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(54) **Title:** COMPOSITIONS AND METHODS FOR CELLULAR REPROGRAMMING

(57) **Abstract:** Disclosed herein are compositions and methods for cellular reprogramming. The compositions comprise one or more miRs and an activator of NF*k*B. Also provided are methods for enhancing or upregulating cardiomyocyte maturation in a cell or a subject and methods for inhibiting or downregulating cardiomyocyte maturation.

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COMPOSITIONS AND METHODS FOR CELLULAR REPROGRAMMING

CROSS-REFERENCE TO RELATED APPLICATION

This patent application claims the benefit of priority of United States Provisional Patent Applications Nos. 62/645,847, filed March 21, 2018, and 62/782,480, filed December 20, 2018, the contents of each are incorporated herein by reference in their entirety.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under 5R01H731814-02 awarded by the National Institutes of Health, DP2OD008586 awarded by the National Institutes of Health, and CBET-1151035 awarded by the National Science Foundation. The government has certain rights in the invention.

BACKGROUND

Heart disease is the number one killer of men and women worldwide. Generally, heart tissue has a limited capacity for regeneration or self-renewal. After a patient recovers from a myocardial infarction, the organ bears a scar and heart function is diminished. The ability to regenerate damaged organs such as the heart remains elusive. As such, there is a pressing need in the art to develop new strategies for the regeneration of damaged organs.

BRIEF SUMMARY OF THE INVENTION

Disclosed herein are compositions and methods for cellular reprogramming. One aspect of the invention is a reprogramming composition comprising one or more miRs comprising a nucleotide sequence having at least 80% sequence identity to miR-1, miR-126, miR-133, miR-133a, mir-206, miR-208, miR-499, mir-499-5p, and combinations thereof; and an activator of NFκB.

Another aspect of the invention is a pharmaceutical composition including an effective amount of the composition of claim 1 and one or more pharmaceutically acceptable carriers, excipients, or diluents.

Another aspect of the invention is a method for enhancing or upregulating cardiomyocyte maturation in a cell comprising contacting the cell with an effective amount of any of the compositions described herein for a sufficient time such that the cell is reprogrammed into a cardiomyocyte.

Another aspect of the invention is a method of enhancing or upregulating cardiomyocyte maturation in a subject comprising administering (i) an effective amount of any of the compositions described or (ii) any of the pharmaceutical compositions comprising the effective amount of any of the compositions described and one or more pharmaceutically acceptable carriers, excipients, or diluents.

Another aspect of the invention is a method for inhibiting or downregulating cardiomyocyte maturation in a cell comprising contacting the cell with an effective amount of a composition comprising a TLR3 inhibitor, a NFκB inhibitor, a ikbkb inhibitor, or a combination thereof for a sufficient time such that cardiomyocyte maturation is inhibited or down-regulated in the cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying figures, which are schematic and are not intended to be drawn to scale. In the figures, each identical or nearly identical component illustrated is typically represented by a single numeral. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention.

Figures 1A-1Eiii show TLR3 inhibition inhibits maturation of reprogrammed fibroblasts into cardiomyocytes. Neonatal cardiac fibroblasts were transfected with negative control miR (negmiR) or miR combo. The day after transfection media was replaced and the cells incubated with either vehicle or the TLR3 pharmacological inhibitor CU-CPT-4a (10μM) for a further 4 days. After incubation with the TLR3 pharmacological inhibitor, cells were cultured in normal growth media for a further 6 days. Quantitative PCR was used to analyze mRNA levels of 13 components of the cardiomyocyte sarcomere.

Figure 1A shows a heat-map overview of the qPCR analysis. Expression values were normalized to the average expression of the negmiR vehicle samples and then averaged (N=3 technical replicates (fibroblasts were derived from single litter and seeded into 3 individual wells). Averages for each gene were then converted to Z-scores. Centroid linkage and Euclidean methods were employed for clustering and distance measurements respectively.

Figures 1Bi-1Ciii show neonatal cardiac fibroblasts were transfected with negative control miR (negmiR) or miR combo. The day after transfection media was replaced and the cells incubated with either vehicle or the TLR3 pharmacological inhibitor CU-CPT-4a (10μM) for a further 4 days. After incubation with the TLR3 pharmacological inhibitor, cells were cultured in normal growth media for a further 10 days.

