plated on feeder cells, and incubated at 37°C and 5% CO2 for 24h. The cells are fed with M15HY medium every 24h.

2.6.2 Splitting of CVPCs

When cells are about 70-80% confluent, they need to be split to prevent overconfluence. For this purpose, the cells and also the feeder cells, where the split CVPCs are subsequently plated out, are fed with M15HY medium 2h in advance. After the 2h, the medium is removed, and the cells are washed with 500µl 1x PBS. After removing the 1xPBS, 200µl of trypsin is added and the plate is incubated at 37°C and 5% CO2 for 20 minutes. After 20 minutes, the reaction of trypsin is inactivated by adding M15HY medium followed by suspension (at least three times the amount of medium). Depending on the cleavage ratio, the appropriate volume of cell suspension is plated onto the pre-fed feeder cells and incubated at 37°C and 5% CO2.

2.6.3 Preadsorption

In order to perform experiments with CVPCs, they must first be detached from the feeder cell layer. For this purpose, the cells are pre-fed 2 h in advance with M5HY medium. For preadsorption, a 10cm and a 24-well plate are prepared with 0.1% gelatin solution. After 2 h, the medium is removed, and the cells are washed with 500µl 1x PBS. Then 200 µl trypsin is added and incubated for 20 minutes at 37°C and 5% CO2. After 20 minutes, the reaction is stopped with 800µl M15HY medium. The cell suspension is pipetted onto the prepared 10cm plate, cautiously agitated, and incubated at 37°C and 5% CO2 for 45 minutes. During this process the feeder cells attach to the gelatin layer and the CVPCs remain in the supernatant. After 45 minutes the plate is carefully swirled, and the supernatant is pooled in a Falcon tube. After proper suspension of the cell solution, the cells are counted in a cell counter and the

cell number is set to 5*10^5. For this purpose, the counted cell suspension is plated on the prepared 24-well plate and incubated at 37°C and 5% CO2 for 24 hours.

2.7 Cardiac Bodies

In preparation for the generation of Cardiac Bodies, A5B8/1 CVPCs are thawed and cultured at least two weeks before the start of the experiment, with the cells being split at 3-day intervals. In this experiment, T0 is the day when the Cardiac Bodies are dropped. On T-1, the cells are prepared by splitting them 1:2. At T0, cells are pre-fed with M15Hy two hours before detachment and the appropriate number of plates are pregelatinized for preadsorption. Cells are preadsorbed (for exact protocol see section 1.9.3. Preadsorption) and brought to a concentration of 4.5 x 104 cells/ml by dilution with M15Si medium. Then, 90 drops of 20 µl volume each are placed onto the lid of a bacterial plate using a multipipette, with the bottom of the dish covered with sterile H2O. In these drops, the Cardiac Bodies grow until day 4.8, after which they are rinsed with M15Si medium onto a pre-gelatinized 10cm plate. After the Cardiac Bodies were well distributed and placed on the plate at regular distances, 5 µM SP600125 was added to the test group and the same volume of DMSO was added to the control group. From T8 onwards, the Cardiac Bodies are regularly photographed and morphologically observed. The medium change is performed according to the following scheme:

Table 4 Feeding scheme for Cardiac Bodies

Day	Medium removed	Medium added	Final Volume
T8	5 ml	8 ml	11 ml
T11	7 ml	8 ml	12 ml
T14	8 ml	8 ml	12 ml
T17	8 ml	10 ml	14 ml

After the medium change, the concentration of the inhibitor was brought back to 5 µM and the same volume of DMSO was added in the control group.

The Cardiac Bodies were lysed on T18, and the proteins and RNA were isolated. For the detailed protocol of protein and RNA isolation see chapter 2.8.1 Lysis and Protein Isolation and chapter 2.10.1 mRNA Isolation.

Every group (Medium, DMSO, Inhibitor) consisted of three biological replicates. For qPCR Analysis, we used all three biological replicates of each two technical replicates were performed. For Western Blot Analysis of samples, the three biological replicated were loaded onto the same gel for every antibody, so that a direct comparison could be drawn.

2.8 Western Blot

2.8.1 Lysis and Protein Isolation

To isolate proteins from CVPCs, they are first pre-fed with M15HY medium two hours before lysis. After two hours, the medium is removed and the cells in the 24-well plates are washed with 500 µl PBS and incubated with 200 µl trypsin. After 20 minutes, trypsinization is stopped with 800 µl of medium and the detached cells are resuspended and pooled in a 15 ml Falcon. The pooled cells are mixed well and a 50 µl aliquot is taken for counting and the cells are counted three times. Meanwhile, the cells are centrifuged at 1000 rpm for 7 minutes. Then, the medium supernatant is removed, the cells are washed with 5 ml PBS and centrifuged again for 7 minutes at 1000 rpm. The supernatant is removed by suction and the cell pellet is resuspended in 1.2 ml PBS and transferred to a 2 ml Eppi. After centrifugation again for 7 min at 1000 rpm, the supernatant is removed, and the pellet is resuspended in appropriate amount of Lysis Buffer. To determine the appropriate amount of Lysis Buffer, the cells were counted, and the total cell number divided by 6000, thus obtaining the volume in milliliters. After addition of Lysis Buffer, cells are inverted at 4°C for 20 minutes. Finally, centrifuge at 14000 rpm for 20 minutes to remove cell debris and transfer the supernatant to a fresh Eppi.

2.8.2 SDS-PAGE

The samples are mixed with SDS-Sample Buffer in the appropriate ratio (1:1, e.g. 20 µl sample + 5 µl 5X SDS-Sample Buffer) and boiled at 95°C for 5 min. The precast gels are removed from the packaging and placed in the Mini tank and filled up to the indicated level with SDS-running Buffer. The samples are pipetted into the slots and the gel is run for 1h 15min at 100V.

2.8.3 Transfer

First, Whatman Paper and Nitrocellulose membrane are cut to the correct size and equilibrated in Harlow Buffer for 15 min before transfer. The gels are removed from the plastic scaffold and equilibrated for 5 min. Now a sponge, two pieces of Whatman Paper and the nitrocellulose are placed on the clear side of the cassette and finally the gel is placed on the membrane without moving it as soon as it touches the membrane. Then two pieces of Whatman paper are placed on the gel and the sandwich is flattened with a roller to avoid air bubbles between the membrane and the gel. Now the cassettes, a stir bar and a cooling block are placed in the tank and filled up with Harlow Buffer until the membrane is fully covered. The transfer runs at 4°C at 350 mA for 2 hours with continuous stirring. The gel is then removed, the membrane labeled, cut and briefly rinsed in water. To fix the proteins in the membrane, the membrane is dried for 30 min between two Whatman papers before blocking.

2.8.4 Total Protein Staining

To normalize the protein amount, the membranes are stained with a Total Protein Stain (Revert 700, LICOR). For this purpose, the membranes are rehydrated in TBS for two minutes after drying. Then 5 ml of Revert700 staining solution is added to the membrane and agitated for 5 minutes. The staining solution is poured off and the same amount of wash solution is added and agitated for 30 seconds. The washing step is repeated a second time. The membrane is then rinsed briefly in water and stored in TBS and photographed with the Odyssey CLx.

2.8.5 Immunoblotting

The membrane is incubated for one hour at room temperature in blocking solution (5% BSA in TBS; 5% NFDM in TBS; Intercept Blocking Buffer, LICOR), either on a rotator or a shaker plate. The blocking solution is poured off and the membrane is then incubated overnight at 4°C in the primary antibody solution. The primary antibody is diluted in the corresponding Diluent (5% BSA in TBS/T; 5% NFDM in TBS/T; Intercept Antibody Diluent, LICOR). The final dilution depends on the information provided by the producer. After this step, the membrane is washed three times in TBS/T for 10 minutes and then incubated in the secondary antibody solution for one hour. The

secondary antibody is also diluted in the same diluent as the primary antibody at a concentration according to the manufacturer's instructions. Depending on the detection method, AP-coupled secondary antibodies or fluorescence-coupled antibodies are used. Ap-coupled Antibodies were incubated at room temperature for 90 minutes, IR-fluorescence-coupled antibodies were incubated for 45-60 minutes. After this incubation step, wash again three times for 10 min. Now the membrane is ready for detection.

2.8.6 Detection

2.8.6.1 Alkaline Phosphatase

For detection by alkaline phosphatase, the membrane is first equilibrated in AP buffer for 5 minutes after the final wash. The buffer is poured off and exactly 10 mL AP buffer is pipetted to the membrane. Then 46.5 µL of NBT and 99 µL of BCIP are added and the reaction is allowed to run in darkness. Photographs are taken after 5, 30, 60, 120 minutes and the next day, respectively.

2.8.6.2 IR-Detection

For visualization by IR, the membrane is briefly washed one last time after the final wash in buffer without detergent and the bands are visualized in LICOR's Odyssey CLx. To do this, the membrane is placed with the protein side facing down directly from the buffer onto the glass plate of the scanner and a frame is drawn around the membrane in the software to indicate the area of scanning. The settings for the scan are: "Auto" for intensity, "lowest" for Quality and 0.0 for the distance to the glass plate. The Total Protein Stain Revert700 is imaged in the 700 nm channel, whereas the protein of interest is imaged in the 800 nm channel.

2.9 Co-Immunoprecipitation of Proteins

2.9.1 Coupling of Antibodies to Dynabeads

For co-immunoprecipitation, the Dynabeads Co-Immunoprecipitation Kit from Invitrogen (catalog number 14321D, 40 reactions) is used.

To achieve optimal antibody coupling, the manufacturer recommends the use of 5-7 µg antibody per milligram Dynabeads. The desired amount of Dynabeads is weighed

and washed with 1 ml C1. To remove the supernatant, place the Eppi in the magnet, allow the beads to collect at the side for about one minute, and pipette off the supernatant. Then the antibody is mixed with C1 according to the manufacturer's instructions and the mixture is pipetted to the beads. Afterwards, the same volume of C2 is added as C1 + antibody. The total volume has to be 100 µl/mg beads. The mixture is incubated overnight at 37°C on a rotator. The next day, the HB and LB wash solutions are first prepared by adding a concentration of 0.01 - 0.1% Tween20. Then, the antibody mixture is removed and washed with 800 µl HB. This is followed by washing with 800 µl LB and three times with 800 µl SB. Finally, a long SB wash step is performed where the beads are incubated on a rotator at room temperature for 15 min. The beads are then resuspended at a concentration of 100 µl SB per mg beads and stored at 4 °C until use. The final concentration of beads is 10 mg/ml.

2.9.2 Lysis and Protein Isolation for Co-IP

For co-immunoprecipitation, the Dynabeads Co-Immunoprecipitation Kit from Invitrogen (catalog number 14321D, 40 reactions) is used.

Initially, the 5X-IP buffer from the kit is diluted with addition of 100 mM NaCl and protease inhibitors (without EDTA). This requires about 2 mL per run + 2mL for washing. Next, the empty Eppis into which the cell suspension is transferred are weighed and noted so that the weight of the cell pellet can be determined later. After that, the cells are trypsinized, pooled, washed 3x with PBS and transferred to the weighed Eppi during the last washing step. After thoroughly removing the supernatant, the Eppi is weighed again, and the weight of the cell pellet is determined. The cell pellet is then resuspended in 1X IP buffer at a ratio of 1:9 and incubated on ice for 15 minutes. The lysate is then centrifuged at 2600g for 5 minutes at 4°C to remove cell debris. The supernatant is transferred to a fresh Eppi, an aliquot is taken (60 µl + 15 µl 5X SDS-SB) and immediately used for co-immunoprecipitation.

