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ECM1 and its role in cardiac remodelling after Myocardial Infarction

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Abbreviations and Definitions

MI – myocardial infarction

ICM – ischaemic cardiomyopathy

LV - left ventricular / left ventricle

DCM – dilated cardiomyopathy

TGF β1 - transforming growth factor β 1

IL – interleukin

TNF – tumor necrosis factor

ACTA2 - α-smooth muscle actin

CCL2 - Monocyte Chemoattractant Protein or MCP-1

TβRI or II – TGFβ type I or II receptor

COL1A1 / COL1A2 – genes of collagen type I, encoding for different components of collagen type I.

TRAF2 - TNF receptor associated factor 2

ECM1 – extracellular matrix protein 1 (gene and protein)

Min – minute

s/sec – second

°C – degree Celsius

Vol - Volume

µl – microlitre

ng – nanogram

NTC – non template control

RPM – rotations per minute

ml – millilitre

mM – milli molar

µg – microgram

h – hour

µm – micrometre

EtOH – ethanol

W – Wat

kDa – kilo Dalton

Ab – anti body

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Zusammenfassung

Nach wie vor bilden Herzerkrankungen und deren Folgen weltweit die Speerspitze der Todesursachen und bedürfen daher besonderer Aufmerksamkeit. Dank der modernen Medizin können heutzutage eine Vielzahl an akut auftretender Herzinfarkte gut behandelt werden, jedoch haben viele Menschen nach Auftreten des Infarkts noch mit dessen Folgeerscheinungen zu kämpfen. In einigen Fällen kann das initiale Event zu chronischer Herzinsuffizienz und Vernarbung (Fibrose) führen, welche den Herzmuskel anhaltend und fortlaufend schwächt und letzten Endes zum Versagen des Organs führt. Hierbei sind verschiedene sich verstärkende Signaltransduktionswege beteiligt. In meiner Arbeit habe ich versucht ein Schlüsselelement dieser Signalwege genauer zu beleuchten und dessen Veränderungen festzuhalten. Die Rede ist hier vom **Extrazellulären Matrixprotein 1**. Dieses steht in Verbindung mit fibrotischen Veränderungen des Herzgewebes und trägt vermutlich maßgeblich zur morphologischen Veränderung des Herzmuskels nach einem Herzinfarkt bei. Um diese genauer zu beleuchten war es notwendig die zugrundeliegenden Signaltransduktionswege, welche durch ECM1 aktiviert werden; zu untersuchen. Hierfür habe ich mit ECM1 behandelte humane Kardiofibroblasten mit qRT-PCR auf bestimmte Gene hin untersucht, die im Verdacht stehen eine fibrotische Reaktion auf den initialen Infarkt zu aktivieren. Im Laufe meiner Arbeit habe ich weiters eine Proteinexpressionsbestimmung mittels Western Blot an humanen Herzproben unternommen um die ECM1 Konzentrationen nach dilatativer Kardiomyopathie sowie nach ischämischen Kardiomyopathien zu untersuchen. Weiters wurde eine Proteinexpressionsbestimmung von mit ECM1 und TGFβ behandelten humanen Kardiofibroblasten mittels Western Blot vorgenommen, um die p-SMAD2 Aktivierung zu untersuchen, welche mit einer erhöhten Kollagenbildung im Zusammenhang steht. Letzten Endes habe ich ECM1 im Mausherzen lokalisiert, nachdem zuvor ein induzierter Herzinfarkt vorgenommen worden ist. Hierfür habe ich mich der Immuno Histochemie bedient und mit fluoreszenzmarkierten Antikörpern gearbeitet.

Die Erforschung von ECM1 und dessen Auswirkungen in der hier vorliegenden Masterarbeit sind von besonderer Bedeutung, da es als Schlüsselstelle für fibrotische Veränderungen im Herzen angesehen wird und somit als Angriffspunkt dienen könnte, um diese aufzuhalten oder deren Fortschritt zu verlangsamen.

Abstract

Heart diseases and their consequences are still the spearhead of the causes of death worldwide and therefore require special attention. Thanks to modern medicine, a large number of acute heart attacks can now be treated well, but many people still have to struggle with the sequelae after the onset of the heart attack. In some cases, the initial event can lead to cardiac fibrosis, which persistently and continuously weakens the heart muscle and ultimately leads to failure of the organ. Various amplifying signal transduction pathways are involved here. In my work I have tried to shed light on a key element of these signalling pathways and to record their changes. We are talking about the **extracellular matrix protein 1**. This is associated with fibrotic changes in the heart tissue and probably contributes significantly to the morphological changes in the heart muscle after a heart attack. To shed light on these in more detail, it was necessary to examine the underlying signal transduction pathways that are activated by ECM1. For this purpose, I examined human cardiac fibroblasts treated with ECM1 using qRT-PCR for certain genes that are suspected of activating a fibrotic reaction to the initial infarction. In the course of my work, I also undertook a protein expression determination using Western Blot on human heart samples in order to examine the ECM1 concentrations after dilated cardiomyopathy and ischemic cardiomyopathy. Furthermore, a protein expression determination of human cardiac fibroblasts treated with ECM1 and TGFβ was carried out by means of Western blot to investigate the p-SMAD2 activation, which is related to the increase in collagen deposition in the heart. In the end, I located ECM1 in the mouse heart after an induced heart attack had previously been performed. For this I used immuno-histochemistry and worked with fluorescence-labeled antibodies.

The research into ECM1 and its effects in this master's thesis are of particular importance, as it is viewed as a key point for fibrotic changes in the heart and could therefore serve as a point of attack to stop or even reverse them.

1. Introduction

Cardiac diseases and heart failure are the clinical manifestations of different forms of underlying cardiovascular disease such as coronary artery disease and are often characterized by reduction of cardiac contractility, chamber remodelling, progressive fibroses, and reduced ventricular compliance. **(1)**

1.1 Ischemia and Myocardial Infarction

Myocardial infarction (MI) is an event where myocardial necrosis occurs after myocardial ischaemia and is one of the major causes of death and invalidity worldwide. It can arise slowly as a manifestation of a chronic progressing coronary artery disease or abruptly after myocardial infarction. The clinical features of myocardial infarction are cell death of cardiac myocytes caused by ischaemia, which is a perfusion imbalance between the supply and the demand of oxygen of the cardiac myocytes. Tissue death itself is characterised pathologically as coagulation and/or contraction band necrosis that arrives from oncosis or cell death. After the beginning of myocardial ischaemia it takes only minutes until cells start dying, however, it takes several hours before myocardial necrosis can be seen by macroscopic or microscopic post-mortem examinations.

Myocardial infarctions are further characterised by different attributes, such as size (microscopic (focal necrosis), small (<10% of the left ventricular [LV] myocardium), moderate (10–30% of the LV myocardium), and large (>30% of the LV myocardium)), location and stages (acute, healing, healed).

Acute MI is characterized by the presence of polymorphonuclear leukocytes that are recruited to the heart minutes after the onset of ischemia and peak few days after MI. The healing infarct is characterised by the presence of mononuclear cells and fibroblasts and little polymorphonuclear leukocytes, whereas the healed infarction is manifested as scar tissue without cellular infiltration. **(2)**

Wound healing after the infarct takes about 5-6 weeks to complete. Infarct reperfusion, which is the reopening of the infarcted vessels, may alter the macro- and microscopic image of the necrotic zone by the production of myocytes with contraction bands and large quantities of extravasated erythrocytes.

Considering all the named conditions and features one can divide the MI in evolving (<6h), acute (6h-7d), healing (7-28d) and healed (29 days and longer). **(3)**

1.2 Dilated Cardiomyopathy

Throughout MI counts as an ischaemic cardiomyopathy there are also other cardiomyopathies that are non-ischaemic such as dilated (or non-ischemic) cardiomyopathy (DCM). DCM arrives with structural and functional myocardial abnormalities such as left or biventricular dilatation and systolic dysfunction in the absence of coronary artery disease, hypertension, valvular disease and congenital heart disease. The pathophysiological consequences are a decrease in blood volume pumped by the left ventricle with each contraction (stroke volume) that directly leads to a smaller amount of blood pumped by the heart (cardiac output per minute). Also impaired ventricular filling appears as well as an increase in end diastolic pressure, which is the measure of how much effort it takes to fill the heart. Next to compensatory changes in the vascular system that are necessary to adapt to the new circumstances also the cardiac preload (stretching of the heart wall before contraction) and the afterload (force for contraction to pump the blood out of the heart) are increased. With the increase in afterload also an increase in wall stress appears. Patients with DCM frequently show evidence of an inflammatory cell filtrate and gene expression patterns that are compatible with immune cell activation.. Immune cells that take part in the remodelling of the myocardium are mast cells, macrophages, T helper 2 (T_H2) and T_H17 cells, as well as B cells in case of autoimmune aetiologies. The immune cells are known to release cytokines like transforming growth factor β 1, IL-4, IL1-β, IL -17A, IL-33 and tumor necrosis factor (TNF) among others that promote remodelling, collagen deposition and fibrosis. The local dysfunction and/or volume overload play a role in increased cardiac workload and wall stress and are consequences and cause of fibrotic remodelling. The replacement of damaged tissue causes stiffening and progression of the dilatation up to heart failure. **(4)**

1.3 The healthy cardiac interstitium and its cellular effectors of cardiac fibrosis

1.3.1 The physiological interstitium

The normal architecture of the myocardium is laminar and comprised of primarily fibrillar collagen. The cardiac matrix network can be divided into three morphologically different constituents called epi-, peri- and endomysium. Located on the endocardial and epicardial surfaces one can find the **epimysium** providing support for the endothelial and mesothelial cells. The **perimysium** encases muscle fibres whiles the perimysial strands connect groups of muscle fibres. Coming from the perimysium we find the **endomysium** surrounding individual muscle fibres. The endomysium tethers muscle fibres together and to their nutrient microvasculature and serves as a connector to cardiomyocyte cytoskeletal proteins across the plasma membrane. The cardiac matrix network is based on collagen and serves as a scaffold for cellular components and plays a pivotal role in the transmission of contractile force. About 85% of the myocardial collagen arrives from **type I** which is associated with thick fibres that confer tensile strength, whereas **type III collagen** makes up about 11% of the total collagen in the heart. It typically forms thin fibres and maintains the elasticity of the matrix. Other than that, the cardiac extracellular matrix also consists of glycosaminoglycanes (for instance hyaluronan), glycoproteins and proteoglycans. The **extracellular matrix** also stores significant amounts of latent **growth factors** and **proteases** that get activated by cardiac injuries and may trigger the fibrotic response. Furthermore, the cardiac interstitium contains cardiac fibroblasts enmeshed in the endomysial interstitial matrix that surrounds cardiomyocytes. As matrix producing cells, they play an important role in preserving the integrity of the matrix network in the heart. Also, vascular cells such as smooth muscle cells, endothelial cells and pericytes can be found in the mammalian heart as well as small numbers of mast cells and macrophages that reside around vessels. **(5)**

Last but not least, there are matricellular proteins to be found in the heart. A Family of structurally unrelated extracellular macromolekules that do not play a primary role in tissue architecture but are induced after injury. Their main purpose is to modulate cell-cell and cell-matrix interactions. As they get released, matricellular proteins associate with growth factors, cytokines and other bioactive effectors and bind to cell surface receptors where they transduce signaling cascades. These proteins are upregulated in the injured and remodeling heart and play a pivotal role in regulation of inflammation, reparation as well as in fibrotic and angiogenetic pathways. While their expression is generally low in normal adult tissue, they get upregulated during development and in response to an injury. **(6)**

1.3.2 The cellular effectors of cardiac fibrosis

1.3.2.1 Cellular composition of the human heart

The human adult heart composites of 11 major cell types, namely: atrial cardiomyocytes, ventricular cardiomyocytes, fibroblasts (FBs), endothelial cells (EC), pericytes, smooth muscle cells (SMC), immune cells (myeloid and lymphoid), adipocytes, mesothelial cells and neuronal cells. The distribution of the cell types vary between atrial and ventricular tissues. The atrial human heart tissues contain 30.1% cardiomyocytes, 24.3% FBs, 17.1% mural cells (pericytes and SMCs), 12.2% ECs and 10.4% immune cells (myeloid and lymphoid), whereas the ventricular regions (apex, interventricular septum and left ventricle) contains 49.2% ventricular cardiomyocytes, 21.2% mural cells, 15.5% FBs, 7.8% ECs and 5.3% immune cells. **(7)**

1.3.2.2 Myofibroblasts, the fibroblasts in action.

The two main causes for the activation of fibrogenic signals in the myocardium are cardiomyocyte death and injurious stimuli like pressure overload or myocardial inflammation that trigger profibrotic pathways. There are several cell types that take part in the fibrotic remodelling process in an either direct way by producing matrix proteins (cardiac myofibroblasts) or indirectly by secreting fibrogenic mediators (macrophages, mast cells, lymphocytes, cardiomyocytes and vascular cells). Myofibroblasts are phenotypically modulated fibroblasts that accumulate at the injury site and have ultrastructural and phenotypical features of smooth muscle cells, such as formation of contractile stress fibres and an extensive endoplasmatic reticulum. One of the most common features is the expression of α-smooth muscle actin (ACTA2) that helps identify differentiated myofibroblasts in injured tissues.

Following cardiac injury an alteration in the matrix environment takes place such as induction and release of growth factors and cytokines and increased mechanical stress. The activation of TGF-β in the cardiac interstitium promotes α-SMA transcription in fibroblasts through activation of the SMAD signalling cascade. The incorporation of α-SMA into stress fibres significantly increases fibroblast contractile activity. Alterations in the composition and mechanical properties of the extracellular matrix leads to the trans differentiation into myofibroblasts through an alteration in the response to mechanical stress.

