Exosomes from G Coupled Receptor Kinase (GRK2) Knock-down Cells Suppress Fibroblast Apoptosis Potential Mechanism by Which GRK2 Inhibition Provides Protection after Myocardial Infarction

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Abstract— Heart failure is a progressive condition where the heart cannot pump the amount of blood that the body needs to survive. After Myocardial Infarction (MI), heart muscle dies and scar tissue forms, reducing the heart's ability to pump blood. These scar tissues are formed due to cardiomyocyte and cardiac fibroblast apoptosis. It has been observed that the expression of G-protein coupled receptor kinase 2 (GRK2) increases following MI and reduction of GRK2 expression in animal models prevents progression to heart failure following cardiac injury. Exosomes are known to be active in cell-to-cell communication in the heart and carry many signals for modulation of gene expression including miRNA. Therefore the hypothesis was made that exosomes carrying signals for GRK2 inhibition will prevent fibroblast apoptosis. Mouse fibroblast NIH 3T3 cells were exposed to exosomes carrying GRK2 specific siRNA or control siRNA. Exosomes with either control or GRK2 siRNA reduced fibroblast cell death, however, GRK2 siRNA containing exosomes were more potent inhibitors of apoptosis. A search of the miRNA database, miRbase, to find naturally occurring miRNAs that may have the same effect as GRK2 siRNA and therefore explain the effect seen with exosomes alone, resulted in only one match, miR-761. This miRNA is known to reduce expression of Mitochondrial Fission Factor (MFF) which regulates cardiac fibroblast apoptosis. This data is the first evidence that GRK2 inhibition may protect cardiac fibroblasts from apoptosis. It also provides evidence that naturally occurring miRNA such as miR-761 encapsulated in exosomes may provide more targeted treatment for patients post myocardial infarction thus lowering a patient's risk of heart failure.

Index Terms— heart failure, fibroblast, GRK2, miRNA, siRNA, exosome, miR-761

1 Introduction

Heart disease has been the number one cause of death in America for nearly 90 years in both men and women. Heart disease kills more people in USA than all the cancers combined [1]. Heart failure is caused by the weakening of heart muscles. These weakened muscles are rendered incapable of pumping blood, resulting in pooling of blood within the chambers of the heart further damaging the heart muscles. Myocardial Infarction (MI) or heart attack often leads to heart failure. Ischemic stress and resulting hypoxia during MI causes massive cardiac cell death or apoptosis. Cardiac fibroblasts are closely intertwined with cardiomyocytes in the extracellular matrix (ECM). Initially these fibroblasts regulate changes in the ECM that are critical to the wound healing response (adaptive remodeling). However; over time, the fibroblasts transdifferentiate into myofibroblasts, produce additional extra cellular matrix to replace the lost cardiomyocytes, causing fibrosis and scar tissue formation. This decreases the heart's capacity to pump blood and therefore increases the chance of progression to catastrophic and irreversible heart failure [1], [2].

Once the process of heart failure begins, it is not reversible but the condition can be managed by changes in lifestyle, drugs and surgical intervention – usually targeted at reducing blood pressure and improving blood flow in the heart and in circulation. Despite a large amount of money spent on therapeutic options, the number of patients admitted to hospitals for heart failure in USA has not decreased in last decade resulting in 500,000 new cases every year and in 610,000 deaths. In fact, there has been an increase in the number of younger patients (<65 year age) going into heart failure [1]. Around half of new cases of heart failure in in this population of patients are due to coronary artery disease and many of these patients develop heart failure in the context of acute myocardial infarction [3].

What if one could prevent patients who go through MI from progressing to heart failure? MI induced cardiomyocyte death is devastating because these cells do not constantly renew to replace lost or damaged cells of their kind. Adult stem cells, usually from the subject's own bone marrow, and Cardiac Progenitor Cells (CPC), found exclusively in the heart, have been used to replace new myocytes into injured hearts in an effort to reverse the damage during heart failure. However, adult stem cells were never able make new cardiomyocytes and therefore the benefit was always short lived [4] and CPCs, although effective in generating new cardiomyocytes in animal models of heart injury [5], are not likely to be as effective clinically since the absolute number is woefully low to make any meaningful contribution towards replacing millions of cardiomyocytes that are lost during heart failure.

