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Cardiac tissue inhibitor of matrix metalloprotease 4 dictates cardiomyocyte contractility and differentiation of embryonic stem cells into cardiomyocytes: Road to therapy☆

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Abstract

Background—TIMP4 (Tissue Inhibitors of Matrix Metalloprotease 4), goes down in failing hearts and mice lacking TIMP4 show poor regeneration capacity after myocardial infarction (MI). This study is based on our previous observation that administration of cardiac inhibitor of metalloproteinase (~TIMP4) attenuates oxidative stress and remodeling in failing hearts. Therefore, we hypothesize that TIMP4 helps in cardiac regeneration by augmenting contractility and inducing the differentiation of cardiac progenitor cells into cardiomyocytes.

Methods—To validate this hypothesis, we transfected mouse cardiomyocytes with TIMP4 and TIMP4-siRNA and performed contractility studies in the TIMP4 transfected cardiomyocytes as compared to siRNA-TIMP4 transfected cardiomyocytes. We evaluated the calcium channel gene serca2a (sarcoplasmic reticulum calcium ATPase2a) and mir122a which tightly regulates serca2a to explain the changes in contractility. We treated mouse embryonic stem cells with cardiac extract and cardiac extract minus TIMP4 (using TIMP4 monoclonal antibody) to examine the effect of TIMP4 on differentiation of cardiac progenitor cells.

Results—Contractility was augmented in the TIMP4 transfected cardiomyocytes as compared to siRNA-TIMP4 transfected cardiomyocytes. There was elevated expression of serca2a in the TIMP4 transformed myocytes and down regulation of mir122a. The cells treated with cardiac extract containing TIMP4 showed cardiac phenotype in terms of Ckit+, GATA4+ and Nkx2.5 expression.

Conclusion—This is a novel report suggesting that TIMP4 augments contractility and induces differentiation of progenitor cells into cardiac phenotype. In view of the failure of MMP9 inhibitors for cardiac therapy, TIMP4 provides an alternative approach, being an indigenous molecule and a natural inhibitor of MMP9.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcard.2015.01.091.

Keywords

Tissue inhibitor of matrix metalloprotease (TIMP); Cardiomyocytes; Contractility; Stem cells; MicroRNA

1. Introduction

The balance of extracellular matrix (ECM) is maintained by the interaction of MMPs (matrix metalloproteases) and TIMPs (tissue inhibitors of matrix metalloprotease) [1,2]. TIMPs are the endogenous inhibitors of MMPs in the cardiac ECM and the imbalance of MMP/TIMP is a hallmark of cardiovascular diseases including myocardial infarction [3–7]. The current study is based on our previous observation that cardiac inhibitor of matrix metalloprotease (~TIMP4) prevents adverse remodeling in volume overload heart failure mouse models and attenuates oxidative stress [8]. We have also established the role of extracellular matrix metalloproteases in end stage heart failure [9]. Initially, the function of TIMPs was confined to MMP inhibition due to limited available literature [10,11] however, emerging studies have indicated the role of TIMP in fine tuning ECM by TIMP mediated receptor signaling and regulating cardiac stem cell differentiation [12–14]. TIMPs consist of four family members (TIMP1–TIMP4) and TIMP4 is the least studied member. The role of TIMP4 binding partners, transcription factors and the signaling mechanisms in the context of heart failure, matrix remodeling and myocardial micro-environment is much needed to be explored.

TIMP4 is highly expressed in heart and mice with deficient TIMP4 succumb to induced myocardial infarction and have reduced survival rate [15]. In other words it can be inferred that, if TIMP4 is absent in the heart, it affects the regeneration capacity against myocardial infarction or the cardiac progenitor cells/stem cells fail to regenerate. However, in this context the studies are lacking on how TIMP4 regulates differentiation of cardiac progenitor cells and protection against myocardial infarction. The goal of this study is to understand the role of TIMP4 in cardiac function by exploring its effect on cardiomyocyte contractility and differentiation of embryonic stem cells into cardiomyocytes. Previous studies from our lab reported that MMP9 gene ablation induces survival and differentiation of cardiac stem cells into cardiomyocytes in the heart of diabetics [16]. In the study diabetic mice (insulin2 (+/−) akita) with MMP9 knock out were used and there was an increase in c-kit expression and survival in double knockout mice which suggested improved survival and differentiation of stem cells. In another report from our lab we showed that MMP activation decreases myocyte contractility in hyperhomocysteinemia [17]. Hence overexpression of TIMP4 should augment contractility since TIMPs are the natural inhibitors of MMPs. We are looking into the mechanisms on how TIMPs can augment contractility by looking into calcium signaling gene serca2a (sarcoplasmic reticulum calcium ATPase2) and microRNA122a.

Embryonic stem cells provide an in vitro model system to study the factors that govern the differentiation of embryonic stem cells into cardiomyocytes. Defining these factors may help in elucidating the corresponding mechanisms in heart that regulate the differentiation of

cardiac progenitor cells after myocardial infarction (death of myocardial tissue). Some of the factors that have been reported in promoting cardiogenesis include: 1) BMPs (bone morphogenetic proteins) that are members of transforming growth factor β (TGF β) superfamily [18,19]; 2) GATA transcription factors (1–6) and their co-activators Nkx-2 [20]; and 3) ventricular myosin heavy chain (vMHC) [21]. Binding of TGF β to the membrane receptors initiates signaling via MAP kinase pathway and phosphorylating smad family of transcription factors Nkx2.5 [22] which is helpful in regeneration. The transcription factor GATA-4 is a zinc finger protein with conserved domain WGATAR and binds to the DNA sequence CGATGG and AGATTA [20]. GATA-4 regulates the expression of cardiac specific genes troponin C, α-MHC and atrial natriuretic peptide [23,24] along with its co-activator Nkx2.5 [25].

Although the other members of TIMP i.e., 1, 2 and 3 are widely expressed, TIMP4 is specifically expressed in heart [26] and mice lacking other TIMP members are not as susceptible to MI as TIMP4 [15]. TIMP-3 has been reported to have beneficial effect in cardiac regeneration after MI [27], but Baker et al. [28] have reported TIMP 3 to cause apoptosis. Hence in the present study we hypothesize that TIMP4 helps in cardiac regeneration by promoting the differentiation of cardiac progenitor cells into cardiomyocytes and reducing contractile dysfunction by regulating serca2a through mir122a. To test this hypothesis, we transfected cardiomyocytes with TIMP4 in parallel with siRNA-TIMP4 and performed contractility studies. We checked the expression of serca2a and mir122a in the transformed cardiomyocytes. Additionally, we treated mouse embryonic stem cells (mESCs) with cardiac extract and cardiac extract minus TIMP4 to study the effect of TIMP4 on differentiation of mESCs into cardiac phenotype. This study provides insight into the role of TIMP4 in cardiomyocyte contractility and promoting cardiogenesis by using mouse embryonic stem cells as they are pluripotent, self-renewing and have the capacity to regenerate the defective myocardium.

2. Material and methods

2.1. Isolation and culture of mouse cardiomyocytes

For isolation and culture of cardiomyocytes, AfCS (The Alliance for Cellular Signaling) protocol (AfCS Procedure Protocol PP00000125 Version 1, 11/05/02) was followed. Proper approval was taken from the institutional IACUC (Institutional Animal Care and Use Committee) committee prior to animal experiments. Briefly, the heart was removed from C57BL/6 mouse, cannulated by aorta on Radnoti perfusion system and perfused with calcium free perfusion buffer for 4 min at 3 ml/min (the remaining blood was flushed from the vasculature and extracellular calcium was removed to stop contractions). The temperature of the perfusion system was maintained at 37 °C. Then the heart was perfused with myocyte digestion buffer containing Liberase blendzyme (Roche Molecular Biochemicals, Indianapolis, IN) for 8 to 10 min at 3 ml/min. The heart became swollen and turned pale. The heart was cut from the cannula below the atria and teased using forceps in a Petri dish containing 2.5 ml of myocyte digestion buffer. The solution was pipetted gently several times with a sterile plastic transfer pipette (2 mm opening) resulting in a homogenous suspension of myocytes. The suspension was transferred to two 15 ml tubes,

one for plating and other for contractility studies. For contractility studies, the myocytes were allowed to settle down for 8–10 min and resuspended in myocyte stopping buffer 2 and calcium was reintroduced at a final concentration of 1.2 mM. The cells were counted using hemacytometer and found to be 90% rod shaped cells (Fig. 1a). The contractility studies were performed using ION OPTIX instrument (LLC, MA 02186, USA). Briefly, 1 ml of the resuspended myocytes were added to the FHD Microscope Chamber System provided by ION OPTIX having two electrodes and connected to the myopacer. The myopacer was set at 1 Hz frequency and 15 V. After the myocytes started contracting, the soft edge acquisition system recorded the contractions and the data was analyzed by ION wizard's analysis function. The myocyte isolation and contractility study was in agreement with Roth et al. [29] and Louch et al. [30]. For myocyte culture, the myocytes were resuspended after settling down in myocyte plating medium and the count was adjusted to 25,000 rod-shaped myocytes/ml of the plating medium in a 50-ml Falcon tube. The plating medium was equilibrated for 2 to 3 h at 37 °C in a 2% CO2 incubator. The cardiomyocytes were plated on laminin coated plates with concentration of laminin as 10 μg/ml. After plating, cardiomyocytes were transfected with TIMP4 plasmid for contractility studies.

