Research Article

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Differentiation of Mouse Embryonic Fibroblasts (MEFs) into Cardiomyocytes Using Human-Derived Cardiac Inducing RNA (CIR)

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ABSTRACT

The present study explores an RNA we have discovered in human heart that induces differentiation of mouse embryonic stem cells and human induced pluripotent stem cells into cardiomyocytes in vitro. We have designated this RNA as Cardiac Inducing RNA or CIR. We now find that CIR also induces mouse embryonic fibroblasts (MEF) to form cardiomyocytes in vitro. For these studies, human-derived CIR is transfected into MEF using lipofectamine. The CIR-transfected mouse fibroblasts exhibit spindle-shaped cells, characteristic of myocardial cells in culture, and express cardiac-specific troponin-T and cardiac tropomyosin. As such, the CIR-induced conversion of the fibroblasts into cardiomyocytes in vitro appears to take place without initial dedifferentiation into pluripotent stem cells. Instead, after CIR transfection using a lipofectamine transfection system, over the next 8 days there appears to be a direct transdifferentiation of >80% of the cultured fibroblasts into definitive cardiomyocytes. Fewer than <7% of the untreated controls using non-active RNA or lipofectamine by itself show cardiomyocyte characteristics. Thus, discovery of CIR may hold significant potential for future use in repair/regeneration of damaged myocardial tissue in humans after myocardial infarction or other disease processes such that affected patients may be able to return to pre-heart-disease activity levels.

Keywords

RNA, Fibroblasts, Cardiomyocytes.

Abbreviations

BSA: bovine serum albumin; CIR: Cardiac Inducing RNA; cTnT: cardiac-specific troponin T; DNA: Deoxyribonucleic acid; DMEM: Dulbecco's Modified Eagle Medium; ESC: embryonic stem cells; FBS: Fetal Bovine Serum; FITC: fluorescein isothiocyanate; iPSC: induced pluripotent stem cells; MEF: Mouse Embryonic Fibroblasts; MIR: myofibril inducing RNA; mRNA: Messenger RNA; NTP: nucleoside 5'-triphosphate; PBS: phosphate-buffered saline; PCR: Polymerase chain reaction; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; RNA: Ribonucleic acid; RT-PCR: Reverse transcription polymerase chain reaction; SGSH: N-sulfoglucosamine sulfhydrolase; WHO: World Health Organization.

Introduction

The major obstacle to heart regeneration after myocardial infarction is that heart muscle cells, or cardiomyocytes, die and are replaced by fibrous scar tissue rather than new cardiac muscle cells. The fibrous scar tissue supports the damaged heart temporarily, but in the long term, it weakens the organ's overall ability to contract normally and increases the risk of a further heart attack which may

lead to heart failure. This is a major reason why heart disease is the leading cause of death worldwide. According to statistics from the World Health Organization (WHO), approximately 17.9 million people die each year from cardiovascular disease, which is 31% of all deaths on Earth. Of these 17.9 million deaths, 15.2 million (or 85%) were due to heart attack or stroke [1]. By 2030, it is estimated that failing hearts alone will kill more than 23 million people every year [2]. Currently, the only option for a severely failing heart is organ transplantation. For almost 20 years, since the early 2000s, scientists have been attempting to use stem cells from an individual's own body to grow new muscle in damaged hearts. Stem cells are capable of producing many of the various types of cells in the body, and the hope is that this will include the ability to generate new cardiomyocytes to replace those damaged by myocardial infarction or other heart disease processes. To date, there has been very limited success with being able to repair damaged areas of the heart following in vivo myocardial infarction or treating heart failure by using stem cells.

In our laboratory, we have discovered three unique Cardiac Inducing RNAs (CIR), one initially in the Mexican axolotl (salamander), *Ambystoma mexicanum* [3], and subsequently, two CIRs, derived from human heart [4-8], that have the ability to turn non-muscle cells into cardiomyocytes with normal myofibrils. We undertook an approach initially developed in our laboratory, to use the axolotl CIR to restore heart development and function in cardiac non-function mutant embryonic axolotls [3]. Also, in our earlier studies, we found that CIR from the axolotl, and subsequently human CIRs, have the capability of directing the fate of non-muscle mouse embryonic stem cells (or ESCs) and human skin-derived induced pluripotent stem cells (iPSCs) to express cardiac-specific proteins and differentiate into cardiomyocyte-like phenotypes in vitro. We cloned and sequenced the active CIR components in the axolotl and two human CIRs. The axolotl CIR was found to be unique in the gene-bank databases [3], while the two human CIRs showed sequence homologies identified with: (1) Clone #6, human exon 8 of N- sulfoglucosamine sulfhydrolase (SGSH) [6-8] and (2) Clone #30, the human COX2 superfamily of genes in the mitochondrial genome [4]. Both human CIRs turned out to be functional homologs of the axolotl (salamander) CIR, since all three of these RNAs promote cardiac myofibril formation in cardiac non-function mutant axolotl hearts as well as in mouse ESCs and human iPSCs in vitro [4,6,7], in spite of a lack of nucleotide primary sequence homology for the three CIRs examined. Thus, neither of the human active CIRs showed sequence homology with the original axolotl CIR or with each other, but both have secondary structures as revealed by the Gene Bee program (use provided courtesy of the Belozersky Institute, Moscow State University, Russia), that are strikingly similar to the active normal axolotl CIR, and to each other, but dissimilar to the non-functional mutant axolotl CIR [4]. We believe that the similar secondary RNA structures may account for their unique abilities to promote myofibrillogenesis and transform non-muscle cells into cardiomyocytes [4]. (Note: we originally termed the axolotl Cardiac Inducing RNA, Myofibril Inducing RNA or MIR; we now refer to all of these RNAs, axolotl and human, as Cardiac Inducing RNA or CIR).

Our earlier published work used a cardiac-mutant axolotl (salamander) embryonic heart bioassay [9,10]. Non-contracting mutant embryonic hearts, ordinarily lacking organized myofibrils, were placed in organ cultures with CIR derived from normal axolotl embryonic anterior endoderm or normal axolotl embryonic heart or human heart. After 48-72 hours, the mutant axolotl hearts in organ culture begin to beat rhythmically, and immunofluorescent staining with contractile protein antibodies (i.e., anti-tropomyosin, anti-cardiac specific troponin-T, anti-α-actinin) revealed the appearance of organized sarcomeric myofibrils of normal morphology. These studies were expanded to mouse embryonic stem cells (ESCs) and human iPSCs (induced pluripotent stem cells) derived from prepuce skin. Immunofluorescent staining of the human iPSCs and mouse ESCs cultured after transfection with CIR using a lipofectamine transfection system and stained with cardiac-specific troponin T (cTnT) and cardiac tropomyosin clearly showed their differentiation into definitive cardiomyocytes. We also found that mutant axolotl hearts [4], mouse embryonic stem cells and human iPS cells [6] respond to the CIR lipofectamine-induced transfection by differentiating into cardiomyocytes with cardiac-specific proteins, characteristic spindle-shaped cardiomyocyte shapes, and cardiac sarcomeric myofibrils of normal morphology in culture. Untreated controls treated with lipofectamine alone or with non-active RNAs in lipofectamine vesicles do not differentiate into cardiomyocytes.