2.9.3 Co-Immunoprecipitation

For Co-IP, a cell amount between 50 mg - 1.5 g is recommended, as well as 1.5 mg of coupled beads. First, a 1X LWB is prepared from 5X LWB (200µl per run) and 0.02% Tween is added. 1.5 mg of coupled Dynabeads are transferred to a fresh Eppi and

washed with 900 µl of 1X IP buffer (already prepared, with protease inhibitor). The lysate is then added and incubated for 30 min at 4 °C on a rotator. The supernatant is then removed and an aliquot of the flow-through is taken. The beads are then washed three times in 200 µl IP, with careful resuspension. This is followed by the wash step with the prepared 1X LWB, where 200 µl is pipetted to the beads, carefully resuspended, and incubated on a rotator for 5 minutes at room temperature. Transfer the entire bead suspension to a fresh Eppi and discard the supernatant (remove an aliquot if necessary). Finally, resuspend the beads in 60 µl Elution Buffer and incubate for 5 min at room temperature on a rotator. The supernatant is collected and transferred to a fresh Eppi. The eluate contains the collected protein and is immediately mixed with 5X SDS Sample Buffer. Aliquots are stored either at -20 °C for a short time or at -80 °C for a longer time. Prior to analysis by Western blot, samples are briefly vortexed and boiled at 95 °C for 5 minutes.

2.10 qPCR

2.10.1 Isolation of mRNA

For isolation of RNA the RNA Mini Kit by FavorGen (Catalog #FATRK001) was used. First a total of 500.000 cells are harvested and washed twice with 1xPBS and then lysed with FARB buffer and β-mercaptoethanol (100:1). The lysed cells are transferred to a sample tube and strongly vortexed for one minute to completely resuspend the cells in the lysis buffer. The sample solution is transferred to a Filter Column Tube and centrifuged at full speed for 2 minutes. The supernatant is transferred to a new microcentrifuge tube, 70% RNAse-free ethanol is added at a 1:1 ratio, and the solution is mixed by vortexing. The sample solution is transferred to a FARB Mini Column and centrifuged at maximum speed for one minute. As the RNA now binds to the column, the flow-through is discarded. The column is washed with 500µl Wash Buffer 1 and centrifuged for one minute, the flow through is discarded. This washing step is repeated twice more with 750µl Wash Buffer 2. The FARB Mini Column is then dried by centrifugation for three minutes and the column is placed in an Elution Tube. 40µl RNase-free ddH20 is dropped into the center of the membrane and incubated for one minute at room temperature. The column is centrifuged at full speed for one minute to elute the RNA. NanoDrop measurement of RNA is used to determine the concentration of isolated RNA.

2.10.2 Generation of cDNA

For cDNA synthesis the LunaScript® RT SuperMix Kit (Cat #E3010) from NEB is used. 4 µl of the Mastermix are mixed with 1 µg of RNA and the corresponding amount of water to reach a total reaction volume of 20 µl. The sample is then placed for 2 Minutes at 25°C, followed by 10 Minutes at 55°C and 1 Minute at 95°C. The synthesized cDNA is then diluted 1:50 for qPCR.

2.10.3 qPCR

The qPCR was performed using the Luna Universal qPCR Master Mix (New England Biolabs, Cat # M3003) according to the following scheme:

Table 5 Components and volumes for qPCR reaction

Component	**20 µl Reaction**	**Final Concentration**
Luna Universal qPCR Master Mix	10 µl	1X
Forward primer (10 µM)	0.5 µl	0.25 µM
Reverse primer (10 µM)	0.5 µl	0.25 µM
Template DNA	variable	< 100 ng
Nuclease-free Water	to 20 µl	

The settings for the thermocycler for qPCR are as follows:

Table 6 Temperatures and times used for the thermocycler in qPCR reaction.

Step	**Temperature (°C)**	**Time (sec)**	**Repeats**
Initial Denaturation	95	60	1
Denaturation	95	15	40-45
Extension	60	30	

For each sample, three biological replicates were taken, of which two technical replicates were created, resulting in N = 6. To analyze the qPCR data, the raw Cq values are exported from BioRad Maestro CFX software and normalized to RPL32 using Excel and the ΔΔCq method, followed by calculation of relative expression of target genes.

3 Results

3.1 JNK1 and JNK2 are present in CVPCs, but not JNK3

Since the function of JNK in cardiac stem cells has been little studied to date, we first wanted to find out by immunoblotting which of the three isoforms of JNK are found in which proportions in CVPCs. For this purpose, undifferentiated wild-type cells (A5B8/1) were cultured for two weeks, lysed, and proteins were isolated. The lysates were blotted and incubated with antibodies against JNK1, JNK2, and JNK3. As shown in Figure 4, only JNK1 and JNK2 could be detected in CVPCs but not JNK3. Furthermore, Western blot analyses indicate that for JNK1, the 46 kDa isoform is present to a greater extent than the 54 kDa isoform. In contrast, for JNK2, the 54 kDa isoform is higher expressed.

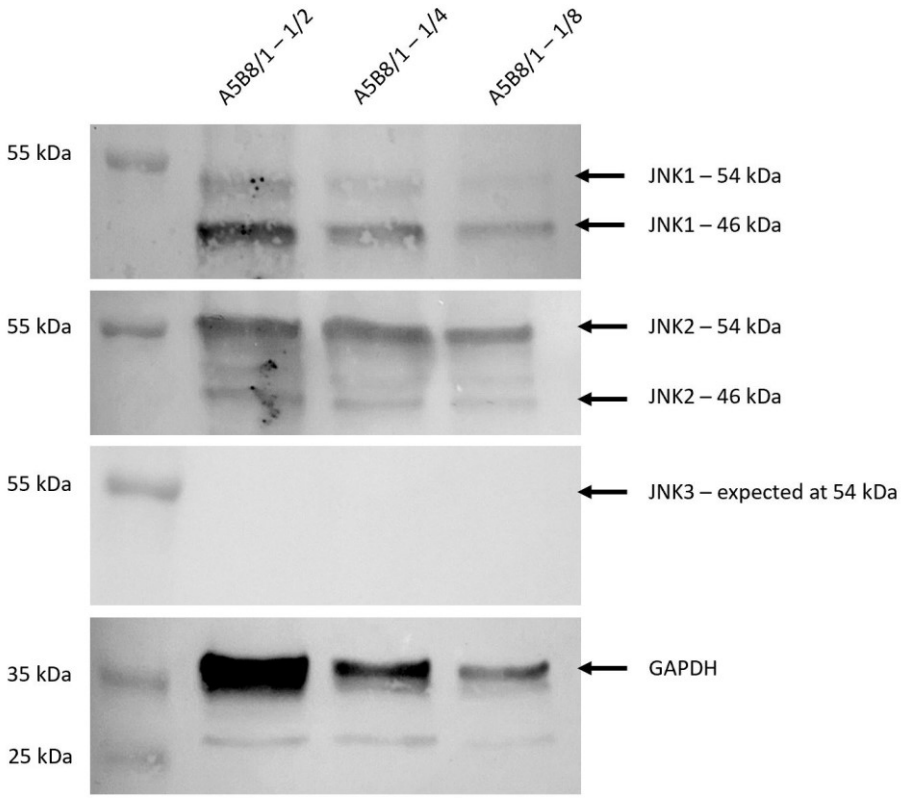

Figure 4 ***Western blot analysis of protein lysates from A5B8/1 CVPCs****. A dilution series was prepared to show which isoforms of JNK were present in CVPCs. ½ indicates a 1:1 dilution of the obtained Lysate, ¼ a 1:2 dilution, 1/8 a 1:4 dilution. Isoform-specific antibodies were used. JNK1 and JNK2 could be detected, but not JNK3. GAPDH served as a loading control in all blots. N = 2.*

3.2 Phosphorylation of JNK correlates with intracellular SPARC levels

Prior to the start of this project, several cell lines were generated that either have a heterozygous knock-out of the SPARC gene, or have an additional, ectopic SPARC allele. These cell lines were used to learn more about the effects of decreased or increased intracellular SPARC levels on protein levels of JNK and its phosphorylation by quantitative Western blot analysis. For this purpose, two cell lines that are heterozygous knock-out lines for SPARC (K11 and K17) were compared with the wild type (A5) and two SPARC-overexpressing cell lines (K4 and K10). All cell lines were cultured simultaneously followed by lysis and protein isolation 24 hours after depriving the CVPs of LIF. To obtain quantitative, comparable results, cell number was normalized before lysing, membranes were later stained with a total protein stain, and protein of interest bands were normalized to it. Alternatively, GAPDH was used as a housekeeping protein for normalization.

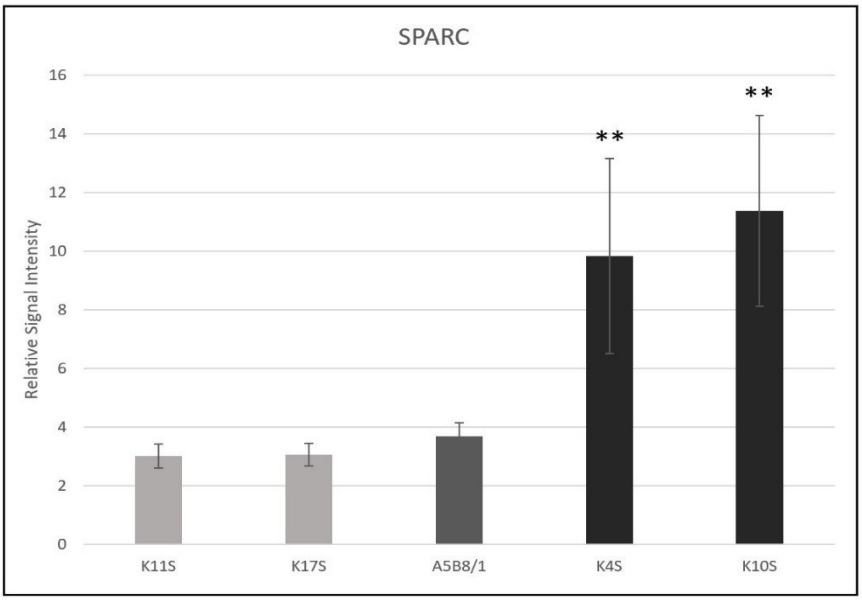

Figure 5 ***Quantitative Western blot analysis of various SPARC knock-out and ectopic cell lines****. Intracellular SPARC protein levels normalized to Total Protein Stain Revert700 were measured. **p < 0.01. Data was generated from two biological replicates and three technical replicates. N = 5*

These quantitative Western blots showed that knockout cell lines K11 and K17 have lower intracellular SPARC protein levels than wild-type A5 and overexpressing cell lines K4 and K10 have significantly higher protein levels (Figure 5). This demonstrated that the mutations indeed have an effect on intracellular SPARC protein levels.

Furthermore, it was tested whether there are different protein levels for JNK1, JNK2 pJNK1/2, and BMP between said cell lines.