2.2. Cloning of TIMP4 in DesRed2 plasmid

TIMP4 gene (GeneBank Accession Number BC064046) was cloned into pIRES2-DsRed2 plasmid (Clontech) which contains the IRES or internal ribosome entry site for enhanced mammalian expression and the DsRed2 gene for fluorescent red color detection (Fig. 1b). The TIMP4 mRNA (7846 bp) was amplified from mouse heart using forward and reverse primers containing restriction sites for EcoR1 and Sac II and cloned in frame into the vector MCS between EcoR1 and Sac II. The TIMP4 gene was confirmed by sequencing and restriction digestion. The expression of TIMP4 was checked by PCR, qRTPCR and Western blot in the transformed cardiomyocytes.

2.3. Transfection of mouse cardiomyocytes with eGFP, TIMP4 and contractility studies

The transfection of cardiomyocytes was performed using Lipofecta-mine 2000 (Invitrogen) at 2.5 μl and 1 μg of the plasmid DNA in OptiMEM medium (GIBCO). Lipofectamine (2.5 μl) and OptiMEM (22.5 μl) were mixed and incubated for 5 min at RT in an eppendorf tube. Similarly, DNA (1 μg–2 μl) and OptiMEM (23 μl) was mixed and incubated for 5 min at RT in another eppendorf tube. Then contents from both the tubes were mixed and incubated further for 5 min to form the liposomes. The resulting solution was then added to cardiomyocytes plated on laminin coated plates in OptiMEM. The transfection was standardized using the GFP plasmid (EF.GFP, plasmid 17616-Addgene). The siRNA transfection was done according to the manufacturer's protocol (Quiagen). The transfection was performed for 3 h, 6 h and 12 h and the expression of GFP, TIMP4 was checked at these time points using Western, Real time PCR and confocal imaging. The contractility studies were performed as explained above using the ION OPTIX (LLC, MA 02186, USA) as per the manufacturer's instructions. For calcium the myocytes were incubated with Fura-2 AM (diluted in incubation buffer to 2 μM) fluorescent dye at 37 °C for 30 min. After incubation, the myocytes were loaded to the FHD Microscope Chamber System provided by ION OPTIX and the calcium measurement was performed similar to contractility measurements.

2.4. Mouse embryonic fibroblasts (MEF) culture

Mouse embryonic fibroblasts were procured from ATCC (American Type Culture Collection Centre, MEF-SCRC1040) and cultured in complete DMEM (Delbecco's Modified Eagle Medium-Gibco Life technologies). To prepare the complete medium, the following were added to DMEM: 1) 10% FBS (ATCC); 2) 1% Penicillin/Streptomycin (Cellgro); 3) 1% Non-essential amino acids (Gibco) and 4) 1% Sodium Pyruvate (Gibco). The media was filter sterilized by passing through a 0.22 μm polyethersulfone sterilizing filter (Corning) inside the biological safety cabinet and stored at 4 °C till further use. The medium was prewarmed at 37 °C before use.

2.4.1. MEF (mouse embryonic fibroblasts) culture—The MEFs were cultured as per manufacturer's instructions. Briefly, the MEF frozen vial was thawed quickly in water bath at 37 °C for 90 s and the contents were poured into 10 ml MEF medium. The vial was centrifuged at 270 ×g for 5 min and the pellet was redissolved in 2 ml MEF medium; the dissolved pellet was plated on T75 flasks coated with 0.1% gelatin under sterile conditions and placed in a cell culture incubator maintained at 37 °C with 5% CO_2. The MEF coated plate was used as a feeder layer for mESCs (mouse embryonic stem cells) after MEF become 70–80% confluent. The MEFs were treated with mitomycin C at a concentration of 10 μg/ml for 3–4 h to mitotically inactivate MEFs. For subculturing, the MEFs were trypsinized (0.25% (w/v) Trypsin, 0.53 mM EDTA solution, ATCC# 302101) as per manufacturer's instructions.

2.5. Mouse embryonic stem cells (mESCs) culture

The mouse embryonic stem cells were procured from ATCC (American Type Culture Collection Centre, mESC-SCRC1002) and cultured in mESC basal medium (SCRR-2011) having the following components: 1) 20% FBS (ATCC); 2) 1% Penicillin/Streptomycin (Cellgro); 3) 1% Non-essential amino acids (Gibco); 4) 1% Sodium Pyruvate (Gibco); and 5). 0.1% β-Mercaptoethanol. To keep the mESCs in the undifferentiated state, LIF (leukemia inhibitory factor) was added in the culture medium at a concentration of 120 μ/ml. The medium was filter sterilized and stored at 4 °C till further use. For differentiating mESC, culture medium without LIF was used.

2.5.1. mESC culture—mESC were cultured on MEFs when they become 70–80% confluent (after growing for 24 h, 55,000 feeder cells/cm^2). Prior to plating the mESCs, the MEF feeder layer was washed with PBS and added 10 ml mESC medium with LIF. The MEF were placed back in the incubator for 1 h. The vial of ES cells was thawed in water bath for 90 s by gentle agitation at 37 °C, keeping the O ring and cap out of the water. The vial's contents were transferred to 5 ml of mESC medium in a 15 ml centrifuge tube and 4 mL of mESC medium was added to bring the total volume to 10 ml. The cells were centrifuged at 270 ×*g* for 5 min and resuspended in 2 ml of mESC medium. The 2 ml of cell suspension was plated on T75 flask containing feeder cells and fresh complete mESC medium. mES cells were plated at a density of 30,000–50,000 cells/cm^2. The plate was incubated at 37 °C in a humidified 5% CO_2/95% air incubator. The plated mESC attached to the MEFs and exhibited small round morphology (Fig. 5). The medium was changed every day with LIF to allow proper growth of mESCs and formation of embryoid bodies (EBs)

which is aggregate of mESCs. The EBs were not allowed to come in contact with each other to keep them in the undifferentiated state. After the EBs attained substantial size (Fig. 5) they were preceded with mESC differentiation.

2.6. mESC differentiation

mESC medium without LIF was used for differentiation. After the formation of embryoid bodies, they were separated from the MEFs and grown on gelatin coated plates for differentiation. Briefly, mESCs growing on MEFs were trypsinized and plated on 0.1% gelatin coated T75 flasks. After incubating for 1 h the MEF settle down leaving the mESCs in the floating form. The supernatant was taken out, centrifuged and the pellet was resuspended in fresh mESC medium without LIF. The suspension culture containing the embryoid bodies was plated on 0.1% gelatin coated plates and incubated for 72–96 h. The media was changed after every alternate day. A flow chart for mESC differentiation has been presented in Fig. 5.

2.7. Treatment groups

The EBs attain considerable size by this time and they are divided into the following treatment groups: 1) no treatment; 2) cardiac extract; 3) cardiac extract with TIMP4 monoclonal antibody (Abcam); 4) TIMP4 purified protein (Abcam). Cardiac extract was formed by grinding the mouse heart in PBS, centrifuging and filter sterilizing the supernatant. The supernatant was then added to the mESC medium without LIF and filter sterilized. Similarly, TIMP4 purified protein and TIMP4 monoclonal antibody were added to the mESC medium and filter sterilized. After incubating for 7 days, embryoid bodies were checked for differentiation into cardiomyocytes by evaluating 1) cardiac specific proteins GATA-4, Nkx2.5 and C kit by Western (Fig. 9); 2) alpha actinin and myosin light chain staining by confocal imaging (Fig. 8); 3) C kit expression by flow cytometry and immunocytochemistry (Fig. 6b) and 4) RT PCR of GATA4, Nkx2.5, CNX 43, MHC and Trop T.

2.8. Immunocytochemistry

For immunocytochemistry the cells were fixed using paraformalde-hyde and permeabilized using Triton X 100 (0.3%). Primary antibodies were added at 1:250 dilution (Abcam) and incubated overnight at 4 °C. After washing with TBS and TBST and applying fluorescently tagged secondary antibodies, the slides were mounted and visualized with a laser scanning confocal microscope (Olympus FluoView1000).

We used alpha actinin and myosin light chain (MYL 7) antibodies for identification of actin–myosin fibers formed in the differentiated stem cells (Fig. 8).

2.9. Western blotting

Protein was extracted from the cardiomyocytes using the Trizol (Life technologies) method and protein estimation was done by Bradford method. The protein samples (30 μg) were denatured in SDS sample buffer (2% SDS, 10 mM dithiothreitol, 60 mM Tris–HCl pH 6.8, bromophenol blue 0.1%) and loaded on 12% PAGE. The protein samples were transferred onto the PVDF membrane and probed with appropriate primary and secondary HRP

conjugated antibodies. The membrane was developed with ECL Western blotting detection system (GE Healthcare, Piscataway, NJ, USA) and the image was recorded in the gel documentation system ChemiDoc XRS system (Bio-Rad, Richmond, CA, USA) (Bio-Rad). The membranes were stripped and reprobed with anti-GAPDH antibody (Millipore, Billerica, MA, USA) as control. The data was analyzed using Image Lab densitometry software (Bio-Rad) and normalized to GAPDH.

