Exogenous miR-451a prevents MMP-9 upregulation in cardiac hypertrophy

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Abstract

Matrix metalloproteinase-9 (MMP-9) degrades extracellular matrix and is increased in several cardiac pathologies. Recently microRNA (miR)-451a has been found to inhibit the expression of MMP-9 in human malignancies. However, its role in regulating MMP-9 in cardiac hypertrophy, has not yet been investigated. We hypothesized that miR-451a would prevent the expression of MMP-9 in cardiac hypertrophy. To test our hypothesis, human induced pluripotent stem cells-derived cardiomyocytes (hiPS-CM) stimulated with endothelin-1 (ET-1) to induce pathological hypertrophy were treated with miR-451a mimic or vehicle control. RT-PCR and MMP-2/MMP-9 activity assay were performed to determine MMP-9 mRNA expression and protein activity, respectively. We found that both mRNA levels of MMP-9 as well as protein activity increased significantly with ET-1 treatment, whereas this increase was prevented by transfecting the cells with miR-451a mimic. Our findings suggest that treating hypertrophic hearts with miR-451a mimic may prevent pathological remodeling, thus serving as a potential novel candidate for targeted treatment of heart failure.

Introduction

Heart failure (HF) has been described as one of the most pressing unmet clinical needs in Europe [1, 2]. The prevalence is estimated to be as high as 1-2% in the western world [3, 4]. HF increases with age [5], and because of our aging population, hospital admissions for HF are predicted to increase by 17 % by 2020 [6]. HF is a severe condition, and in chronic HF one and five year mortality rates are about 20% and 50%, respectively [7]. Despite the therapeutic benefits of numerous treatment options, the prevalence of heart failure continues to increase, underscoring the need for new therapeutic strategies.

HF is a result of hypertrophic growth of cardiomyocytes and pathological remodelling of extracellular matrix (ECM), due to injury or increased workload [8]. ECM is the framework of the myocardium and includes proteins as collagens, laminins, fibronectins and matricellular proteins, which are important in maintenance of normal structure and function of the heart [9]. Matrix metalloproteinase-9 (MMP-9) has a crucial role in degradation of ECM and remodeling of the heart. It is a member of the MMP family, which consists of zinc-dependent endopeptidase enzymes, each of them able to cleave at least one ECM protein. The MMP family is divided into five groups based on the enzyme's structure and function: collagenases, stromelysins, gelatinases, matrilysins and membrane-type MMPs. MMP-9 is, together with MMP-2, a part of the gelatinase group, meaning it can cleave gelatins. MMPs are inhibited by their tissue inhibitors (TIMPs). TIMP-1 is crucial in regulating MMP-9 [10-12]. MMP-9 is involved in physiological processes like embryo implantation [13], but it is also elevated in many pathological conditions including inflammatory processes like cancer, arthritis and diabetes [14]. In cardiovascular conditions such as hypertension, atherosclerosis, myocardial infarction (MI) and heart failure, MMP-9 has been shown to be

upregulated [15-19]. Some studies show that reducing the levels of MMP-9 in the heart, causes reversal of cardiac remodelling and reduced infarct size [20, 21]. This is of great clinical significance, as pathologicalremodelling of the heart following a MI, is one of the main causes of developing HF. Because of the high prevalence of HF and the unmet clinical need for better targeted treatment, the cardiac research field has grown extensively over the last decades. Many molecular mechanisms and intracellular pathways in HF have therefore already been described [22]. Recently microRNAs (miR), small (around 21-nucleotide-long) non-coding RNAs, that regulate protein expression by inhibiting translation or promoting degradation of the target mRNAs, have been shown to play an important role in controlling cardiac homoeostasis. Several studies show miRs to be dysregulated in pathological processes [23, 24]. miR-451a and miR-144 have been shown to have protective effects against simulated ischemia/reperfusion-induced cellular injury [25]. miR-451a has also been found to be one of the most downregulated miRs in rats with cardiac hypertrophy induced by transverse aortic constriction surgery (TAC) [24], as well as in human hypertrophic cardiomyopathy [26]. Furthermore, an ongoing study in our lab (Group of Molecular and Cellular Cardiology, Institute of Circulation and Medical Imaging, Faculty of Medicine) at NTNU, found significantly reduced expression levels of miR-451a (73%, P=0.0001) in left ventricle myocardium samples collected from patients undergoing elective coronary artery bypass grafting (CABG) with post MI heart failure compared to patients with no previous MI and no clinical signs of HF. The specific role of miR-451a in regulating cardiac hypertrophy is sparsely investigated, but recent studies showed that miR-451a regulates MMP-9 in hepatocellular carcinoma [27], lung cancer [28] and glioma [29]. Overexpression of miR-451a in these studies decreased the expression level of MMP-9, suppressing cell proliferation and migration. Given these data, and the fact that MMP-9 plays a crucial role in cardiac remodelling, we hypothesized that overexpression of miR-451a in hypertrophic hiPS-CM would decrease the expression of MMP-9.