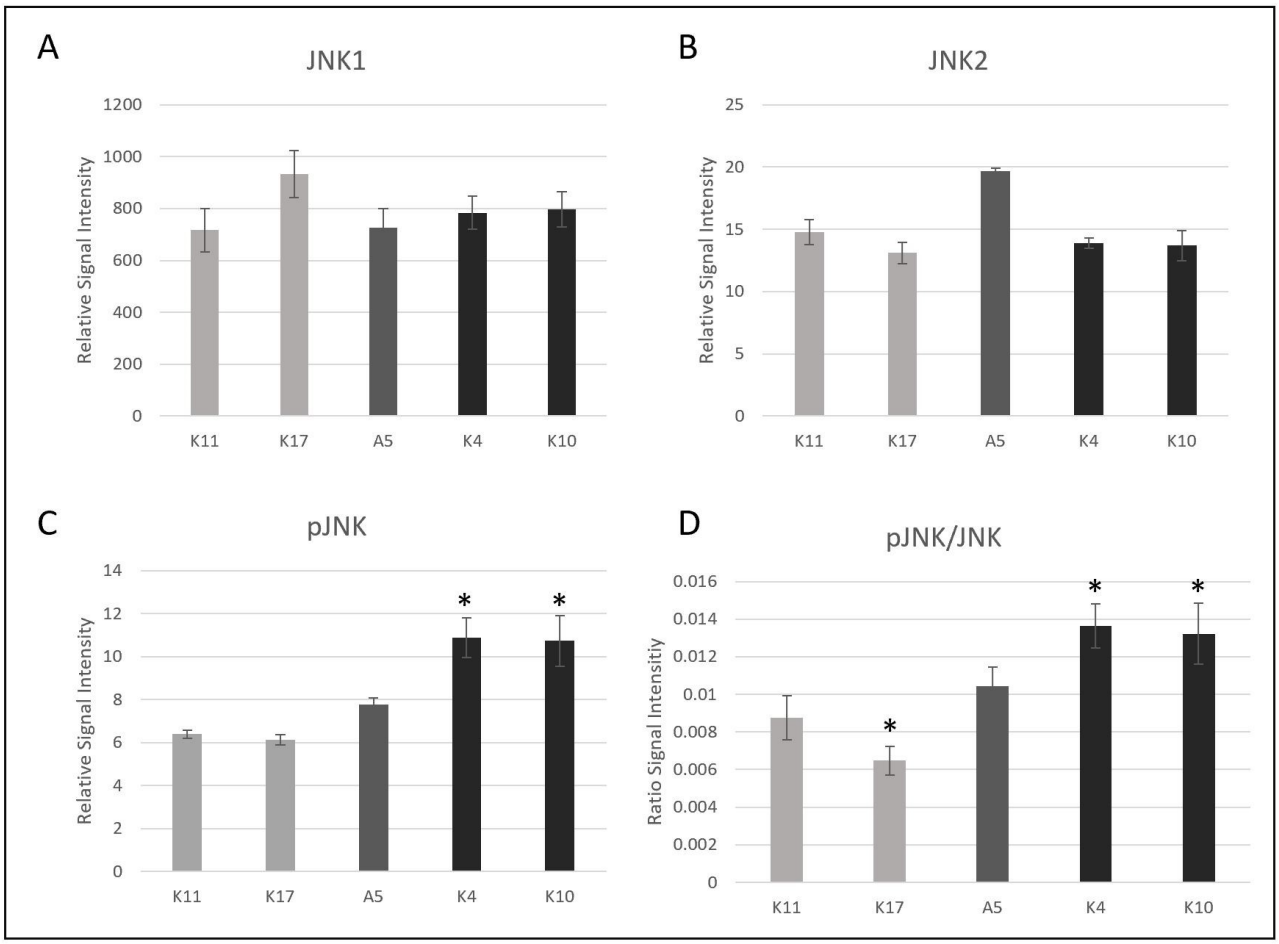

Figure 6 ***Summary of quantitative Western blot analyses of various SPARC knock-out and ectopic cell lines.*** *The intracellular protein levels of the following proteins were tested: (A) JNK1, (B) JNK2, (C) pJNK. (D) shows the normalization of pJNK to the total amount of JNK, which is the sum of JNK1 and JNK2. *p<0.05. Data was obtained from two biological replicates and two technical replicates. N = 2.*

Figure 6 shows no significant difference between the cell lines for the JNK1 and JNK2 isoforms in the unphosphorylated state. However, when phosphorylated JNK is observed, it is evident that pJNK is significantly higher in the SPARC ectopic cell lines than in the wild type. The correlation between the phosphorylation level of JNK and the intracellular SPARC amount is evident both for total pJNK and when pJNK values are normalized to total JNK protein.

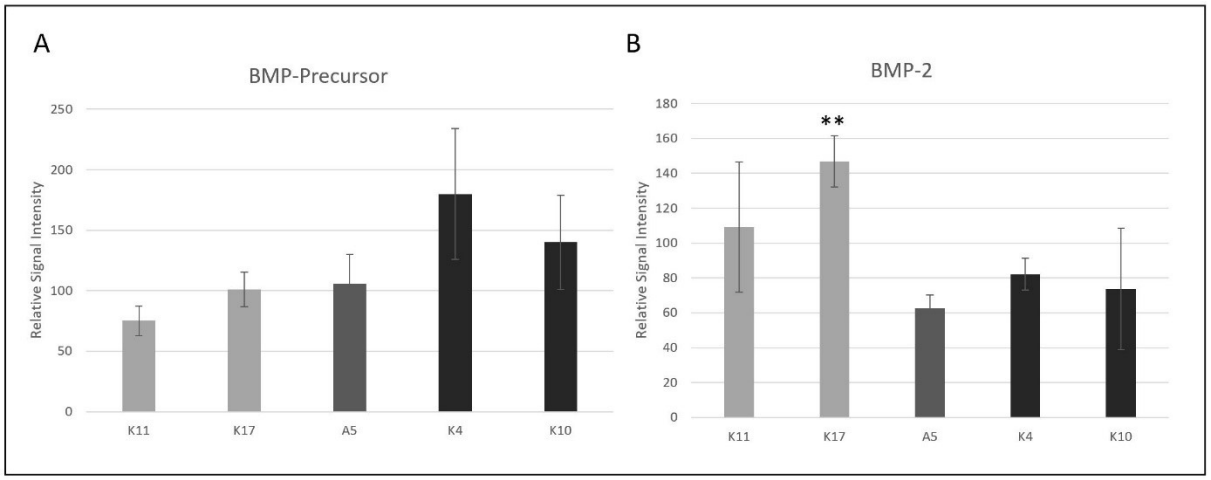

Figure 7 ***Quantitative Western blot analysis of (A) BMP precursor and (B) BMP-2*** *protein levels in SPARC KO & ectopic cell lines. **p<0.01. Data was obtained from one biological replicate and two technical replicates. N = 2.*

The same trend is observed for the uncut precursor protein of BMP, which is lower in the knockout cell lines than in the wild type and the highest levels were found in the SPARC-overexpressing cell lines. This is in contrast to the processed, active BMP-2 protein, for which the knockout cell lines show significantly higher levels (Figure 7).

3.3 Desmin K.O. and overexpressing cell lines do not show expected protein levels

Previous experiments of our group gave evidence that the intracellular Desmin protein level correlates with the amount of SPARC in the cell. To verify this, different cell lines presenting a heterozygous knock-out for the Desmin gene (1d, 2H) were compared with A5B8/1 wild-type cells and cell lines exhibiting an ectopic, additional Desmin allele and therefore expected to overexpress Desmin (K4, K12). Cell lines were again cultured simultaneously, and protein lysates were prepared 24 hours after LIF deprivation.

Quantitative Western blot analysis showed that genotype did not correlate with Desmin protein levels. Heterozygous knock-out cell lines were not found to have less intracellular Desmin than wild-type or cell lines with an additional allele.

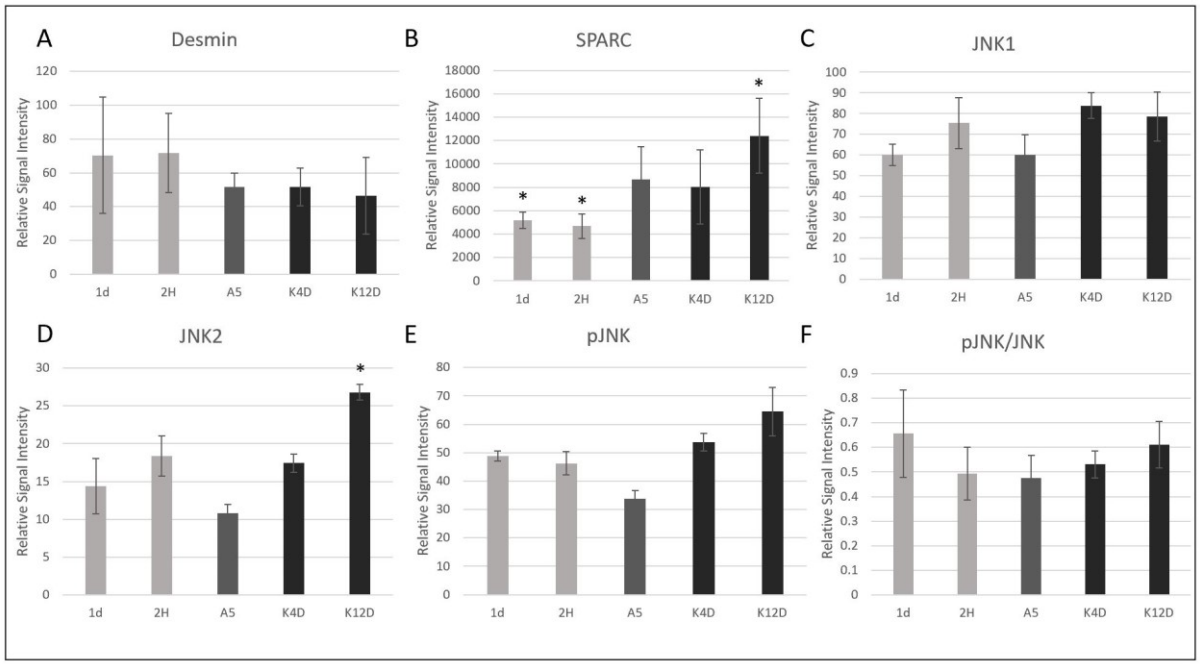

Figure 8 ***Overview of quantitative Western Blot analysis of Protein Levels*** *of (A) Desmin, (B) SPARC, (C) JNK1, (D) JNK2, (E) pJNK. (F) shows the normalized pJNK values to total JNK, which is the sum of JNK1 and JNK2. Data was obtained from one biological replicate. For Desmin, SPARC and pJNK two technical replicates were generated, for JNK1 and JNK2 one technical replicate was done. For each technical replicate, three analytical replicates were produced. *p<0.05.*

Furthermore, although cell lines 1d and 2H were shown to have lower SPARC levels than the wild-type, the correlation between an ectopic Desmin allele and higher SPARC protein levels is ambiguous because although the overexpressing cell line K12 has the highest SPARC levels, the second overexpressing cell line K4 shows lower levels than the wild type A5 (Figure 8, B).

Desmin overexpressing cells as well as knock-outs and wild type do not differ significantly with respect to protein levels of JNK1 (Figure 8, C). The trends for JNK2 and pJNK are very similar in that wild type has the lowest amount, KO lines 1d and 2h are similar to overexpressing line K4, and K12 has significantly more protein (Figure 8, D, E). Adding the values for JNK1 and JNK2 to determine the total JNK protein of the cell and normalizing the pJNK values to this, it can be seen that the levels in the Desmin cell lines only slightly differ, and no trend is discernible (Figure 8, F).

3.4 Levels of the Housekeeping Protein Vinculin are not uniform in Desmin cell lines

A successful normalization strategy is essential to obtain reliable quantitative Western blot data. At the beginning of this study, the housekeeping proteins GAPDH and vinculin were used to normalize Western blots. However, after several blots with cell lysates of Desmin knock-out and overexpressing cell lines on which vinculin was used as a housekeeping protein, it became clear that the results showed similar trends no matter which target proteins were blotted for. To verify that the different Desmin cell lines had equal vinculin levels, blots were normalized with a total protein stain in addition to Vinculin. This revealed that Desmin knockout cell lines consistently had lower vinculin levels than Desmin overexpressing cell lines (Figure 9). Although Vinculin is frequently used as a housekeeping protein for normalization, this data revealed, that Desmin mutants expressed Vinculin at different levels, which made it useless as a normalization strategy.