Materials and Methods

Cell Culture, Reagents, Cell Monitoring During Culture Period

The mouse embryonic fibroblast (MEF) cell line, obtained from the American Type Culture Collection (Manassas, Virginia) (STO, ATTC®CRL-1503) was expanded and grown routinely in growth medium on DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) [11]. Cells in culture were regularly passaged when they reached full confluence. The transcription reaction mixture was prepared using a MAXIscriptW T7 Kit, Ambion # AM1314M, as follows: 1 µg of DNA from the PCR product, 2 µL of X10 transcription buffer, 2 µL of T7 Enzyme Mix and 1 µL of 10 mM NTP and the volume was adjusted to 20 µL using nuclease-free water. The reaction was allowed to proceed for 2 hours at 37ºC. The CIR was extracted and purified using ethanol precipitation and resuspended in nuclease-free water. The concentration of CIR was determined spectrophotometrically by the Nano drop method; the cells then were transfected with the active CIR using a lipofectamine transfection system at concentrations of 1, 6 and 12ng CIR (RNA)/ul in Opti-MEM medium as follows [12-14]. In 15 ml tubes, 564 µl of Opti- MEM® I Reduced Serum Medium (Invitrogen #31985062) was combined with 36 µl of Lipofectin® Transfection Reagent (Life Science, #18292011). The required amount of RNA to make a final solution concentration of 12 ng/µl in 6 ml of medium was prepared and incubated for 30 min at 37ºC. The volume then was adjusted with Opti-MEM to a final volume of 6 mL RNA/Opti-MEM solution and filtered through a 0.2 µm Millipore filter to prevent bacterial or other microorganism/microparticle contamination. The culture medium was aspirated from the cells and replaced with RNA/Opti-MEM solution

and incubated in a cell culture incubator (at 37ºC and 5% CO_2) overnight and for an additional seven days. On a daily basis the cell morphologies of the CIR-transfected control cultures were monitored using an Olympus inverted microscope. After seven full days, the morning of the eighth day following CIR-transfection, the cells in the cultures were fixed, immuno-stained and processed for observation using an IX73 Inverted Olympus fluorescent microscope and an Olympus FV3000 Laser Confocal Microscope System.

Fixation, Staining and Confocal Microscopy of Cultured Cells

CIR-treated, non-inducing RNA-treated, lipofectamine-only treated or completely untreated control cells in culture were fixed in 4% paraformaldehyde for 30 min, rinsed in phosphate-buffered saline (PBS) with 3% bovine serum albumin (BSA) for 3 min, permeabilized in 0.1% Tween-20 and 3% BSA, and stained overnight at 4ºC with the specific primary mouse-derived hybridoma antibodies diluted 1:75 with PBS [9-13]. The cells were then rinsed with PBS and 3% BSA for 3 min and stained with the secondary fluorescently-labelled goat anti-mouse polyclonal antibody diluted 1:75 with PBS at 4ºC in a dark environment for 1 h. The cells were immunofluorescently stained for cardiac tropomyosin and cardiac specific troponin-T. The primary antibodies were monoclonal antibodies from mouse, and the secondary antibodies were Goat F (ab) antimouse monospecific polyclonal antibodies labelled with a fluorescein isothiocyanate (FITC) tag excited at 490 nm (Abcam, Cambridge, MA). The cells were imaged and analyzed using an Olympus IX73 Inverted fluorescent microscope to identify and localize the presence of tropomyosin and cardiac troponin-T in the cells. To obtain more detailed images and quantitation of the immunofluorescently-stained cardiac specific troponin-T and cardiac tropomyosin, an Olympus FluoView® FV3000 Laser Confocal Microscope was used. Intensity of fluorescence staining detected in the confocal microscope was quantified by using ImageJ software.

Results

Mouse Embryonic Stem Cells Transfected with Cardiac Inducing RNA-6 (CIR-6)

For positive controls in our current experiments designed to test the effects of CIR-6 (derived from active clone #6 in our cDNA library) transfection on differentiation of mouse embryonic fibroblasts into cardiomyocytes in vitro, mouse embryonic stem cells (ESCs) transfected with CIR-6 were used as in our earlier experiments [6-8]. The mouse ESCs are seen to form tight stem cell colonies with smooth edges in vitro when viewed by phase contrast microscopy [6,7] (not shown here). After transfection of the cells with CIR using a lipofectamine transfection approach, by 4 days in culture, the mouse ESCs differentiate into characteristic spindle-shaped morphologies (Figure 1A) and express cardiac-specific troponin-T (Figure 1B) as illustrated by immunofluorescent staining. When the mouse ESCs are transfected with non-active RNAs or lipofectin only, the stem cells do not show characteristic cardiomyocytes shapes in culture, but rather show random shapes, mostly non-spindle in nature (Figure 1C). In addition, the stem cells treated with the non-active RNAs or lipofectin only, do not show significant staining for cardiac specific troponin-T or tropomyosin, but rather show only background staining for these cardiac myofibrillar proteins—cardiac troponin-T staining is illustrated here (Figure 1D). As the cells continue to differentiate in culture after CIR transfection, using a functional homologue of CIR-6 (RNA-499C) [7] as with CIR-6 the ESCs show organized myofibrillar structures when immunostained with cardiac anti-troponin-T (Figure 1E), anti-tropomyosin (Figure 1F) and anti-α-actinin (Figure 1G) by 8 days in culture. Mouse ESCs cultured in the same way, but treated with lipofectin only or non-active RNAs (derived from clones #4 and #291) do not develop these typical cardiomyocyte shapes in culture nor do they have significant staining for cardiac-specific troponin-T, cardiac tropomyosin, or α-actinin [6,7].

Morphological Studies

Mouse Embryonic Fibroblasts Transfected with Cardiac Inducing RNA (CIR)

Lipofectamine transfection of mouse embryonic fibroblasts (MEFs) with CIR-6 causes the fibroblasts to react very similarly to mouse ESCs in that the mouse embryonic fibroblasts also show an induced expression of cardiac protein markers, including cardiac-specific troponin-T (Figures 2A,B,C) and cardiac tropomyosin (Figures 2D,E,F), as revealed by immunohistochemical staining. Moreover, transfection with the active CIR-6 changes the typically branched fibroblast morphologies (Figure 2A) into more characteristic spindle-shaped cardiomyocytes-like structures (Figures 2B,C). The mouse fibroblasts begin to express cardiac specific troponin-T and cardiac tropomyosin after 4 days in culture, even while they still exhibit characteristic branched fibroblast morphologies (Figure 2A,B). As differentiation of the fibroblasts appear to progress toward definitive myocardial-like phenotypes, the cells lose their exaggerated branched appearances and take on more spindle shapes while beginning to form definitive myofibrillar structures (Figures 2A,B,C,D,E,F). Early sarcomeric myofibril staining patterns are evident by 8 days in culture (Figures 2D,E,F). It is clear from the present study, that the cultured fibroblasts from mice react similarly to the way mouse ESCs do when transfected with CIR-6, using our in vitro lipofectamine transfection approaches previously published [6,7] (Figure 1). Fibroblast cells not transfected with active CIR do not express significant amounts of the cardiac marker proteins as shown by only background fluorescent staining with the cardiac-specific troponin-T and cardiac tropomyosin antibodies.