2.10. Flow cytometry

Flow cytometry for embryonic stem cells was done using the Accuri CFlow Plus system (Ann Arbor, Michigan, USA) with gating applied. Prior to flow cytometry, live cells were counted by Bio Rad TC10 automated cell counter using Trypan Blue solution. The embryonic stem cells were grown on MEF in chambered slides and stained with C-kit FITC antibody (Abcam) (1:100). The number of C-kit positive cells were counted from the forward scattered vs. side scattered graph. C-kit Positive cells were also observed under laser scanning confocal microscope (Olympus FluoView1000) (Fig. 6b). The stem cells were also checked for the expression of Oct 4 (Fig. 6a) which is pluripotent marker by using anti-Oct4 antibody (1:100) along with the negative control (rabbit IgG).

2.11. RT-PCR and real time PCR

RNA was extracted from the cardiomyocytes using Trizol reagent (Life Technologies) according to the manufacturer's instructions. The cDNA was synthesized using the MultiScribe reverse transcriptase kit (Quiagen) using 2 μg total RNA and random primers. Semi-quantitative PCR was performed using the gene specific primers in Bio-RAD thermocycler. For real time PCR sybr green (Quanta Fast low Rox) was used in the Stratagene M×3000P real-time PCR machine.

For microRNA 122a the Quiagen Primer assay and miscriptII microRNA kit was used. The fold expression was calculated by the delta C_t method after normalizing the genes with 18 s rRNA or snoRD-72.

2.12. Statistical analysis

We used SPSS 16.0 for performing all data analysis. The data is presented as mean ± SEM. The statistical significance among experimental groups was determined by one way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. The comparison between two groups was done by Student's independent t test and $p < 0.05$ was considered as significant. Multiple comparison between groups was performed by two way ANOVA.

3. Results

3.1. The transfected cardiomyocytes expressed eGFP and TIMP4

The expression of eGFP and TIMP4 was evaluated using confocal microscopy. The cardiomyocytes transfected with eGFP showed green color while those transfected with TIMP4 showed red color in confocal microscopy (Fig. 2a). The expression of TIMP4 was further confirmed by RT-PCR, Western and real time PCR (Fig. 2b & c). The expression was evaluated at 0 h, 6 h and 12 h and there was elevated expression of TIMP4 RNA as

determined by RT-PCR and real time PCR as well as in the protein levels from 0–12 h as compared to the untransformed cells in Western blots. When we used TIMP4 siRNA, the level of TIMP4 went down and became lower than the wild type in both Western and RT-PCR.

3.2. Augmented contractility in TIMP4 transfected myocytes

Contractility studies were performed in both WT and transfected cardiomyocytes (after 6 h and 12 h of transfection) cultured on laminin coated plates using the ION OPTIX instrument and the data was analyzed by using the ION OPTIX software. We found that the contractility pattern was different in the TIMP4 transfected cardiomyocytes as compared to the untransformed after 6 h and 12 h of transfection. The value of amplitude was more negative in TIMP4 overexpressed cardiomyocytes and the length vs time peak was deepened and narrowed (Fig. 3a & b). To the contrary, the cells which were transfected with TIMP4 siRNA showed a broadened peak and lesser amplitude values. We also measured overall calcium distribution in the transfected myocytes (Supplementary Fig. 4). There was increase in the calcium up-take and release in the TIMP4 transfected myocytes as compared to the siRNA-TIMP4 transfected myocytes.

3.3. The expression of serca-2a and mir122a in TIMP4 overexpressed cardiomyocytes

Next, we attempted to investigate the mechanism, how overexpression of TIMP4 can lead to increased contractility. When we checked the expression of serca-2a we found elevated levels by Western blots, RT PCR and qRT PCR (Fig. 4a, b & c). When we used siRNA to inhibit TIMP4 we found that there was down regulation of serca-2a in the TIMP4 inhibited cardiomyocytes. Bioinformatics analysis [31] showed that mir122a was closely associated with serca-2a due to sequence similarity. So we checked the expression of mir122a and found that mir122a was down regulated in the TIMP4 overexpressed cardiomyocytes while in the TIMP4 inhibited cells, the levels of mir122a were increased 2–3 folds (Fig. 4d). We also checked the expression of TIMP1/TIMP2 (Supplementary Fig. 1), MMP9 (Supplementary Fig. 2) and ryanodine receptor (Supplementary Fig. 3). We found non-significant changes in the expression of TIMP1/TIMP2 and TIMP3 (data not shown) in the TIMP4 transfected cells. There was decrease in the expression of MMP9 in the TIMP4 overexpressed myocytes while MMP9 was upregulated when TIMP4 was inhibited. Although we found increase in the expression of ryanodine receptor in the TIMP4 overexpressed myocytes as compared to diminished expression in the siRNA-TIMP4 myocytes, this may be due to the compensatory effect by upregulated serca2a expression and increase in the levels of calcium in the sarcoplasmic reticulum.

3.4. The differentiated cells express more C kit and less Oct 4

A schematic diagram for differentiation of stem cells is presented in Fig. 5. From 0–5 days the embryoid bodies expressed Oct 4 which gradually decreased after 15 days of differentiation (Figs. 6a & 7). To check the role of TIMP4 in the differentiation of embryonic stem cells to cardiomyocytes we treated the embryoid bodies which were maintained in the undifferentiated state with and without cardiac extract. We found that there was increase in the number of C-kit positive cells after 15 days in the cardiac extract treated cells (Figs. 6b & 8). We used monoclonal antibodies in the cardiac extract against

TIMP4 to check whether TIMP4 is the factor responsible for the conversion of embryonic stem cells into cardiomyocytes. There was no significant increase in the number of C-kit positive cells when the cells were treated with cardiac extract minus TIMP4 (Fig. 9a).

3.5. Expression of alpha actinin and myosin light chain

We stained the immobilized and differentiated stem cells using alpha actinin and myosin light chain antibodies and observed the formation of actin-myosin fibers in the differentiated stem cells (Fig. 8). We found the expression of alpha actinin and myosin light chain in the cells treated with cardiac extract (Fig. 8a) while the cells that were treated with cardiac extract + TIMP4 antibody or untreated cells did not show the formation of actin-myosin fibers (Fig. 8b).

3.6. The differentiated cells express cardiac specific genes and proteins

To establish the conversion of mouse embryonic stem cells to cardiac phenotype we checked the expression of cardiac specific genes GATA4, Nkx2.5, MHC and Trop T at day 0, day 5, day 10 and day 15. The results showed increased expression of cardiac specific genes from day 0–15 (Fig. 7). However there was decreased expression of Oct 4 which is a pluripotency marker gene. Additionally, we checked the expression of GATA4 and Nkx2.5 proteins which are cardiac specific transcription factors and found elevated expression of the proteins in the cells treated with cardiac extract as compared to untreated or cells treated with cardiac extract + TIMP4 (Fig. 9a, b & c).

4. Discussion

This study aims at finding the role of TIMP4 in alleviating contractility and differentiation of cardiac progenitor cells to cardiomyocytes. Our previous study showed that cardiac inhibitor of metalloproteinase protein (~TIMP4) attenuates oxidative stress and cardiac remodeling when administered intraperitoneally in volume overload heart failure mice models [8]. TIMP4 is primarily expressed in the heart [26] and is diminished in congestive heart failure as compared to other TIMPs [32]. Our data suggests that overexpression of TIMP4 in the myocytes leads to downregulation of MMP9 levels with non significant changes in the levels of TIMP1/2. We speculate that administration of cardiac specific TIMP4 can be cardio protective for two reasons: 1) It augments contractility by increasing the expression of serca2a through microRNA 122a and 2) it induces the differentiation of cardiac progenitor stem cells into cardiomyocytes. TIMP4 can be cardio protective for two other reasons: 1) it decreases the MMP activity and 2) TIMP4 protein contains amino acid residues that can sequester ROS [33].

Our results show augmented contractility in the cardiomyocytes transformed with TIMP4 as compared to untransformed or TIMP4-siRNA transformed cardiomyocytes. There was increased uptake and release of calcium in the TIMP4 transformed myocytes as compared to the siRNA-TIMP4 myocytes. This is a novel report showing augmented contractility in TIMP4 overexpressed cardiomyocytes. Additionally, the data suggests that TIMP4 is the factor that helps in the differentiation of cardiac progenitor cells to cardiomyocytes, as the stem cells treated with cardiac extract differentiate into cardiac phenotype but not the cells

treated with cardiac extract without TIMP4 (Fig. 8). Previous reports suggest that mice deficient in TIMP4 have poor regeneration capacity after myocardial infarction [15], though the mechanisms were poorly understood. Targeted overexpression of TIMP4 in heart after myocardial infarction improves cardiac remodeling [34]. Takawale et al. have reported that myocardial recovery from ischemia reperfusion is compromised in the absence of TIMP4 [35]. We have reported from our laboratory that activation of MMPs decreases contractility in hyper-homocysteinemia [17]. When MMPs are ablated or decreased, it leads to better regeneration capacity by directing the differentiation of cardiac progenitor cells to cardiomyocytes [16] and improves cardiac function in diseased hearts [36–38]. MMP inhibition has been proposed as a therapy for vascular diseases [39]. We also found downregulation of MMP9 in the TIMP4 overexpressed myocytes as compared to the TIMP4 inhibited myocytes. Owing to the potential regenerative effects on heart failures, MMP9 inhibitors were used in clinical trials i.e., the double-blinded, placebo controlled trial of "PREMIER" Prevention of Myocardial Infarction Early Remodeling-using the MMP inhibitor, PG11680. However the clinical trials were ineffective and difficult to implement. TIMP4 provides an alternative approach where it can act as MMP inhibitor — as an endogenous molecule and a cardio protective agent by scavenging ROS. Studies do show that TIMP4 has an additional role apart from MMP inhibition [8] and it can mediate receptor signaling [14].