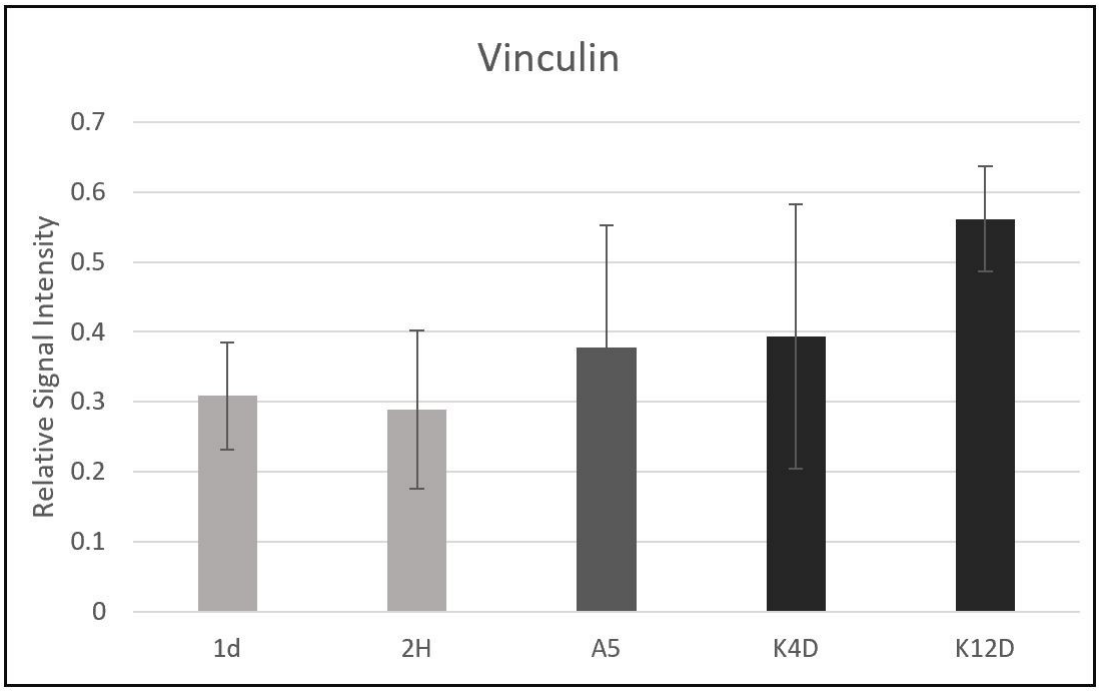

Figure 9 ***Quantitative Western Blot Analysis*** *of Vinculin in Desmin knock-out an overexpressing cell lines. Data is obtained from two biological replicates and in total four technical replicates (N = 6).*

3.5 Both SPARC and Desmin physically interact with pJNK

Since the results of the previous quantitative Western blot experiments were not conclusive, we hoped to gain more clarity on how the proteins of interest interact by performing binding studies by means of co-immunoprecipitations (co-IP). To perform the co-IPs, magnetic beads were used to which antibodies were covalently bound. Initially, attempts were made to bind appropriate antibodies against SPARC, Desmin, and JNK to the beads and the experiment was performed with lysates from wild-type A5B8/1 cells. Unfortunately, these co-IPs were unsuccessful, and the results were masked by unbound antibodies that later resulted in false-positive bands on the membrane. To circumvent this problem, SPARC-mCherry (SmC K5-6) and Desmin-mCherry (DmC[N28]) expressing cells were subsequently used and a mCherry antibody was covalently bound to the beads. Because the mCherry antibody was raised in a different species than the antibodies of our proteins of interest, the problem of masking bands could be circumvented.

As a control, another experiment was performed with cells expressing only mCherry (mC-K8) to rule out the possibility of a target protein interacting with the mCherry protein.

These interaction studies showed that JNK2 was detected in the eluate of the Desmin-mCherry pulldown, but not JNK1. It was also found that the phosphorylated form of JNK also binds to Desmin. The eluate was also analyzed for other proteins of interest, such as TGF-beta 1&2, (p-)Smad2/3, but no interaction was detected (Figure 10).

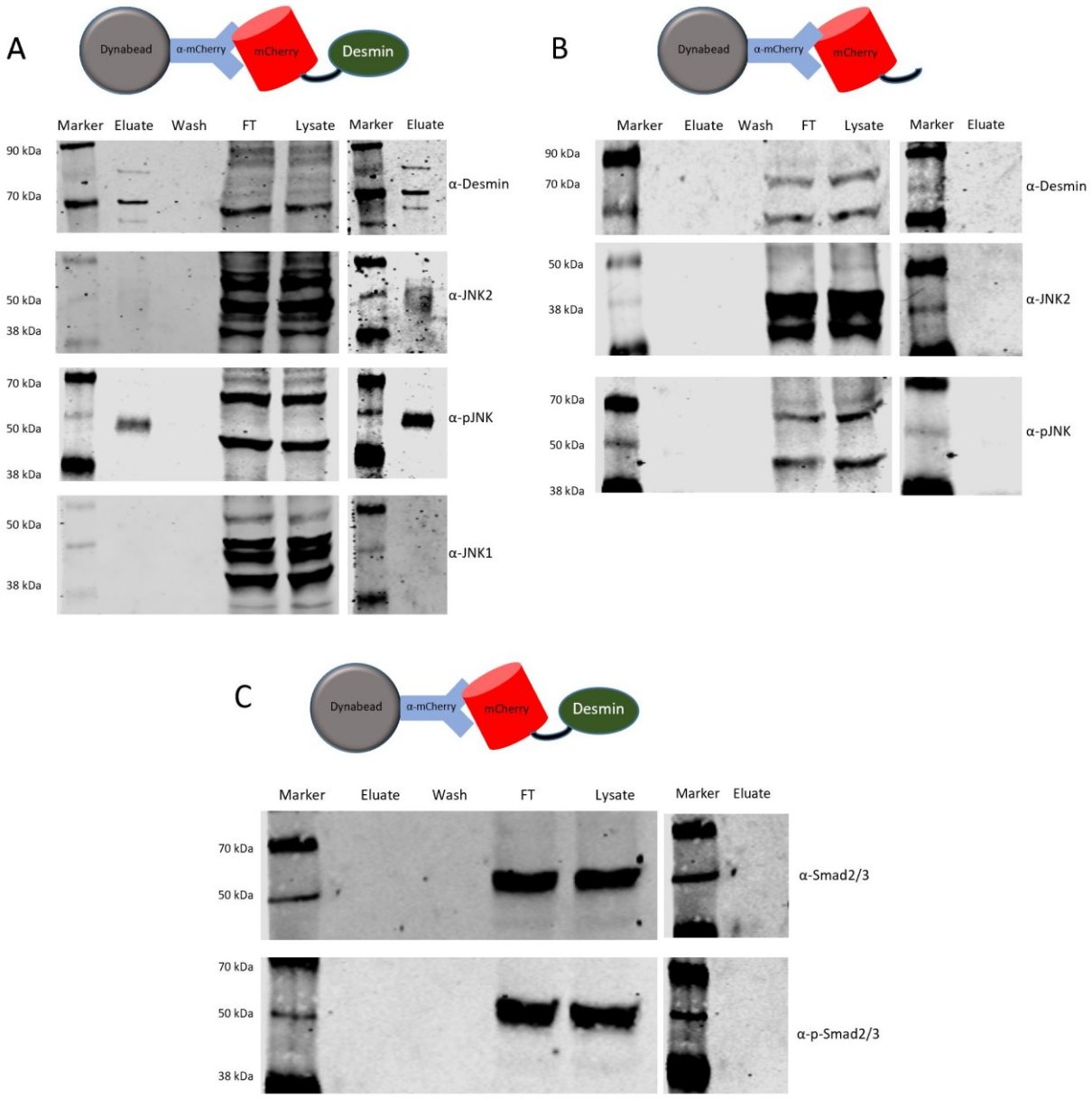

Figure 10 ***Co-immunoprecipitation*** *with lysates from (A)(C) DmC(N28) cell line expressing the fusion protein Desmin-mCherry. (B) Lysates from mCherry expressing cell line mC-K8 as a negative control. (A) Western blot analysis of Co-IP samples blotted with antibodies against Desmin (positive control), JNK2, JNK1, pJNK. (B) Samples blotted with antibodies against Desmin, JNK2, pJNK to show that the proteins of interest do not bind to mCherry. (C) Western blot analysis of Co-IP samples with antibodies against Smad2/3 and p-Smad2/3. Marker = protein ladder; Eluate = eluted protein; Wash = final wash step; FT = flow-through after incubation with Dynabeads; Lysate = cell lysate, starting material. The section on the right shows the marker and the eluate with a 5X increase in signal intensity.*

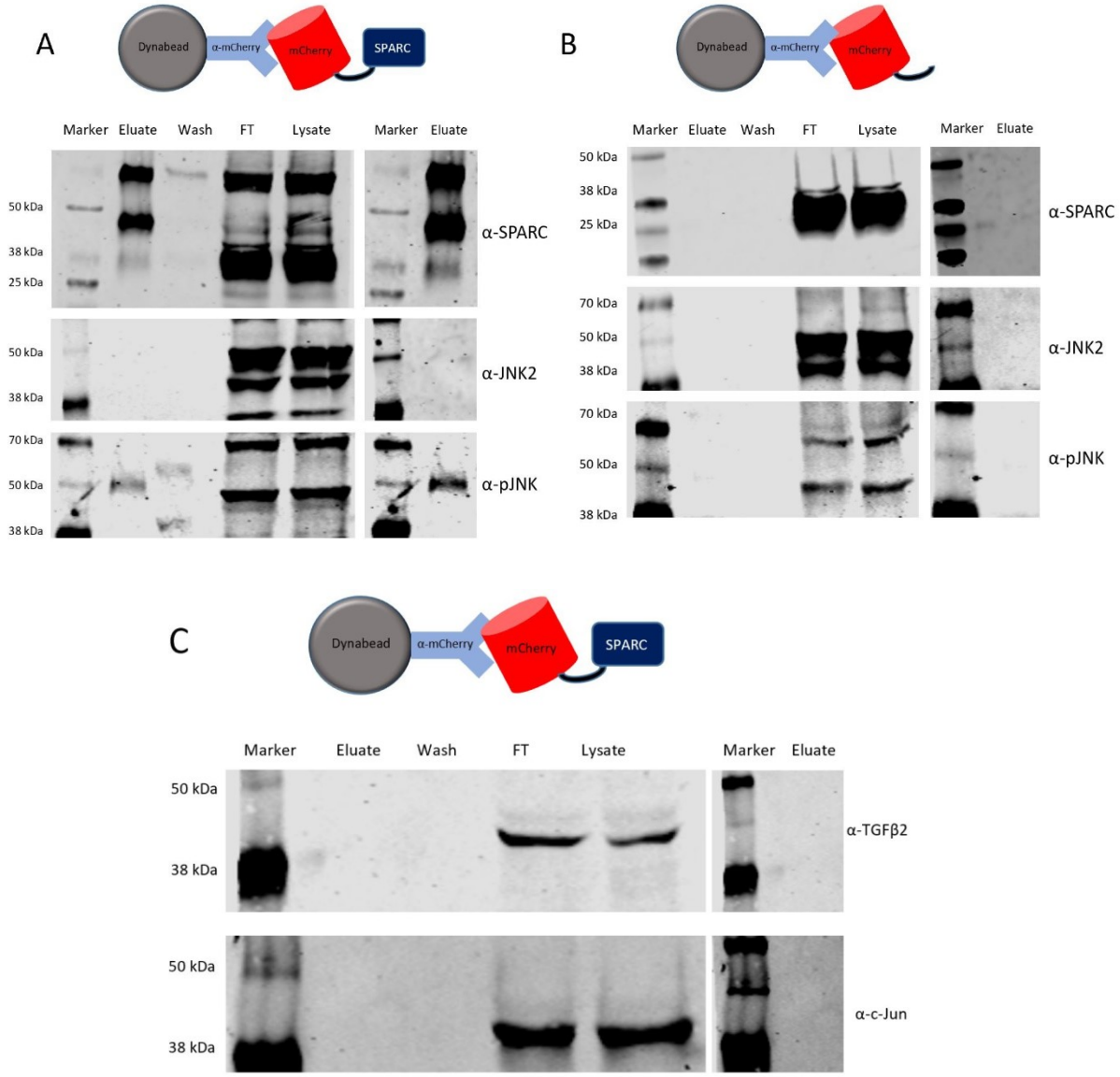

Figure 11 ***Co-immunoprecipitation*** *with lysates from (A)(C) SmC cell line expressing the fusion protein SPARC-mCherry. (B) Lysates from mCherry expressing cell line mC-K8 as a negative control. (A) Western blot analysis of Co-IP samples blotted with antibodies against SPARC (positive control), JNK2, pJNK. (B) Samples blotted with antibodies against SPARC, JNK2, pJNK to show that the proteins of interest do not bind to mCherry. (C) Western blot analysis of Co-IP samples with antibodies against TGFβ2 and c-Jun. Marker = protein ladder; Eluate = eluted protein; Wash = final wash step; FT = flow-through after incubation with Dynabeads; Lysate = cell lysate, starting material. The section on the right shows the marker and the eluate with a 5X increase in signal intensity.*