1.3.2.3 Innate immune cells and their role in myocardial infarction

Neutrophils are the first to migrate to the impacted area post MI. Neutrophil numbers peak a couple of days post-MI and decrease gradually. Some of the neutrophils remain at the injury site once the **inflammatory phase** is over. They induce the release of ROS and pro – inflammatory cytokines. Further they promote the infiltration and proliferation of **monocytes**, dendritic cells, natural killer cells, Th1 (T helper cell type 1), Th17 (T helper cell type 17) and B-lymphocytes as well as additional neutrophils. Neutrophils also serve as a source of matrix degrading enzymes such as MMP-8 and MMP -9 (matrix metallopeptidase -8 and -9), collagenase and neutrophil elastase. Their main task is to phagocyte the degraded matrix components and cells coated in complement. Throughout MCP-1 (monocyte chemoattractant protein 1) produced by neutrophils and endothelial cells, **monocytes** become drawn to the injury site. **(8)**

Monocytes are short-lived circulating cells that can be divided into the classical and non-classical subset. In humans they are defined by CD16 (cluster of differentiation 16) expression. Classical monocytes described as CD14+CD16neg in humans are implicated in inflammation. They contribute to the innate immune response via production of TNF-α and nitric oxide, as well as by antigen presentation to T cells. On the other hand, non classical monocytes $CD14^{+}CD16^{+}$ are thought to persist in the circulation, they patrol the endothelium and may play a role in immune surveillance. **(9)**

Infiltrating monocytes turn into **M1-type macrophages** in response to high levels of **IFN- γ, TNF-α and DAMPs (damage-associated molecular patterns)** at the site of injury. M1-macrophages are usually detected around 24 hours post MI and peak four days later, while the gradual shift towards M2 polarization starts five days post MI. M1-macrophages release the pro-inflammatory cytokines TNF-α, IL-1β, CXCL10 (chemokine C-X-C motif ligand 10), IL-6, IL-12 and IL-23. High plasma levels of TNF-α post MI is an indication of the severity of MI damage and usually comes with increased risk of heart failure. Furthermore, **CXCL10** serves as a **chemoattractant** of other **macrophages**, dendritic cells, NK cells (natural killer cells) and TH1 lymphocytes. M1-macrophages also release MMP-9 that degrades basement membranes, facilitating the entry of additional immune cells. Overall, it can be said that M1- macrophages promote a pro – inflammatory milieu. After digesting apoptotic neutrophils M1- macrophages decrease their production of pro inflammatory IL-1β, IL-6 and TNF-α, and start to increase their production of IL-10 and **TGF-β** (transforming growth factor beta), indicating a shift towards the M2-type. They also reduce their expression of IL-23, resulting in decreased IL-17, and inhibition of additional neutrophil recruitment. M2-type macrophages are likely involved in the induction of the apoptosis of myofibrobalsts and play a role in the maturation phase post MI. **(8)**

M2-type macrophages are induced by IL-4 or IL-13 and express high levels of IL-10 and participate in resolution of inflammation and angiogenesis**. (10)**

1.3.2.4 Cells of the adaptive immune response in MI

CD4+ T cells

Among the adaptive immune cells, CD4+ helper T lymphocytes which interact with antigens presented on MHC-II molecules (on antigen presenting cells) seem to play a role in post ischemic cardiac remodelling. The so called CD4+CD28^{null} T cells expand in diverse chronic inflammation diseases but cannot be found in healthy individuals. They secrete IFN-γ and TNF-α, as well as cytotoxic mediators. CD4+CD28^{null} T cells are present in the blood in the context of acute coronary events during several months. Another CD4+T subset that has been described were immune suppressive natural regulatory T cells (Tregs). These cells limit autoimmunity and maintain self-tolerance through the suppressing of activated effector T-cells, directly or through inhibition of antigen -presenting cells. Therefore a few "resident" Tregs are present in the healthy heart, but they rapidly infiltrate the myocardium in the context of acute ischemia in a CCR5 – dependent manner with a peak after 24h in a re-perfused model of MI or day 7 in a permanent artery ligation model. According to previous studies Tregs seem to have a beneficial role. Treg depletion was described to worsen cardiac inflammation, infarct size and left ventricular dysfunction following MI. In contrast Treg expansion improved survival and myocardial wound healing. Tregs therefore limit pathogenic CD8+ and CD4+ T cells recruitment in the heart and turn the monocyte/macrophage polarization towards a pro – fibrotic phenotype. Treg expansion further increases pro collagen α-1 (I) and procollagen α-1 (III) mRNA expression, as well as collagen I and collagen III protein levels in the heart tissue. At later stages of remodelling Tregs interact with fibroblasts and promote a matrix preserving cardiac fibroblast phenotype. It has also been shown that Tregs can limit cardiomyocyte apoptosis and can induce their proliferation through the release of several soluble factors such as IL-10 and IL-33. **(11)**

CD8+ T cells

Another important player in the adaptive immune response are CD8+ T cells. They are able to directly kill virally infected or damaged cells. The activation of CD8+ T cells requires the interaction with antigen – presenting cells with MHC I. CD8+ T cells are then able to lyse the target cells through the secretion of perforin and granzymes and to a less extend through the engagement of membrane bound death inducing ligands such as Fas-ligand. One subtype that needs to be mentioned in context of MI are the CD8+AT2R T cells that have no cytotoxic activity, suggesting a potential cardioprotective role in ischemia by infiltrating the peri-infarct zone and downregulation of pro-inflammatory cytokines expression. **(11)**

B cells

The exact mechanism of B cell recruitment and activation are yet under investigation but in experimental MI in mice $B220^{+}IgM^{+}$ B cells peaked in the heart tissue at day 5 after the onset of ischemia. $CD20^{+}$ B cells have further been found in human heart biopsy from MI patients at day 1 and day 6, following MI. B cells secrete several cytokines like B cell – derived lymphotoxin-α and TNF- α that control the development of follicular DCs and the formation of B cell follicles in the spleen. In MI at the acute phase, the specific CCL-7 production by mature B cells orchestrates monocyte mobilisation from the bone marrow to the blood and furthermore their recruitment into the ischemic heart. **(11)**

1.3.2.5 The three phases of fibrosis

The cellular response to myocardial infarction can be divided into three overlapping phases: inflammatory phase, proliferative phase, and maturation phase.

Cardiomyocyte death triggers the activation of innate immune pathways which leads to the expression of cytokines, chemokines and adhesion molecules that initiates the inflammatory phase and leads to the infiltration of the infarct zone with leukocytes. The inflammatory phase is characterized by two major events. First, cardiac extracellular matrix faces extensive fragmentation. Generation of low molecular weight hyaluronan fragments in the infarct may exert pro-inflammatory actions and activate fibroblasts through CD44-mediated pathways. Second, a highly dynamic matrix is formed that creates a scaffold for infiltration, migration, and proliferation of the already mentioned leukocytes that facilitate the reparative process.
After the clearance of the infarct zone of dead cells the inflammatory leukocytes become apoptotic and the activation of inhibitory mediators suppresses the inflammatory reaction which leads to the proliferative phase. The activation of macrophages may stimulate trans differentiation of cardiac fibroblasts into myofibroblasts which are the key cells in scar formation. Furthermore, activation of angiogenic pathways results in formation of a microvascular network in the healing infarct that is necessary to provide oxygen and nutrients to the reparative cells.
Transition to the maturation phase follows with activation of anti-fibrotic signals (to prevent uncontrolled fibrosis) and the crosslinking of extracellular matrix proteins in the infarct zone. Most fibroblasts and vascular cells then become apoptotic. The mature scar, comprised of dense

cross-linked collagen, enhances tensile strength while increasing passive stiffness that contributes to diastolic dysfunction. In the infarct border zone and the remote remodelling myocardium fibroblasts and inflammatory cells may persist as pressure and volume loads may still provide stimulatory signals. **(5)**

1.4 Molecular pathways involved in cardiac fibrosis

1.4.1 Cytokines

Pro-inflammatory cytokines are potent regulators of collagen metabolism and affect fibroblast phenotype and gene expression. Specially IL- 1β and IL6 decrease collagen synthesis in isolated cardiac fibroblasts. IL- 1β is also increasing the concentration dependent cardiac fibroblast migration and has potent antiproliferative effects on cardiac fibroblasts.

As source of IL-1β pro-inflammatory monocyte subsets were identified, as well as the "inflammasome" and neutrophils. **(12)**

IL 6, on the other hand, can be produced by most tissues of the body as well as almost all cells of the immune system (specially neutrophils). It is involved in the regulation of acute-phase response and inflammation. **(13)**

Furthermore, there are the anti-inflammatory and pro-fibrotic cytokines named IL-10 and TGF-β 1. While IL-10 is secreted by M2-type macrophages TGF-β 1 is expressed by myofibroblasts, vascular smooth muscle cells, endothelial cells and macrophages. It is inhibiting lymphpocyte proliferation and modulates differentialtion and apoptosis of T cells. **(5,14)**

1.4.2 Chemokines

1.4.2.1 CCL2 and CXCL 2 and its role in Cardiac Fibrosis

Monocyte Chemoattractant Protein or MCP-1 (CCL2) is produced by a variety of cells including macrophages, fibroblasts, epi- and endothelial cells and is thought to play a pivotal role in pathophysiological processes in the remodelling after MI. CCL2 is increased in rat heart during reperfusion after MI but not after 1-2 hours of permanent infarction. It was also found in human cardiac myocytes and human cardiac fibroblasts under cytokine stimulation but not under hypoxia in vitro. This suggests that CCL2 is more associated with inflammation than with hypoxia/ischemia. It was identified in the ischemic myocardium after repetitive cycles of brief ischemia/reperfusion (IR) to be associated with increased collagen content, macrophages infiltration, interstitial remodelling, and LV dysfunction. CCL2's profibrotic actions are thought to be associated with decreased macrophage recruitment. Furthermore, CCL2 is capable of decreasing hypoxia induced cell death in cultured cardiac myocytes. CCL2 chemotactically attracts mononuclear cells. These cells are known to be a source of fibrogenic mediators like TGF-β and Fibroblast Growth Factor. Further it induces macrophage synthesis of TGF-β1 and collagen. It is also reported to directly modulate fibroblast activity and phenotype. The activation of CCL2/CCR2 pathway induces monocyte-mediated inflammation and several related processes. On the other hand, cardiac overexpression of CCL2 reduces infarct area and scar formation and therefore improving LV dysfunction and remodelling. **(15)**

1.4.3 TGF-β

TGF-β is perhaps the best characterized fibrogenic growth factor and it is found in three isoforms (TGF-β1,2,3) encoded by three distinct genes in mammalian. All three isoforms signal through the same cell surface receptors, but they exhibit distinct patterns of expression. The TGF-β 1 isoform is the predominant form in the cardiovascular system and is ubiquitously expressed in myofibroblasts, vascular smooth muscle cells, endothelial cells, and macrophages. In the normal heart it is found as a latent complex, unable to associate with its receptors. **(5)**

It is secreted initially bound to latent associated peptides in an inactive form that has to be activated by cell-cell interaction, acidification, and enzymatic cleavage. TGF-β1 acts primarily as an immunosuppressant, inhibiting lymphocyte proliferation in the presence of interleukin-2 as well as modulating differentiation and apoptosis of T cells. After an injury it is stimulating

the migration of neutrophils, monocytes, and fibroblasts to the injury zones. It subsequently enhances the expression of the extracellular matrix proteins from fibroblasts. **(14)**

After cardiac injury, the extracellular concentration of TGF-β is regulated primarily through conversion of the latent form to the active form, in which a relatively small amount of latent TGF-β is sufficient to induce a maximal cellular response such as trans differentiation of cardiac fibroblasts into myofibroblasts. Once the active form of TGF-β binds to the constitutively active type II receptor ***(TβRII)*** on the cell surface the resulting complex trans phosphorylates the cytoplasmatic domain of the type I receptor (TβRI). Subsequently TGF-β signals through downstream intracellular signals like the SMAD-pathway or SMAD-independent pathway. In fibrotic remodelling of MI Smad3 signalling plays an essential role. **(5)**

1.4.3.1 The TGF-β1- SMAD pathway

TGF-β1 binds to a dimerized complex comprised of TGF-β1 receptor 1 and 2. This is followed by a phosphorylation of SMAD proteins, which are conserved transcriptional proteins that act as second messengers for TGF-β1. The phosphorylated heteromeric complex regulates the expression of certain DNAs. SMAD proteins in general can be categorized into three groups: receptor-activated SMAD (SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8), co-mediator SMADs (SMAD4 and SMAD10) and inhibitory SMADs (SMAD6 and SMAD 7). In the heart the effects of TGF-β1 are primarily mediated through SMAD2 and SMAD3. Once phosphorylated SMAD 2 forms a complex with SMAD3 and 4 and trans locates into the nucleus where it binds to SMAD -binding oligonucleotides present in the regulatory region of specific genes such as collagen type I (COL1A1 and COL1A2 (encoding for different components of collagen type I)) and type III in the heart, followed by an alteration of gene expression levels.
Inhibitory SMADs such as SMAD6 and 7 inhibit the action of TGF-β1 by preventing SMAD2 phosphorylation (SMAD6) and disrupting SMAD complex formation (SMAD7). Inhibitory SMADs such as SMAD7are found to be downregulated post MI. **(14)**