Figures 1Bi-1Biii show RNA levels of the cardiomyocyte sarcomere components Myh6 (αmyosin heavy chain) (Fig. 1Bi), Actn2 (αsarcomeric actinin) (Fig. 1Bii), and Tnni3 (cardiac troponin-I) (Fig. 1Biii) was determined by qPCR. N=4 independent experiments.

Figures 1Ci and **1Cii** show cells provided miR combo (Fig. 1Ci) or miR combo and TLR3 antagonist (Fig. 1Cii) fixed and stained with anti-Actn2 antibodies (red). Nuclei were stained with DAPI (blue). Scale bar 50 microns.

Figure 1Ciii shows quantification of immunostaining. Cells expressing Actn2 were counted and expressed as a percentage of the total cell population. N=6 independent experiments.

Figures 1D -1Eiii show neonatal cardiac fibroblasts were first transfected with either a control siRNA or a siRNA that targeted TLR3. Two days later, the cells were transfected again with either the negative control miR negmiR or miR combo. The day after transfection with miRNAs the media was replaced and the cells cultured in normal growth media for 14 days.

Figure 1D shows quantification of TLR3 knockdown by qPCR. N=3 independent experiments.

Figures 1Ei-1Eiii show RNA levels of the cardiomyocyte structural proteins Myh6 (αmyosin heavy chain) (Fig. 1Ei), Actn2 (αsarcomeric actinin) (Fig. 1Eii) and Tnni3 (cardiac troponin-I) (Fig. 1Eiii) was determined by qPCR. N=4 independent experiments. Data represented as Mean ± SEM. ***P<0.001, **P<0.01, *P<0.05, ns: not significant. For A, B and D comparisons are made between miR combo and negmiR for each group. For C, comparison is made between control siRNA and siRNA targeting TLR3.

Figures 2Ai-2Biv show neither TLR3 inhibition nor TLR3 activation affects early stage cardiac reprogramming.

Figures 2Ai-2Aiv show neonatal cardiac fibroblasts were first transfected with either a control siRNA or a siRNA that targeted TLR3. Two days later, the cells were transfected again with either the negative control miR negmiR or miR combo. The day after transfection with miRNAs, the media was replaced and the cells cultured in normal growth media for 3 days. RNA levels of the cardiomyocyte-lineage commitment factors Gata4 (Fig. 2Ai), Hand2 (Fig. 2Aii), Tbx5 (Fig. 2Aiii), and Mef2C (Fig. 2Aiv) was determined by qPCR. N=9 independent experiments. Comparisons are made between miR combo + control siRNA and miR combo + TLR3 siRNA, ns: not significant. Data represented as Mean ± SEM.

Figures 2Bi-2Biv show neonatal cardiac fibroblasts were transfected with negative control miR (negmiR) or miR combo. The day after transfection media was replaced and the cells incubated with vehicle or the TLR3 agonist Poly(I:C) LMW (low molecular weight Poly(I:C)) for a further 3 days. RNA levels of the cardiomyocyte-lineage commitment factors Gata4 (Fig. 2Bi), Hand2 (Fig. 2Bii), Tbx5 (Fig. 2Biii), and Mef2C (Fig. 2Biv) was determined by qPCR. N=6 independent experiments. Comparisons are made between miR combo + vehicle and miR combo + TLR3 agonist, ns: not significant. Data represented as Mean ± SEM.

Figures 3Ai-3Diii show NFκB is important for miR combo reprogramming.

Figures 3Ai-3Biii show neonatal cardiac fibroblasts were transfected with negative control miR (negmiR) or miR combo. The day after transfection media was replaced and the cells incubated with vehicle or the NFκB antagonist Bay 11-7085. After one day of treatment, the media was replaced with normal growth media and cells cultured for a further 12 days.

Figures 3Ai-3Aiii RNA levels of the cardiomyocyte structural proteins Myh6 (αmyosin heavy chain) (Fig. 3Ai), Actn2 (αsarcomeric actinin) (Fig. 3Aii), and Tnni3 (cardiac troponin-I) (Fig. 3Aiii) following treatment with the NFκB antagonist Bay 11-7085 was determined by qPCR. N=3 independent experiments.