Another option would be to "increase the protection" of the remaining cardiomyocytes so that they do not keep on dying. A third option would be to ensure that appropriate cardiac fibroblast-mediated cardiac remodeling occurs. G protein-coupled receptors (GPCR) have been identified to regulate almost all processes of cardiomyocytes and cardiac fibroblasts including cell proliferation and cell death. In fact, the most popular class of drugs for heart disease, beta-adrenergic receptor blockers, targets some of these GPCRs [6]. The activity of these receptors is regulated by G protein-coupled Receptor Kinases (GRKs). One of the most abundant GRKs is GRK2, which regulates cardiomyocyte death signaling [7]. Large amounts of GRK2 are expressed in cardiomyocytes and also in cardiac fibroblasts during MI and subsequent heart failure. Increased GRK2 expression and activity in surrounding heart tissue at the time of damage enhances the damage in the form of the fibrosis and scarring of heart muscle, and the more prone a patient becomes to develop heart failure [8].

Research over the past two decades has shown that inhibition of GRK2 expression in the cardiomyocytes and in cardiac fibroblasts has significant protective results in animal models of myocardial infarction and leads to better recovery. Much of this research has been done by creating mouse "knock-out" and transgenic "over-expressor" lines of GRK2. These lines have allowed researchers to observe that GRK2 knockout mice presented with decreased fibrosis in the infarcted area in mice that have undergone MI [8, 9]. It is; however, not so easy to alter the genes of human beings to reflect the knockout of GRK2 expression in animal models. Also, fibroblast function is required for cardiac tissue repair initially so a complete absence of fibroblasts would also not be helpful. One hypothesis that is being tested is whether exosomes secreted by heart cells could be used to regulate GRK2 function.

Exosomes are tiny micro-vesicles released by almost all cells including cardiomyocytes and fibroblasts. Exosomes were initially characterized as a means to shuttle 'cellular garbage' out of the cells [11]]. Recent work has provided evidence of a broader role for exosomes in regulating various cellular and molecular processes including cell-cell communication. Typically, exosomes are 30-100nm in size and are produced by a number of cell types [12]. One of the most fascinating characteristics of exosomes is their ability to carry cell type specific microRNAs (miRNAs) as payload [13].

MicroRNAs (miRNA) are small (approximately 22 nucleotides long) non-coding RNAs that induce gene silencing by binding to target sites found within the 3'UTR of the targeted messenger RNA (mRNAs). Binding of miRNAs to their target mRNAs prevents protein production by suppressing protein synthesis and/or by initiating mRNA degradation. It is thought that individual microRNAs may target as many as 100 different mRNAs [14].

Exosomes secreted by one cell are quickly taken up by other cells [15] and deliver their payload from one donor cell to the recipient cells, thus they may be used to deliver miRNAs to the site of action for a transient effect. A recent study has demonstrated that exosomes, irrespective of their cell of origin, are themselves able to induce cardioprotection after acute myocardial injury [16]. In the same study the authors evaluated the role of a miRNA 144/451 that is known to be elevated during cardiac injury. However, this miRNA, when delivered via the exosomes, had no further cardioprotective impact compared to exosomes alone [16]. As discussed before, inhibition of GRK2 in fibroblasts has been shown to be cardioprotective in animal models [8], [9], [17]. However the exact mechanism of this observation is not understood. It is known that both cardiomyocytes and cardiac fibroblasts secrete exosomes. It has been shown that exosomes secreted by cardiac fibroblasts carry miRNAs to cardiomyocytes causing cellular hypertrophy [18]. Is it feasible then for exosomes secreted by fibroblasts and by cardiomyocytes to also carry signals that impact cell death? What would be the impact of using exosomes to carry GRK2 siRNAs to cardiac fibroblasts? Are

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there naturally occurring miRNAs that could inhibit GRK2 expression and be carried by exosomes?