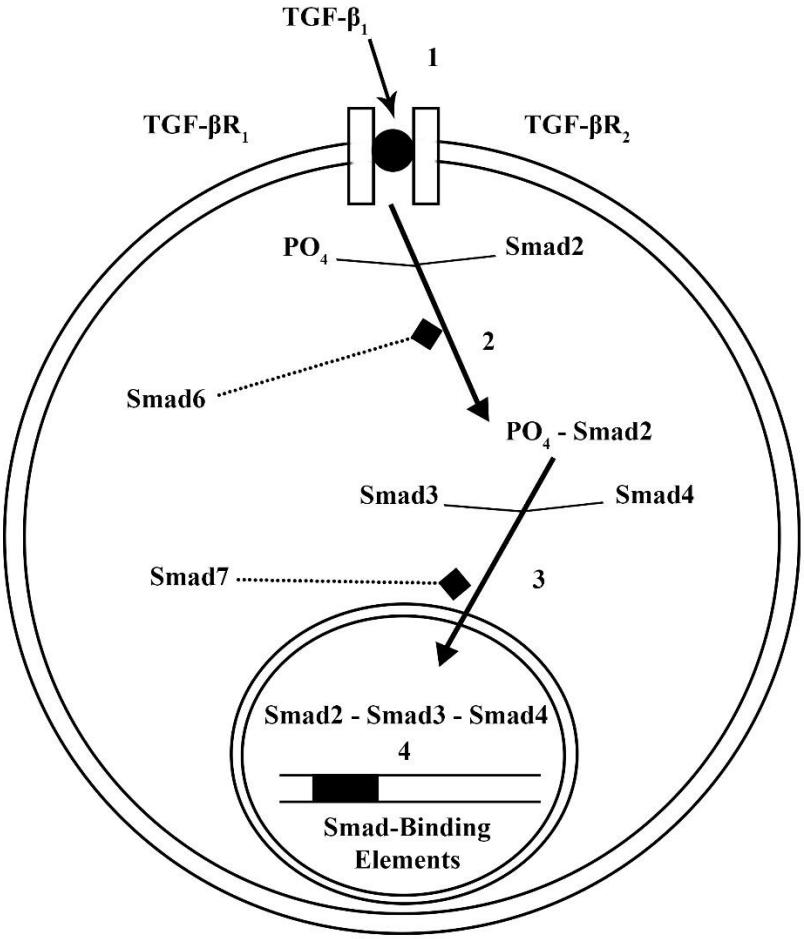


Figure 1: Signal Transduction of TGF-β1 in the SMAD pathway
(1) TGF-β1 binds to a dimerized complex of TGF-β1 receptor 1 and 2 followed by (2) a phosphorylation of SMAD 2. SMAD 2 then forms a complex (3) with Smad3 and 4 and trans locates (4) into the nucleus where it binds to SMAD-binding oligonucleotides present in the regulatory region of specific genes such as collagen type I (COL1A1 and COL1A2) and type III, followed by an alteration of gene expression levels.
Inhibitory SMADs preventing SMAD2 phosphorylation (SMAD 6) and disrupting SMAD complex formation (SMAD 7).

1.4.4 ECM1 and its role in myocardial infarction

Extracellular matrix protein 1 (ECM1) is a multifunctional protein that interacts with many other extracellular matrix proteins and enhances extracellular matrix (ECM) protein binding throughout its interaction. It also mediates a variety of processes in many tissues. It is therefore widely expressed with a molecular weight of 85kDa. ECM1 is a glycoprotein encoded by the ECM1 gene located at the chromosome 1q21 in humans. ECM1 is found to be upregulated in the aging and infarcted heart (but not in the pressure overload cardiomyopathy). The upregulation of the ECM1 mRNA and protein takes place early in the infarct zone whereas the border zone shows upregulation in ECM1 mRNA but not protein.

It has also been shown that ECM1 is not expressed by any resident cell type in the heart (primary human CFbs, human CFb cell line, human cardiac myocytes, human coronary artery endothelial

cells, human coronary artery smooth muscle cells and differentiated human coronary artery smooth muscle cells) under standard culturing conditions. However, this data is limited yet as ECM1 has not been studied in detail in the heart. Further investigations have shown that ECM1 is expressed in bone marrow cells, especially in the granulocyte cell fraction but also in the monocyte fraction. As ECM1 expression changes are resolved by day 28 post MI it is suggested that ECM1 plays an early inflammatory role in post MI fibrotic healing. **(16)**
In the liver tissues of mice, it was found that ECM1 stabilizes extracellular matrix-deposited TGF-β in its inactive from by interacting with integrins. There was also an inverse correlation to be found between the level of ECM1 and the severity of fibrosis in liver tissues of patients and mice with CCL4 induced liver fibrosis. **(17)**

Furthermore, ECM1 was found to play an important role in the structural and functional biology of the skin. Therefore, ECM1a utilizes different regions to bind to a variety of ECM components. ECM1 serves as a basement membrane protein in human skin and is part of the network-like suprastructures containing perlecan, collagen type IV and laminin 332 as constituents. It enhances the binding of collagen IV to laminin 332 and is therefore involved in the dermal-epidermal junction and interstitial dermis. ECM1 plays a pivotal role in the extracellular matrix formation, cell adhesion, cell signalling and regulation of tissue differentiation and/or maturation. **(18)**

Besides that ECM1 has an important role in promoting follicular helper T cell differentiation and antibody production where it is a positive regulator in T-follicular helper (T_{FH}) differentiation by repressing IL2-STAT5-Bcl6 signaling pathway. ECM1 is an effective enhancer of T_{FH} differentiation, germinal center response as well as in the neutralizing antibody production. It was found that secreted ECM1 is critical for T_{FH} differentiation and antibody response by showing that a lack of ECM1 inhibits T_{FH} cell development and impairs GC B-cell reactions and antigen-specific antibody production. **(19)**

1.4.5 TRAF 2 and its cardioprotective role

TNF receptor associated factor 2 (TRAF2) is a critically regulator of RIP1-RIP3-MLKL necroptotic signalling. Necroptosis (programmed necrosis) is induced by the activation of RIP1 and RIP3 (receptor-interacting protein 1 and 3) which forms the necroptotic cell death complex called the necrosome. This necrosome then engages MLKL through RIP3-dependent phosphorylation. MLKL once phosphorylated oligomerizes and hinds to phospholipids to disrupt membrane integrity causing necroptotic cell death.

Traf2 plays an important role in signal transduction from receptors of the TNFR superfamily and the interleukin-1/Toll-like receptor superfamily, the regulation of cell proliferation, inflammation, immune response, survival, and apoptosis. It also mediates mitochondrial autophagy, which gives protection against hypoxia/reoxygenation injury in cardiomyocytes. Cardiac restricted expression of low levels of Traf2 in mice were shown to protect against ischemia reperfusion injury.

Traf2 expression is also markedly increased in mouse hearts after MI injury for 2 weeks, suggesting a potential role of Traf2 in myocardial response to pathological stress.

Furthermore, histological analysis of mice hearts with a cardiomyocyte specific deletion of Traf2 could show high levels of myocardial fibrosis and cardiomyocyte dropout. **(20)**

1.4.6 WNT5a and heart failure

In heart failure (HF) WNT signalling is dysregulated and may promote cardiac hypertrophy, fibrosis and inflammation. Blocking WNT5a in animal models had a preventing effect on HF. In failing human hearts, a WNT5a protein correlation with interleukin (IL)-6 and tissue inhibitor of metalloproteinases (TIMP)-1 could be found. Furthermore, it could be shown that in primary mouse and human cardiac fibroblasts a recombinant WNT5a dose dependent upregulation of mRNA and protein expression of IL6 and TIMP-1 was present. WNT5a induction of IL-6 and TIMP-1 in cardiac fibroblasts might promote myocardial inflammation and fibrosis and thereby contribute to HF progression. **(21)**

2. Material and Methods

2.1 Human cardiac fibroblast cell culture

The cell culture of commercially sourced human cardiac fibroblast (HuCFb) cells (Sigma Aldrich, C-12375) were performed with recommended reagents as per the manufacturer's protocols. Therefore, the cells were quickly thaw in a 37°C water bath and resuspended. Afterwards the suspension (1ml) was put into a T75 flask (Sigma Aldrich, SIAL0641) containing 15ml of Cardiac Fibroblast Growth Medium (Sigma Aldrich, 316-500). The flask was then given into a humidified incubator at 37°C with 5% CO_2 for 24h. After that the medium was changed to fresh medium every day unit the cells reached 60% confluency. At 60% confluency the medium got doubled until the culture reached 80% confluency and was ready to use. HuCFbs were serum starved for 16 hours/overnight in DMEM/F12 Glutamax (Thermo Fisher, 10565018) supplemented with 0.5% FBS (Thermo Fisher, A4766801), then treated with recombinant ECM1 (RNDSYSTEMS, 3937-EC-050) (20ng/ml) for 3, 6 or 16 (qPCR) hours and 5, 10 or 30min (Western blot) to investigate pathway activation. For the Western blots were further 10ng/ml recombinant TGF-β1 (Sigma Aldrich, H8541) used. Protein and mRNA were extracted from cells for use in Western Blot and qPCR.

2.2 RNA extraction and reverse transcription for qPCR of ECM1 treated human cardiac fibroblasts.

Protocol for the purification of total RNA, including small RNAs, from animal tissue – miRNeasy Mini Handbook 03/2013 was used.

Procedure:

Human cardiac fibroblasts cells were disrupted and homogenized in QIAzol Lysis Reagent. After 5 min of rest to allow dissociation of nucleoprotein complexes, chloroform was added to the tube with the homogenate and was vortexed for about 15 s. After another rest of 2-3 min the tubes were centrifuged for 15 min at 1200x g at 4°C. After centrifugation, the samples separated into 3 phases. The top phase containing the RNA was transferred to a new collection tube and 1.5 vol of 100% ethanol was added. The total volume was then transferred into a RNeasy Mini spin column in a 2ml collection tube and was centrifuged at about 8000xg

(10000rpm) for 15s at room temperature and the flow through was discard. 700 µl of RWT Buffer was then add to the RNeasy Mini spin column and again centrifuged at 8000x g for 15s to wash the column. The flowthrough was discarded again. Then 500 µl of RPE Buffer was add to the RNeasy Mini spin column with the same centrifugation step followed. Flowthrough was discarded again. After adding another 500 µl RPE Buffer to the column a centrifugation step of 2mins at 8000x g followed to dry the column membrane. The column was then transferred to a new 1.5 ml collection tube and about 50 µl RNase-free water was add on the column's membrane. With a centrifugation step of 1 min at 8000x g the RNA was then eluted. The concentration of the RNA was finally measured by nanodrop.

2.2.1 Reverse Transcription

For the reveres transcription of our freshly isolated RNA the Qiagen Reverse Transcriptase Kit (Cat No./ID: 205311) was used.

After the evaluation of the concentration of the freshly isolated RNA the RNA was diluted to 300 ng/ µl. For the reverse transcription gDNA has to be eliminated, therefore a gDNA wipe out buffer 7x , the template RNA (1µg) and RNAse free water were mixed together according to the manufacturer script and incubated for 2 min at 42 °C to get rid of the gDNA. After that Quantiscript RT, Quantiscript RT buffer and RT primer mix were mixed and used as our RT Master Mix. 6 µl of the Master Mix and 14 µl of template RNA were put together and loaded on the thermocycler for a 15 min incubation at 42°C followed by a 3 min incubation at 95°C. The freshly synthesised cDNA was then stored at -20°C until it was used for the qPCR.

For NTC gDNA wipe out buffer and RNAse free water were put together without the template RNA. RT minus control was generated through adding 1 µl of RNAse free water instead of Quantiscript RT.

2.2.2 qPCR Protocol

For the qPCR I have used 0,48 µl of DNAse free Water, 5 µl of SYBR GREEN Super Mix (Biorad #172-5274) and 0,26 µl of Forward - and Reverse Primer (Tab.1) each. In the End cDNA was added to the master mix with a volume of 4 µl. The total Volume was 10 µl (6 µl Master Mix, 4 µl cDNA) per well. The plate was then centrifuged for 1 minute at 900 RPM before it was given into the Biorad CFX 96 Lightcycler (Runningprotocol Fig.2).

After an initial denaturation at 95 °C for 30 sec 43 cycles were run with a denaturation at 95 °C for 15 sec and an annealing with 62 °C for 30 sec. Extension and fluorescence read were performed at 72 °C for 30 sec. In the end the plate was hold at 4°C.

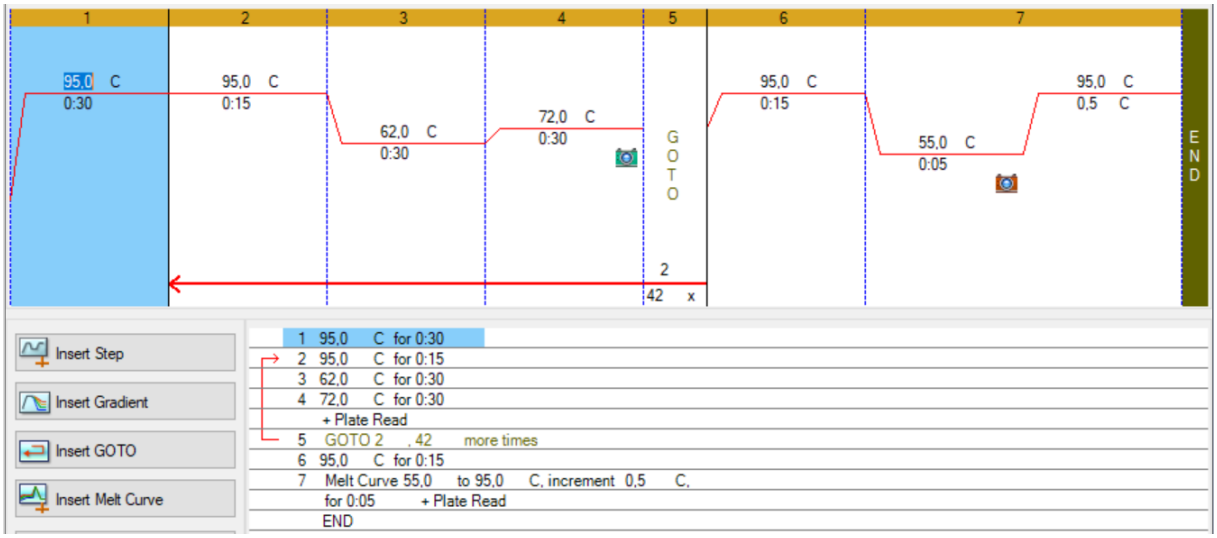


Figure2: qPCR Amplification cycles. For the qPCR an initial denaturation phase of 30 seconds at 95°C was chosen. After the initiation 43 cycles were run with a denaturation phase of 15 sec at 95°C, an annealing phase of 30 sec at 62°C and an extension phase of 30 sec at 72°. In the end the plate was hold at 4°C.