The pulldown with SPARC-mCherry also detected pJNK in the eluate, but not JNK2 or JNK1. This suggests that SPARC can bind only to the phosphorylated form of JNK (Figure 11). The Immunoblot Analysis of SPARC-mCherry Co-IP showed very faint bands for BMP-Precursor and BMP-2 protein. To confirm the presence of the bands in the eluate, Empiria Studio Software was used to calculate the pixel values. Figure 12, B shows that compared to the “Wash” fraction, there can be faint bands detected in the “Eluate” fraction. These bands correspond at around 60 kDa to the BMP Precursor Protein and at around 25 kDa to the processed and active BMP-2 protein (Figure 12).

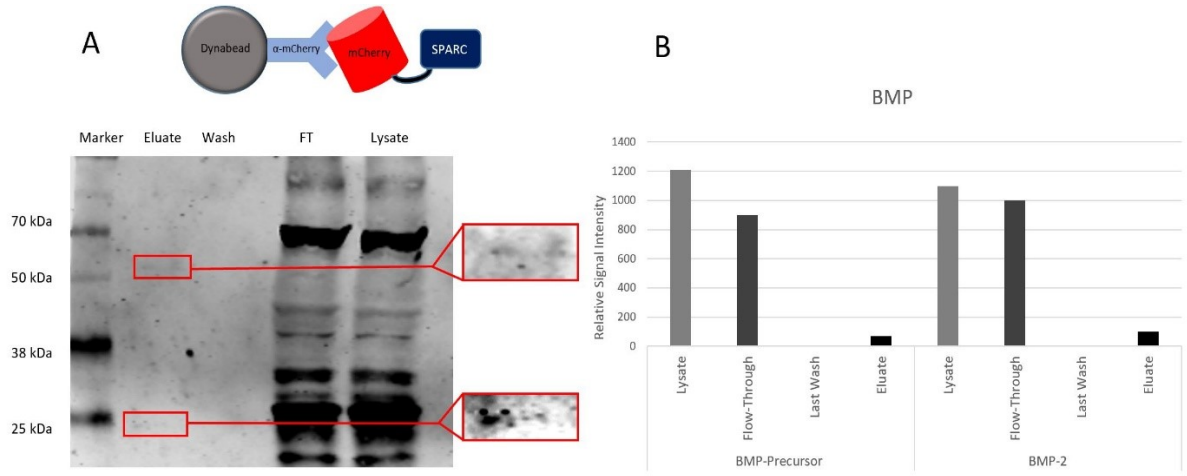

Figure 12 ***Co-Immunoprecipitation of Lysates from SmC cells using mCherry Antibody as a bait****. (A) shows Immunoblotting results of Co-IP samples incubated with BMP-Antibody. (B) shows quantification of BMP-Precursor and BMP-2 bands by Empiria Studio software. Marker = protein ladder; Eluate = eluted protein; Wash = final wash step; FT = flow-through after incubation with Dynabeads; Lysate = cell lysate, starting material. The boxes show putative BMP-Bands in the eluate with increased Signal Intensity and contrast.*

To obtain further information on the binding relationship between JNK with Desmin and SPARC, the co-IPs were performed in the presence of the JNK inhibitor SP600125. Here, the inhibitor was added 24h before lysis of the cells and was also present in the Lysis Buffer at a concentration of 5 µM. With the addition of the inhibitor, neither JNK2 nor pJNK could be detected in the eluate fraction of the Desmin-mCherry co-IPs. Also, no pJNK was found in the eluate of the SPARC-mCherry co-IP (Figure 13).

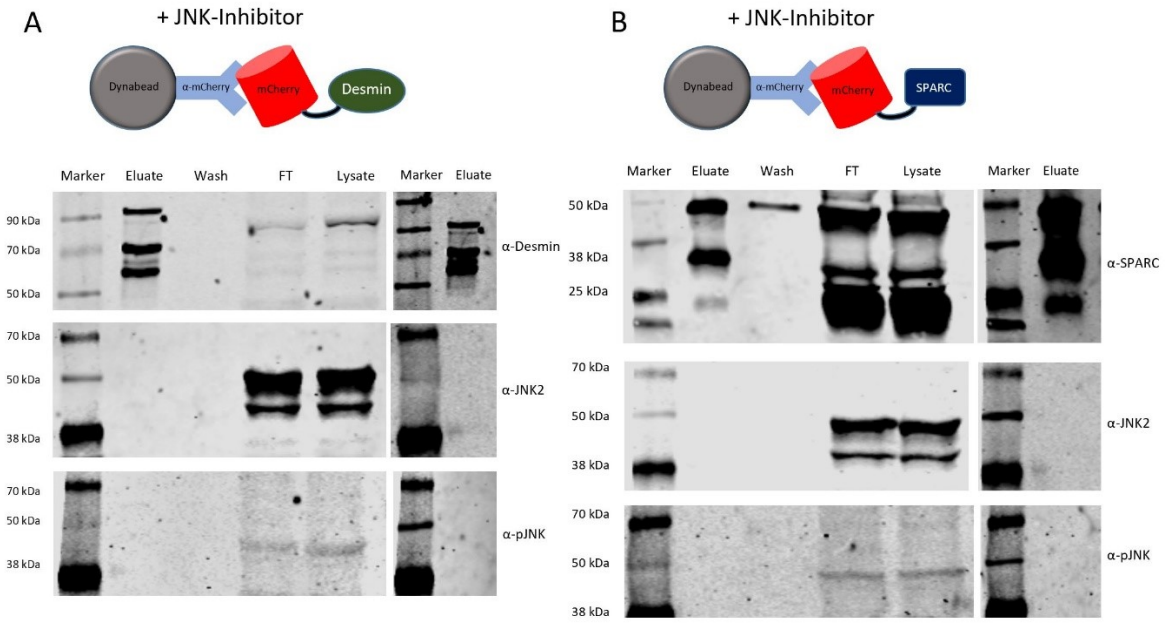

Figure 13 ***Co-immunoprecipitation with SP600125*** *with lysates from (A) DmC cell line expressing the fusion protein Desmin-mCherry. (B) Lysates from SmC cell line expressing SPARC-mCherry. Both Lysates were pre-treated with JNK-Inhibitor SP600125 (c = 5 µM) and the same concentration of SP600125 was also added to the Lysis Buffer. (A) Western blot analysis of Co-IP of DmC + SP600125 samples blotted with antibodies against Desmin (positive control), JNK2, pJNK. (B) Samples of SmC + SP600125 Co-IP blotted with antibodies against SPARC, JNK2, pJNK. Marker = protein ladder; Eluate = eluted protein; Wash = final wash step; FT = flow-through after incubation with Dynabeads; Lysate = cell lysate, starting material. The section on the right shows the marker and the eluate with a 5X increase in signal intensity.*

3.6 Desmin exhibits sequence homologies with JNK substrate c-Jun

Since co-immunoprecipitation experiments demonstrated that Desmin and (p-)JNK2 bind to each other, it was of interest to determine whether Desmin had sequence or structural similarities to other binding partners or substrates of JNK. This search revealed that Desmin has 42% sequence similarity to the JNK substrate c-Jun. The homology sites are located at positions 117-144 for Desmin, in the region of coil 1A, and at positions 275-302 for c-Jun, in the region of the leucine zipper bZIP at the C-terminus.

3.7 JNK Inhibitor delays differentiation of developing Cardiac Bodies

3.7.1 SP600125 concentrations from 10 µM slow down cell proliferation

To investigate the effect of the JNK inhibitor SP600125 on cardiomyogenesis, cardiac bodies were grown from wild-type cells (A5B8/1). For this purpose, undifferentiated cells are first incubated in hanging droplets for 5 days so that they can assemble into a 3D structure. These droplets are then washed onto a gelatinized surface so that they can adhere. Thus, further differentiation into adult cardiac cells is promoted. Since this was a pilot experiment and there was nothing known in the literature about the appropriate concentration of JNK inhibitor for differentiating CSCs, a time series experiment was first performed with different concentrations of the inhibitor and cell proliferation was observed. After 24 hours, cell number was determined. None of the concentrations tested resulted in the cells being unable to divide, but it was apparent that proliferation was already slowed after 24 hours at a concentration of 10 µM (Figure 14). After 48 hours, hardly any differences can be seen, but the cells were not provided with fresh medium during this period, which is why the effects can be attributed to starvation of the cells.

Based on these data, the following experiment was performed with an inhibitor concentration of 5 µM.

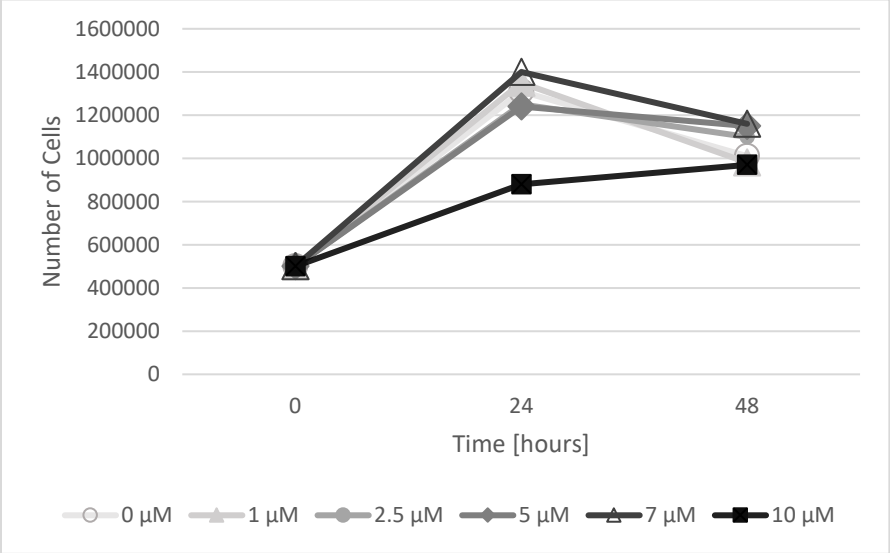

Figure 14 ***Cell numbers of A5B8/1 cells treated with different concentrations of the JNK inhibitor SP600125 at 0, 24, and 48 hours.*** *Cell number for the groups of 0 – 7 µM declines after 24 hours due to deprivation of fresh medium and starvation of the cells.*

3.7.2 Treated Cardiac Bodies are morphologically indistinguishable from control

The differentiating Cardiac Bodies were photographed regularly to determine whether morphological differences between the groups could be observed by the addition of the inhibitor. This would indicate that the chosen concentration of inhibitor is too high and cell division is inhibited. For this purpose, photographs from day 10 to day 14 were compared for size and shape of the cardiac bodies. No morphological differences could be observed between the groups based on the photographs (Figure 15).