The hypothesis for this study is that exosomes from cells where expression of GRK2 is silenced by small interfering RNA (siRNA) treatment will protect fibroblasts from hypoxia induced cell death or apoptosis. To test this hypotheses, mouse fibroblast cell line NIH 3T3 (ATCC) were used as a model for cardiac fibroblasts and the effect of miRNAs was simulated using small interfering RNA (siRNA). Exosomes carrying GRK2 specific siRNA were used to knock down (KD) the level of GRK2 expression in fibroblasts that secreted these exosomes. Fibroblasts treated with exosomes with or without the GRK2 specific siRNAs were tested in a hydrogen peroxide stimulated cell death assay to simulate hypoxia mediated cell death during MI. The goal was to determine whether exosomes from GRK2-KD cells would inhibit NIH3T3 cell death. Finally, the sequence of the GRK2 siRNA was used to search a miRNA database (miRbase) to evaluate if naturally occurring miRNAs can have the same effect as the siRNAs.

2 Material and Methods

2.1 Cells and cell culture:

NIH3T3 cells were grown in Dulbecco's Modified Eagle's (DME) medium with 10% Bovine serum supplement at 37°C. To split cells into multiple plates or wells, media was suctioned off and cells were incubated with 1 mL of Trypsin EDTA for 1-2 minutes at 37°C. 1 mL of DMEM 10% bovine serum media was then added to stop detachment. The resultant mixture was centrifuged for 2 minutes at 2000 rpm. The cell pellet was re-suspended in 2 mL DMEM 10% media. One mL cell mixture was then pipetted carefully into each of 2 new plates. Nine mL DMEM 10% media was pipetted over cells to fill each plate. Prior to hypoxia simulation study, cells were washed in serum free DMEM.

2.2 siRNA transfection:

For GRK2 knock down, 12 nanomole mouse GRK2 siRNA-sense strand sequence: GCUCAGUUUCAUCCUGGAUtt, antisense strand sequence: AUCCAGGAUGAAACUGAGCtt, or a scrambled sequence mis-match siRNA (control) was mixed with HiPerfect Transfection Reagent and used to transfect NIH3T3 cells according to manufacturer's instructions (Qiagen, Valencia, CA).

2.3 Exosome preparation and treatment of NIH3T3 cells with exosomes:

Exosomes from culture media of either control NIH3T3 cells or NIH3T3-GRK2 KD cells were isolated using ExoQuick kit (System Biosciences) according to manufacturer instructions. Briefly, cells were allowed to grow overnight at 37°C and culture media was collected and centrifuged at 3000 rpm for 15 minutes to separate out cells and debris. The supernatants were transferred to new tubes and 2 mL ExoQuick TC solution was added to each tube. Tubes were refrigerated overnight and then centrifuged 1500 rpm for 30 minutes. The clear supernatants were removed, and the pellet at bottom of tube was re-centrifuged at 1500 rpm for 5 minutes to get rid of excess liquid. The exosome pellet was re-suspended in 100 microliters of Phosphate Buffered Saline (PBS) and stored at 4°C.

Approximately 100,000 NIH3T3 cells were exposed to exosomes isolated from either control NIH3T3 cells or NIH3T3-GRK2 KD cells (as described above) for 24 hours in 10% DMEM with serum. NIH 3T3 cells were either treated with 50 micromolar (μM) H_2O_2 for 24 hours to mimic hypoxia or left untreated. Following H_2O_2 treatment, cells were immediately harvested for PCR, fluorescence microscopy and TUNEL cell death assay.

2.4 Flourescence microscopy to detect exosome uptake

Exosomes are difficult to visualize and quantitate using bright-field microscopy due to their small size. NIH3T3 fibroblasts treated with siRNA were therefore labeled with the red fluorescent dye PKH26 (Sigma-Aldrich) according to manufacturer's instructions. PKH26 labeling occurs by partitioning of this lipophilic dye into cell membranes, staining is nearly instantaneous. Briefly, exosome treated cells were trypsinized, re-suspended in PBS and centrifuged at 400 X g for 5 minutes. A 2X cell suspension was prepared by adding 1 mL of Diluent C (Catalog Number G8278) to the cell pellet and re-suspending with gentle pipetting to insure complete dispersion. Immediately prior to staining, a 2X Dye Solution was made (4×10^{-6} M) in Diluent C by adding 4 mL of the PKH26 ethanolic dye solution to 1 mL of Diluent C. The 1 mL of 2X Cell Suspension was rapidly added to 1 mL of 2X Dye Solution and mixed by pipetting. Images were taken using a fluorescence microscope immediately after staining.