To elucidate MMP independent side of TIMP4 we looked into calcium channel gene serca2a that may be affected by overexpression of TIMP4. We found that there was increase in the expression of serca2a with overexpression of TIMP4. Serca2a is a calcium channel present in the sarcoplasmic reticulum and it promotes cardiac relaxation and contractility by sequestering and releasing calcium in the myocytes [40]. In myocardial infarction and human heart failure, there is a decrease in the expression of serca2a which is hallmark of cardiac myocyte dysfunction [40–43]. Studies depict that decrease in the expression of serca2a leads to decrease in contractility in rats after MI and increase in the expression of serca2a can lead to augmented contractility in cardiovascular diseases [44,45]. Ryanodine receptor in the sarcoplasmic reticulum works in conjunction with the serca2a and helps maintaining calcium levels by calcium efflux [46]. The upregulated expression of serca2a was accompanied by increased expression of the ryanodine receptor in our study which may be due to the compensatory effect mediated by increased levels of calcium in the sarcoplasmic reticulum. The expression of serca2a is tightly regulated by microRNAs and bioinformatics analysis and expression studies on microRNAs have shown that mir122a tightly regulates serca2a [31]. When we checked the expression of mir122a, we found a negative correlation between mir122a and serca2a. The data suggested that when TIIMP4 is overexpressed in cardiomyocytes, there is decrease in the expression of mir122a which upregulates the serca2a expression.

We further looked into the role of TIMP4 in differentiation of cardiac progenitor cells into cardiomyocytes by using mouse embryonic stem cells. We differentiated mouse embryonic stem cells into embryoid bodies and added cardiac extract with and without TIMP4. The embryoid bodies that were treated with cardiac extract having TIMP4 showed differentiation into cardiac phenotype which was confirmed with the cardiac specific expression of GATA4 and NKx2.5 which are cardiac specific transcription factors (Fig. 9b & c). There was

increase in the number of Ckit + cells in the group treated with cardiac extract (Fig. 6b). The embryoid bodies that were treated with cardiac extract with TIMP4 monoclonal antibody to block TIMP4, did not show profound differentiation into cardiomyocyte lineage and showed poor expression of CKit, GATA4 and Nkx2.5 (Fig. 9a, b & c). In our previous study we have shown the culture and differentiation of mouse embryonic stem cells and the expression of MMP9 in the differentiated stem cells [47]. Although various growth factors e.g., TGFβ1 [48], bFGF [49], retinoic acid [50], BMP2 and FGF [51] have been reported for the differentiation of embryonic stem cell into cardiomyocytes but studies are lacking to show the role of TIMP4 in the differentiation of stem cells to cardiomyocytes.

This is a novel study which provides evidence for the role of TIMP4 in cardiomyocyte contractility and differentiation of mouse embryonic stem cells to cardiomyocytes. The rationale behind looking into the expression of serca2a is its involvement in contractile function by regulating calcium levels in the sarcoplasmic reticulum and augmented contractility with TIMP4 transfection. Since the clinical trials for MMP9 inhibitors have not been so far successful, it provides and alternative approach for cardiac therapies, as administration of TIMP4 has been beneficial in terms of MMP inhibition, differentiation of cardiac progenitor cells and scavenging of ROS. Additionally, TIMP4 is an indigenous molecule which omits the use of synthetic agents that may cause further side effects (Fig. 10).

4.1. Limitation

Although we could not establish how TIMP4 regulates serca2a but TIMP4 overexpression was accompanied by increase in serca2a levels and decrease in mir122a, a microRNA which can target serca2a, GATA4 and Mef2c. There is induction of the two transcription factors GATA4 and Mef2c that can trigger the differentiation of cardiac progenitor cells to cardiomyocytes. Hence, downregulated levels of mir122a suggest two points: 1) TIMP4 is upregulated along with serca2a which augments contractility and 2) the transcription factors GATA4 and Mef2c are upregulated which can induce differentiation of progenitor cells. In our future studies, we are looking into other genes and proteins that are associated with contractility which comprise calmodulin, calmodulin kinase, beta adrenergic receptors, phospholamban, G coupled receptors (GPCR), sodium/calcium exchanger, calcium/potassium ATPase in the mitochondria and the effect of mitochondrial fusion and fission on contractility. It is evident from our study that TIMP4 instigates the differentiation of cardiac progenitor cells into cardiac phenotype but we could not establish whether the differentiated cells were fully functional cardiomyocytes. Also, TIMP4 needs cardiac environment to differentiate cardiac progenitor cells into cardiomyocytes. Combining the results of our previous study and the current study, TIMP4 emerged as a potential candidate for future cardiac therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AfCS	The Alliance for Cellular Signaling
ATCC	American Type Culture Collection Centre
BMP	bone morphogenetic protein
EB	embryoid bodies
ECM	extracellular matrix
GFP	green fluorescent protein
LIF	leukemia inhibitory factor
MCS	multiple cloning site
MEF	mouse embryonic fibroblasts
mESCs	mouse embryonic stem cells
MI	myocardial infarction
MMPs	matrix metalloproteases
PBS	phosphate buffered saline
ROS	reactive oxygen species
Serca2a	sarcoplasmic reticulum calcium ATPase 2
TBS	tris buffered saline
TGFβ	transforming growth factor β
TIMP	tissue inhibitor of matrix metalloprotease
vMHC	ventricular myosin heavy chain

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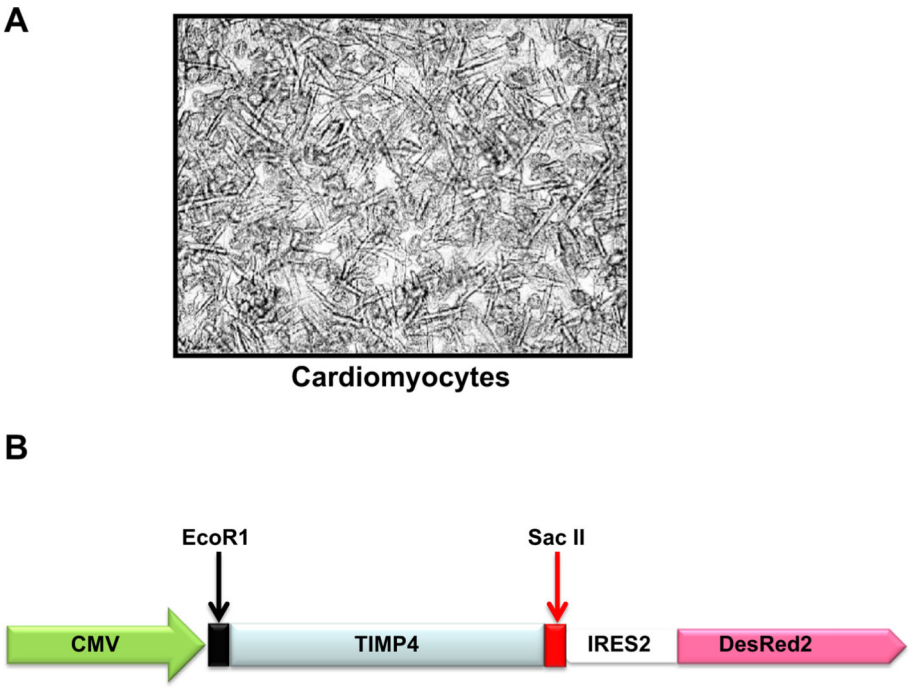

Fig. 1.

A) Cardiomyocytes were isolated from mouse (C57BL/6) heart and represented 90% rod shaped cells. B) Schematic representation of cloning of TIMP4 in pIRES2DsRed2 Vector. TIMP4 was cloned at the multiple cloning site between EcoR1 and Sac II and confirmed by sequencing.