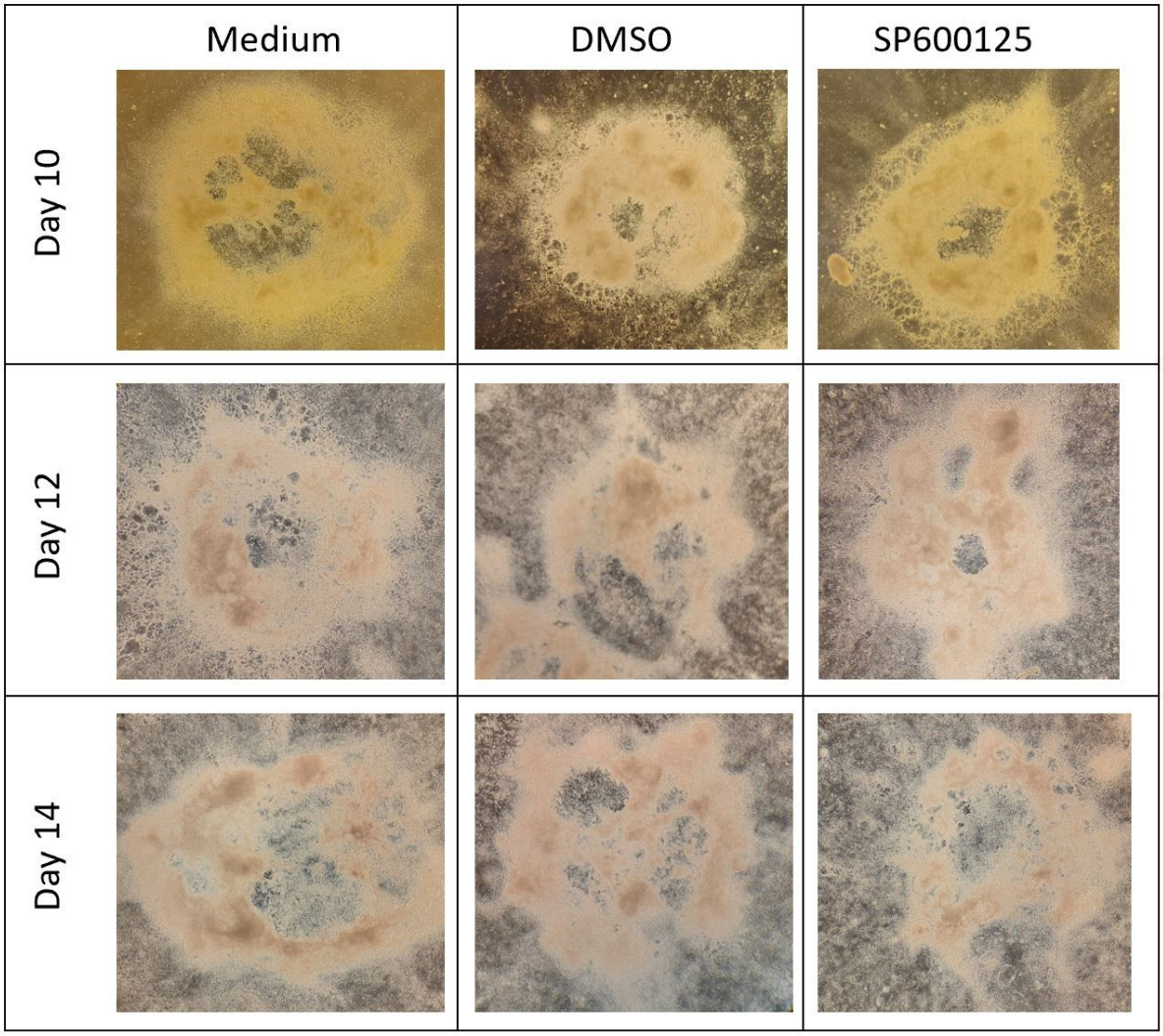

Figure 15 ***Photos of Cardiac Bodies from A5B8/1 cells****, controls with medium and DMSO, test group with 5 µM JNK inhibitor SP600125. Photos were taken on day 10, 12 and 14 at similar times. Photos were taken through the 4X objective of a light microscope.*

3.7.3 Differentiation of Cardiac Bodies is delayed by the JNK Inhibitor

From day 9, the differentiating cardiac bodies were observed and counted daily. The number of beating CBs and the number of contracting centers were recorded. From day 10, a clear difference between the control group and the DMSO and inhibitor group in the proportion of beating CBs could already be observed, but not yet in the total number of beating centers (Figure 16, A, B). From day 12 onward, the inhibitor-treated CBs show a markedly slowed differentiation, with no difference between the two control groups, medium and DMSO. From day 15, the three groups are indistinguishable in terms of proportion of contracting CBs and number of contracting centers, which remains so until the end of the experiment.

The contracting centers beat irregularly and arrhythmically in the first days, therefore the beating frequency was measured only from day 13. Here it can be seen that both the medium and DMSO groups are in the range of 60-70 beats per minute, while the inhibitor treated cells beat significantly slower. On day 17, all groups show a similar beating frequency. On day 18, only the frequency of the medium group could be measured, since both the DMSO group and the inhibitor group already exhibited arrhythmic beating (Figure 16, C).

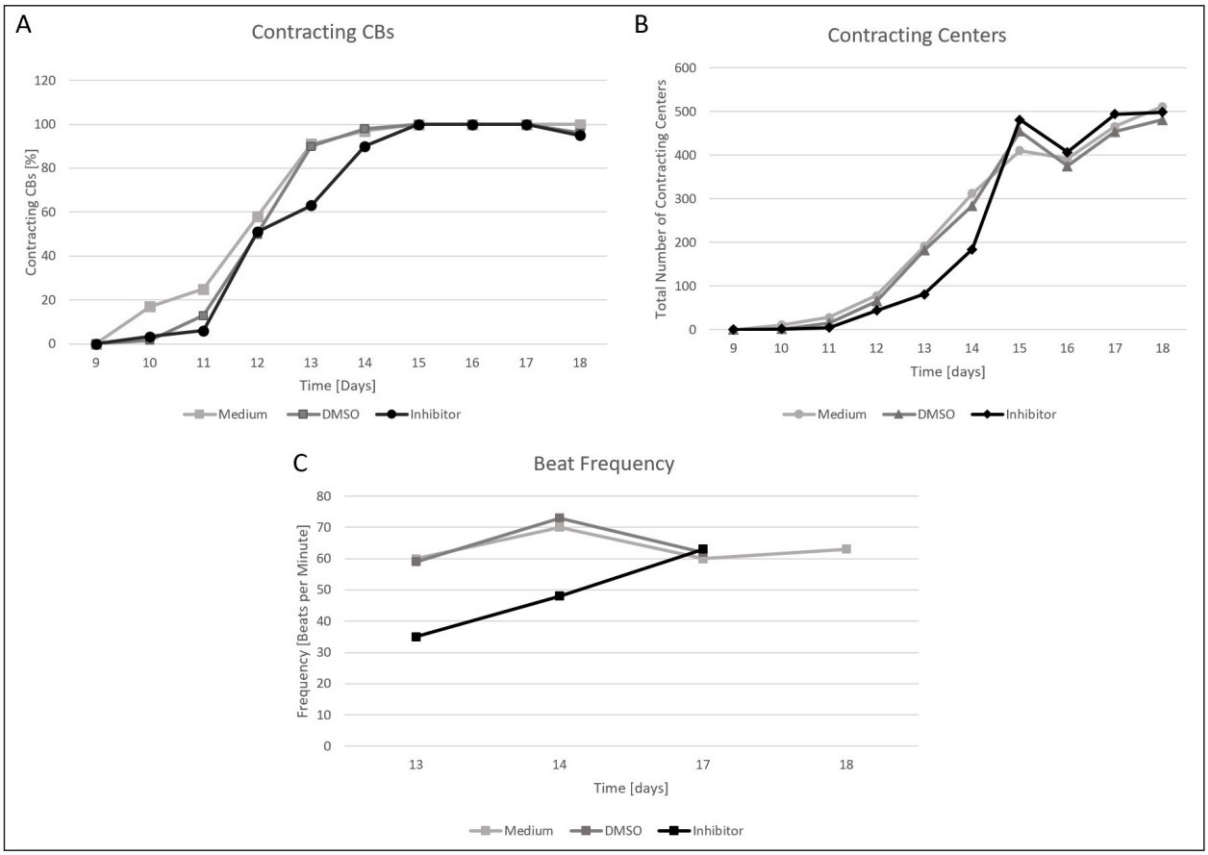

Figure 16 ***Overview of different parameters of cardiac body differentiation****. (A) Percentage of cardiac bodies that contract, i.e., in which at least one contracting center was detected. (B) Total number of contracting centers. (C) Beating frequency of the contracting centers. To determine this, the beating frequency in beats per minute was determined for at least three contracting centers per plate, and the average was calculated. No data could be obtained for the DMSO and inhibitor group after day 17 because the contracting centers were already arrhythmic. Each data point shows the average of three biological replicates (N = 3).*

3.7.4 JNK-Inhibitor influences Gene Expression and Protein Levels of Target Proteins

CBs were lysed at day 18 and both RNA and protein were isolated. qPCR and Western blot analyses showed that JNK1 was slightly upregulated by the JNK inhibitor at both the mRNA level and the protein level (Figure 17, C, D). JNK2 was decreased at the protein level by addition of the JNK inhibitor but not at the mRNA level (Figure 17, E, F). As expected, phosphorylation of JNK is strongly suppressed by the inhibitor (Figure 18, A, B).