Tab1. Primers used for qPCR(Company: Microsynth)

h-TRAF2 rev2:	5’ GGA GAA GAT GGC GGG TAT G
h-TRAF2 fwd2:	5’ GCT GAC TTG GAG CAG AAG GT
h-IL1 β rev2:	5’ GGC AGA CTC AAA TTC CAG CTT
h-IL1 β fwd 2:	5’ GAT AAG CCC ACT CTA CAG CTG
h-TGF β 1 rev1:	5’ ATT TCC CCT CCA CGG CTC AA
h-TGF β 1 fwd1:	5’ ATT CCT GGC GAT ACC TCA GC
h-TPT1 rev:	5’ CGC AGG GAT TTC TTT CTT TTG
h-TPT1 fw:	5’ GGC ATG GTT GCT CTA TTG GA
h-TGF β 2 rev1:	5’ TGA GCC AGA GGG TGT TGT
h-TGF β 2 fwd1:	5’ GAG AGG AGC GAC GAA GAG TA
h-WNT5a fw2:	5’ CTT TGG GGA TGG CTG GAA G
h-WNT5a rev2:	5’ GGG TTA TTC ATA CCT AGC GAC
h-ACTA2 fwd1:	5’ AGC GTG GCT ATT CCT TCG TT
h-ACTA2 rev1:	5’ CCC ATC AGG CAA CTC GTA ACT
h-CXCL2 fwd2:	5’ CAC CCC AAG AAC ATC CAA AG
h-CXCL2 rev2:	5’ CTT AAC TAT GGG GGA TGC AGG
h-IL6 rev1:	5’ GTG AGT GGC TGT CTG TGT GG
h-IL6 fwd1:	5’ TTC GGT CCA GTT GCC TTC TC
h-COL1A2 fwd1:	5’ TCA GAA CAT CAC CTA CCA CTG
h-COL1A2 rev1:	5’ CCC CAT TCA TTT GTC TTT TTA GAG C
h-CCL2 fwd1:	5’ AGC AGC AAG TGT CCC AAA GA
h-CCL2 rev1:	5’ GTG GAG TGA GTG TTC AAG TC

2.3 Western Blots of human heart samples and human cardiac fibroblast samples.

2.3.1 Tissue homogenisation of human heart samples for ECM1 Western Blot

Human heart tissue samples with a weight of about 80 mg were homogenised in a RIPA Buffer with the Fisherbrand™ Pellet Pestle™ Cordless Motor**.** After homogenisation, the samples were centrifuged at 4°C for 15 min at 18 000 rcf. The supernatants were then kept as cytosolic enriched fraction (pellet = myofilament enriched fraction).

Table 1: RIPA Buffer Recipe for 45 mL adjusted at pH 8

Stock Concentration	Stock Volume (10ml)	Final Concentration	Volume		Chemical
-	-	150 mM	438	mg	NaCl (Roth,3957.1)
10%	1 g	0.5%	2,5	ml	Na Deoxycholate (Millipore,1.06504)
10%	1 g	0.1%	500	µl	SDS (Sigma-Aldrich, 436143)
-	-	1%	500	µl	Triton X-100 (Thermo Scientific, 85111)
-	-	50 mM	394	mg	Tris-HCl (Roth, 9090.3)
-	-	-	41,5	ml	MilliQ H2O
-	-	-	1	Tablette/10ml	Phosphostop (Roche, 04906845001)
-	-	-	1	Tablette/10ml	Complete (Roche, 11873580001)

2.3.2 Determination of protein concentration

Pierce™ BCA Protein Assay Kit Catalog Numbers 23225 and 23227 thermoscientific.

The determination of the protein concentration was made by using Pierce™ BCA Protein Assay Kit (Catalog Numbers 23225 and 23227 by Thermoscientific) according to manufacturer's manual.
Therefore, I have prepared the working reagent by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. Then the cytosolic fractions of the human heart samples were diluted 1:5 and were pipetted into a microplate (25 µl) (Thermo Scientific™ Pierce™ 96–Well Plates, Product No. 15041). Also, the manufacturers BSA (Bovine Serum Albumin) Standard was add to the plate (25 µl). Then the working reagent was added to each well (200 µl) and the plate was shaken softly for about 30s. After an incubation time of 30 min at RT the plate was imaged at 545 nm.

2.3.3 Western Blot

After protein concentration was determined 20 µg of the samples were mixed with 6,25 µl of sample buffer (4x Biorad 161-0791), 1,25 µl XT reducing agent (20x Biorad 161-0792) and filled up with ddH20 to a final volume of 25 µl per sample. After boiling for 5 min at 95°C the samples were loaded on a 4-12 % Bis Tris Gradient Gel (Biorad, # 345-0124). As running Buffer, I used XT Mops 20x (Biorad 161-0788), furthermore I have used Precision Plus Protein All Blue Standards (Biorad 161-0373) as a standard. The gel was then running for 2,5 h at 120 V in cold room. After 2,5 h I started the transfer of the proteins from the gel onto a nitrocellulose membrane (Amersham TM Protran TM 0,45 µm NC, GE healthcare life science, Lot Nr 25679266) for 2 h at 400 mA in cold room again. When the transfer was done the nitro-cellulose membrane was blocked with a 5% Milk solution in TBST for 1 h at RT, followed by 2 washing steps à 10 min in TBST at RT. The primary AB of ECM1 (1:000; Abcam 126629, EPR6701) was then loaded in a 0,5 % Milk solution in TBST for incubation over night at 4°C, just like GAPDH (D16H11 rabbit mab 1:1000).
The same western blot was re-incubated with sigma prestige HPA027241 ECM1 antibody 1:497 in 5% BSA (Bovine Serum Albumin) in TBST to reach the same concentration as used with the Abcam antibody to compare the results. Whereas the psmad2 (1:500 cell signalling

18338S) or total SMAD 2/3 (1:1000 cell signalling 3102S) were loaded in 5% BSA in TBST at 4°C over night

2.4 Immuno histo chemistry of mouse heart, liver and oesophagus

For immune histo-chemistry hearts and livers of mice were used that either got a ligation of a coronary artery to induce a myocardial infarction or have had a so-called SHAM where the whole operative procedure was performed without ligating a coronary artery. Then after three days the mice were sacrificed and the heart and liver were taken out to be first fixed in 4% paraformaldehyde for 24 h and then embed in paraffin or go directly into Sakura OCT cryo tissue embedding compound (Tissue-Tek).

2.4.1 Paraffin fixation

For the paraffin embedded samples an antigen retrieval was necessary. Therefore, after tissue slices were made and put on a microscopy slide, the samples were put into an incubator for 15 mins at 60°C. After that, the samples were put in 100% Xylene 3x5min, 100% EtOH, 95% EtOH, 70% EtOH and ddH20 each for 5 mins. This was to deparaffinise and rehydrate the specimen. As the rehydration was performed the samples were boiled in 1mM NaEDTA at pH 8 in the microwave at 900W for 20 min. Out of the microwave, the samples were put into ddH2O for a quick washing step before I put them into PBS for 5 min. The following steps were then performed in a humid chamber. For permeabilization the samples were covered in 0,2% Triton X in PBS for 30 min followed by a blocking step with 1% BSA, 10% Goat Serum (Abcam) and 0,2 % Triton X in PBS for 60 min. As soon as the blocking step was done the samples were then washed with PBS for 5 min. To safe some antibody and to better address the area of interest on the microscopy slide the specimen was circled with a Super Pap Pen (Invitrogen # 00-8899). Primary antibody (HPA027241 – Sigma, Prestige Antibody ECM1 (host rabbit, specificity human)) as well as Igg control (Rabbit IgG isotype control (Invitrogen lot: UA276761)) were then thaw on ice and a dilution of 0,0005 mg/ml in blocking buffer was made. The specimen was then incubated with the Primary antibody or Igg control over night at 4°C. On the next day I washed the microscopy slides 3 times á 5 min in PBS with 0,2% Triton X. Afterwards, the secondary antibody solution was made with a dilution of 1:1000 in PBS with

0,2 % Triton X. For Secondary Anitbody Anti-rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor® 488 Conjugate) #4412S was used. The incubation was then carried out in the closed humid chamber under exclusion of light at room temperature for an hour. After the incubation, the slides were washed once in PBS with 0,2% Triton – X for 5 min, which was followed by another incubation step with a WGA (Wheat Germ Agglutinin, Alexa Fluor™ 555 Conjugate Catalog number: W32464) solution that was made from a stock solution of 1 mg/ml in PBS. Therefore, I have taken 10 ul of stock solution and 990 ul of PBS per slide. After an incubation time of 15 min under the exclusion of light I washed the samples 3 times à 5 min in PBS and covered them with DAPI to mount the coverslip on the microscopy slide. Finally, I let them dry overnight under the exclusion of light at 4°C and sealed the microscopy slides the next day with clear nail polish. Imaging took place right afterwards to avoid fading of the fluorophores. The imaging was made with a basic fluorescence microscope and a CLSM.

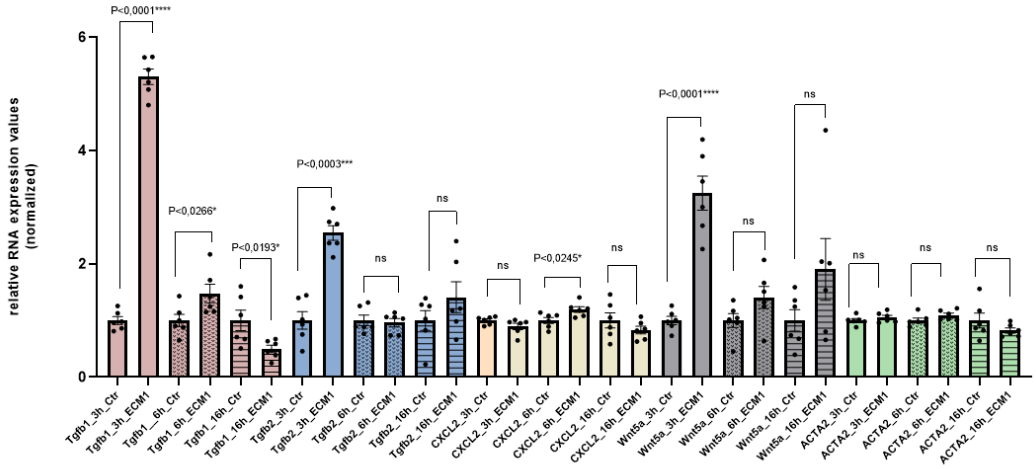
2.4.2 Cryo-fixation

The cryo sections were cut in slices of 2-3 µm and were air dried for about 45 min until they were completely dry. The specimens were fixed in 4% PFA for 5 min and washed 3 times à 5 min in PBS. After the washing steps I put the samples right into the humid chamber and started the permeabilization steps just like I did for the paraffin fixed samples. From there on the protocol follows the steps in the “paraffin fixed samples” protocol.

3. Results

3.1 qPCR of ECM1 treated human cardiac fibroblasts

To check which inflammatory and fibrotic pathways got turned on after stimulating human cardiac fibroblasts with ECM1 in cell culture for 3h,6h and 16h I have performed a qRT-PCR on several genes. As human cardiac fibroblasts turn into myofibroblasts in cell culture quick, I was not able to see any changes for ACTA2 at any given time point as the levels were high from the beginning. ACTA2 serves as an indicator of the trans differentiation of human cardiac fibroblasts into myofibroblasts as it is phenotypic for myofibroblasts. As ECM1 is known to play a role in early inflammatory processes post MI I was able to see a significant increase in IL1 β and IL6 after 3h and 6h of treatment. For IL6 I have seen an attenuation at the 16h timepoint compared to the control (untreated cells). Since TRAF2 plays a role in signal transduction of the IL1 receptor family I was also able to see an increase of TRAF2 for 3h and 6h after treatment. As WNT5a is positively correlated with IL6 I was further able to see a significant increase in WNT5a for the 3h timepoint. For TGF β 1 and 2 I have seen a significant increase for the 3h timepoint, whereas TGF β 1 stays up increased for the 6h timepoint as well, compared to the control group. For the 16h timepoint I found a significant decrease for TGF β 1 only. These findings are mirrored when talking about COL1a. Here I found a significant decrease for 3h and 6h whereas, for the 16h timepoint, an increase can be seen. This might be due to the delayed signal transduction through TGF β going over the SMAD pathway to activate COL1a. Stimulated by ECM1, CCL2 showed a decrease for the 3h and 6h timepoint, here ECM1 could play a role in a negative feedback loop as CCL2 attracts mononuclear cells known as source of fibrogenic mediators like TGF β. For CXCL2 on the other hand an increase for the 6h timepoint was found.



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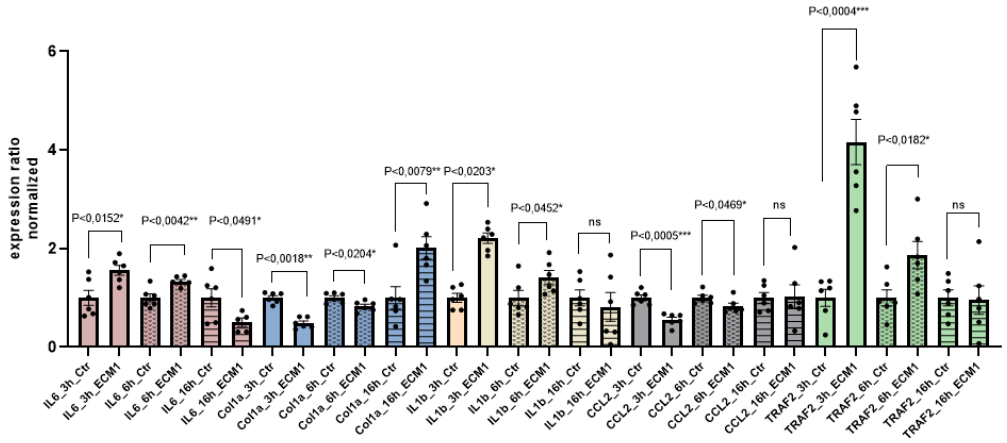

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Figure 2: qRT-PCR of ECM1 treated and untreated human cardiac fibroblasts: Cell culture of commercially sourced human cardiac fibroblast (HuCFb) cells (Sigma Aldrich) were performed with recommended reagents as per the manufacturer's protocols. HuCFbs were serum starved for 24 h, then treated with recombinant ECM1 (20ng/ml) for 3, 6 or 16 hours. RT-qPCR was performed to examine expression levels of A: TGF-β 1, TGF-β 2, CXCL2, Wnt5a, ACTA2 and B: IL6, Col1a, IL1 β , CCL2, TRAF2. Statistical significance: *P ≤ 0.05, **P < 0.01, ***P < 0.001.