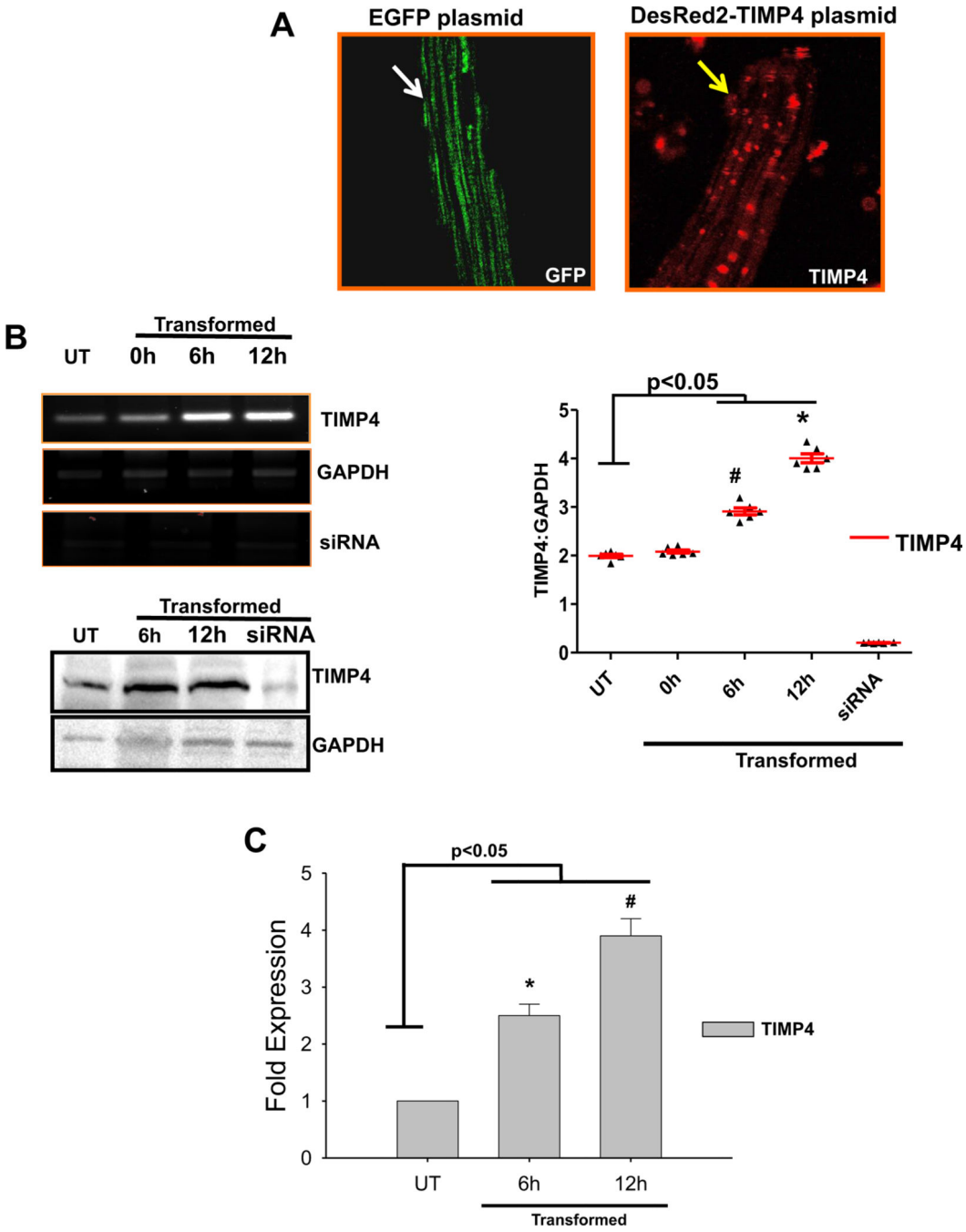

Fig. 2.

Transfection of TIMP4 and GFP in mouse cardiomyocytes: GFP and TIMP4 were transfected into mouse cardiomyocytes using lipofectamine 2000 (Invitrogen) and imaged by confocal microscopy. A) The TIMP4 transfected cardiomyocytes appeared red in color due to the DeRed2 fluorescent tag and the GFP transfected cardiomyocytes appeared green in color (arrows). B). TIMP4 expression in cardiomyocytes was confirmed by RT-PCR and Western blots. In cardiomyocytes transfected with TIMP4-siRNA, there was negligible expression of TIMP4. The expression of TIMP4 was observed at different time points: 0 h, 6

h and 12 h and there was increase in the expression with time (UT — untransformed; *p = 0.01 as compared to untransformed or 0 h-Student's t-test; n = 6). C). Real time expression of TIMP4 in the transformed cardiomyocytes and in untransformed, at 6 h and 12 h. The real time PCR showed increase in the expression at different time points which confirmed the Western results. *p = 0.001 as compared to untransformed, Student's t-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

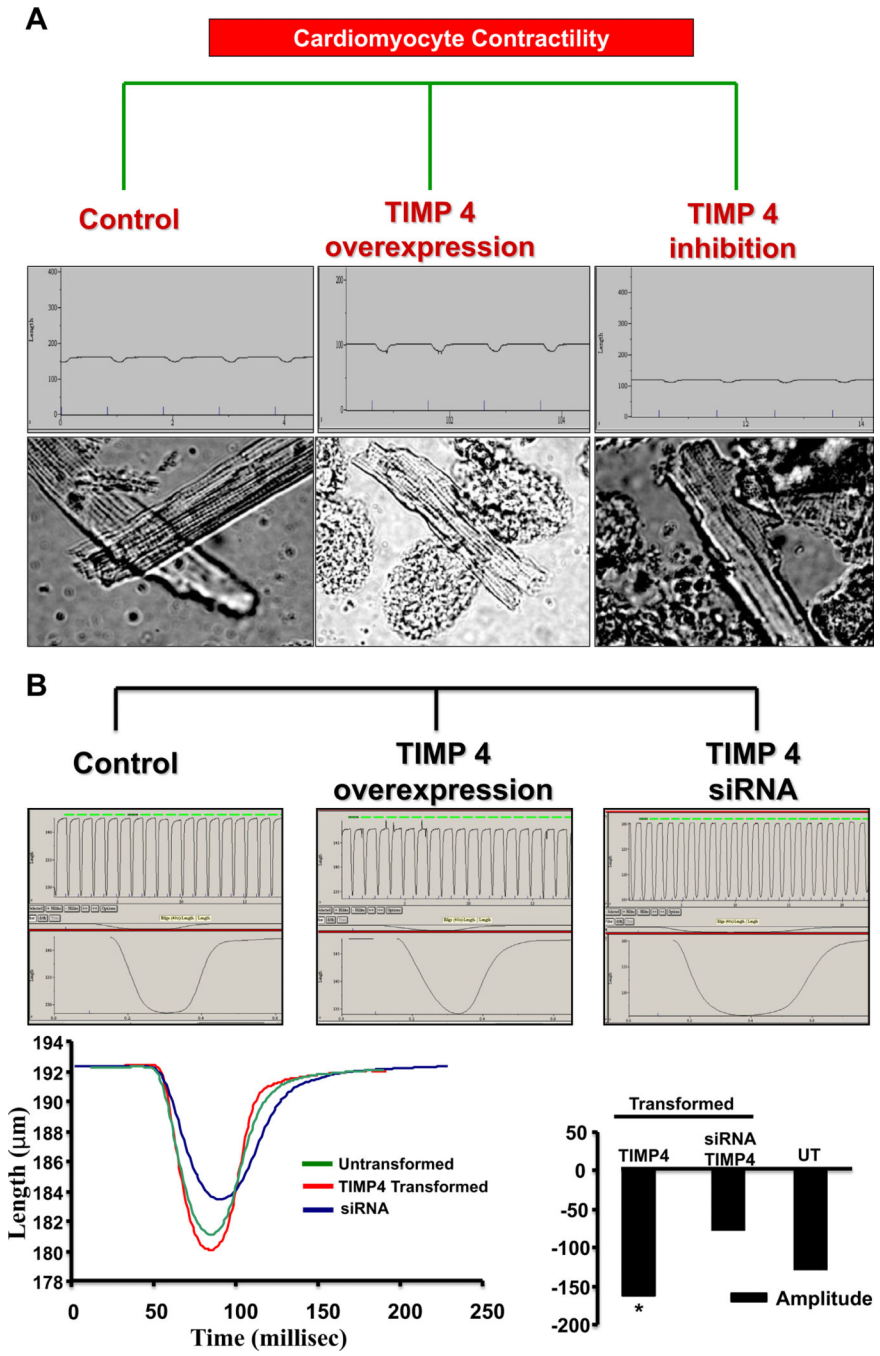

Fig. 3.

The contractility studies in the transformed cardiomyocytes by ION OPTIX: We performed the contractility studies in the cardiomyocytes which were transformed with TIMP4 and TIMP4-siRNA after 6 h and 12 h of transfection. There was increase in contractility in the TIMP4 transformed cardiomyocytes as depicted from the graphs generated by the ION OPTIX measurements (for contractility measurements, n = 20). A). Representative length vs time graphs (as generated by ION OPTIX) showing contractility in the untransformed, TIMP4 transformed and TIMP4-siRNA transformed cardiomyocytes. The data shows the

average of such 20 recordings in different cardiomyocytes. B). The representative length vs time graphs and the amplitude graphs of the untransformed, TIMP4 transformed and TIMP4-siRNA transformed cardiomyocytes. n = 20 and for significance *p = 0.01 as compared to untransformed, Student's t-test.