Methods

Seeding of hiPS-CM

hiPS-CM (Cor. 4U, Axiogenesis, Germany) were seeded in 300µL Cor.4U media on a 48 well plate at a density of 30 000 cells per well. The wells had previously been coated with 0.1% gelatin in PBS solution. Gelatin was used instead of fibronectin, because of fibronectin's well known effect on cell hypertrophy [30]. The cells were incubated at 37°C in 5% CO2.

Endothelin treatment and transfection of miR-451a mimics

After four days, the cells were transfected with either miR-451a mimic or scramble (miRIDIAN microRNA Mimic, Dharmacon, Colorado, USA). Lipofectamine® RNAiMAX Transfection Reagent (ThermoFisher, Massachusets, USA) was used according to manufacturer's instructions [A]. Four hours later medium was removed and replaced with a starving medium containing: Williams E medium supplemented with hepatocyte maintenance pack (cocktail B). 48 hours after transfection the cells were treated with 10 nM endothelin-1 (ET-1) (E7764 Sigma-Aldrich, Missouri, USA) to induce cell hypertrophy [31, 32].

RNA isolation and RT-PCR

Cells were harvested 18 hours after ET-1 treatment. Total RNA isolation was performed using miRNeasy Mini Kit (Qiagen, Germany) according to manufacturer's protocol [B].

Reverse transcription for mRNA was performed using QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's instructions [C]. Reverse transcription for miRNA was performed using miScript RT II Kit (Qiagen, Germany) following manufacturer's instructions [D]. 200ng RNA was used in each reaction.

PCR for mRNA quantification was performed using QuantiTect SYBR® Green PCR Kit (Qiagen, Germany) according to manufacturer's instructions [E]. MMP-9, NPPB and HPRT Quantitect Primer Assays (Qiagen, Germany) were used. Following oligonucleotide pairs were used:

B2M **F:** CTCACGTCATCCAGCAGAGA **R:** TCTTTTTCAGTGGGGGTGAA,

MMP-2 **F:** GAAGGATGGCAAGTACGGCT **R:** ACAGGCTGTACCCTTGGTCA,

TIMP-1 **F:** ATGCACAGTGTTTCCCTGTTT **R:** CGGGCAGGATTCAGGCTATC and TIMP-2 **F:** CAGATGTAGTGATCAGGGCCA and **R:** AGGGCACGATGAAGTCACAG

The housekeeping genes HPRT (hypoxanthine guanine phosphoribosyl transferase) and B2M (β-2-microglobulin) were used as references.

PCR for miRNA quantification was performed using miScript SYBR® Green PCR Kit as per manufacturer's protocol [F]. miR–451a and RNU 6-2 miScript Primer Assays (Qiagen, Germany) were used. All the PCR reactions were run in a C 1000 Thermal Cycler with CFX96 RT-PCR detection system (Bio-Rad, California, USA). The PCR results were analyzed using the ΔΔCT method [33] to find the expression level relative to control.