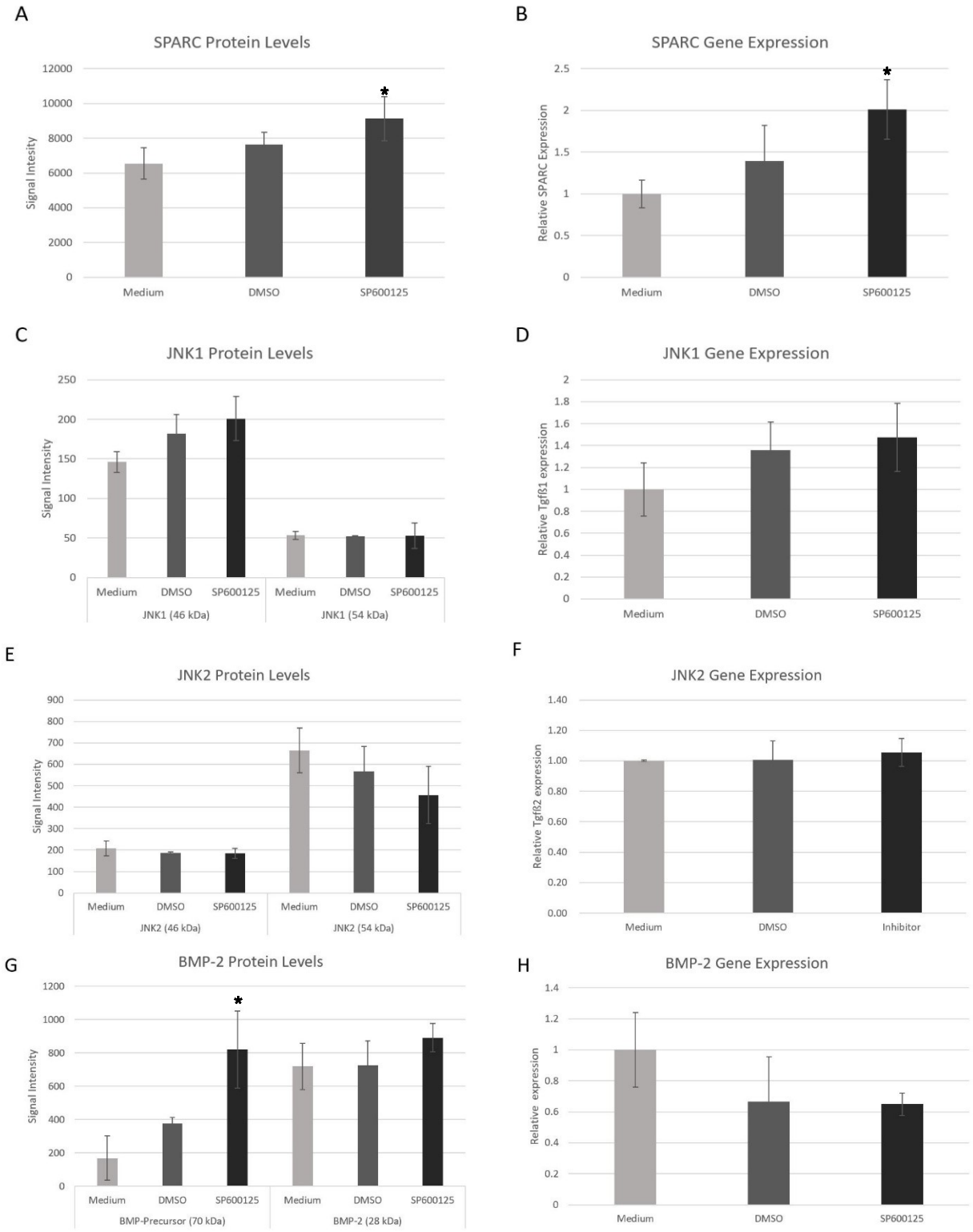

Figure 17 ***Overview of both protein levels and gene expression of various targets****. Protein Levels were determined by quantitative Western blot analysis and signals of target proteins were normalized to the Total Protein Stain Revert 700. Gene expression data were determined by qPCR and normalized to housekeeping gene RPL32. (A) SPARC intracellular protein levels. (B) SPARC gene expression. (C) JNK1 protein level. (D) JNK1 gene expression. (E) JNK2 protein level. (F) JNK2 gene expression. (G) BMP protein amount. (H) BMP-2 gene expression. Western Blot Data was obtained from three biological replicates and one technical replicate (N = 3). qPCR data consists of three biological replicates and two technical replicates of each (N = 6). *p<0.05.*

SPARC is upregulated at both the mRNA level and the protein level by addition of the inhibitor (Figure 17, A, B).

BMP-precursor and active form are also increased at the protein level by addition of the inhibitor compared with controls. Gene expression of BMP is reduced in the DMSO control to the same extend as in the inhibitor group (Figure 17, G, H).

Also, protein levels of the JNK downstream target and AP1 complex member c-Jun are significantly lower in the inhibitor group than in the medium or DMSO group, this is also observed for the phosphorylation of c-Jun (Figure 18, C, D).

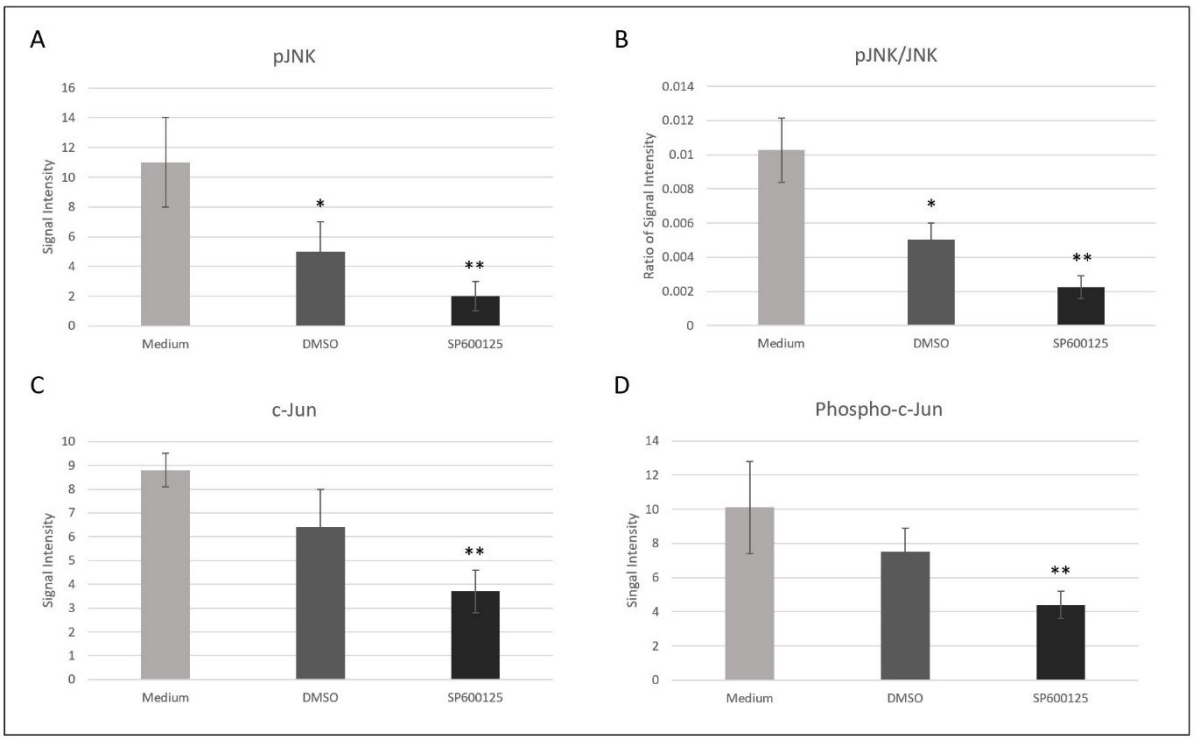

Figure 18 ***Effects of JNK inhibitor SP600125 on Phosphorylation of JNK and c-Jun****. (A) pJNK protein amount tested by quantitative Western blot analysis. (B) Normalization of pJNK to the total amount of JNK in the cell. (C) Protein amount of c-Jun. (D) Protein amount of phosphorylated c-Jun. *p<0.05, **p<0.01. Western Blot Data was obtained from three biological replicates and one technical replicate (N = 3).*

Gene expression of the transcription factor Klf4 is also significantly upregulated by SP600125 treatment. At the mRNA level, no difference was detected between groups for other genes of interest (Tgfb1, Tgfb2, GATA4, Nkx2.5, and Desmin) (Figure 19).

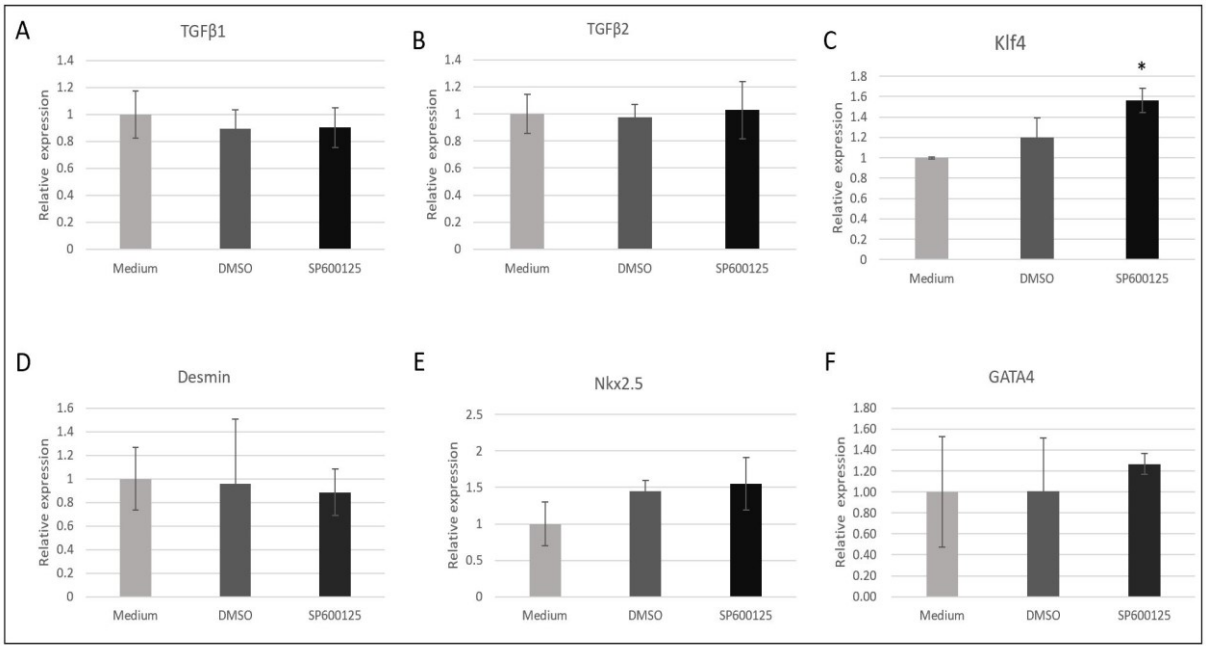

Figure 19 ***Gene expression analyses of various target genes*** *applying SP600125 tested by qPCR. (A) TGFβ1, (B) TGFβ2, (C) Klf4, (D) Desmin, (E) Nkx2.5, (F) GATA4. *p<0.05. qPCR data consists of three biological replicates and two technical replicates of each (N = 6).*

4 Discussion

4.1 SPARC shows a dichotomy in regulating growth-factor dependent signaling pathways

SPARC is a matricellular protein that is highly expressed in the developing heart and plays a role in the differentiation of CSCs into adult cardiac cells. Several signal transduction pathways are thought to be modulated by SPARC, but it is not clear how it functions in the differentiation of CVPCs.

The results of this study have shown that increased intracellular SPARC levels correlate with increased phosphorylation of JNK. This agrees with the results of Wang et al. (2019), who showed that SPARC silencing decreased the phosphorylation of ERK, JNK, p38, NF-κB, and IKKβ. Another study found evidence that SPARC promotes self-renewal of limbal epithelial stem cells via the JNK signaling pathway (Zhu et al., 2020). Activation of the JNK signal transduction pathway by SPARC also plays a role in cell pathology. For example, in some cancers where SPARC is overexpressed compared to healthy tissue, JNK phosphorylation is increased (Sailaja et al., 2013).

However, a recent finding of ours is that SPARC physically interacts with the phosphorylated form of JNK in the cell. An indication that SPARC indeed plays a regulatory role in the JNK signaling pathway is the fact that only the activated, phosphorylated form binds to SPARC.

The interaction studies in this work found indications that SPARC physically interacts with BMP, which agrees with previous unpublished data from our group. This interaction is proposed to happen extracellularly. We have used whole cell lysates for our interaction studies, however, there is a possibility that the extracellular matrix was not washed away, and SPARC bound to ECM binds to BMP. This is also a possible explanation for why only such weak bands were obtained. Both TGF-beta and BMP can non-canonically activate the JNK pathway. Rivera and Brekken (2011) have shown that SPARC regulates TGF-beta signaling, but in this case through direct contact with the receptor endoglin. Follistatin-like 1 (Fstl1), a SPARC family protein, plays an important role in vertebrate development by inhibiting BMP signaling (Sylva et al., 2013). Another protein related to SPARC, SMOC 1 (SPARC-related modular calcium binding 1) regulates BMP-2 signaling by binding to the BMP receptor and thus

preventing phosphorylation of other downstream target proteins (Wang et al., 2022). These data show that SPARC is an important player in many developmental biology settings when it comes to cell differentiation. However, the modes of action are not consistent, as both activating and inhibitory effects on growth factor receptors are found. SPARC thus appears to have various effects on signaling molecules and receptors of the TGF-beta family.