Quantitative Studies

Cardiac Troponin-T and Tropomyosin Protein Accumulation in CIR Transfected Fibroblasts

In quantitative studies based upon immunofluorescent staining for cardiac tropomyosin and cardiac specific troponin-T using ImageJ software, in combination with immunofluorescent laser confocal microscopy, transfection of cells with escalating concentrations of 1, 6 and 12 ng/µl of CIR-6 show increasing levels of both cardiac specific troponin-T protein expression (Figure 3 and 4) and cardiac tropomyosin protein expression (Figures 5 and 6) visible as increased emitted fluorescent staining intensity. Also,

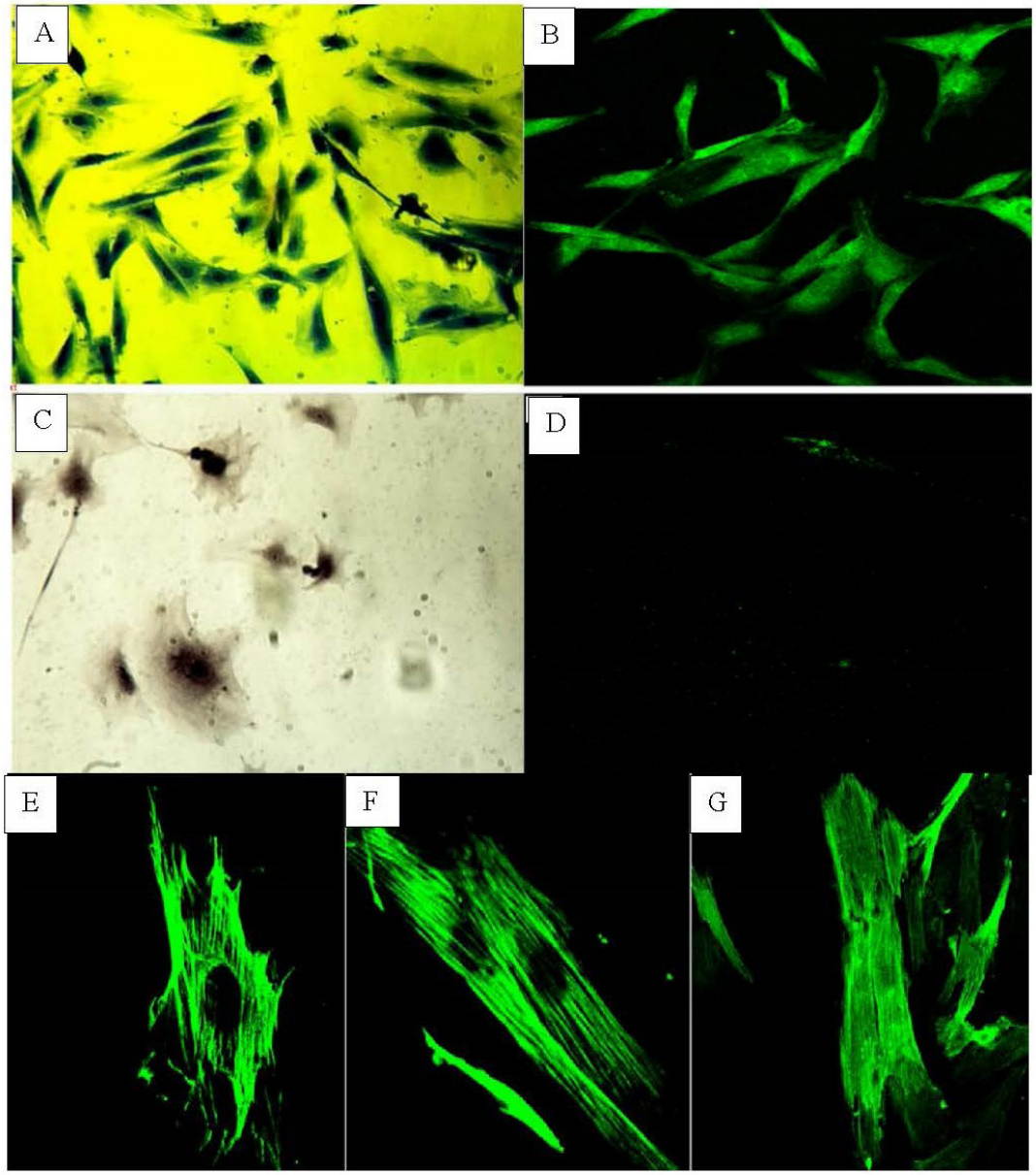

Figure 1: Cultured mouse ESCs after transfection with CIR-6 using lipofectamine take on spindle shapes (A) and stain positively using anti-cardiac troponin-T antibodies (B). The mouse ESCs treated with lipofectamine only, but lacking CIR-6 do not display characteristic early cardiomyocyte shapes in vitro, but rather show variable undifferentiated cell morphologies (C). Furthermore, the ESCs not transfected with CIR-6 do not express significant amounts of cardiac contractile proteins-staining for troponin-T as illustrated here (D). However, mouse ESCs transfected with CIR-6 using lipofectamine express cardiac specific proteins including cardiac specific troponin-T (E), cardiac tropomyosin (F) and α-actinin (G) as evidenced by immunofluorescent microscopy. In fact, when the CIR-6 transfected mouse ESCs are immunostained for the above cardiac contractile proteins, they show significant staining for contractile proteins in greater than >80% of the cells after 8 days in culture. In addition, CIR transfected ESCs cultured for 8 days show myofibrillar structures oriented longitudinally and, usually, exhibit spindle and rectangular shapes characteristic of definitive cardiogenic lineages in vitro (E, F, G). (Note: Figures E,F,G are adopted from reference 7 with permission of the authors).

we created Excel graphs of the staining intensities in correlation with the quantities of transfected RNA to estimate the amounts of cardiac troponin-T protein expression (Figures 3 and 4) and cardiac tropomyosin protein expression (Figures 5 and 6). There is a clear direct dose-response correlation with the quantities of CIR transfected into the cells via lipofectamine vesicles present in the cultures and the quantities of the cardiac proteins expressed in the cells (Figures 3,4,5,6).

In our studies of accumulated cardiac specific troponin-T and cardiac tropomyosin proteins in the trans-differentiating fibroblasts, as revealed by ImageJ quantitation software used in combination with epifluorescent immunofluorescent microscopy and laser confocal microscopy, we have discovered that the fibroblasts in culture transfected with 12 ng/ul of CIR-6 in lipofectamine vesicles show more than >80% cardiac cell phenotypes of the cultured cells based on the expression