MMP-2/ MMP-9 activity assay

To determine activity of MMP-2/MMP-9 we performed an additional experiment using a new vial of hiPS-CM. All protocols for seeding and treatment for hiPS-CM were similar to previous experiments. As MMPs are secreted to the ECM, *in vitro* studies should be performed by measuring enzyme activity in cell medium. Therefore, we collected cell medium 18, 42 and 66 hours after ET-1 treatment. MMP-2/ MMP-9 activity in cell medium was measured using CBA003 InnoZyme Gelatinase (MMP2/MMP9) Activity Assay, Fluorogenic Kit (Merck Millipore, Massachusetts, USA) according to manufacturer's instructions [G]. Fluorescence signal was recorded at Ex/Em = 320/420 nm using the FLUOstar® Omega plate reader.

Statistical analysis

All data are presented as means ± SEM. To determine statistical differences between groups we used ANOVA, followed by post hoc analyses using the Bonferroni method of multiple comparisons. Differences between groups were considered significant at $P < 0.05$. GraphPad Prism was used for all statistical analysis.

References to manufactors methodological descriptions

A http://tools.thermofisher.com/content/sfs/manuals/Lipofectamine_RNAiMAX_Reag_protocol.pdf

B https://www.qiagen.com/us/resources/resourcedetail?id=d9c17dc7-6a0d-4728-9b04-9a6c9bc9e0e3&lang=en

C https://www.qiagen.com/us/resources/resourcedetail?id=a7889bfb-cb1b-4e23-a538-9e4f20fdca91&lang=en

D https://www.qiagen.com/us/resources/resourcedetail?id=b112855a-7ce0-4b30-b493-a5683ee130fd&lang=en

E https://www.qiagen.com/us/resources/resourcedetail?id=1eadd69a-137c-424f-8863-0b4ff6ceac85&lang=en

F https://www.qiagen.com/us/resources/resourcedetail?id=11896a97-6d43-4efd-9793-773ba2d2af73&lang=en

G https://www.merckmillipore.com/NO/en/product/InnoZyme%E2%84%A2-Gelatinase-%28MMP-2%2FMMP-9%29-Activity-Assay-kit%2C-Fluorogenic,EMD_BIO-CBA003?ReferrerURL=https%3A%2F%2Fwww.bing.com%2F&bd=1#anchor_USP

Results

ET-1 treatment that was used to induce pathological hypertrophy, resulted in an approximately 4-fold increase (Fig. 1A, $P < 0.001$) in natriuretic peptide B (NPPB) expression, which is a well-known marker for cardiac hypertrophy [34].

ET-1 treatment gave an approximately 2-fold increase in MMP-9 expression (Fig. 1B, $P < 0.05$), whereas treatment by miR-451a mimic in ET-1 stimulated cells prevented the increase of MMP-9. Treatment by miR-451a mimic did, however, not reduce the basal level of MMP-9 in the control group any further.

Furthermore, we tested the expression of MMP-2, as it is a member of the same MMP group as MMP-9, and has been found regulated by miR-451a in previous studies [28, 29]. We found no effect on expression levels of MMP-2 following ET-1 stimulation or miR-451a- mimic (Fig. 1C).
We found TIMP-1, which is the main inhibitor of MMP-9 [10-12], to be expressed in a similar pattern to MMP-9 (Fig. 1D), but this was not statistically significant.
The expression of TIMP-2, which is another inhibitor of the MMP family, especially important to regulate the activity of MMP-2, was not found altered in the different groups (Fig.1E).

To verify if ET-1 treatment reduced miR-451a, as previously reported in hypertrophic models [24], we analyzed relative abundance of miR-451a in scramble, ET-1 treated group (Fig.1F). We found the mean fold change to be lower in this group (0,5) than in the scramble, control group (1.0), but this was not statistically significant. Furthermore, we found the transfection of miR-451a mimics to hiPS-CM successful in both groups, as the miR-451a level was approximately 250 and 570 fold ($P<0.05$) increased.

ET-1 treatment caused hypertrophy regardless of miR-451a transfection. Despite a significant increase in NPPB, we did not observe any obvious morphological alterations between the different conditions in hiPS-CM (Fig. 2).