2.5 PCR for GRK2 knock-down:

Total RNA was isolated from NIH3T3 cells transfected with siRNA using TRIzol (Life Technologies) and cDNA was synthesized by reverse transcription of the RNA with Superscript II (Life Technologies) as recommended. Real-time PCR was performed in duplicate with a 1:100 dilution of the cDNA on a MyIQ real-time PCR detection system (Bio-Rad) with the SYBR Green PCR Master Mix (Applied Biosystems). The oligonucleotide primers to examine expression of mouse GRK2 were - forward primer 5'-CCCTCTCACCATCTCTGAGC-3', reverse primer 5'-

CGGTTGGGGAACAAGTAGAA-3'. For normalization, 18S rRNA was used - forward primer 5'-TCAAGAACGAAAGTCGGAGG-3', reverse primer 5'-GGACATCTAAGGGCATCAC-3'). Real-time PCR conditions were as described earlier [19]. Specificity of PCR products was confirmed by gel electrophoresis. Real-time PCR data analysis was carried out using the ΔΔct method [20] and relative RNA levels (GRK2 vs. 18s RNA) were calculated.

2.6 TUNEL Cell Death assay:

Cells were fixed with 4% paraformaldehyde (PFA) and stained using the DeadEnd™ Fluorometric TUNEL System kit (Promega) per manufacturer instructions. This kit measures the fragmented DNA of apoptotic cells by catalytically incorporating TMR_{red}-12-dUTP at 3´-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, enzyme (rTdT), which forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. The TMR_{red}-12-dUTP-labeled DNA then can be visualized directly by fluorescence microscopy. Briefly, after fixation with paraformaldehyde, cells were washed with PBS and permeabilization solution was added for 2 minutes on ice. Cells were re-washed with PBS and incubated with TUNEL reaction mixture for 60 minutes at 37 °C. Cells were counter-stained with DAPI to identify the nuclei and analyzed for TUNEL staining using a fluorescence microscope (wavelength range of 570-620 nm for red dye detection and 358-461 nm for DAPI) after being re-washed with PBS.

2.7 Statistical analysis:

All values in the text and figures are presented as mean ± SEM of independent experiments. Statistical significance was determined by student's t-test. Probabilities of 0.05 or less were considered to be statistically significant.

2.8 miRNA database query:

The GRK2 siRNA sequence was used to query miRbase (http://www.mirbase.org/)

3 Results

Prior publications have indicated that in the setting of cardiac injuries, including myocardial infarction (MI), increased levels of GRK2 (for example, in transgenic GRK2 overexpressing mice) results in increased fibrosis and reduced heart function in mice, while removal of GRK2 specifically in the heart by gene knock out technique (GRK2-KO mice) renders mice somewhat protected by MI-induced injury and fibrosis [9], [10]]. GRK2 specific small interfering RNA (siRNA) was used to knock down the level of GRK2 in NIH3T3 fibroblast cells. Before isolating exosomes, it was ascertained using RT-PCR that the siRNA specifically reduced GRK2 mRNA levels in NIH3T3 cells (Fig 1). As shown in Figure 1, GRK2-siRNA transfection reduced the level of GRK2 mRNA by 64% compared to control cells.