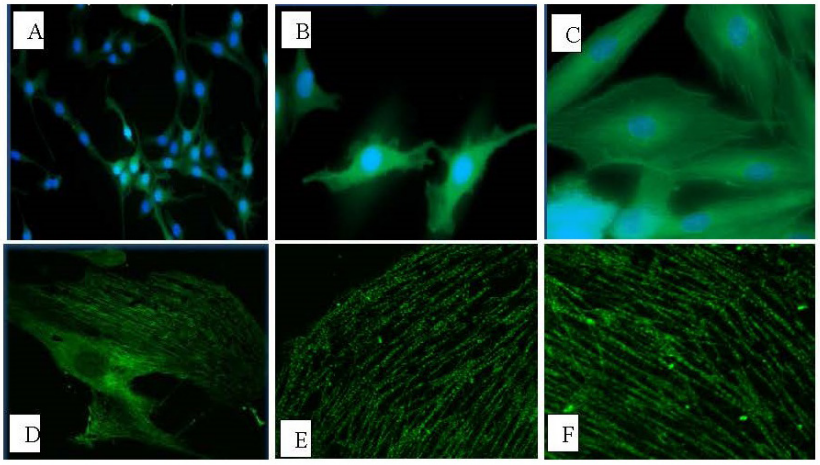

Figure 2: Cultured mouse embryonic fibroblasts after transfection with CIR-6 using lipofectamine to introduce the CIR-6 into the cells in vitro. A. Mouse fibroblasts transfected with CIR after 4 days in culture, immuno-stained using cardiac-specific troponin-T monoclonal antibodies. The mouse fibroblasts express cardiac specific troponin-T (shown) and cardiac tropomyosin (not shown) after 4 days in culture, even while the cells clearly still exhibit characteristic fibroblast-type branching morphologies; B. With progressing development in culture after CIR-6 transfection, the fibroblasts progress toward more definitive cardiomyocytes spindle-shaped morphologies and most of the long fibroblast-like extensions appear to be "withdrawn" into the central part of the cell and at the same time, the staining intensity for cardiac specific proteins (cardiac-specific troponin-T, shown, and tropomyosin, not shown) have increased in the cell cytoplasm, first around the area of the nuclei and then distributed throughout the cytoplasm of the cell; C. The fibroblasts by 6-8 days CIR post-transfection have clearly transformed into spindle-shaped cardiomyocytes-type morphologies and most of the cardiac specific troponin-T (shown here) and tropomyosin-stained cells show thin smooth nascent myofibril-like structures; D, E, F. By the 8th day post CIR-transfection, more than 80% (>80%) of the cultured fibroblast cells have been transformed into cardiogenic cells in culture with organized nascent, sarcomeric, myofibrils when immuno-stained with cardiac specific troponin-T or cardiac sarcomeric tropomyosin (shown). Fibroblast cultures without lipofectamine transfection show very little staining for cardiac-specific troponin-T or cardiac tropomyosin and the cells do not show in vitro myocardial cell morphologies, but rather remained fibroblast-like in appearance and morphology throughout the 8 day experimental period and beyond.

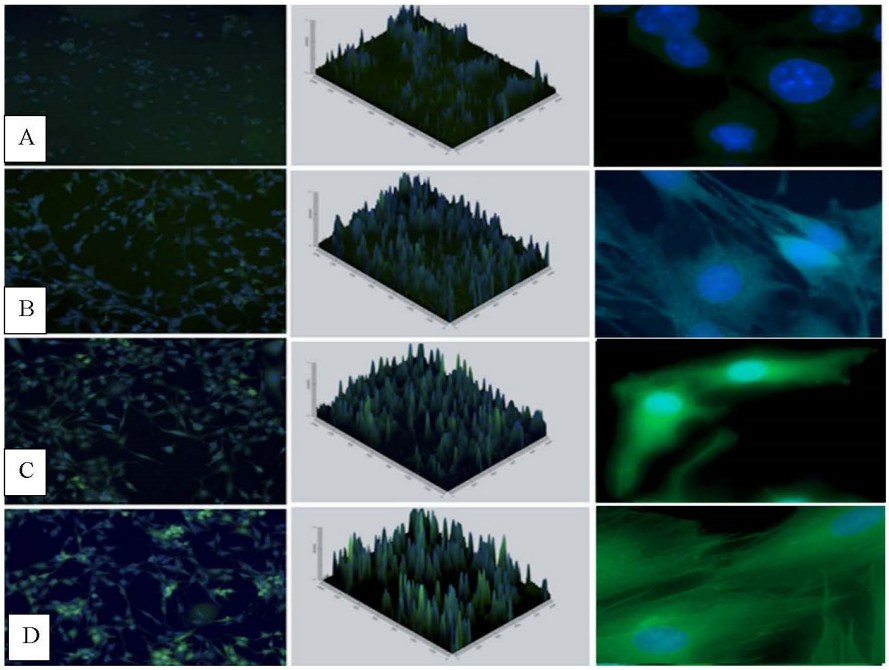

Figure 3: Low magnification micrographs (10X) are shown in the left hand vertical column while high magnification images (40X) are shown in the right vertical column. The images in the center represent quantifications of the fluorescently stained cells in culture using ImageJ software to analyze the laser confocal microscopy FITC immunofluorescence troponin-T staining and the blue nuclear staining with DAPI. Thus, the green fluorescence shows staining with anti-troponin-T antibodies: A. Control mouse fibroblasts without CIR-6 treatment show no detectable troponin-T expression; B. Mouse fibroblasts transfected with 1ng/ul of CIR-6 in culture show slight levels of troponin-T staining; C. Mouse fibroblasts transfected with 6ng/ul of CIR-6 in culture express an intermediate quantity of troponin-T and multiple cells with cardiomyocytes-like morphologies; D. Mouse fibroblasts transfected with 12 ng/ul of CIR-6 show significant expression for troponin-T and have a large percentage of the fibroblasts showing cardiomyocyte-like morphologies.

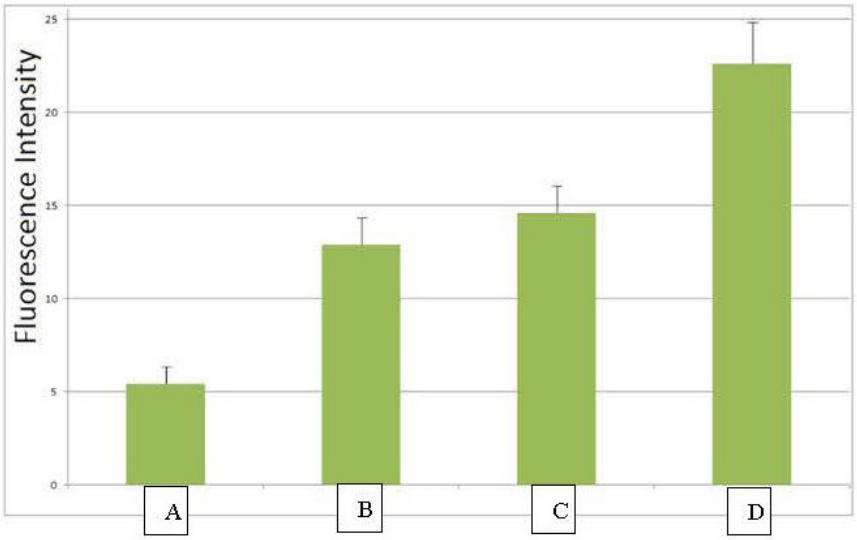

Figure 4: The relative fluorescence intensity of cells stained with anti-troponin-T antibodies was calculated for the escalating concentrations of CIR-6 transfecting the fibroblasts (A=0 ng/ul, B=1 ng/ul, C=6 ng/ul, D=12 ng/ul). There is a clear direct dose-response relationship between increased troponin-T expression and increased CIR-6 transfected into the fibroblasts.