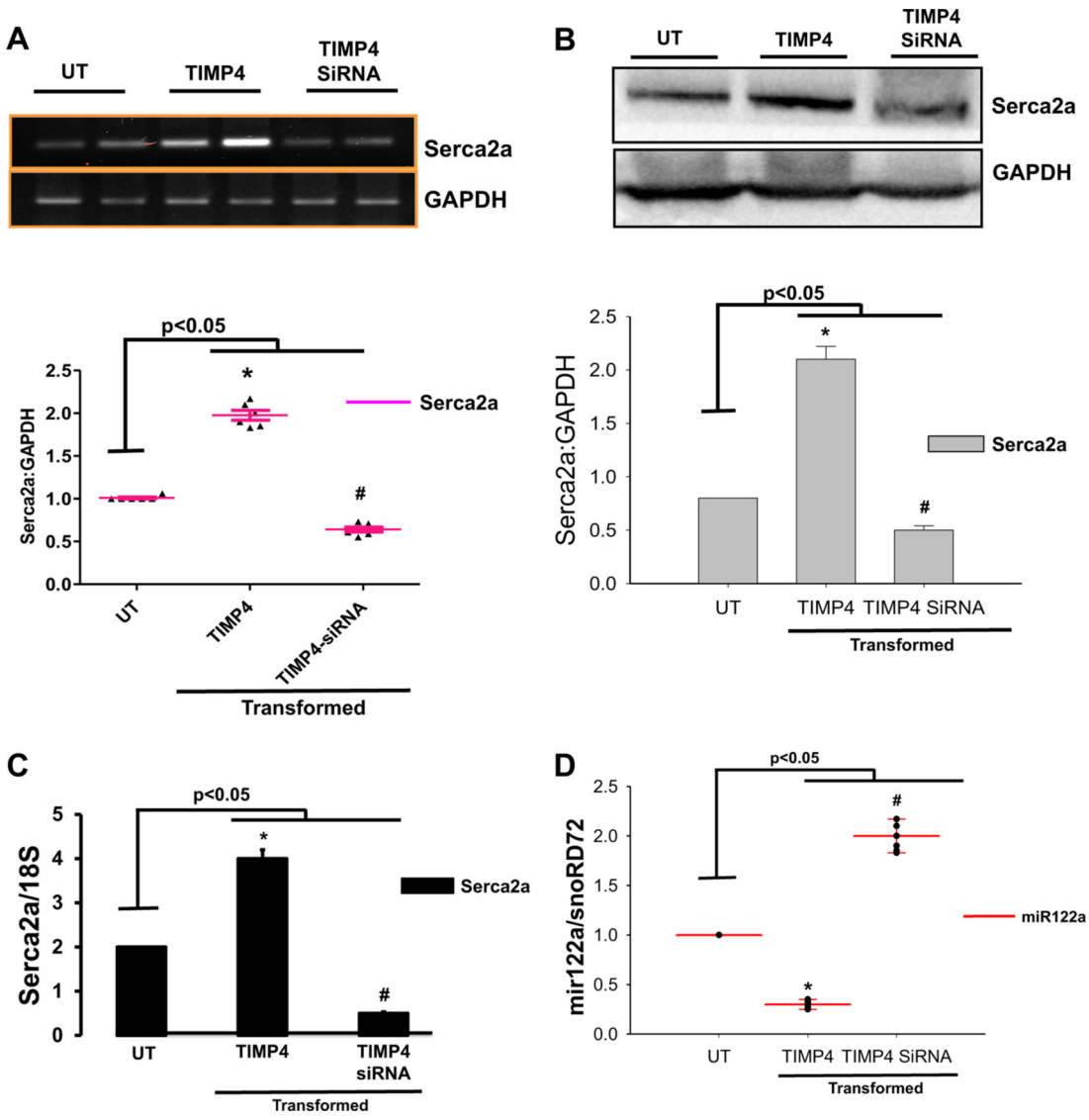

Fig. 4.

The expression of serca2a in TIMP4 transformed cardiomyocytes and mir122a: Serca2a or sarcoplasmic reticulum calcium ATPase2a is a calcium channel in myocytes and it regulates contractility by regulating the levels of calcium. We examined the effect of TIMP4 overexpression on serca2a and found increase in the expression seerca2a with TIMP4. A). RT-PCR showed increase in the expression of serca2a in the TIMP4 transformed cardiomyocytes while in the TIMP4-siRNA transformed myocytes, the serca2a levels were decreased. B). Western blots showed an increase in the expression of serca2a with TIMP4 C). Real time PCR confirmed an increase in the expression of serca2a in the TIMP4 transformed cardiomyocytes as compared to TIMP4-siRNA transformed myocytes. D). The expression of serca2a is tightly regulated by mir122a so we looked into the expression of mir122a in the TIMP4 transformed myocytes. There was a decrease in the expression of mir122a in the cells transformed with TIMP4 as compared to the TIMP4-siRNA transfected cells. #, *p = 0.004 as compared to untransformed; n = 6.

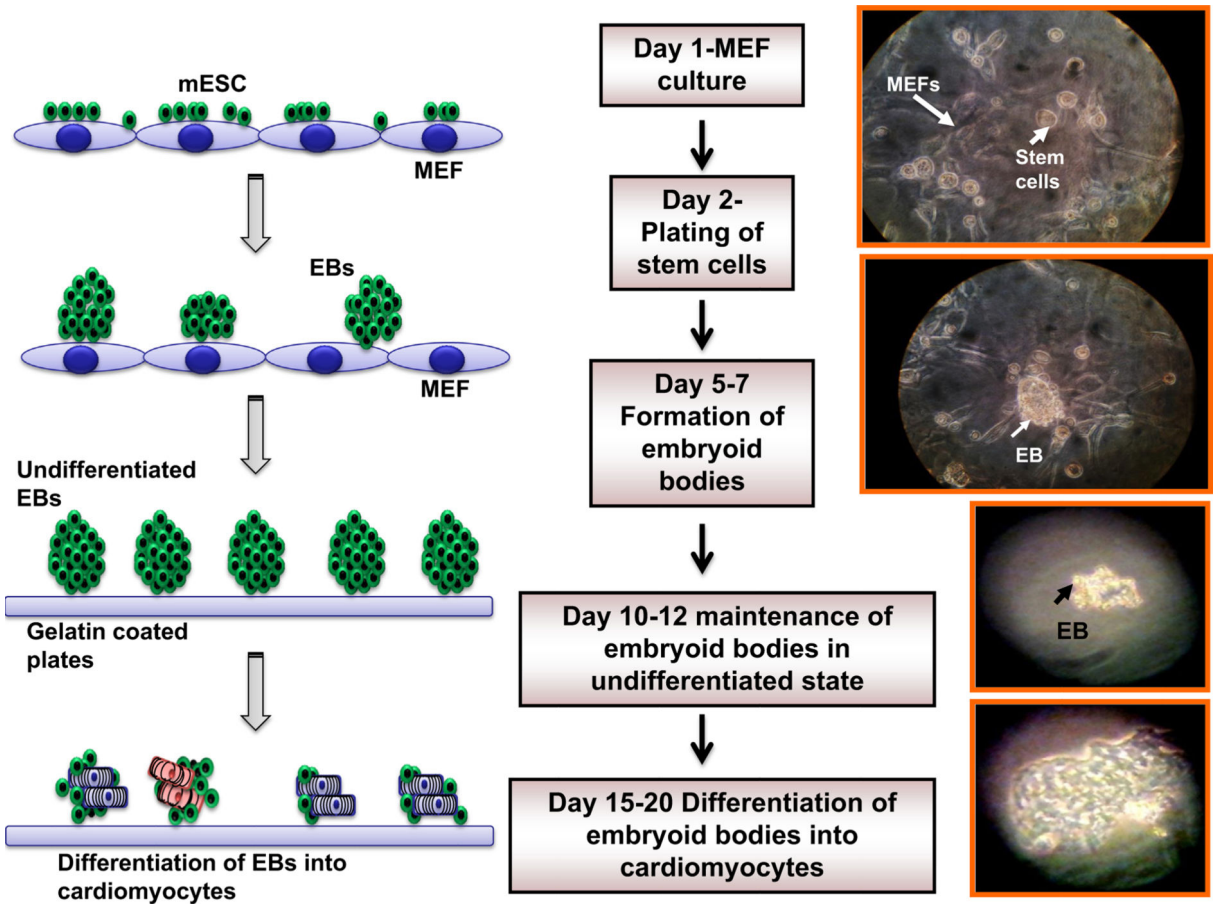

Fig. 5.

Schematic presentation of growth and differentiation of mouse embryonic stem cells to cardiomyocytes. A). The mouse embryonic stem cells were cultured on mouse embryonic fibroblasts in a similar manner as described earlier [47]. To determine whether TIMP4 helps in the differentiation of embryonic stem cells to cardiomyocytes, we treated the embryoid bodies with cardiac extract with and without TIMP4. We used monoclonal antibody for TIMP4 to block the effect of TIMP4 in the cardiac extract. We observed that cells treated with cardiac extract showed distinct phenotypes of cardiomyocytes (the extreme right panel shows pictures obtained from UV–visible microscope-LEICA at 40×). EB-embryoid bodies, MEF-mouse embryonic fibroblasts, mESC-mouse embryonic stem cells.