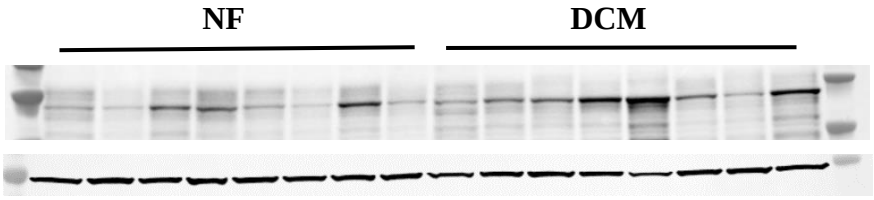
3.2.1 Western Blot of ECM1 in non-failing, dilative and ischemic human heart samples.

For the western blot of the non-failing human heart samples and the dilative human heart samples normalised with GAPDH I received a non-significant increase of the dilative group compared to the non-failing hearts which will further be called the control group. The significance was determined with the students t.test (p= 0.07) as well as with the Mann-Whitney test (p= 0.065). For the western blot of the ischemic hearts and the control group normalised with GAPDH I also got a non-significant increase which was however not as prominent as in the western blot of the dilative group. Also, for the second blot students t. test (p=0.36) and the Mann-Whitney test (p=0.3282) were performed.

The same membranes were then stripped and re-incubated with Sigma Prestige anti ECM1 antibody (HPA027241) to double check the specificity of the antibodies and make the results more comparable with the immune histo chemistry staining.

For the Sigma Prestige anti ECM1 antibody I saw a non-significant decrease in ecm1 for the dilative hearts for the 75kDa band but an increase for the 150 kDa band, also non-significant,

which is supposed to be the dimeric form of ECM1. Also, the ischemic heart samples showed the same results with a non-significant decrease for the 75kDa band compared to the control group and a non-significant increase for the 150 kDa band compared to the non-failing hearts.



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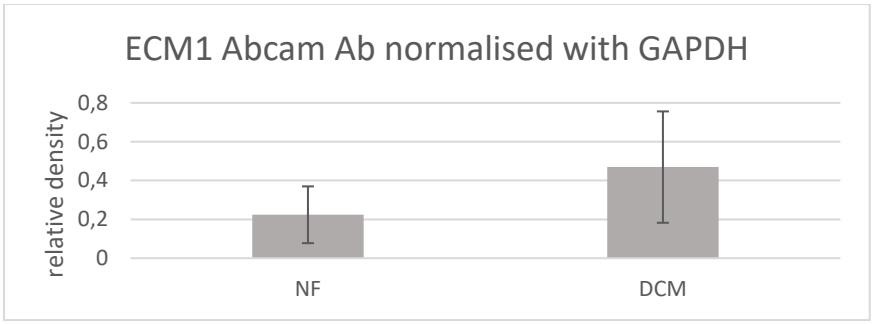

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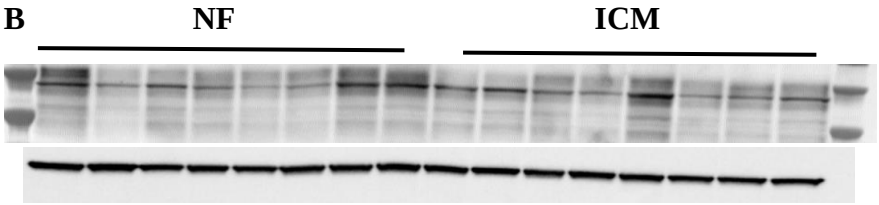

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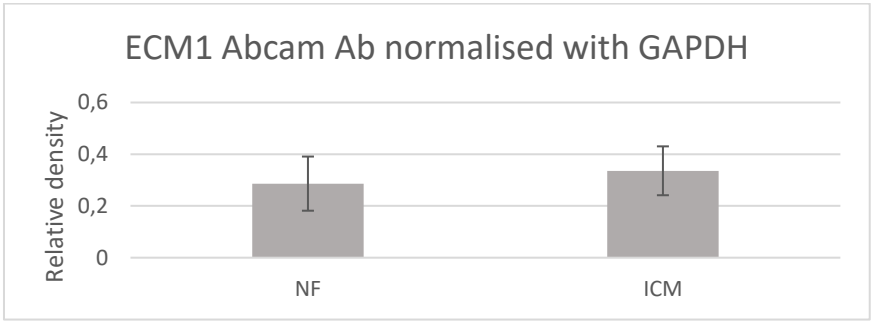
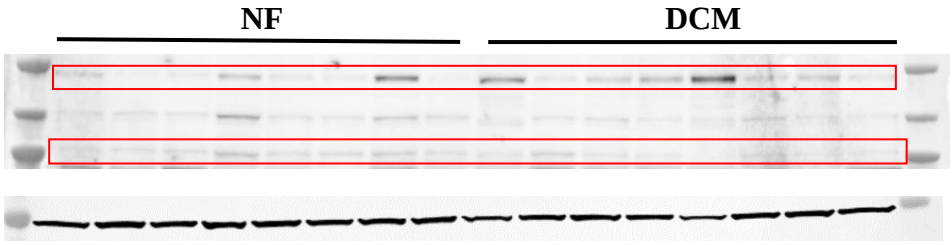
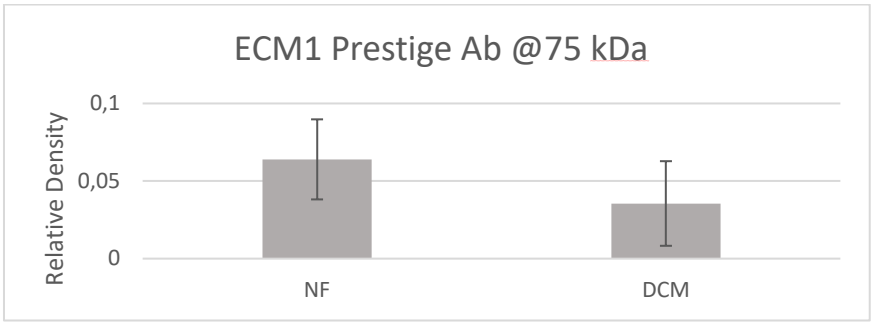


D

Figure 3: Detection and Quantification of Western Blot with patients' heart samples of non-failing hearts, dischemic and ischemic hearts (Abcam Ab). After western blotting, the membrane was incubated with ECM1 antibody (Abcam). A Detected signals of western blot membrane by chemiluminescence of NF and DCM hearts. B Quantification of measured intensities of Western Blot of the 61 kDa band normalised with Gapdh. C Quantification of measured intensities of western blot of NF and ICM hearts. D Quantification of measured intensities of western blot of the 61 kDa band normalised with Gapdh.


NF
DCM

A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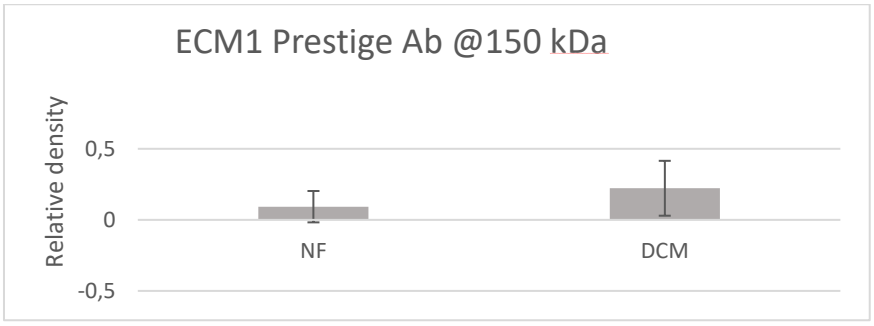
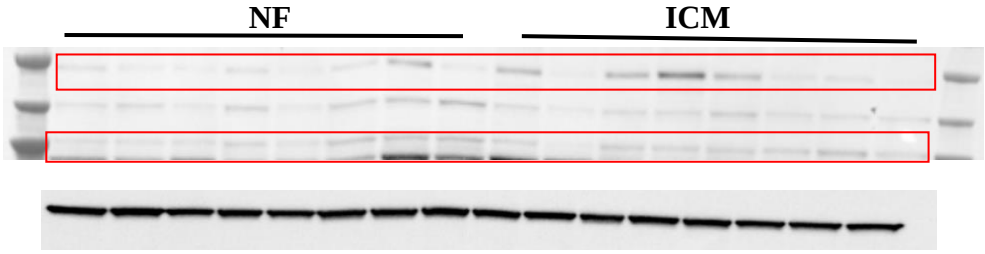
Relative Density
0,1
0,05
0
NF
DCM

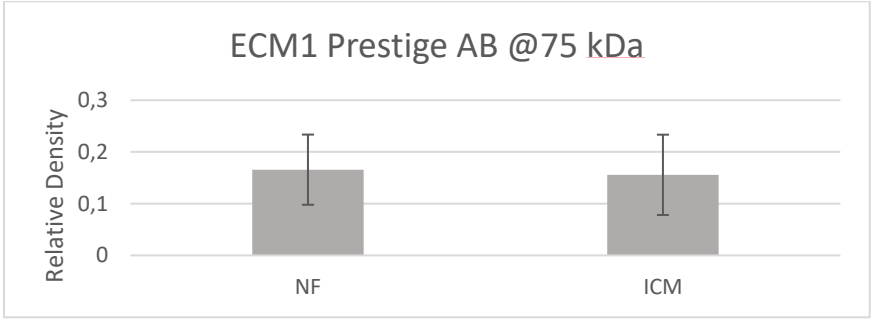
B


ECM1 Prestige Ab @150 kDa
Relative density
0,5
0
-0,5
NF
DCM

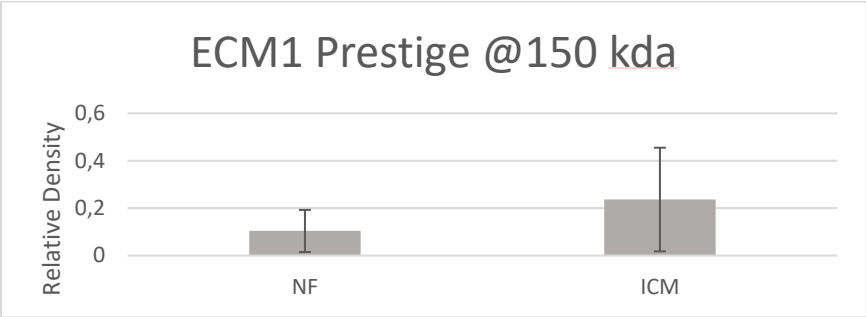
C


NF
ICM

D


ECM1 Prestige AB @75 kDa
Relative Density
0,3
0,2
0,1
0
NF
ICM

E



F

Figure 4: Detection and Quantification of Western Blot with patients' heart samples of non-failing hearts, dischemic and ischemic hearts (Sigma Prestige Ab). After western blotting, the membranes of Figure3 were stripped and re-incubated with ECM1 antibody (HPA027241). A Detected signals of western blot membrane by chemiluminescence of NF and DCM hearts. B Quantification of measured intensities of Western Blot of the 75 kDa band. C Quantification of measured intensities of western blot of the 150 kDa band. D Detected signals of western blot membrane by chemiluminescence of NF and ICM hearts. E Quantification of measured intensities of western blot of the 75 kDa band. F Quantification of measured intensities of western blot of the 150 kDa band.

3.2.2 Western Blot of p-SMAD2 and total SMAD in human cardiac fibroblasts treated with ECM1, TGF β and double-treatment

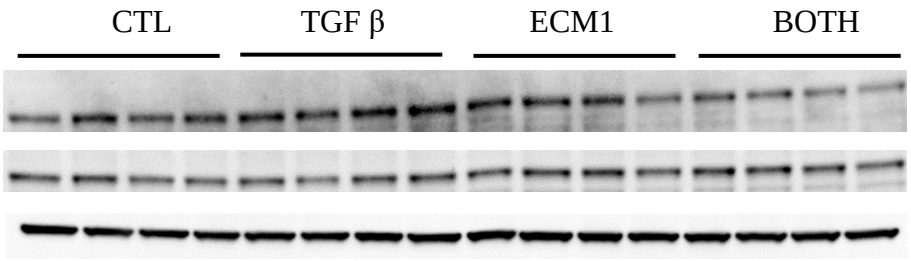
The blots were performed on cells that were treated with ECM1, TGF β and both as a double-treatment and harvested at 3 different timepoints. After cell harvest and purification, I have performed western blots with the three different treatments and a non-treated control group. Therefore, I have shown that for the 5 minutes timepoint the p-smad2 concentration significantly decreased for the double treatment group (TGF β = 10ng/ml and ECM1= 20ng/ml) compared to the other groups. The psmad2 was normalised against total SMAD. Furthermore, I have also incubated the blot with GAPDH and normalised with it, which showed the same results. Here psmad2 was also in the ECM1 treated group significantly decreased compared to the TGF β group.

For the 10 minutes timepoint I was able to show that p- SMAD normalised against total SMAD increased for the TGF β treated group compared to the control group and also the double treated group showed an increase in p- SMAD compared to the control.

If normalised with GAPDH the results change massively and did not show any significance at all.