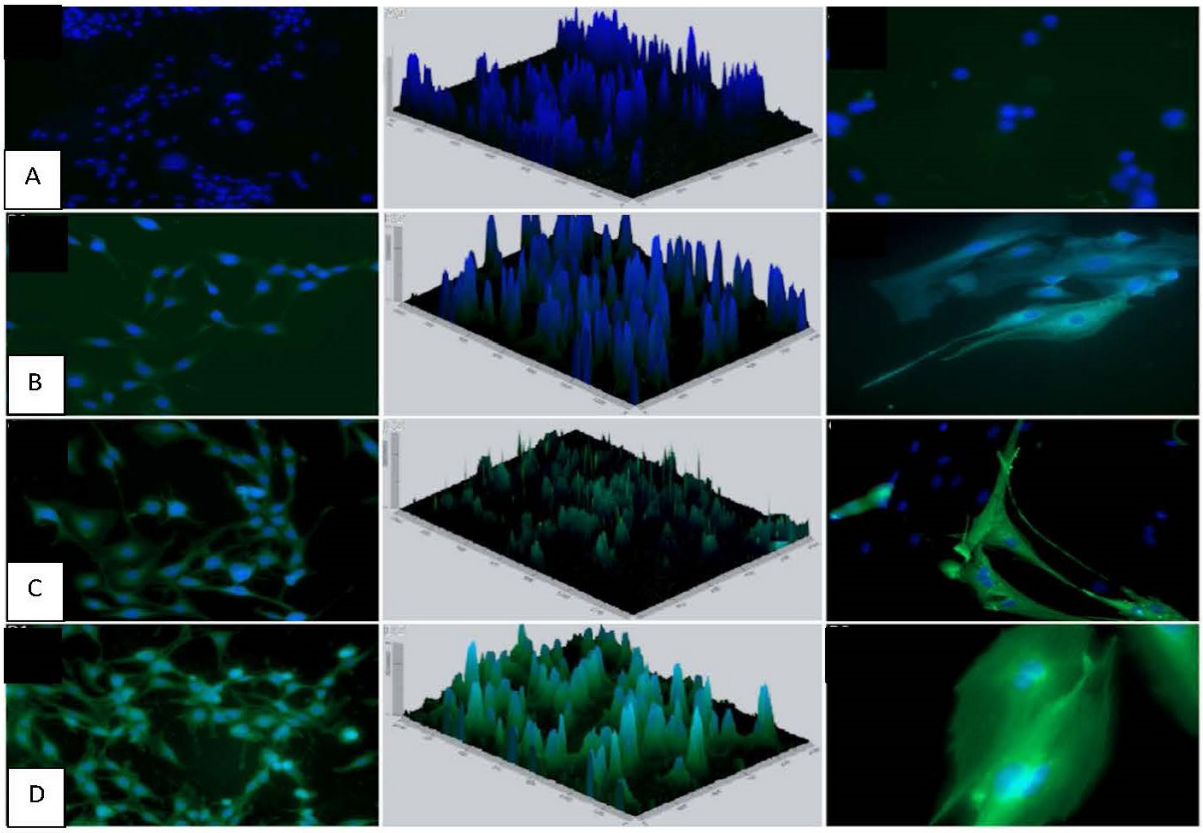

Figure 5: Low magnification micrographs (10X) are shown in the left hand vetrical column while high magnification images (40X) are shown in the right vertical column. The images in the center represent quantifications of tropomyosin in the FITC immunofluorescently stained cultured cells using ImageJ software analyzed laser confocal microscopy. The green staining shows cells stained with FITC-labelled antitropomyosin antibodies and the blue staining shows nuclei stained with DAPI. The micrograph staining is as follows: A. Control mouse fibroblasts without CIR-6 treatment show no tropomyosin expression; B. Mouse fibroblasts transfected with 1ng/ul of CIR- 6 in culture show slight levels of tropomyosin staining; C. Mouse fibroblasts transfected with 6ng/ul of CIR-6 in culture express an intermediate quantity of tropomyosin and multiple cells with cardiomyocyte-like morphologies; D. Mouse fibroblasts transfected with 12 ng/ul of CIR-6 show significant expression for tropomyosin and have a large percentage of the fibroblasts showing cardiomyocyte-like morphologies.

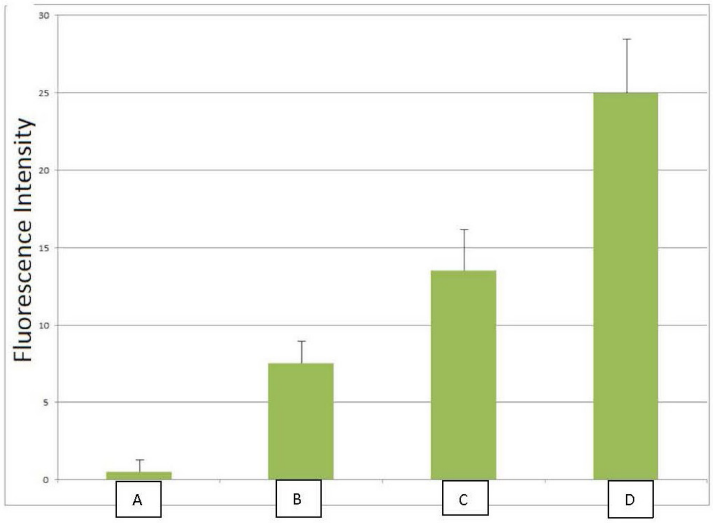

Figure 6: Emitted FITC fluorescent intensity of immunofluorescent staining for tropomyosin in fibroblasts cultured for 8 days after an initial transfection of increasing quantities 1ng/ul to 12ng/ul of CIR-6 using our lipofectamine transfection protocol. The fluorescent intensity shows clear dose response increases in tropomyosin expression relative to transfecting the CIR-6 in increasing concentrations. A. 0 ng/ul control shows low background fluorescent staining; B, 1 ng/ul has a modest level of staining; C, 6ng/ul shows intermediate fluorescent staining, D, 12ng/ul CIR-6 shows a significantly higher level of staining intensity indicating an increased level of accumulated cardiac tropomyosin protein compared to lower quantities of transfected CIR-6 into the mouse fibroblasts at the lower transfected concentrations.