As MMP-9 expression was found to be downregulated by miR-451a, we performed MMP-9/MMP-2 activity assay to measure if MMP-9 activity would differ in our different groups of hiPS-CM. As expected, MMP-9/ MMP-2 activity was higher in hiPS-CM treated with ET-1. MMP-9/MMP-2 activity was significantly lower in the group both transfected with miR-451a and treated with ET-1, compared to the group only treated with ET-1 (Fig.3, $P < 0.05$).

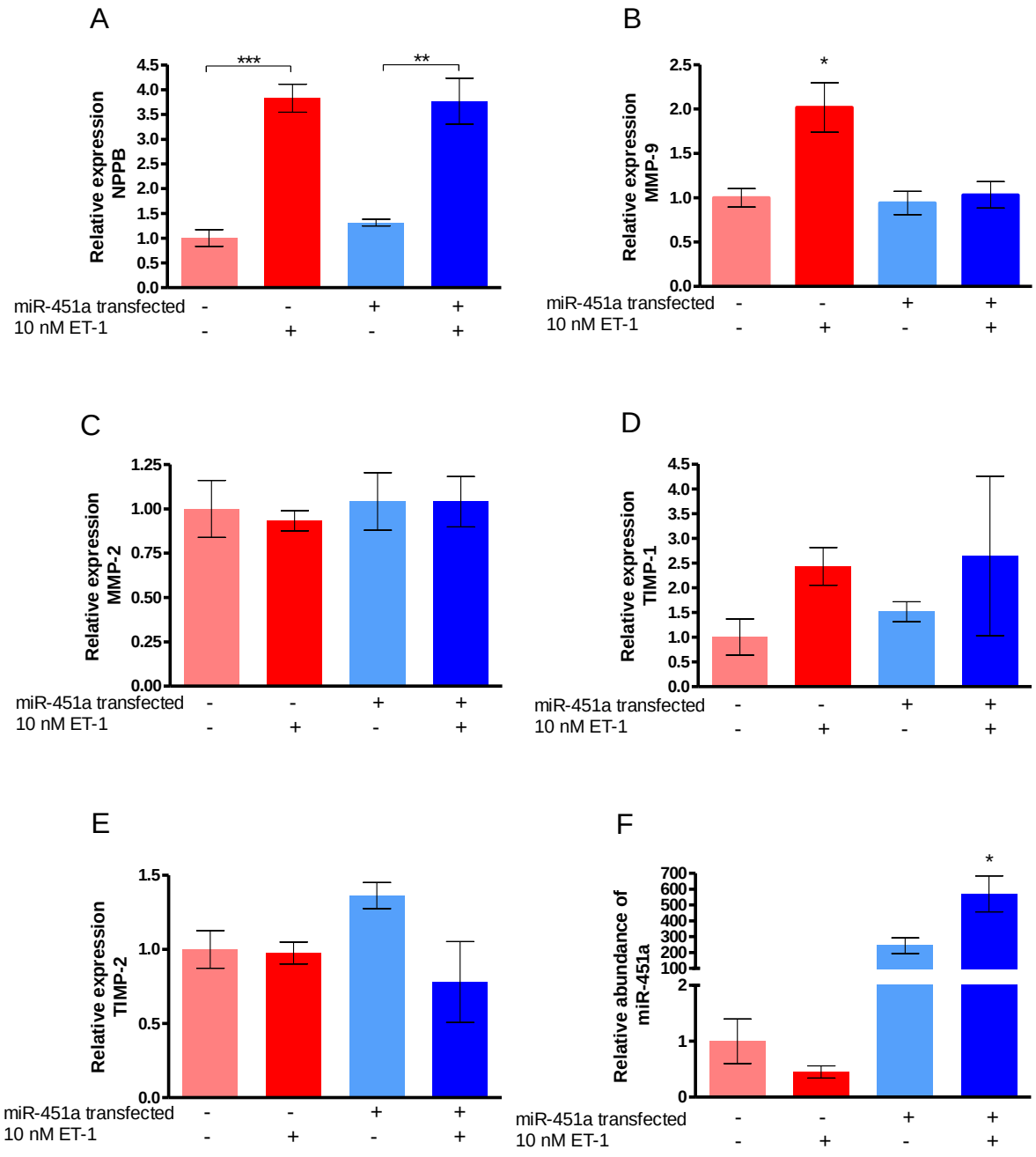

Fig.1. *PCR- results 18 hours after ET-1 treatment. Relative expression of: NPPB (A), MMP-9 (B), MMP-2 (C), TIMP-1 (D) and TIMP-2 (E). Relative abundance of miR-451a (F). Data are expressed as means ± SEM. * P < 0.05, ** P < 0.01, *** P < 0.001*