4.2 Desmin might exhibit a regulating function as a scaffold for JNKs

Since Desmin is one of the earliest expressed cardiac muscle-specific proteins, and Desmin K.O. mice show severe cardiac deficits, it appears to play an important role in cardiomyogenesis. As an IF protein, it has scaffolding functions; however, it has already been shown that the regulatory effect of Desmin goes beyond that of a mere scaffold.

The results of this study have shown that Desmin physically interacts with JNK2 and pJNK and that this interaction is extinguished in the presence of the JNK inhibitor SP600125. It should be noted here that presumably no binding between Desmin and pJNK could be detected any longer since SP600125 already after 24 h causes the amount of pJNK in the cell to decrease to such an extent that the interaction is no longer detectable simply by decreasing the amount of pJNK. However, the fact that binding to JNK2 can also no longer be detected when the inhibitor is used may have the following reasons: 1) Desmin generally only binds to the phosphorylated form of JNK2 and this is also recognized by the JNK2 antibody. 2) Binding between Desmin and JNK2 is prevented by the ATP analog SP600125, which blocks the binding site. The binding of Desmin to JNK2 has already been shown by Javed et al. (2020). Furthermore, they demonstrated that overexpression of Desmin increases the phosphorylation of JNK. Other studies have shown that in skeletal muscle cells exposed to stress, JNK is strongly phosphorylated. However, this phosphorylation does not occur in Desmin knockout cells, indicating that Desmin plays an important role in signal transduction (Palmisano et al., 2014). These data provide evidence that Desmin serves as a scaffold and as a regulator in signal transduction from JNK to its substrates.

Another indication of this is the sequence homology observed between Desmin and the AP1 complex member c-Jun, which is considered the main substrate of JNK. A

region of Desmin and c-Jun matches in 42% of its sequence, which according to Pearson (2013) suggests that they share similar functions. What is interesting here is that the homologous sequence in c-Jun is located in the region of the leucine zipper, which is responsible for DNA binding on the one hand, but is also essential for interaction with JNK according to May et al. (1998). This study has shown that while the delta region of c-Jun is essential for docking to JNK, it is not sufficient, as mutations in the leucine zipper region mean that c-Jun and JNK can no longer interact (Figure 20). Now, precisely the Leucine zipper of c-Jun is shares homology with the Coil 1a motif of Desmin. Thus, there is a possibility that Desmin binds to JNK via the Coil 1a motif. Furthermore, c-Jun also forms homodimers via the leucine zipper. Desmin could thus also bind directly to c-Jun and thus serve as a third interaction partner and scaffold, for the interaction between JNK and c-Jun. However, this is all speculative. It is also not known whether Desmin is involved in the interaction between JNK and upstream proteins that phosphorylate JNK.

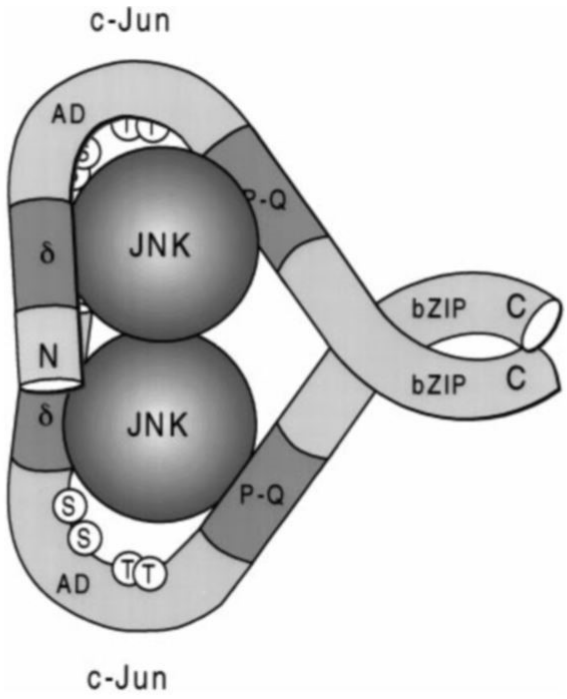

Figure 20 ***Proposed scheme of binding between JNK and c-Jun dimers.*** *This graphical representation shows a JNK dimer binding to both the P-Q regions and the δ-regions of c-Jun. Meanwhile, the c-Jun dimer forms a bond at its C-terminus in the region of bZIP. Figure taken and adapted from May et al. (1998).*

4.3 JNK signaling pathway inhibition delays cardiomyogenesis

Previous studies have shown that JNK signaling plays a role in cell differentiation. To investigate this role in CVPCs, we inhibited the JNK signaling pathway during differentiation of Cardiac Bodies. It was shown that although the Cardiac Bodies treated with the inhibitor initially exhibited decreased cardiomyogenesis, this effect was no longer observable at day 15 and the groups showed no significant difference from then on. This means that either the inhibitor concentration used was not sufficient to produce a more pronounced effect, or the cells activated an alternative signaling pathway as a rescue route. What argues against the inhibitor concentration being too low is the fact that JNK phosphorylation was greatly reduced compared to the control groups. Also, the protein level of the JNK substrate c-Jun dropped in the test group compared to the controls.

The upregulation of the SPARC gene in response to JNK inhibition is a controversial result. It is known that c-Jun binds to the SPARC promoter as a transcription factor and thus regulates its expression. Studies have found that overexpression of c-Jun correlates with upregulation of the SPARC gene (Briggs et al., 2002, Kögel et al., 2005). Nevertheless, there is also evidence that c-Jun downregulates SPARC gene expression in embryonic fibroblasts (Vial and Castellazzi, 2000). Thus, c-Jun is able to affect the expression of SPARC both positively and negatively depending on the cell type and the state of the cell.

This dichotomy of SPARC activity also exists in human cancers, where for some tumor types, such as breast cancer, prostate cancer, and melanoma, it has been shown that overexpression of SPARC leads to more aggressive and invasive cancer cells (Graham et al., 1997, Sturm et al., 2002, Thomas et al., 2000). In contrast, overexpression of SPARC in fibrosarcoma and ovarian cancer leads to slowing of tumor progression (Colombo et al., 1991, Yiu et al., 2001). This theme of dichotomous behavior of SPARC thus runs through many different cellular processes, suggesting that SPARC is a protein that acts primarily in a situation-dependent and cell type-dependent manner. It is of central interest for many research areas to elucidate these different functions of SPARC.

Our results have shown that while c-Jun protein levels strongly decrease upon application of the JNK inhibitor, SPARC increases at both the mRNA and protein levels. As a possible explanation for this, it was shown by Florian Steiner that the transcription

factor Klf4 also binds to the promoter of SPARC. Klf4 was upregulated at the mRNA level in the inhibitor group, proportional to SPARC. That means that the upregulation of SPARC might either be the result of transcriptional activation by Klf4 instead of c-Jun, or the absence of an otherwise present negative feedback loop involving SPARC and c-Jun.

The upregulation of Klf4 by inhibition of JNK is consistent with the results of Yao et al. (2014), which showed that JNK1 and JNK2 are able to phosphorylate Klf4, whereupon the transcription of Klf4 is significantly decreased. It was known that Klf4 undergoes various post-translational modifications, but it is not known what effects phosphorylation has on Klf4 activity. Thus, our results fit this picture, as inhibition of JNK promotes Klf4 activity.

The protein levels of BMP-2 and the BMP precursor protein were also increased in the treated cardiac bodies. As mentioned previously, the BMP-2 signaling protein represents a potential site for regulation of the TGF-beta signaling pathway by SPARC. Here, there might be a regulatory negative feedback loop that is omitted by inhibition of JNK, which is why BMP protein levels are increased. However, this is pure speculation and further experiments are necessary to understand the connections between these proteins.

5 Summary and Outlook

The hypothesis of this study is that SPARC and Desmin communicate witch each other through the JNK signaling pathway and influence the regulation of cardiomyogenesis through this pathway. Evidence was found that SPARC and Desmin both interact and communicate with the JNK signaling pathway. First, in SPARC-overexpressing cells that exhibited increased intracellular SPARC protein levels, increased phosphorylation and thus activation of JNK was also detected. Furthermore, co-immunoprecipitation studies revealed that Desmin physically interacts with JNK2 and pJNK and that this interaction is abolished in the presence of the JNK inhibitor and ATP analogue SP600125. What was surprising here was that SPARC also has the ability to bind to pJNK, which provides a possible regulatory site. In addition, indications that SPARC interacts with BMP were found, which is consistent with previous unpublished data from our group. This extracellular interaction represents another opportunity for SPARC to influence the activation of the TGFβ/BMP signaling pathway, which noncanonically relays its signals via JNK. Evidence that the JNK signaling pathway plays a role in the regulation of cardiomyogenesis is the fact that differentiation of cardiac bodies treated with SP600125 is slowed. Moreover, in Cardiac Bodies treated with the JNK inhibitor, SPARC is upregulated, suggesting that not only does SPARC affect the JNK pathway, but also the JNK pathway affects the expression of SPARC. There appears to be a feedback loop here, but a dichotomy of SPARC behavior still prevails.

A next step to untangle this network would be to investigate the role of SPARC in rescuing the JNK inhibition phenotype by performing studies not only in wild-type cells but in SPARC knock-out and overexpressing cells. Here, one could also add exogenous SPARC and observe whether this abolishes the slowing of cardiomyogenesis. Further co-immunoprecipitations could be used to identify additional binding partners that may play a role in this network. It would also be interesting to explore which players act upstream of JNK and phosphorylate JNK and whether these can also be bound and regulated by SPARC or Desmin.

In conclusion, despite some discrepancies in the results, this work does its part to gain further understanding of the complex regulation of cardiomyogenesis in CVPCs.

6 Zusammenfassung

Herz-Kreislauf-Erkrankungen sind weltweit die häufigste Todesursache. Während man früher dachte, das Herz sei ein rein postmitotisches Organ, weiß man heute, dass das Herz verschiedene Populationen von kardiovaskulären Vorläuferzellen beherbergt. Trotz des starken wissenschaftlichen Interesses an Stammzellen ist das Wissen über die Regulation der Kardiomyogenese in CVPCs begrenzt. Es gibt Hinweise darauf, dass das Intermediärfilament-Protein Desmin und das matrizelluläre Protein SPARC an der Regulation der Kardiomyogenese beteiligt sind, aber wie sie kommunizieren und interagieren, ist unbekannt. Vorläufige Literaturrecherchen haben den JNK-Signalweg als mögliche Schnittstelle dieser beiden Proteine ins Licht gerückt. In dieser Studie wurden die Auswirkungen der intrazellulären SPARC-Level auf die JNK-Phosphorylierung mittels quantitativer Western-Blot-Analyse untersucht. Die physische Interaktion zwischen Desmin, SPARC und Akteuren des JNK-Signalwegs wurde durch Co-Immunopräzipitation aufgeklärt. Um die Auswirkungen einer Hemmung des JNK-Signalwegs auf die Kardiomyogenese zu untersuchen, wurden Cardiac Bodies gezüchtet und mit dem JNK-Inhibitor SP600125 behandelt. Diese Experimente zeigten, dass in SPARC-überexprimierenden Zellen die JNK-Phosphorylierung erhöht ist. Darüber hinaus wurde gezeigt, dass Desmin physisch mit JNK2 und pJNK interagiert, und diese Bindung wurde in Gegenwart von SP600125 aufgelöst. Die Hemmung der JNK-Signalübertragung hat auch eine verlangsamende Wirkung auf die Kardiomyogenese in Cardiac Bodies. Zusammenfassend lässt sich sagen, dass SPARC und Desmin in der Lage sind, die JNK-Signalisierung zu beeinflussen, und dass umgekehrt die JNK-Signalisierung die SPARC-Genexpression und die Proteinmenge beeinflusst, was darauf hindeutet, dass der JNK-Signalweg eine vielversprechende Schnittstelle für die Kommunikation von SPARC und Desmin bei der Regulierung der Kardiomyogenese darstellt.

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