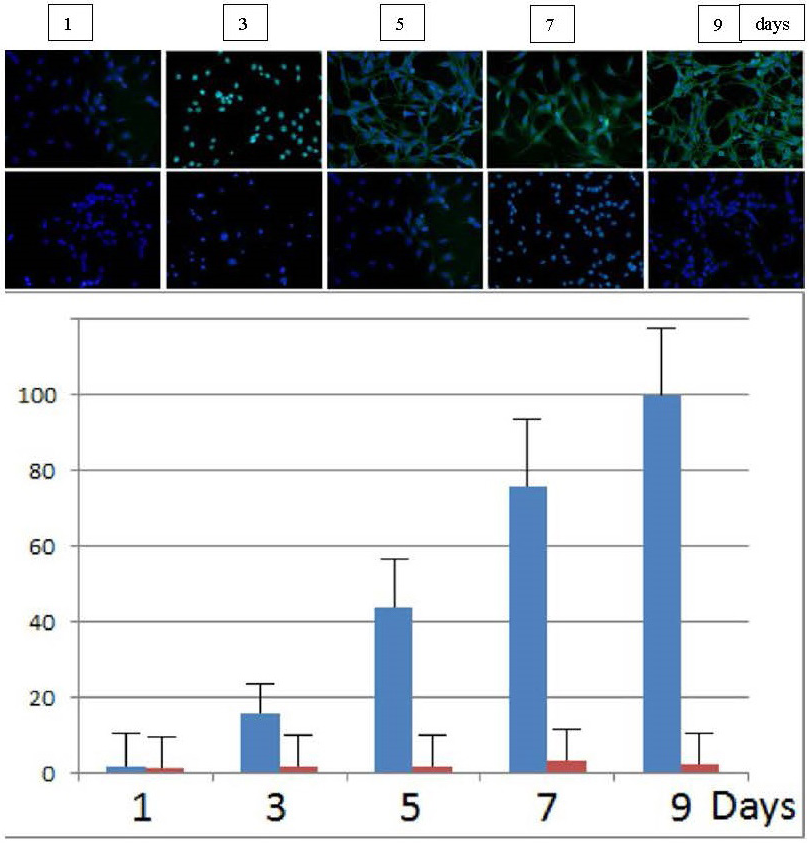

Figure 7: Time course experiment in which mouse fibroblasts were transfected with CIR-6 using lipofectamine at a concentration of 12 ng/ul and then culturing the cells for a total of 8 full days. The cells were evaluated for cardiac troponin-T quantities using ImageJ software after 1, 3, 5, 7 and 9 days in culture. The highest level of fluorescent staining was, as expected, found to be at the end of 8 days (collected on Day 9). The highest level was assigned a value of 100% in the graph and earlier values were indicated as a percentage of the 100% value. It is clear that the CIR-6 transfected fibroblasts express cardiac specific troponin-T in increasing amounts with progressing time through the 8-day period studied.

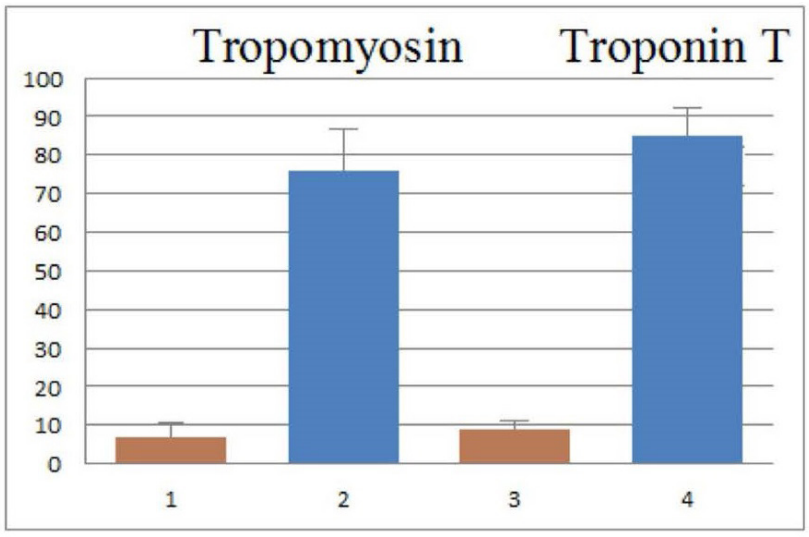

Figure 8: Expression of mRNAs for cardiac troponin-T and tropomyosin were evaluated by using quantitative RT-PCR after transfection of CIR-6 (12 ng/ul) into mouse embryonic fibroblasts (2 and 4) and culturing the cells for 8 full days (harvesting the cells on the 9th day). Controls (1 and 3) were treated with lipofectamine only. The mouse fibroblasts transfected with CIR-6 expressed significant amounts of mRNAs for both cardiac tropomyosin (2) and troponin-T (4), while the control fibroblasts treated with lipofectamine only showed very low levels of mRNAs for the cardiac proteins.

of cardiac specific troponin-T (Figures 3 and 4) and cardiac-specific tropomyosin (Figures 5 and 6).

Time-Course Experiments for Cardiac Protein Expression in CIR-Transfected Fibroblasts

We conducted time-course experiments by incubating the mouse fibroblasts in vitro with 12 ng/ul of CIR followed by quantitation of troponin-T after immunofluorescent staining and analysis using ImageJ quantitative software, after 1, 3, 5, 7 and 9 days in culture. Expression of cardiac specific troponin-T estimated in these experiments increased significantly by the 5th day in culture and continued to increase further on the 7th and 9th days of incubation indicating continued differentiation of the fibroblasts into definitive cardiomyocytes (Figure 7). As Figure 7 shows, there was a continuing increase in cardiac accumulation for cardiac troponin-T (shown) and tropomyosin (not shown) during the time period of 1, 3, 5, 7 and 9 days. The highest level for the troponin-T and tropomyosin was, as expected, after the full 8 days (analyzed beginning of the 9th day) in culture after CIR-6 transfection. The highest quantity of troponin-T was arbitrarily set at 100 for the 9^{th} day in culture and the earlier time points were calculated as a percentage of the 9^{th} day protein levels for troponin-T; tropomyosin levels showed similar increases over the 1–9-day period. On the contrary, without CIR-6 treatment, the fibroblasts showed small insignificant increases during the time course of the cultures. Very similar observations were made relative to the quantitative expression of tropomyosin (data not shown).

Quantitative RT-PCR Analyses of Tropomyosin and Troponin-T mRNAs

In addition to quantitating the cardiac specific troponin-T and tropomyosin proteins, we also quantitatively analyzed the mRNAs for tropomyosin and cardiac troponin-T expression using real time quantitative RT-PCR experiments. After transfecting fibroblasts with CIR-6, then culturing the cells for 8 days, total nucleic acids were extracted and DNA was digested. Analysis of both tropomyosin and troponin-T mRNA expression showed significant increases in the quantities of mRNAs after treatment with the CIR-6 after 8 days in culture while with lipofectin treatment only and no CIR-6, there were very small quantities of tropomyosin and troponin-T mRNAs after the full 8 day period (Figure 8).

Discussion

Treatment of mouse embryonic fibroblasts with Cardiac Inducing RNA induces expression of cardiac markers in vitro revealed by immunohistochemistry very early in the culture process. CIR transfected cells show positive staining for cardiac troponin-T and cardiac tropomyosin after only 4 days in culture. Even though these same cells exhibit typical fibroblast-type morphologies, staining with cardiac-specific troponin-T and cardiac tropomyosin antibodies by the classical definition of a “cardiac muscle cell”, defines these cells as “cardiac muscle cells” showing cardiac phenotypes. As time progresses in vitro, the CIR-transfected cultured fibroblast cells produce increasing quantities of cardiac troponin-T and tropomyosin. Simultaneously, the cells transform from a fibroblastic-type morphology to a more typical early cultured, in vitro cardiac cell-type morphology that shows spindle shapes (Figure 2A,B,C). In addition, the contractile proteins (cardiac troponin-T and cardiac tropomyosin) examined in this study begin to align longitudinally forming nascent myofibril-like structures in the early spindle-shaped cells; the cells continue to form myofibril-like structures that by day 8 in culture show striated sarcomeric patterning organizations after antibody staining for both cardiac troponin-T and tropomyosin contractile proteins (Figures 2D,E,F).