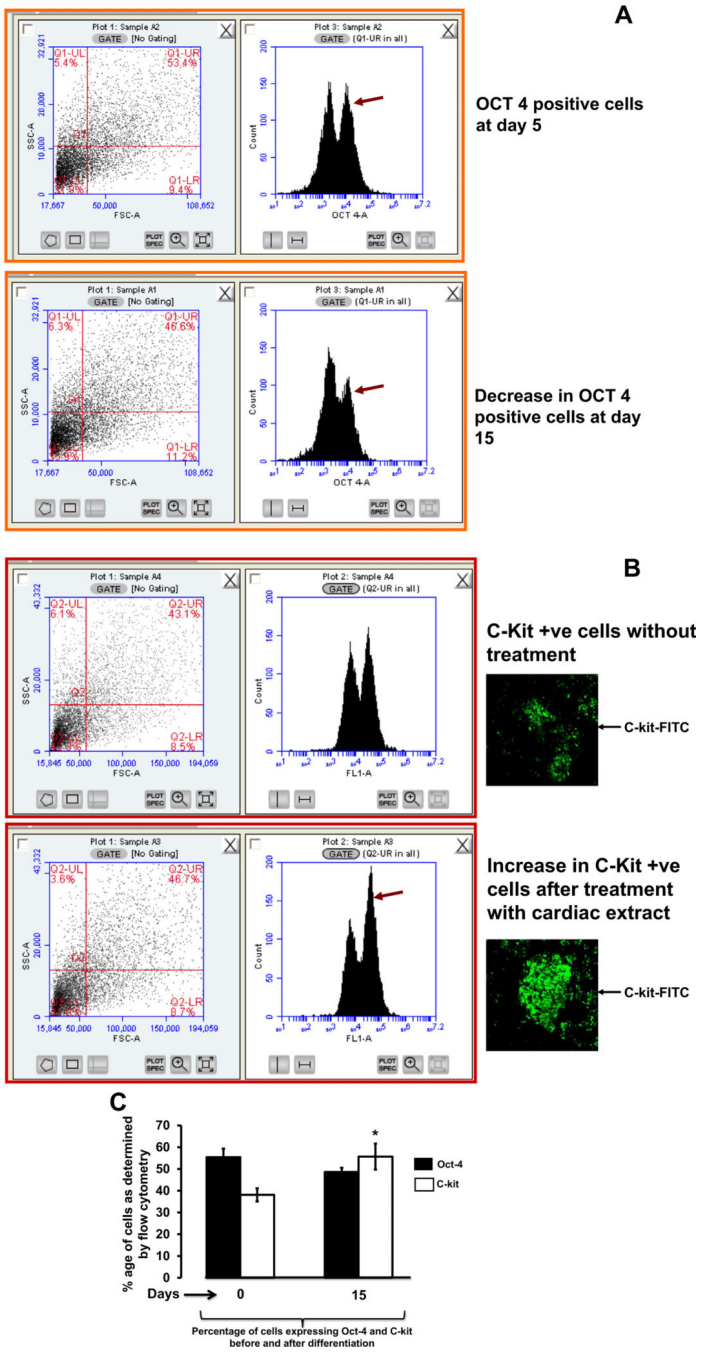

Fig. 6.

Flow cytometry analysis for the determination of Ckit and Oct4 positive cells. To determine the pluripotency of stem cells, we used anti-Oct 4 antibody (rabbit-1:100) along with negative control (rabbit IgG). We used anti-Ckit for determining the differentiation of stem cells into cardiomyocytes. A). There is a decrease in the number of cells expressing Oct 4 after 15 days of differentiation (arrow). B). The cells that were treated with cardiac extract showed increased expression of Ckit as compared to those treated with cardiac extract minus TIMP4 (arrows). The cells stained in green show Ckit-FITC. C). Graph presenting the

number of cells expressing Oct 4 and Ckit as determined by Flow cytometry. *p = 0.01 as compared to 0 day treatment.

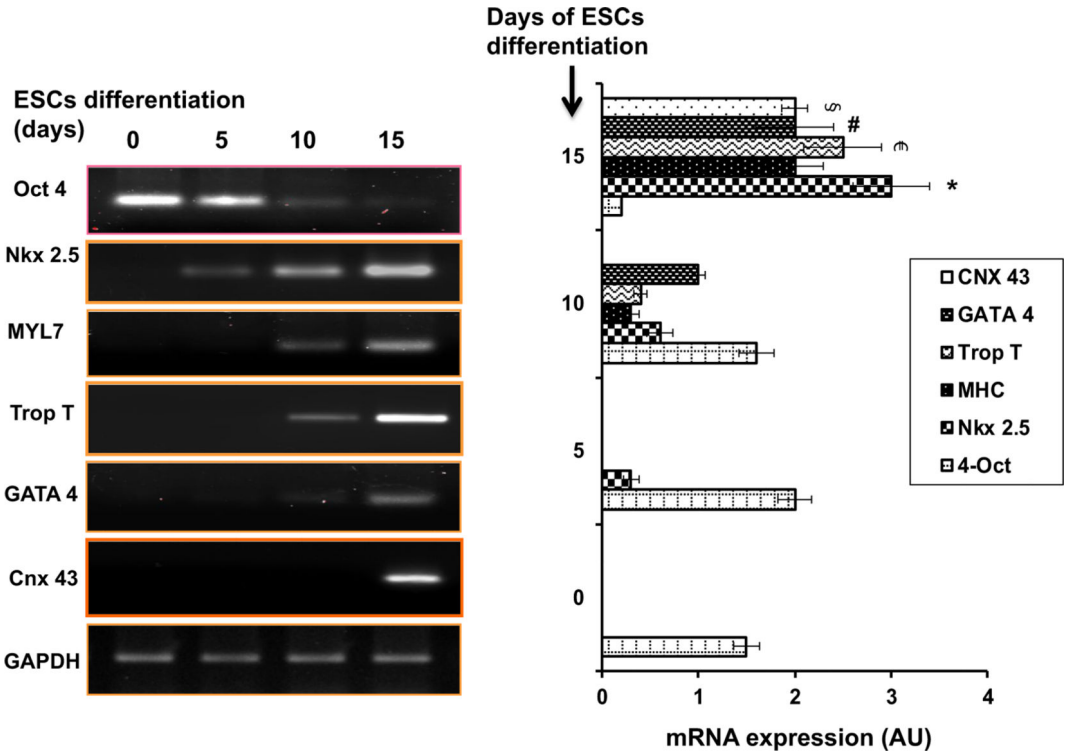

Fig. 7.

Expression of cardiac specific genes. The expression of cardiac specific genes and transcription factors was checked by RT-PCR at days 0, 5, 10 and 15. There was an increase in the expression of GATA4, Nkx2.5, myosin light chain (MYL7), Troponin T and connexin 43 genes. *, #, §, € $p < 0.05$ as compared to 0 day treatment.

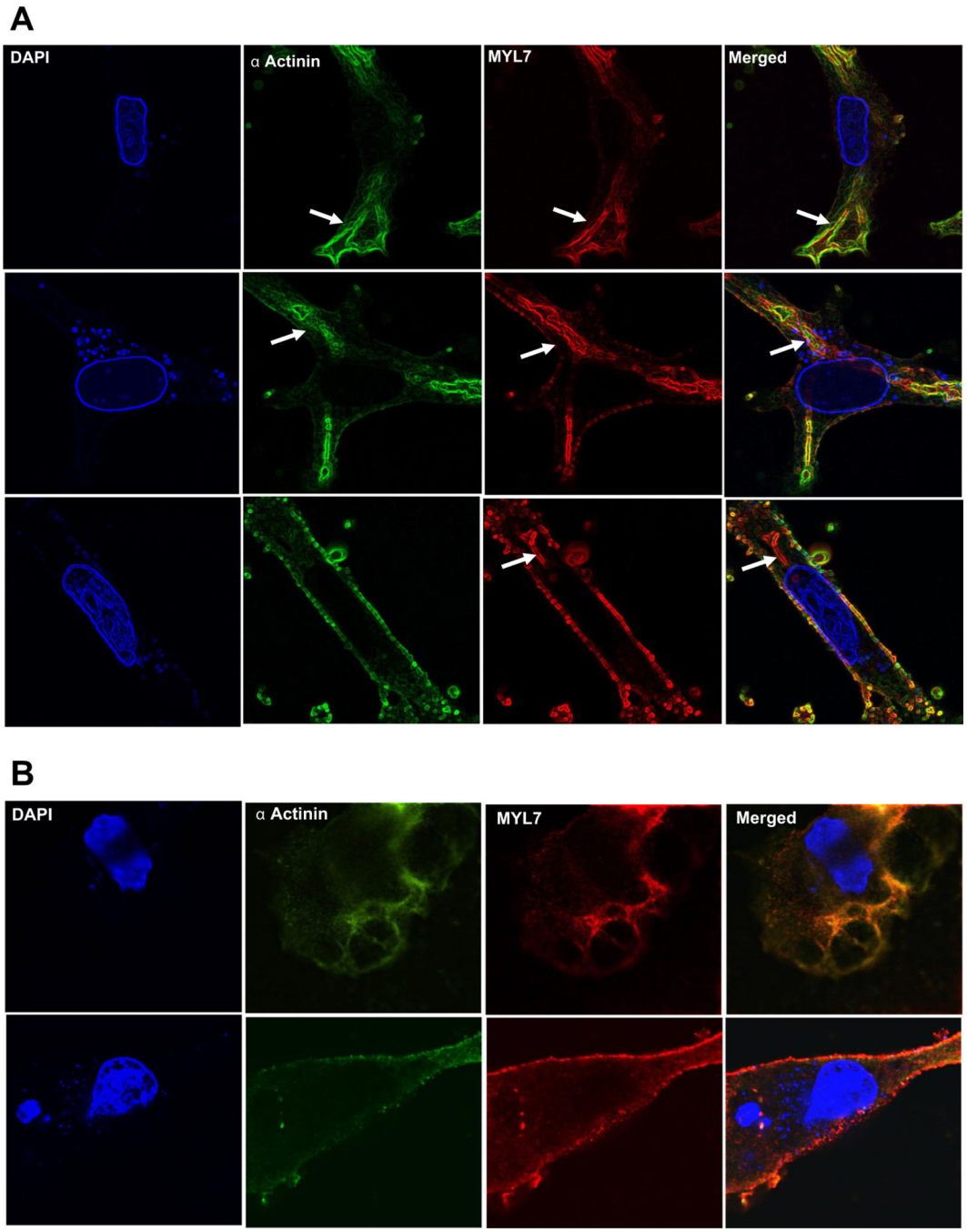

Fig. 8.