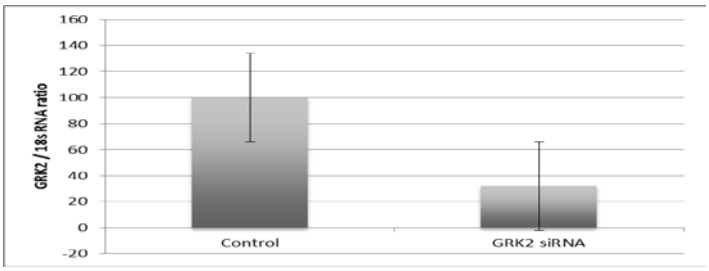

Fig 1. GRK2 mRNA expression is decreased in GRK2 KD-NIH3T3 cells. GRK2 and 18s RNA levels were determined by RT-PCR. Data is shown as a ratio ± SEM (p=0.001)

The second step was to ensure that the isolated exosomes were intact, were not impacted by the transfection of siRNA, and could be taken up by cells. Exosomes secreted into the culture media of NIH3T3 cells transfected with control or GRK2 siRNA were isolated using the ExoQuick kit (System Biosciences) according to manufacturer instructions and as described in the material and methods section of this paper. Exosomes from both cell types were labeled with a red-fluorescence dye, PKH26, prior to incubation with NIH3T3 cells for 1 hour (Fig 2). Fluorescence microscopy was performed to visualize the exosome internalization by the cells. Exosomes from both types of cells were efficiently internalized by the cells as shown in figure 2. These data show that knock down of GRK2 does not affect the cell internalization property of the exosomes.

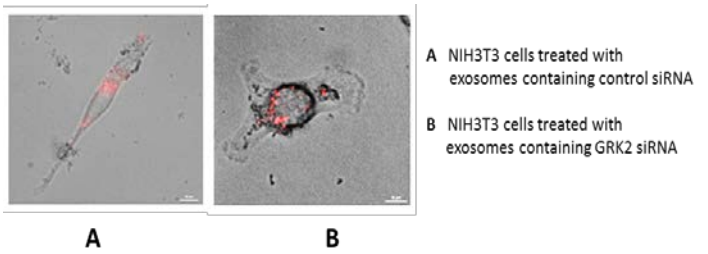

Fig 2. Exosomes are internalized by NIH3T3 cells. NIH3T3 fibroblasts were treated with exosomes containing control and GRK2 siRNA and labeled with the red fluorescent dye PKH26. Images were taken using a fluorescence microscope and show uptake of the red dye labeled exosomes into NIH3T3 cells.

Next, the ability of exosomes from control and GRK2 knockdown (KD) cells to protect the fibroblasts from hypoxia mediated programmed cell death (apoptosis) was investigated. Apoptosis was induced in NIH3T3 cells by treating them with

hydrogen peroxide (50 μM H_2O_2,) for 24 hours. Cells were simultaneously treated with either control or GRK2-KD cell derived exosomes or left untreated. Cells not treated with H_2O_2 served as control for cell death. Percentage of apoptotic cells were determined by TUNEL staining as described in methods section (TUNEL positive, red-pink stained nuclei are apoptotic cells, blue stain depicts presence of intact nuclei). Under the fluorescence microscope, several images of each well were captured around clusters of cells present. After imaging was done for each of the 12 wells, all images were collected and organized by which of the four groups they fell into. Figure 3 shows representative images (2 per group) of the four experimental groups.

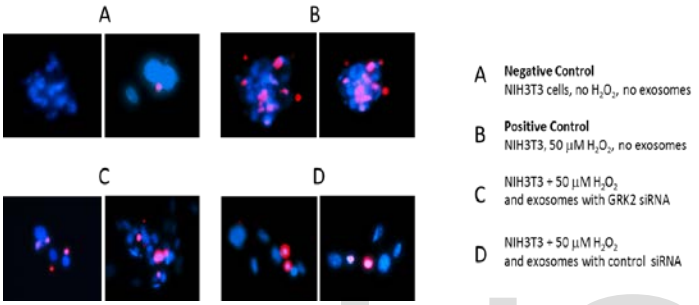

Fig 3: Representative images of TUNEL stain (2 images per group shown) (A) NIH3T3 fibroblasts without H_2O_2 or exosomes (negative control for ischemia-induced cell death) (B) NIH3T3 fibroblasts exposed to 50μM H2O2 but no exosomes (positive control), (C) NIH3T3 fibroblasts exposed to 50μM H2O2 and exosomes with GRK2 siRNA (D) NIH3T3 fibroblasts exposed to 50 μM H2O2 and exosomes with control siRNA. Blue color (DAPI) stain shows the presence of cell nuclei. Red-pink color stain shows TUNEL positive nuclei.