For the 30 minutes treated samples I then received similar results like in the 5 minutes treated group when normalised with total SMAD. The double treated samples showed a strong and

significant decrease in p- SMAD 2 compared to the control and to the other groups. The ecm1 treated group showed also a strong and significant decrease in psmad2 compared to the TGF β treated group and the TGF β treated group has shown an increase compared to the control.
For the calculation normalised with GAPDH I have also seen a strong decrease in psmad2 for the double treated group compared to all the other groups. I have also seen a significant decrease of psmad2 in the ecm1 treated group when compared to the TGF β treated group.
To have an eye on the p- SMAD values of the different TGF β treated groups (5,10 and 30 min) I have done another western blot where I compared the three groups with the control group. Here I have seen a significant increase of p- SMAD 2 for the 30-minute timepoint compared to the 5 and 10 minute timepoint when normalised with total SMAD. If normalised with GAPDH the 30 minutes timepoint shows a significant increase over all the other groups including control. The statistical test performed was the students t.test (significance ≥0,04).



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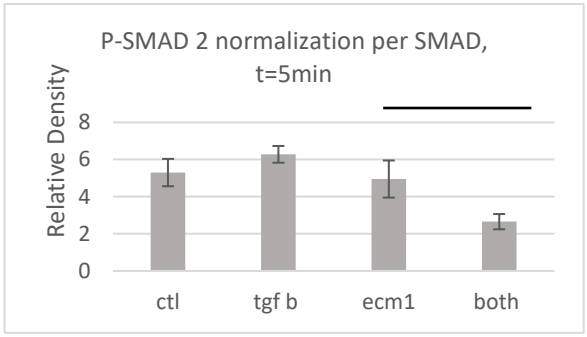
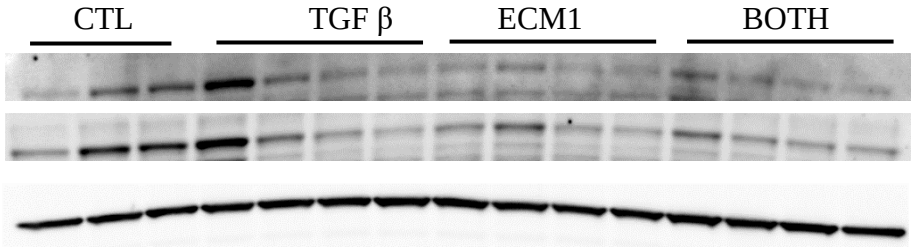


B



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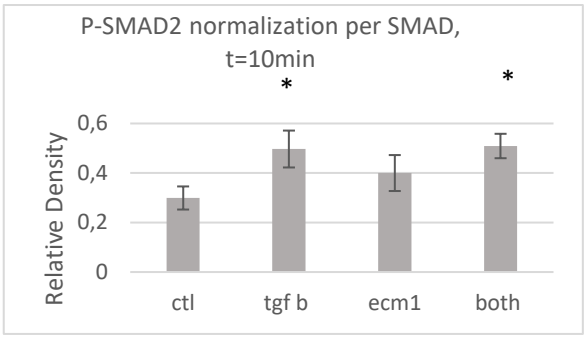
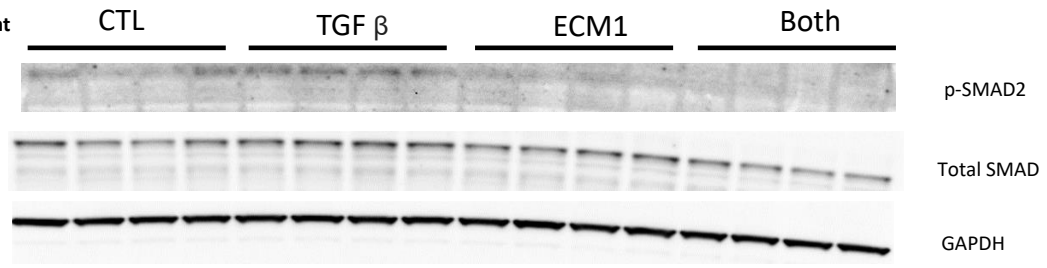


D

30 Minute time Point



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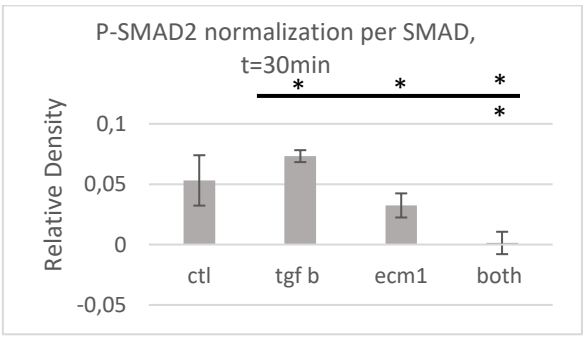

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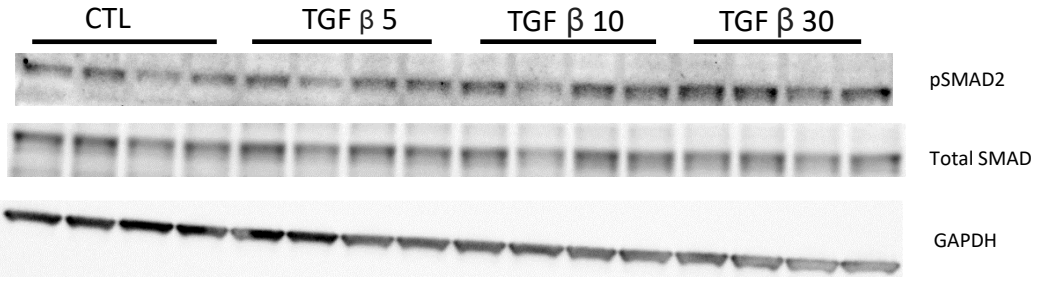

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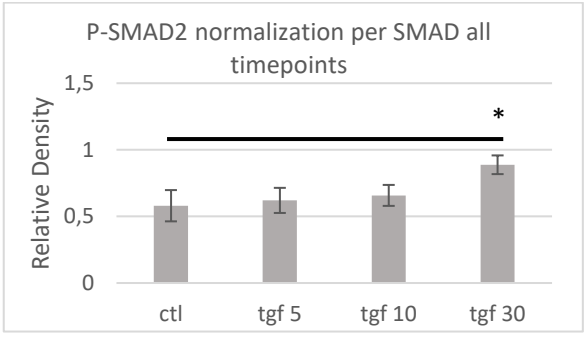

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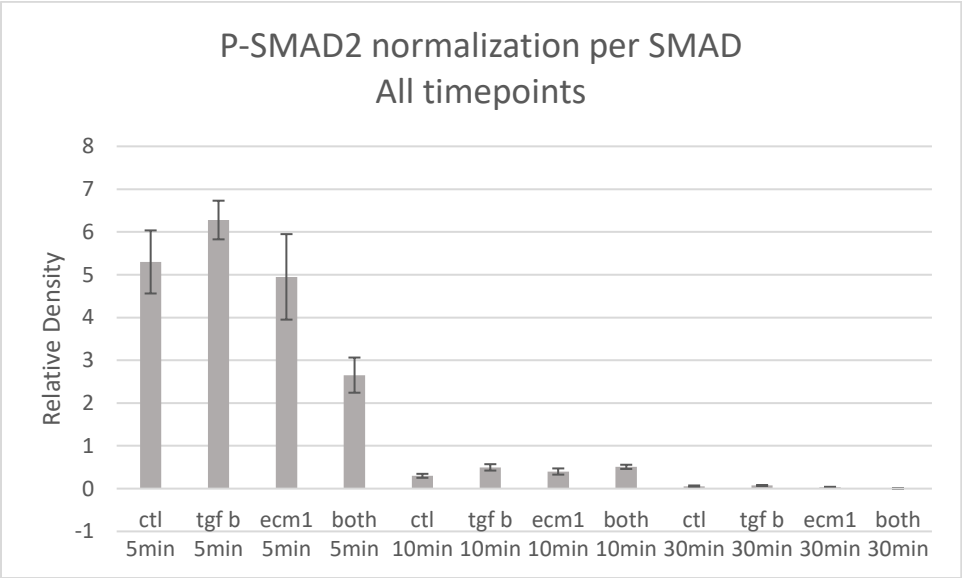

I

Figure 5 A-I: Quantification and detection of Western Blot with human cardiac fibroblasts, treated with either ECM1 (20ng/ml), TGF-β (10ng/ml) or both at 5, 10 and 30 minutes time point to investigate their effect on P-SMAD pathway. Also, a non-treated group was used as a control. After western blotting, membranes were incubated with psmad2, total SMAD and GAPDH antibodies. Loading of equal amounts of proteins was determined by GAPDH-antibody. Statistical significance: *P ≤ 0.04

3.3 Immuno histo chemistry

For the immuno histo chemistry of induced MI mice hearts I have sacrificed mice after 3 days post MI and took out their heart (liver and oesophagus as a control). After preparation I checked for ECM1 activity by anti ECM1 Sigma Prestige Antibody in heart, liver and oesophagus. Liver and oesophagus served as a positive control as it is known that ECM1 is expressed in those tissues under physiological conditions. As ECM1 is not expressed in any resident cardiac cells I have checked the MI hearts for ECM1 expression of invading bone marrow derived cells. Therefore, I have used Cryosections as well as paraffin sections, since the latter are able to preserve the tissue structure in a more accurate way. To make sure the signals I got are real I also doublechecked with a rabbit IgG isotype control that showed no significant signal in any of my images. For the cryosections I then became a strong signal for heart, oesophagus and liver sections. In the paraffin sections I was able to achieve more detailed images of the histology of the tissues when using the basic fluorescence microscope. Especially for the heart images (Figure 9) one can see, that there is ECM1 activity at the infarct zone as well as in the pericardium, the border zones. These pictures show the first overview before increasing the magnification and resolution by using confocal laser scanning microscopy (CLSM). By using CLSM (Figure 10) I was able to show, that the main ECM1 activity in the infarct zone takes

place in the intercellular space, were it fills out damaged areas to gain stability and reinforce those areas of the infarct.

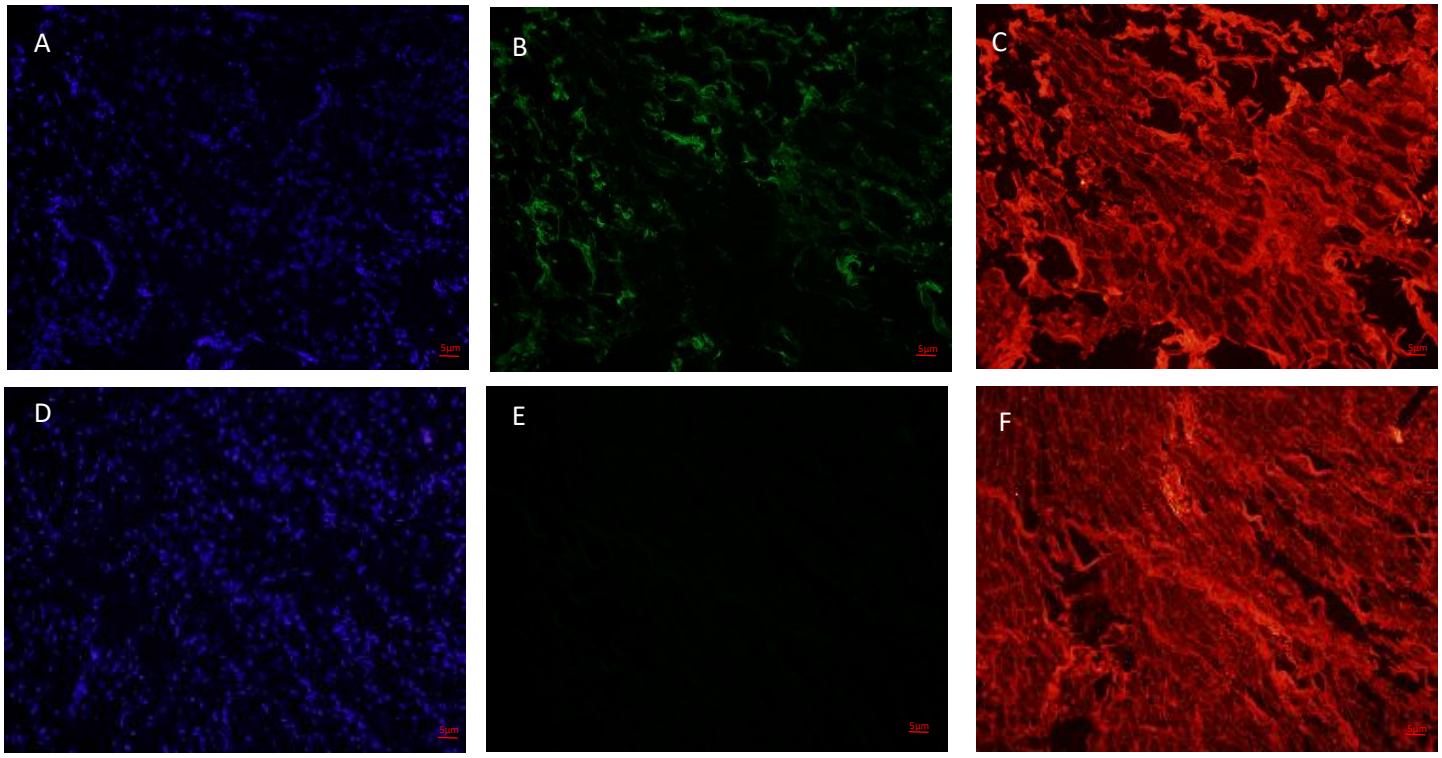

Figure 6: Immunohistochemistry staining of cryofixed mouse heart. A-C: A Dapi, B ECM1 Antibody immune fluorescence staining C WGA staining - of mouse heart tissue. D-F: Negative Control with D Dapi, E Rabit Isotype IgG and F WGA. All images were taken with a magnification of 20x and 15ms excitation.