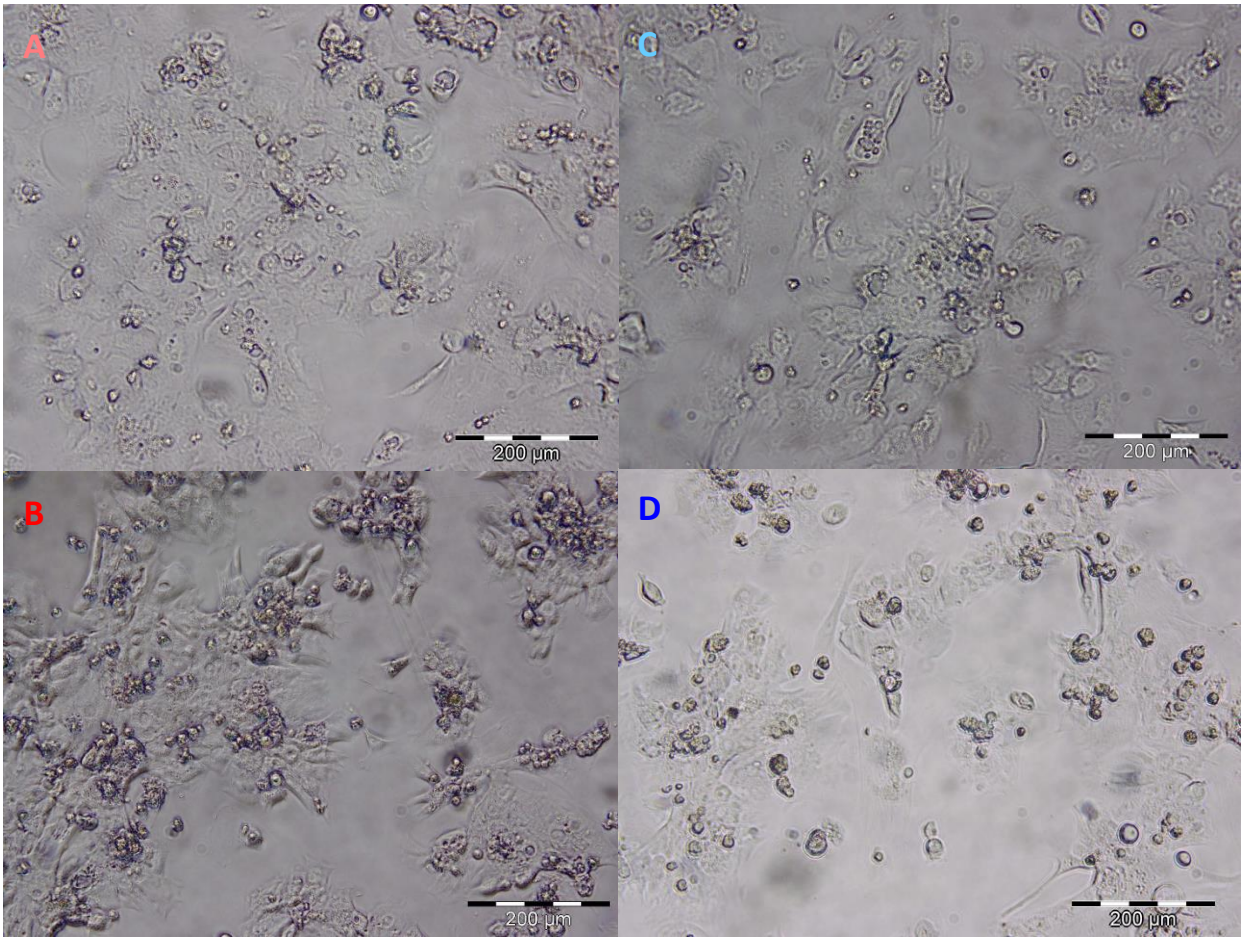

Fig.2. *Photo of iPS cells immediately before RNA isolation.* ***A****: scramble, control* ***B****: scramble, ET-1.* ***C****: miR- 451a, control.* ***D****: miR- 451a, ET- 1.*

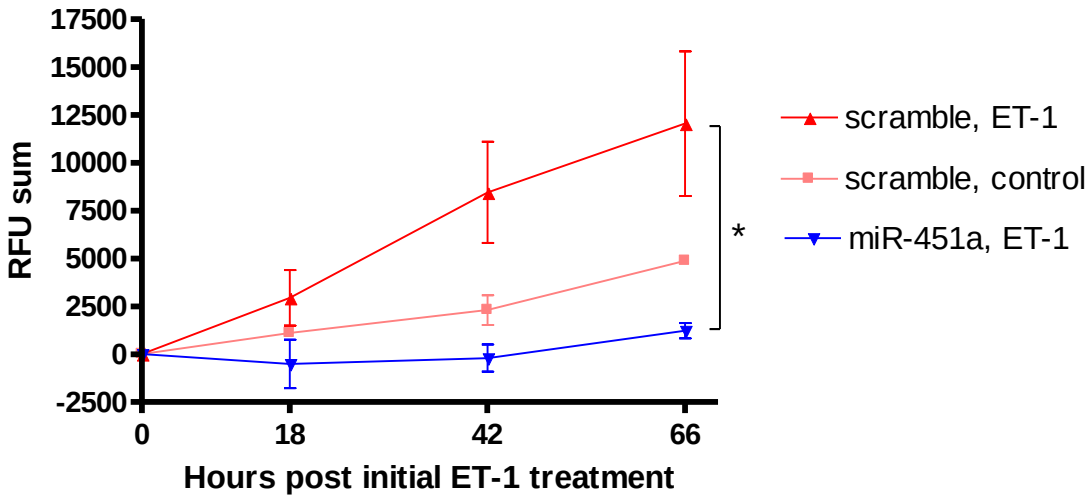

Fig.3. *MMP-9/MMP-2 activity in cell medium 18, 42 and 66 hours after ET-1 treatment measured as sum of relative fluorescence units (RFU). * $P < 0.05$*

Discussion

This study found MMP-9 to be regulated by miR-451a. MMP-9 expression was increased approximately 2-fold in an in vitro model of cardiac hypertrophy, but this increase was inhibited by transfection with miR-451a. This is of possible clinical significance as MMP-9 plays a crucial role in pathological remodeling of the heart. Investigating the effect of miR-451a treatment is an important step in finding new targeted treatments for heart failure. The exact mechanism of this regulation is not yet understood, and should be investigated further.

We found that ET-1 treatment led to an increase in the expression of NPPB, the gene encoding brain natriuretic peptide (BNP), which is a well-known marker for cardiac hypertrophy. Despite the increased NPPB we did not see any obvious changes in cell morphology between ET-1 treated and untreated hiPS-CM. miR-451a treatment did not cause any change in NPPB expression, which might indicate that miR-451a does not regulate cardiac hypertrophy, only cardiac remodeling. However, our data are from 18 hours after ET-1 treatment, which is a relatively short period. It is therefore possible that miR-451a has an effect on chronic hypertrophy, and should be investigated in further studies.

TIMP-1 (tissue inhibitor of metalloproteinase-1) can form high-affinity noncovalent 1:1 enzyme–inhibitor complexes with MMP-9 and prevent it from degrading substrates [12]. TIMP-1 deficiency is associated with a hypertrophic response and increased remodeling in experimental acute myocardial infarction [35].