After 8 days in culture, the data show that >80% of the mouse fibroblasts transfected with 12ng/ul of CIR, by using the classical definition of cardiac specific troponin-T and cardiac tropomyosin expression to define cells as "cardiac muscle cells", appear to have differentiated directly from fibroblasts into definitive cardiomyocytes. In the control groups, less than <7% of the lipofectin-only or non-active RNA-transfected control fibroblast cells in culture show evidence of cardiac-specific troponin-T or display cardiac tropomyosin expression, or cardiac morphological characteristics. Thus, it is clear that the Cardiac Inducing RNA (CIR) is able to promote mouse embryonic fibroblasts to transdifferentiate directly into cardiomyocytes in vitro, without any obvious de-differentiation, pluripotent stem cell transitioning and/or re-differentiation phases apparent. Our findings are in agreement with other studies which show that some RNA species apparently have the ability to re-program non-muscle cells, including cardiac- or dermal-derived fibroblasts toward cardiogenic lineages without going through a progenitor state [15,16]. Fibroblasts are purported to be not fully differentiated cells, which may facilitate their direct conversion into other cell types in vitro, including mesodermal cells [17]. Our results support this concept and strongly suggest that the Cardiac Inducing RNA (CIR-6) we have discovered can stimulate and promote the direct conversion of the mouse fibroblasts into cardiogenic cells that express cardiac specific contractile proteins and promote overall the differentiation of a cardiogenic morphological phenotype to these fibroblasts in vitro.

In previous studies using simple recessive cardiac non-function mutant embryonic axolotls (salamanders, *Ambystoma mexicanum*), we showed that a salamander-derived CIR functional homolog of human CIR derived from normal embryonic salamander anterior endoderm (a potent embryonic heart inducing tissue) is essential for cardiac muscle to differentiate in normal embryonic axolotls [3]. Homozygous recessive cardiac non-function mutant axolotl embryos result from a single point mutation in recessive gene c and cardiac non-function mutant embryos in homozygous condition (c/c) do not produce normal axolotl Cardiac Inducing RNA (CIR); but rather, a simple point mutation makes the mutant CIR secondary RNA structure abnormal rendering the homozygous cardiac mutant CIR non-functional [3]. Thus, the scientific premise for the present study is based on our findings of a unique RNA secondary structure, initially discovered in the Mexican axolotl and then in humans, that can induce non-muscle cells to differentiate into contracting cardiomyocytes with structurally normal-appearing myofibrils. As already mentioned, we named this RNA, Cardiac Inducing RNA or CIR [3-8,10,18].

In our earlier published work, we employed the simple recessive mutant gene salamander model, the cardiac non-function mutant axolotl (salamander) system, to create embryonic heart bioassay experiments [9,10,19,20]. Non-contracting cardiac non-function mutant embryonic hearts, lacking organized myofibrils, were placed in whole organ cultures with cardiac inducing RNA (CIR) from normal axolotl anterior endoderm (a potent embryonic heart-inducing tissue) or from cardiac inducing RNA (CIR) from human heart RNA library clones #6 and #30 (now referred to as CIR-6 and CIR-30). After 48-72 hours, the non-beating mutant axolotl hearts begin to beat rhythmically, and immunofluorescent staining with muscle contractile protein antibodies (e.g., anti-cardiac tropomyosin, anti-cardiac specific troponin-T, anti-α-actinin, anti-myosin) revealed the appearance of organized sarcomeric myofibrils of normal morphology in the mutant axolotl hearts which ordinarily do not form organized myofibrils [4,6,7]. These studies were expanded to the transfection of mouse embryonic stem cells (ESC) and human iPS cells with CIR-6 and CIR-30. We found that using the human iPS cells and mouse ESCs transfected with CIR and immunofluorescently stained for cardiac-specific troponin-T (cTnT) or cardiac tropomyosin revealed organized sarcomeric myofibrils confirming that these non-muscle cells in the non-functional mutant hearts had been induced to differentiate into contracting cardiomyocytes with organized sarcomeric myofibrils and the mutant hearts now beat normally in the organ cultures [6,7]. Thus, in these earlier studies, we demonstrated that cardiac non-function mutant axolotl hearts, human iPS cells and mouse embryonic stem cells all respond to the two cloned human CIRs (CIR-6 and CIR- 30) by differentiating into cardiomyocytes that express cardiac-specific proteins, form characteristic spindle-shaped cardiomyocyte shapes in culture, and cardiac sarcomeric myofibrils of normal morphology making them capable of contraction [4,6,7].

In the present research, we have expanded our studies by transfecting human Cardiac Inducing RNA derived from clone #6 (CIR-6) directly into mouse embryonic fibroblasts using lipofectamine to determine if the CIR-6-derived human RNA could also induce fibroblasts to differentiate directly into myocardial-like spindle-shaped cells that express cardiac specific proteins and associated mRNAs, including cardiac-specific troponin-T and cardiac tropomyosin, as detected by immunohistochemical staining and by quantitative RT-PCR. Expression of these contractile proteins showed organization into sarcomeric myofibrils characteristic of nascent striated cardiac muscle cells. Interestingly, computer analyses of the RNA secondary structures of the active human-derived CIR-6 reveal significant similarities to the Cardiac Inducing RNA (CIR) originally described for the salamander (formerly termed MIR in salamander for Myofibril Inducing RNA) that also promotes non-muscle cells to differentiate into cardiac muscle. Thus, the human- and salamander-derived CIRs, though from broadly different vertebrate animal classes of *Mammalia* and *Amphibia*, appear to have evolutionarily highly conserved secondary RNA structures suggesting that these RNAs may play major roles in all vertebrate heart development and, particularly, in the differentiation of cardiomyocytes from non-muscle cells during early embryonic development. One of the human-derived fetal heart RNAs (CIR-6) reveals that it is a fragment of N-sulfoglucosamine sulfohydrolase and the caspase recruitment domain family member 14 precursor gene [6]. Molecular analyses of the second CIR (CIR-30) derived from fetal heart RNA is associated with the mitochondrial cytochrome c oxidase subunit II gene [4]. In our previous studies, we transfected mouse embryonic stem cells (mESCs) and human induced pluripotent stem cells (hiPSCs) with both CIR-6 and CIR-30 and found that

with either CIR, both mESCs and hiPSCs readily differentiated into characteristic early developing cardiomyocytes and expressed cardiac contractile protein markers including cardiac specific troponin-T, tropomyosin and α-actinin [6,7]. That these active CIR RNAs seem to be derived from portions of larger genes may indicate that they function as non-translating epigenetic components in the cells. Further detailed analyses will be required to verify the intriguing possibility of this preliminary hypothesis.

In the present study, we have determined that mouse embryonic fibroblasts (MEFs) have essentially the same ability as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) to differentiate directly into cardiac muscle cells without being first converted into "conventional" pluripotent stem cells (i.e., iPSCs). Surprisingly, the time frame for the mouse fibroblasts to express cardiac contractile proteins, cardiac specific troponin-T or cardiac tropomyosin, is very similar to mouse ESCs and human iPSCs in vitro. The fibroblasts express cardiac troponin-T and tropomyosin after 4 days in vitro which is essentially the same as the mouse ESCs and human iPSCs [6,7]. In addition, all of these different cell types, including the mouse embryonic fibroblasts, show that >80% of the cells express significant amounts of the cardiac proteins, including cardiac specific troponin-T and cardiac tropomyosin after transfection with the CIR followed by 8 days in culture. Our observations in the present and earlier studies indicate that ESCs and embryonic fibroblasts from mice may differentiate at a slightly faster rate than do the human iPSCs [6,7]. We believe that this observation probably results from a higher/faster metabolic rate genetically inherent in differentiating mouse cells versus differentiating human cells. This is not a particularly surprising observation given that mouse embryos are fully developed and born after approximately 21 days of gestation compared to about 280 days in humans. Moreover, the heart begins to beat in mouse embryos at ~8 days post-fertilization while in human, the heart begins to contract at ~22 days post-fertilization. More detailed studies will be required to confirm our preliminary observations that mouse embryonic stem cells or fibroblasts differentiate into myocardiocytes more rapidly than human-derived iPSCs or fibroblasts in vitro after CIR transfection (Unpublished observation, L. Lemanski).