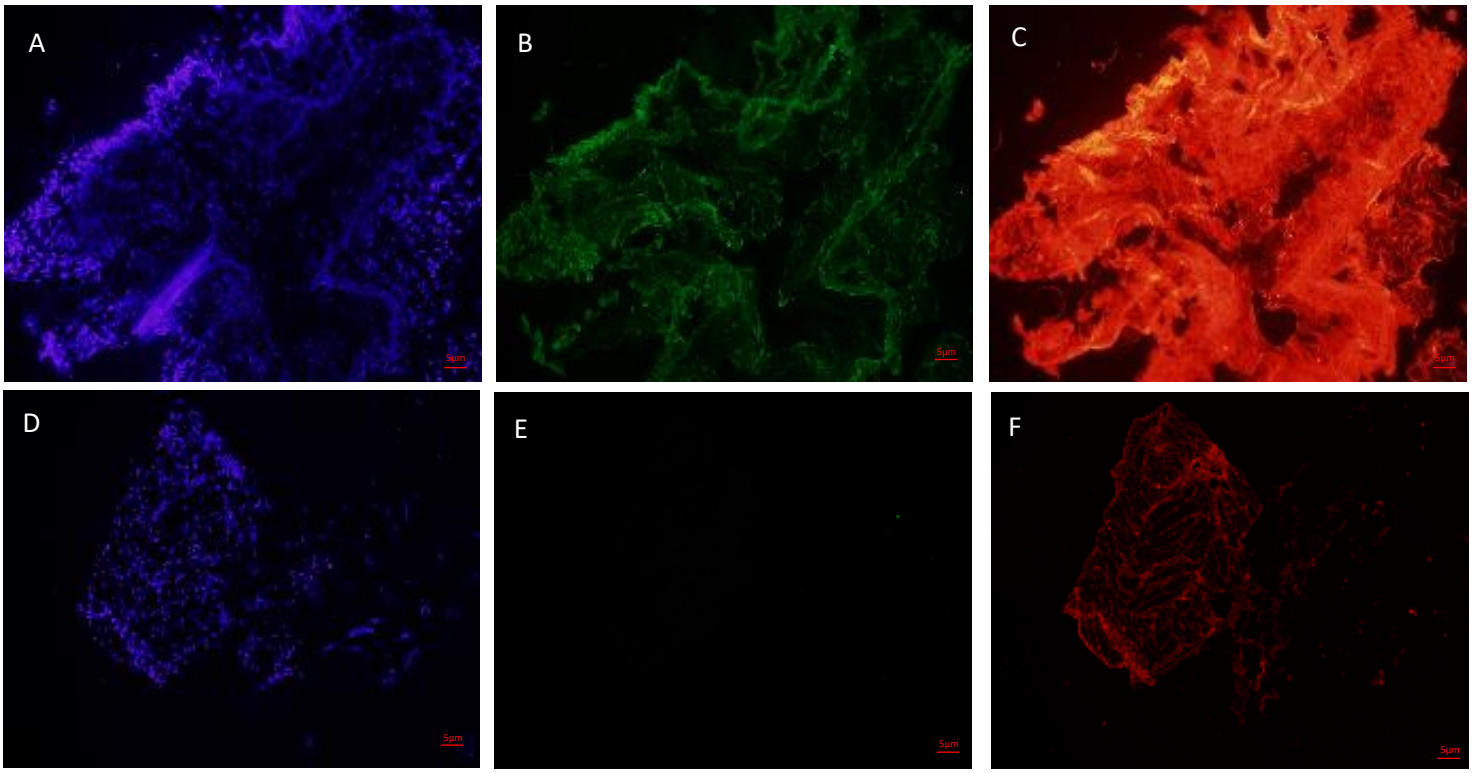

Figure 7: Immunohistochemistry staining of cryofixed mouse Esophagus. A-C: A Dapi, B ECM1 Antibody immune fluorescence staining C WGA staining - of mouse esophagus tissue. D-F: Negative Control with D Dapi, E Rabit Isotype IgG and F WGA. All images were taken with a magnification of 20x and 15ms excitation as well as 60ms for ECM1 (B).

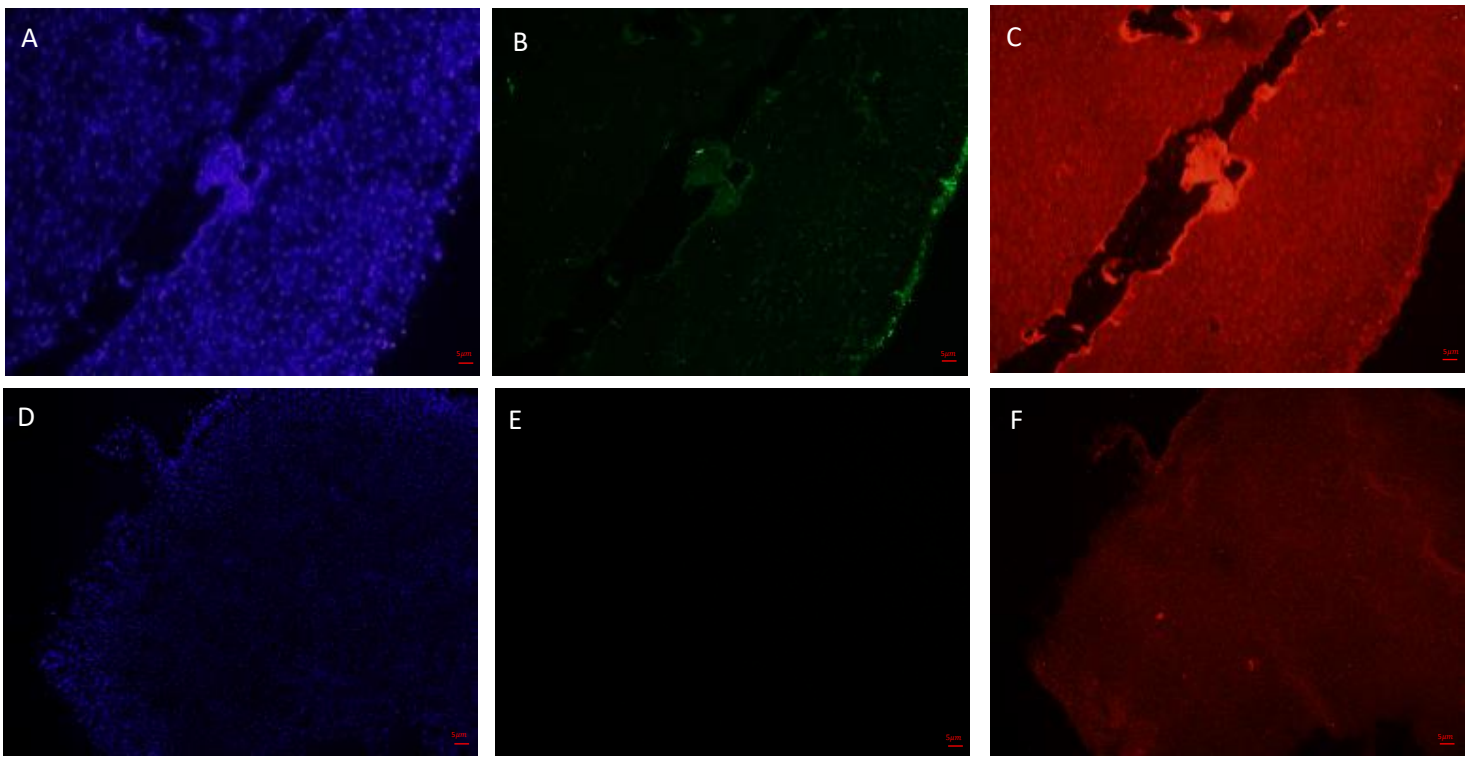

Figure 8: Immunohistochemistry staining of cryofixed mouse liver. A-C: A Dapi, B ECM1 Antibody immune fluorescence staining C WGA staining - of mouse liver tissue. D-F: Negative Control with D Dapi, E Rabit Isotype IgG and F WGA. All images were taken with a magnification of 20x and 15ms excitation or 60ms (B).

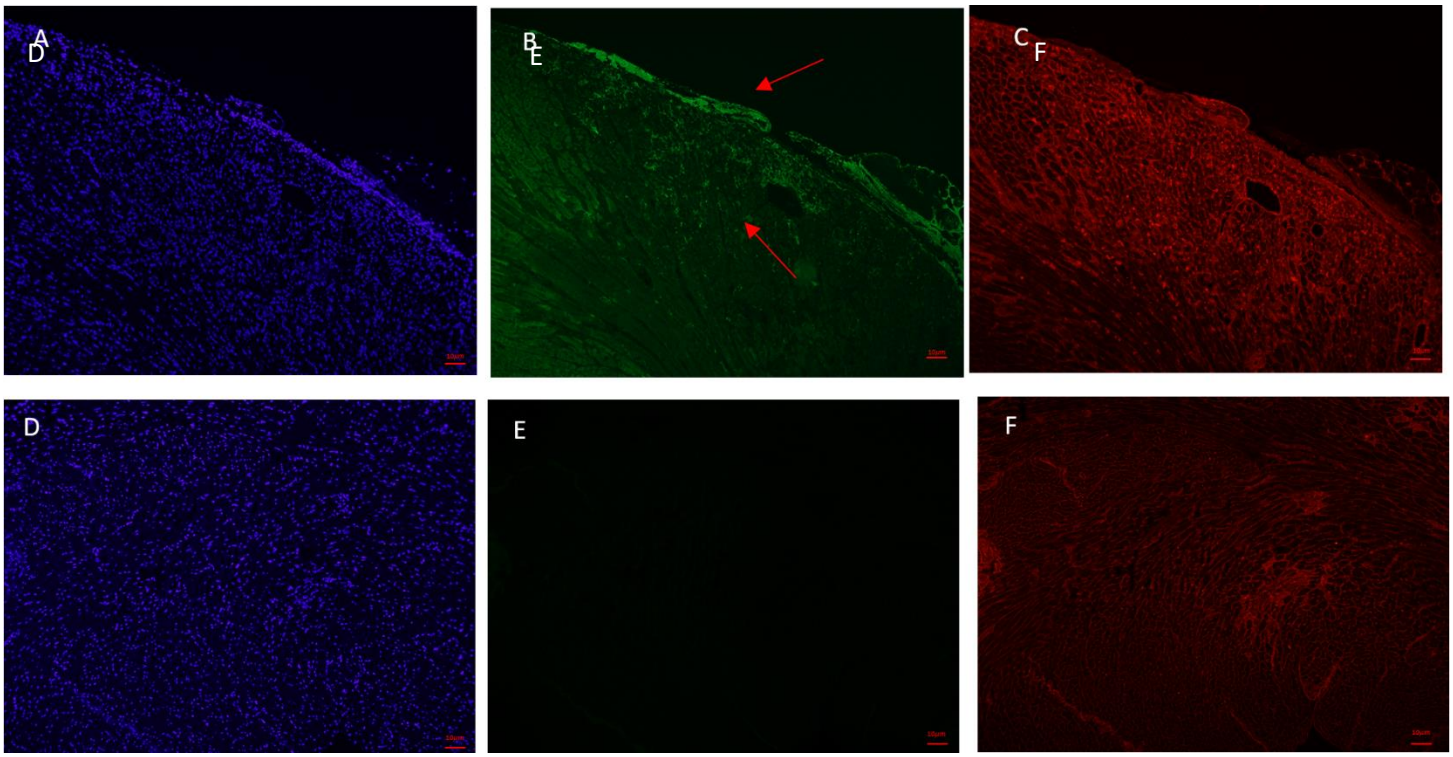

Figure 9: Immunohistochemistry staining of paraffin fixed mouse heart. A-C: A Dapi, B ECM1 Antibody immune fluorescence staining, the red arrows indicate the infarct zone with the pericardium as the border. C WGA staining - of mouse heart tissue. D-F: Negative Control with D Dapi, E Rabit Isotype IgG and F WGA. The pictures were taken with a magnification of 10x and 15 ms (A and C) as well as 300ms for B.

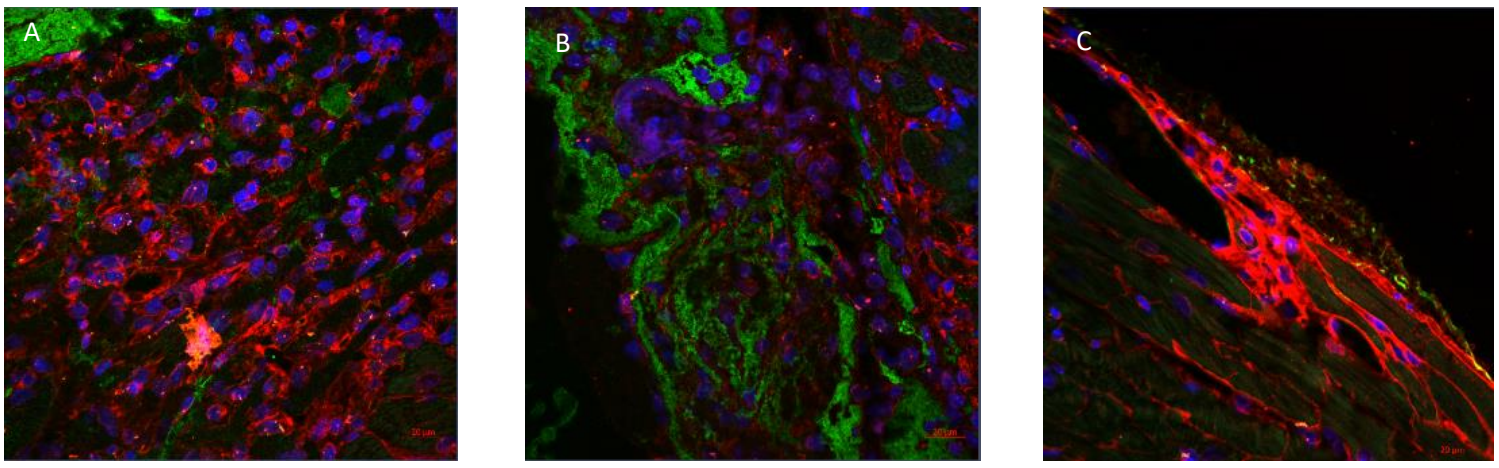

Figure 10: Immunohistochemistry staining of paraffin fixed mouse heart imaged with CLSM (ECM1 Ab). ECM1 expression is detectable in the intracellular space in the infarct zone. A-C shows different spots of the infarct with different strength of ECM1 deposition. Blue shows the cell nuclei, red the cell membranes and green the ECM1 deposition. Magnification 40x.