The presence of TUNEL positive cells is indicative of cell death. TUNEL positive cells in each group were counted manually across 24 visual fields (wells) and the means were converted into percentage of all cells in the field. This data is shown in figure 4.

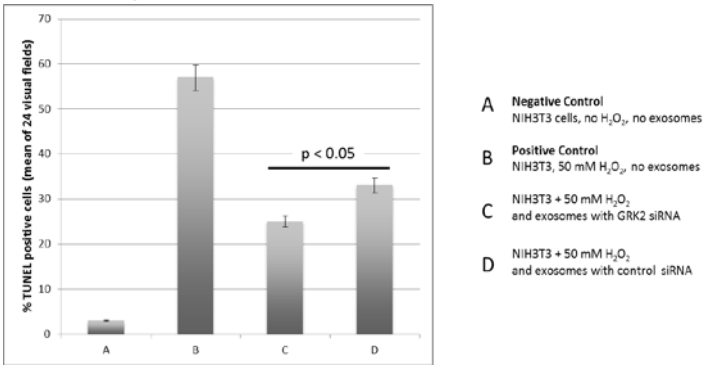

Fig 4: Comparison of percentage cell death in control and experimental groups as evaluated by TUNEL analysis.

Group A, which was exposed to neither H_2O_2 or to exosomes, was the negative control, and was expected to show very little cell death if any. True to expectation, only 1.92% of the cell count for this group was TUNEL positive or dead. Group B was the positive control, exposed only to 50 μM H_2O_2. This group was expected to have the most severe cell death, and this was displayed well in the data. 57.66% of the cells in-group B were dead. Group C was exposed to the 50μM H_2O_2 and then to GRK2siRNA knockdown exosomes. Group D was also exposed to the 50μM H_2O_2, and then was exposed to the control siRNA transfected NIH3T3 exosomes.

As hypothesized, cells exposed to GRK2 siRNA exosomes had far lower percentages of hypoxia mediated cell death than untreated cells. It was surprising to note that control siRNA exosomes also protected fibroblasts from apoptosis, although cells exposed to the GRK2 siRNA knockdown exosomes were more effective. Group C (GRK2 siRNA exosomes) had a cell death percentage of 25.66%, while Group D (control siRNA exosomes) showed 34.33% cell death.

In this study, siRNA is used as a synthesized simulation for naturally occurring miRNAs that could target GRK2. It was observed that exosomes that were transfected with control siRNA still had a protective effect on the NIH 3T3 fibroblasts. Several naturally occurring miRNAs are expressed in the heart and are known to regulate gene expression in cardiac cells [21]. Therefore it is possible that the control exosomes carried a naturally produced miRNA that has the capacity to regulate cardiac fibroblast apoptosis. Using the sequences of the sense and antisense strands of the selective siRNA against GRK2 a BLAST alignment search for known miRNAs was conducted in miRbase. Only one known miRNA (both human and mouse sequences) matched the GRK2 siRNA sequence (figure 5). This miRNA; miR-761 targets and reduces expression of the Mitochondrial Fission Factor (MFF) gene. MFF has been shown to regulate cardiomyocyte apoptosis [22]. Therefore, a plausible explanation for the protective properties of exosomes is that they contain a naturally occurring miRNA; miR-761 or another as yet un-identified miRNA capable of regulating cardiac cell apoptosis.