TIMP -1 increases with high expression of MMPs, and has been found to be increased in hypertensive heart failure rats [36], and end stage human heart failure [19]. We found, however, only a tendency of increased TIMP-1 expression following ET-1 stimulated cell hypertrophy that was prevented by miR-451a treatment. Lack of statistical significance may be explained by our low number of biological replicates.

We found no change in the expression of TIMP-2 and MMP-2. TIMP-2 is both an activator and inhibitor of MMP-2 [37]. We did not find any increase in MMP-2 expression in cell hypertrophy, although it has been found increased in HF in left ventricular human samples [19] and in human serum [38]. Another study has found MMP-2 to continue to increase with the progression of heart failure in patients undergoing aortic valve replacement [39]. Since progression of HF increases MMP-2, it might be that acute ET-1 treatment was not sufficient to increase the expression of MMP-2. miR-451a was not found to alter the expression of MMP-2 in our study although some cancer studies have found miR-451a to regulate both MMP-9 and MMP-2 [28, 29]. An explanation to our different result, might be that ET-1 treatment did not increase MMP-2 expression, as a consequence the levels of MMP-2 might be too low to show any further downregulation with miR-451a treatment.

MMP-9 has been found to be involved in the development of several human malignancies and found to facilitate tumor progression, invasion, angiogenesis and metastasis [40-43]. miR-451a has been found to regulate MMP-9 in lung cancer [28], liver cancer [27] and glioma [29], so in the future it may be possible to develop a drug treating both HF and some cancer types.

The MMP-2/MMP-9 activity assay confirmed our PCR results. It proved that MMP-9/MMP-2 activity is increased in the medium of ET-1 treated cells without miR-451a mimic, compared to the medium of ET-1 treated cells with miR-451a mimic. PCR results only give information about transcription of genes, and as there are many steps from transcription to final enzyme activation, several factors may be involved in regulation of these complex processes. Enzyme activity measurements can be more closely related to function than quantitative protein detection, as activity can be affected by many things like posttranslational modification.

Limitations

The most critical limitation in this study was the low number of biological replicates. There were three replicates of every condition, which is a minimum. Low number of replicates cause low statistical power and decreased confidence of the conclusions drawn in the experiment. This may explain that some of our data are not statistically significant. hiPS-CM are very expensive and this is why we decided to have only

three replicates. This study can therefore be considered a pilot study, which will be repeated with more hiPS-CM in further studies.

Another limitation of our experiment was the use of 420 nm emission filter in MMP-2/MMP-9 activity assay, which is indeed within the wavelength needed for quantifying our data. The ideal emission filter for this experiment would, however, been 405 nm. Potentially, we may therefore have lost maximum intensity of the signal, something that could have improved the quality of our data further.

It would be interesting to separate MMP-9 and MMP-2 activity. This was not possible, as they have the same substrate specificity. It would also be interesting to look at the accumulation of MMP-9 / MMP-2 after 44 and 66 hours in cell medium. As the medium was removed after 18, 44 and 66 hours, we lost the possibility to investigate enzyme accumulation.

miR-451a has the same precursor RNA (pri-miRNA) as miR-144 [44], and was found to give a synergistic protective effect with miR-144 in simulated ischemia/reperfusion-induced cardiomyocyte death [25]. Therefore, it would be interesting to test if miR-144 also regulates MMP-9, and if the miR-144/451 cluster would give a synergistic effect on MMP-9 regulation. This will be investigated in further studies in our lab.

As the study is an *in vitro* study, miR-451a must be extensively tested *in vivo* in animal models of cardiac hypertrophy to evaluate if it is safe and effective, before it can be eventually considered for human clinical trials.

Conclusion

The present study found that the upregulation of MMP-9 expression and activity in hypertrophic hiPS-CM, is prevented by transfection with miR-451a mimic. MMP-9 is involved in pathological remodeling of the heart, which contributes to the development of HF. miR-451a may therefore be a potential novel candidate for targeted treatment of HF.

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