Expression of α-actinin and MYL7 (myosin light chain) as determined by immunocytochemistry. The differentiated stem cells showed the expression of α-actinin and MYL7 (myosin light chain) which is the specific property of cardiomyocytes. A) All panels show the cells treated with cardiac extract that differentiate into cardiomyocytes and develop actin and myosin fibers (arrows). B). The first row shows untreated cells and the second row shows cells treated with cardiac extract minus TIMP4 that do not develop actin–myosin fibers.

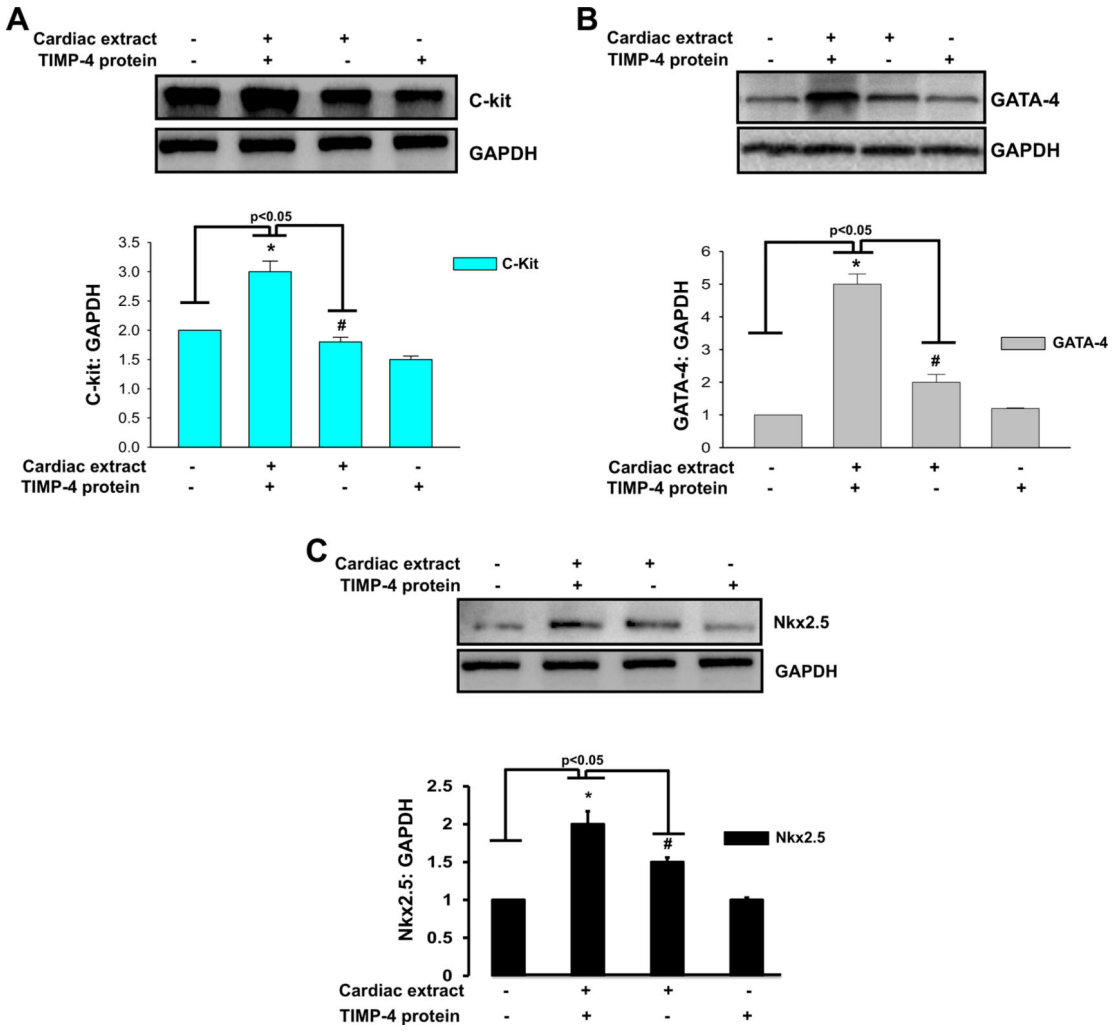

Fig. 9.

Expression of cardiac specific transcription factors in the differentiated stem cells A). Western blot showing CKit expression B). Western blot showing the expression of GATA4 which is a cardiac specific transcription factor in cells treated with cardiac extract. C). Western blots showing the expression of NKx2.5 which is also a cardiac specific transcription factor similar to GATA-4. *p < 0.05 as compared to untreated cells; #p < 0.05 as compared to cells treated with cardiac extract.

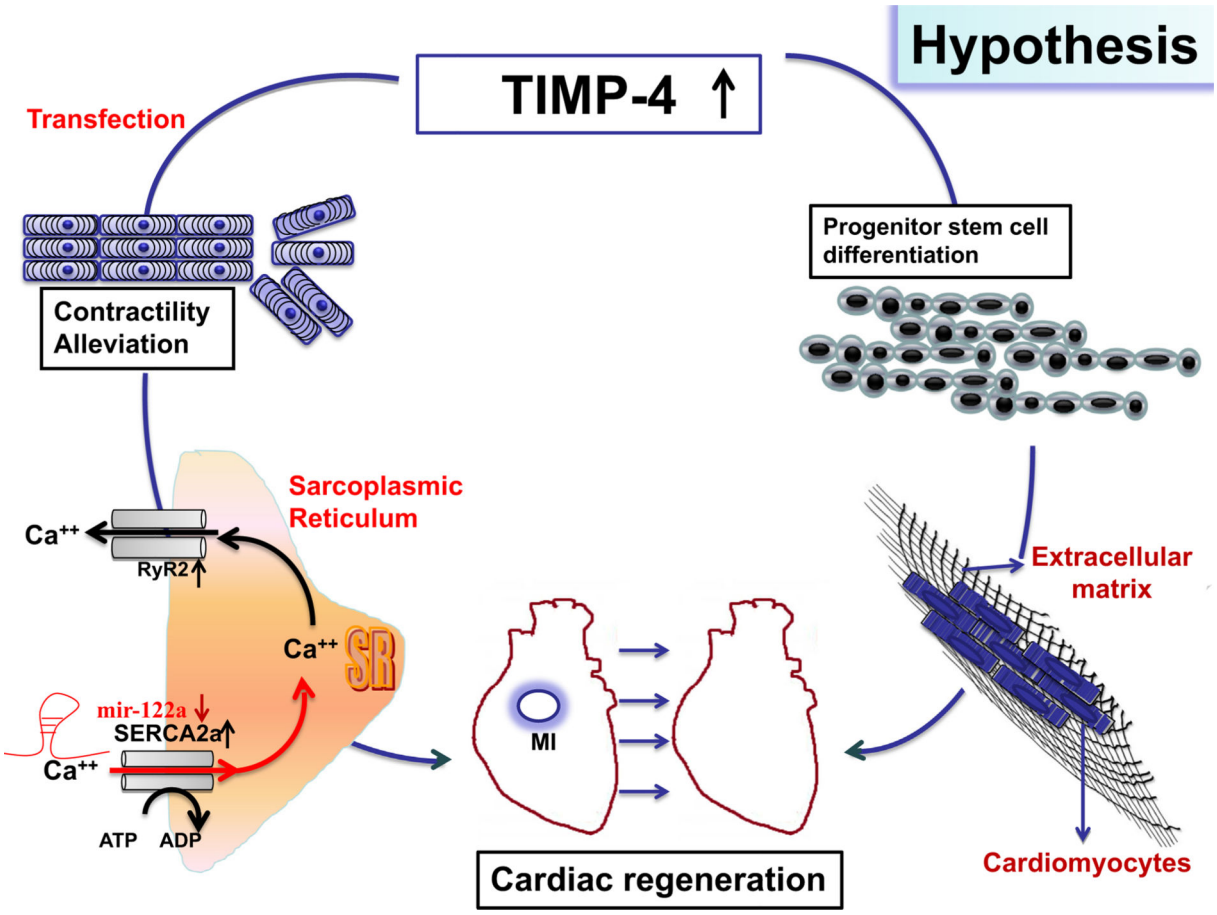

Fig. 10.

Overall picture of how TIMP4 can be effective in cardiac regeneration by alleviating contractility and inducing the differentiation of cardiac progenitor cells into cardiomyocytes.