Accession	ID	Query start	Query end	Subject start	Subject end	Strand	Score	Evalue	Alignment
MIMAT0012768	mdo-miR-761	4	18	5	19	+	66	2.6	Align
MIMAT0003893	mmu-miR-761	4	17	5	18	+	61	6.8	Align
MIMAT0009373	bta-miR-761	4	17	5	18	+	61	6.8	Align
MIMAT0009936	cfa-miR-761	4	17	5	18	+	61	6.8	Align
MIMAT0010364	hsa-miR-761	4	17	5	18	+	61	6.8	Align
MIMAT0012793	mml-miR-761	4	17	5	18	+	61	6.8	Align
MIMAT0012817	ptr-miR-761	4	17	5	18	+	61	6.8	Align
MIMAT0012853	rno-miR-761	4	17	5	18	+	61	6.8	Align
MIMAT0012923	eca-miR-761	4	17	5	18	+	61	6.8	Align

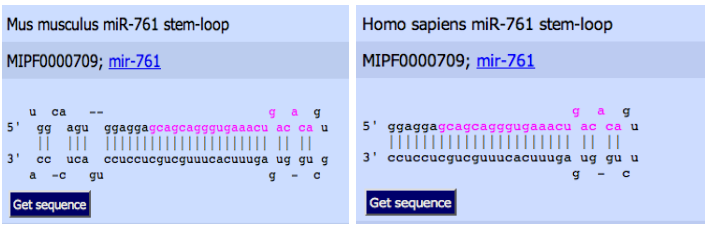

Fig 5. miRbase sequence alignment results show that GRK2 siRNA sequence matches only the miR-761 sequence across species. Sequence alignment with mouse and human miR-761 against the GRK2 siRNA sequence (pink script) is shown.

4 Discussion

GRK2 has been shown to be a key regulator of cardiac contractile function. Up-regulation of GRK2 is an early event after myocardial injury and silencing myocardial GRK2 expression can prevent or rescue progression to heart failure in certain animal models [23]. However, the mechanism by which GRK2 promotes cell death in the heart is not fully understood. The data presented in this manuscript shows that GRK2 mRNA knockdown reduces apoptosis of NIH3T3 cells in culture, a model for cardiac fibroblasts. [9],[10]

An interesting observation in this experiment is that exosomes from cells transfected with control siRNA which have intact GRK2 levels also demonstrate the ability to reduce hypoxia induced fibroblast apoptosis. It has been observed that exosomes alone, irrespective of the cell of origin, can provide cardio-protection and reduce cardiomyocte death in an ischemia-reperfusion model [16]. The data presented in this paper adds evidence for the potential impact of exosomes on cardiac fibroblasts.

Cardiomyocyte regeneration is very limited and a slow process. Given that nearly 25% of cardiomyocyte can die within hours of a single MI event, repair after such a catastrophic event is primarily attributed to fibroblast mediated scar formation, rather than cardiomyocte regeneration. Thus exposure to exosomes with GRK siRNA could be an interesting targeted approach to prevent cardiac cell death following MI.

Many miRNAs are thought to regulate cardiac function and even cardiac fibroblast reprogramming [24]. Since the GRK2 siRNA had a strong impact on fibroblast apoptosis, one of the queries in this paper was whether there might be naturally occurring miRNA that could share sequence identity with the siRNA used in this study. A search of the miRNA sequence database (miRbase) identified only one complete match – miR-761, which is one of the many miRNAs that is linked to cardiomyocyte apoptosis [22]. It is interesting that miR-761 is responsible for the regulation of mitochondrial fission factor, which plays a role in mitochondrial fission via inhibition of mitochondrial fission factor (MFF). MFF expression is induced during hydrogen peroxide exposure and ischemia-reperfusion injury and it has been shown that miR-761 protects cardiomyocytes from hypoxia mediated apoptosis by decreasing MFF expression [22]. Thus miR-761 is present in the cardiomyocyte and cardiac fibroblast environment during oxygen stress conditions such as MI. It is well known that exosomes act as carriers of many miRNAs including perhaps miR-761. This may explain the protective effect of exosomes alone on fibroblasts in this study, although driven by a completely different mechanism of action. This hypothesis needs to be evaluated in in future studies, potentially in animal models of MI.

On a broader scale, this data opens up a variety of options to explore new therapies such as miRNAs, aimed at reducing the likelihood of progression to heart failure following a MI event. miRNAs usually target multiple genes and may have detrimental effects if delivered systemically. Exosomes may provide an interesting naturally occurring delivery tool for miRNA that could be delivered directly to the heart at the most appropriate time.

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