Conclusions

Our finding that greater than >80% of the mouse embryonic fibroblasts differentiate into cardiogenic cells after 8 days in culture after lipofectamine transfection of human-derived CIR-6, suggests that CIR may hold great promise for use in human medicine, in combination with an individual patient's own fibroblast cells for the repair of damaged myocardial infarcted tissues in vivo. By injecting CIR transfected fibroblasts or CIR alone into areas of damaged heart after myocardial infarction or other disease processes, we believe that this approach very well may be able to promote the differentiation of new muscle tissue in the damaged areas of the heart and regenerate those damaged areas into normally functioning cardiac muscle again. If, indeed, this can be applied to humans, whereby a patient having had a myocardial infarction could be treated by the simple harvesting of that same patient's dermal fibroblasts (e.g., by needle biopsy), treatment of these fibroblasts in vitro for a few days with CIR, and then injection of these newly-created cardiogenic-lineage cells into the damaged area of the diseased heart might allow a patient to recover heart function and return to pre-heart-attack activity levels. This clearly would represent a major step forward in the development of a treatment and potential cure for heart failure. The research described in this paper is based on a completely novel approach, using fibroblasts and a specific human Cardiac Inducing RNA derived from clone #6 in our Human RNA library (CIR-6) that induces fibroblasts to differentiate into cardiomyocytes in culture. Furthermore, the approach is very novel in that the CIR-6 we have discovered from human heart has the unique ability, in a single step, to transform >80% of the fibroblasts in vitro into cardiogenic cells that express cardiac-specific proteins and show cardiomyocyte morphologies. As such, the CIR recently has been issued as US Patent (Cardiac Myofibril Induction, #10,413,617 [09/17/19]) by the United States Patent and Trademark Office (Inventors: Larry F. Lemanski [Lead Inventor]; Andrei Kochegarov [Associate Inventor]; Ashley Arms [Associate Inventor].

Thus, the human Cardiac Inducing RNA (CIR) discovered in our laboratory, in the near future, may hold great promise for the repair of damaged myocardial tissue resulting from myocardial infarction or other disease processes in human hearts. We believe that the research presented here has the potential for rapid translation to human clinical medicine whereby individuals who have suffered from myocardial infarction, after treatment, may be able to return to pre-heart-attack activity levels again and individuals with terminal heart failure may be able to fully recover. If this could be accomplished, cardiac transplantation would no longer be the only method available to cure heart failure, rather, treatment with CIR-6 transfected stem cells or fibroblasts might be able to replace the heart transplant treatment option by providing an effective treatment to repair/regenerate cardiac muscle damage after heart attacks and provide a complete and definitive cure for heart failure in humans.

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References

1. https://www.who.int/en/news-room/factsheets/detail/cardiovascular-diseases-(cvds)
2. https://www.heart.org/idc/groups/ahamahpublic/@wcm/@sop/@smd/documents/downloadable/ucm_470704.pdf
3. Zhang C, Jia P, Huang X, et al. Myofibril-inducing RNA (MIR) is essential for sarcomeric tropomyosin expression, the organization of myofibrils and the initiation of cardiac contractile function in Mexican axolotls during development. J Biomed Sci. 2009; 16: 81.

4. Moses-Arms A, Kochegarov A, Arms J, et al. Identification of a human mitochondrial RNA that promotes tropomyosin synthesis and myocardial differentiation. Invitro Cell Dev Biol Anim. 2015; 51: 273-280.
5. Kochegarov A, Moses A, Lian W, et al. A new unique form of microRNA from human heart, microRNA-499c, promotes myofibril formation and rescues cardiac development in mutant axolotl embryos. J Biomed Sci. 2013; 20: 20.
6. Kochegarov A, Moses-Arms A, Lemanski LF. A fetal human heart cardiac inducing RNA (CIR) promotes the differentiation of stem cells into cardiomyocytes. In Vitro Cell Dev Biol Animal. 2015; 51.739-748.
7. Kochegarov A, Moses-Arms A, Hanna M, Lemanski LF. Micro RNA-499c induces the differentiation of stem cells into cardiomyocytes. International Archives of Medicine. 2015; 8: 1-9.
8. Kochegarov A, Lemanski LF. New Trends in Heart Regeneration: A Review. J Stem Cells Regen Med. 2016; 12: 61-68.
9. Zhang C, Dube D, Huang X, et al. A point mutation in bioactive RNA results in RNA secondary structure alteration and failure of mutant heart correction in Mexican axolotls. Anat Embryol. 2003; 206: 495-506.
10. Rueda-de-Leon E, Kochegarov A, Lian W, et al. Human heart RNA promotes tropomyosin synthesis and myofibrillogenesis in mutant axolotl hearts. MD-Medical Data. 2011; 3: 223-227.
11. Lemanski LF, Zhang C, Kochegarov A, et al. Protection of mouse embryonic stem cells from oxidative stress by methionine sulfoxide reductases. In: Oxidative Stress: Molecular Mechanisms and Biological Effects; InTech, Rijeka, Croatia. 2012; 23: 197-230.
12. Zhang C, Jia P, Jia Y, Weissbach H, et al. Methionine sulfoxide reductase A (MsrA) protects cultured mouse embryonic stem cells from H2O2-mediated oxidative stress, J.Cell Biochem. 2010; 111: 94-103.
13. Zhang C, Jia P, Jia Y, et al. Anoxia, acidosis and intergenic interactions selectively regulate methionine sulfoxide reductase transcriptions in mouse embryonic stem cells. J.Cell. Biochem. 2011; 112: 98-106.
14. Jia P, Zhang C, Jia Y, et al. Identification of a truncated form of methionine sulfoxide reductase A expressed in mouse embryonic stem cells. J. Biomed. Sci. 2011; 18: 46.
15. Jayawardena T, Egemnazarov B, Finch E, et al. MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibrobasts to cardiomyocytes. Circ Res. 2012; 110: 1465-1473.
16. Pratico ED, Feger BJ, Watson MJ, et al. RNA-mediated reprogramming of primary adult human dermal fibroblasts into ckit(+) cardiac progenitor cells. Stem Cells Dev. 2015; 24: 2622-2633.
17. Bar-Nur O, Gerli MFM, Di Stefano B, et al. Direct 24 reprogramming of mouse fibroblasts into functional skeletal muscle progenitors. Stem Cell Reports. 2018; 10: 1505-1521.
18. Lemanski LF, Kochegarov A, Arms A. Cardiac Myofibril Induction (Issued Patent, United States Patent and Trademark Office) Number 10,413,617. 2019
19. Lemanski LF, LaFrance SM, Erginel-Unaltuna N, et al. The cardiac mutant gene c in axolotls: cellular, developmental and molecular studies. Cell Molec Biol Res. 1995; 41: 293-305.
20. Lemanski LF, Nakatsugawa M, Bhatia R, et al. A specific synthetic RNA promotes cardiac myofibrillogenesis in the Mexican axolotl. Biochem Biophys Res Com. 1996; 229: 974-981.