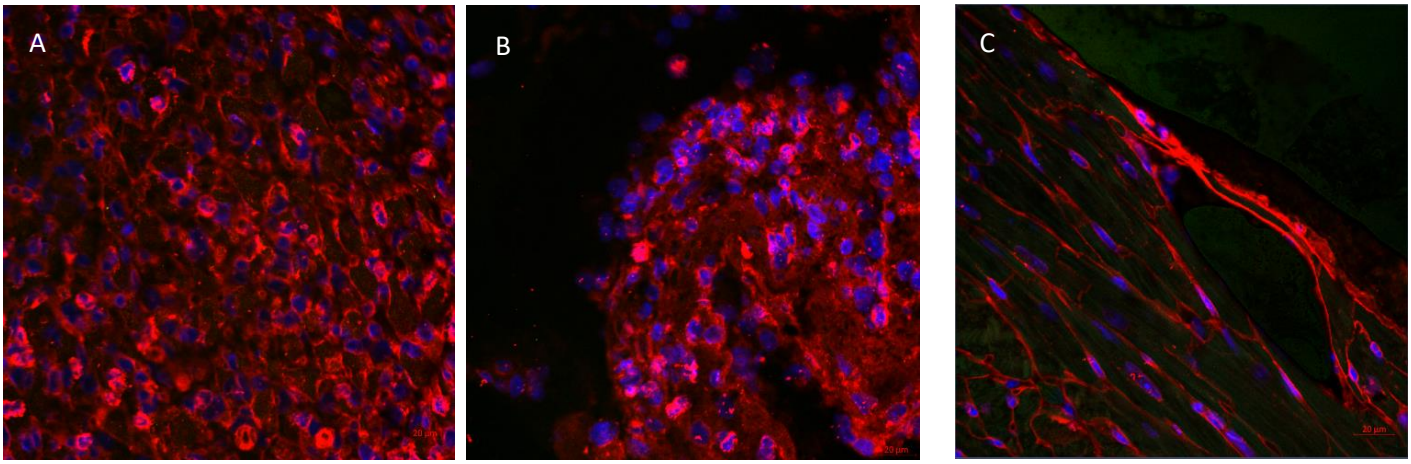

Figure 11: Immunohistochemistry staining of paraffin fixed mouse heart imaged with CLSM (IgG Control). A-C shows different spots of the infarct, stained with Dapi (blue) for the cells nuclei, WGA (red) for the cell membranes and Rabit Isotype IgG as a negative control. Magnification 40x

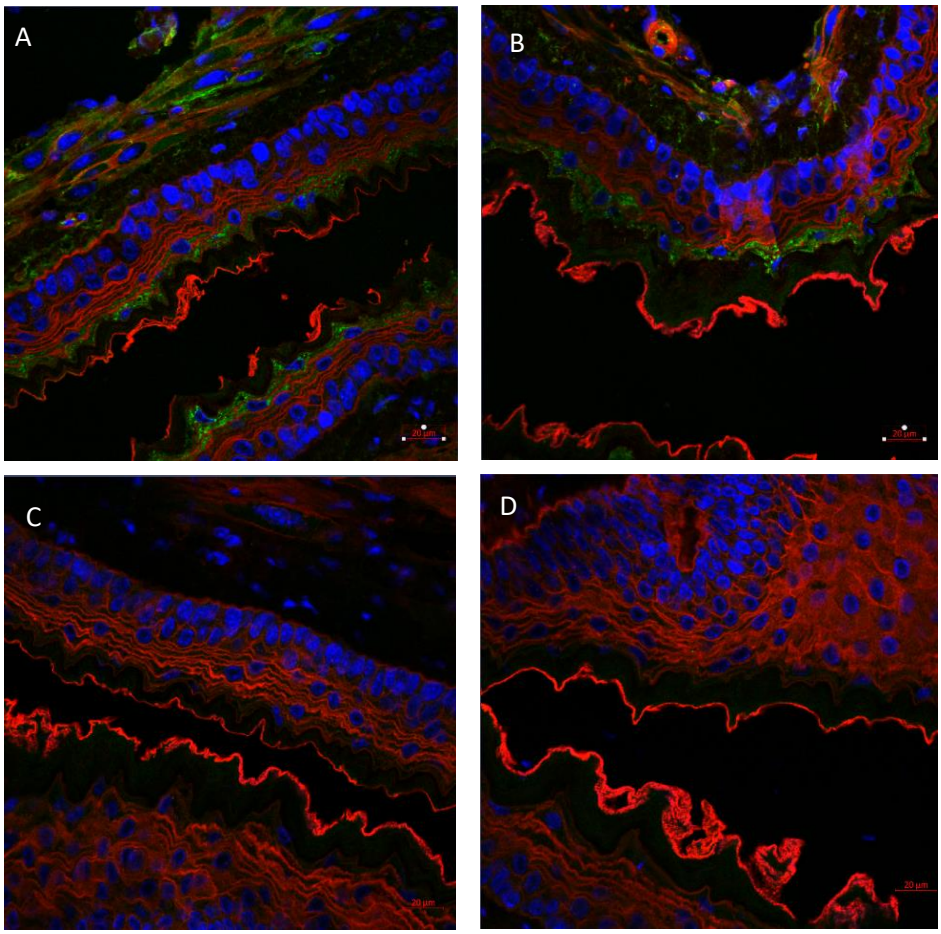

Figure 12: Immunohistochemistry staining of paraffin fixed mouse oesophagus. A-B shows different spots of the oesophagus, stained with Dapi (blue) for the cells nuclei, WGA (red) for the cell membranes and ECM1 Antibody immune fluorescence staining (green). C-D is stained with Dapi (blue), WGA (red) and Rabit Isotype IgG as a negative control. Magnification 40x.

4. DISCUSSION

The goal of my master thesis was to investigate the ECM1 expression in the heart to figure out its role in post MI heart remodelling. Therefore, I have used human cardiac fibroblast cells incubated with ECM1 and harvested at different timepoints (3h,6h,16h), followed by observing different gene expression patterns by using qPCR. I also looked for changes in p-SMAD protein expression patterns when treated with ECM1, TGF β and double treatment by western blot of human cardiac fibroblasts. Furthermore, I tried to find differences of ECM1 protein expression in different heart disease conditions (dilated cardiomyopathy and ischaemic cardio myopathy) by western blot. In the end I localised ECM1 in mouse heart of post MI hearts in the intracellular space of the infarct zone and the border zone by immunohistochemical staining.

4.1 The effect of ECM1 on the signalling pathways of human cardiac fibroblast cells.

Human cardiac fibroblasts were serum starved for 16 hours/overnight and then treated with recombinant ECM1 (RNDSYSTEMS, 3937-EC-050) (20ng/ml) for 3, 6 or 16 (qPCR) hours to investigate pathway activations of several inflammatory and pro fibrotic genes, as well as cell differentiation markers.

I was able to show a strong increase of TGF β1 and 2 for the 3 hours timepoint lasting until the 6h timepoint for TGF β1 and a decrease of TGF β1 expression for the 16h timepoint. As TGF β1 signals through downstream intracellular signals like the SMAD-pathway that leads to alterations in collagen gene expression levels I was also able to show a downregulation for Col1a with 3h and 6h but a strong upregulation of Col1a for the 16h timepoint. The late expression of Col1a with the 16h timepoint might be caused by a time shift due to the SMAD-pathway. An ECM1 related downstream collagen I production has also been shown by previous studies of Sean Hardy. **(16)**

Furthermore, I was able to show an increase of WNT5a for the 3 hours timepoint as well as IL6. IL6 was upregulated for the 6h timepoint as well and showed a decrease for the 16h timepoint. In previous studies a correlation between WNT5a and IL6 levels was shown already which might promote myocardial inflammation, fibrosis and heart failure. (10)U Also IL1b was upregulated with the 3h and 6h timepoint.

CCL2 showed a downregulation from 3h to 6h timepoint which might be due to an early antifibrotic effect of ECM1 on CCL2 expression, since CCL2 acts pro fibrotic through decreasing macrophage recruitment. **(15)**

On the other hand, TRAF2 showed a strong increase for 3 and 6h timepoint. TRAF2 is known as a response mechanism for pathological stress and possibly decreases myocardial fibrosis as well as cardial myocyte dropout. **(20)**

All the findings combined together suggest an early inflammatory effect of ECM1 on human cardiac fibroblasts signalling pathways.

4.2 ECM1 levels in non-failing, dilative and ischemic human hearts

In my second experiment I tried to investigate ECM1 expression on protein level in healthy human heart as well as in dilative and ischemic human hearts by using western blots with ECM1 antibody. Here I had to check if ECM1 is expressed on the same level in the two conditions dilative and ischemic human hearts or if there is a significant difference.

After the blotting I have used ECM1 Abcam Antibody on both of the membranes (NF+DCM and NF+ICM). For the blot with NF+DCM human heart samples I got an almost significant increase of ECM1 whether for the NF+ICM human heart samples there were no changes to see. It could be worth to redo the NF+DCM blot with a new pool of samples to check for significance.

After the first incubation with the ECM1 Abcam Antibody I stripped both membranes and reincubated them with Sigma Prestige anti ECM1 antibody (HPA027241). For this antibody I became a non-significant decrease in the NF+DCM at the 75kDa band and a non-significant increase of ECM1 for the 150 kDa band. For the NF+ICM blot I received no significant changes within the two groups for the 75kDa band and a non-significant increase for the 150kDa band.

Since all of these findings were non-significant and also the Sigma Prestige anti ECM1 antibody was incubated after I have striped the blot it would be useful to redo the Sigma Prestige anti ECM1 antibody (HPA027241) incubation with a new set of blots, to make sure that the original signal of the Abcam Antibody is not affecting the signal and also to guarantee that there is still enough protein left on the membrane since stripping is taking of protein from the membranes

too, which further weakens the signal. This is also what we seen when looking at the signal of Sigma Prestige anti ECM1 antibody (HPA027241).

4.3 P-SMAD2 levels in human cardiac fibroblasts treated with ECM1, TGF β and double-treatment

Since the activation of the SMAD-pathway plays a pivotal role in cardiac remodelling after MI by altering the gene expression levels of COL1A1 and COL1A2 I tried to investigate the possible role of ECM1 on SMAD-signalling. First, I was able to show a time dependent decrease in P-SMAD2 from 5 to 30 minutes timepoint in all of the tested groups as depictured in Figure 5 I. For the 5 minutes timepoint I was able to show a decrease in P-SMAD2 with the double treatment of TGF β and ECM1. As TGF β is supposed to upregulate P-SMAD2 expression the decrease of P-SMAD2 in the double treated group suggests that there might be another pathway involved in P-SMAD signalling that ECM1 interacts with. Further the activation of P-SMAD2 might take some time to start effecting the concentration of it. For the 10 minutes timepoint I was then able to show the upregulation of P-SMAD2 within the TGF β treated group compared to the control group, though the double treated group was upregulated as well. The upregulation of the double treated group could be due to high background noise and therefore deserves some further investigations. For the 30 minutes timepoint I was able to show that there is a clear decrease of P-SMAD2 in the ECM1 treated group as well as in the double treated group. Looking at the TGF β treated group I was able to show a significant increase in P-SMAD2 not for Figure 5F but for Figure 5H that represents a western blot with all of the TGF β treated groups with increasing timepoints. Comparing all of the findings, I was able to show that there is a time dependent decrease in P-SMAD2 in the untreated groups as well as in all of the treated groups. Furthermore, I was able to show that ECM1 itself, but specially in combination with TGF β is able to decrease P-SMAD2 concentrations over the given time course, suggesting that ECM1 is playing a yet unidentified role in SMAD signalling. The double treatment shows further downregulation compared to the single treatment with ECM1 probably due to a negative regulatory effect of TGF β administration compared with ECM1s ability to keep latent TGF β in its inactive form within the extracellular matrix. Further studies on this will be needed to illuminate the exact mechanisms beyond the effect of the double treatment.

4.4 Localizing ECM1 in mouse MI samples by Immunofluorescence CLSM

To localize ECM1 in the mouse heart with induced MI immunofluorescence confocal laser scan microscopy was performed. The establishment of an optimized immune histo-fluorescence protocol enabled the localisation of ECM1 in mouse heart, oesophagus and liver. First with cryofixed samples and a basic fluorescence microscope to get a quick overview, then by the use of paraffin fixed samples under a confocal laser scan microscope for the right magnification and resolution for the MI heart samples. With the help of CLSM I was then able to show ECM1 expression within the intracellular space in the Infarct zone of the mouse heart. By examining Figure 9 one can also see that the ECM1 expression is localized in the infarct zone and pericardium, but not in infarct remote zones. In the infarct zone ECM1 was surrounding dead or destroyed cells and filling up the space they left behind. Also, in oesophagus and liver I was able to show ECM1 expression in the intracellular space. They were used as a positive control tissue.

5. OUTLOOK

ECM1 was found to be sufficient for fibroblast stimulation and collagen production in vitro and is thought to be a new intermediary between inflammation and fibrosis and therefore to be a potential novel therapeutic target. In my master thesis I was able to show the effects of ECM1 on different genes within human cardiac fibroblasts, and the effect on the p-SMAD signalling pathway. Furthermore, I was able to show the deposition of ECM1 in post-mi murine hearts. To investigate further effects of ECM1 it could be beneficial to check for further genes effected by ECM1 as well as to redo the experiment of ECM1's effect on different cardiac disease condition with a bigger pool of samples.

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