Figures 3Bi-3Bii shows cells provided miR combo (Fig. 3Bi) or miR combo and NFκB inhibitor (Fig. 3Bii) were fixed and stained with anti-Actn2 antibodies (red). Nuclei were stained with DAPI (blue). Scale bar 100 microns. Inset pictures are at 5x magnification.

Figures 3Biii shows quantification of immunostaining. Cells expressing Actn2 were counted and expressed as a percentage of the total cell population. N=3 independent experiments.

Figures 3C-3Diii show neonatal cardiac fibroblasts were transfected with microRNAs (negmiR or miR combo) and siRNA (control siRNA or a siRNA that targeted Ikbkb). The day after transfection with miRNAs the media was replaced and the cells cultured in normal growth media for either 4 days (to assess knockdown efficiency) or 14 days (to assess RNA levels of cardiomyocyte structural proteins).

Figure 3C show quantification of Ikbkb knockdown by qPCR. N=3 independent experiments.

Figures 3Di-3Diii show RNA levels of the cardiomyocyte structural proteins Myh6 (αmyosin heavy chain) (Fig. 3Di), Actn2 (αsarcomeric actinin) (Fig. 3Dii), and Tnni3 (cardiac troponin-I) (Fig. 3Diii) was determined by qPCR. N=3 independent experiments.

Data represented as Mean ± SEM. Comparisons are made between miR combo and negmiR for each group, $^{**}P<0.01$, $^{*}P<0.05$, ns: not significant.

Figures 4A-4Dii show RelA mediates the effects of NFκB. Neonatal cardiac fibroblasts were first transfected with either a control siRNA or a siRNA that targeted the NFκB subunit RelA. Two days later, the cells were transfected again with either the negative control miR negmiR or miR combo. The day after transfection with miRNAs, the media was replaced and the cells cultured in normal growth media for 13 days.

Figure 4A shows quantification of RelA knockdown by qPCR.

Figures 4Bi-4Bii show cells provided miR combo and control siRNA (Fig. 4Bi) and miR combo + RelA siRNA (Fig. 4Bii) fixed and stained with anti-Actn2 antibodies (red). Nuclei were stained with DAPI (blue). Scale bar 100 microns. Inset pictures are at 5x magnification.

Figure 4Biii shows quantification of immunostaining. Cells expressing Actn2 were counted and expressed as a percentage of the total cell population. N=3 independent experiments.

Figures 4Ci-4Ciii show RNA levels of the cardiomyocyte structural proteins Myh6 (αmyosin heavy chain) (Fig. 4Ci), Actn2 (αsarcomeric actinin) (Fig. 4Cii), and Tnni3 (cardiac troponin-I) (Fig. 4Ciii) was determined by qPCR. N=3 independent experiments.

Figures 4Di-4Dii show RNA levels of the endodermal marker Gata6 (Fig. 4Di) and the general marker of differentiation Tgfb2 (Fig. 4Dii) were determined by qPCR. N=3 independent experiments.

Data represented as Mean ± SEM. Comparisons are made between miR combo and negative control miR (negmiR) for each group, $^{**}P<0.01$, $^{*}P<0.05$, ns: not significant.

Figures 5Ai-5Bviii show the NFκB subunit RelA binds to the promoters of cardiomyocyte maturation genes.

Figures 5Ai-5Aviii show neonatal cardiac fibroblasts were transfected with negmiR or miR combo. After 7 days, chromatin DNA was subjected to ChIP analysis. Primers were designed to target the first 1Kb of the indicated cardiomyocyte sarcomere genes Actn2 (Fig. 5Ai), Myh6 (Fig. 5Aii), Mypn (Fig. 5Aiii), Tnni3 (Fig. 5Aiv), Ttn (Fig. 5Av), Myoz2 (Fig. 5Avi), Tnnc1 (Fig. 5Avii), and Tnnt2 (Fig. 5Aviii), (represented by -01Kb). Results are presented as the fold enrichment in RelA binding where percent input of the negmiR control was taken to be 1. N=3 independent experiments. Data represented as Mean ± SEM. Comparisons are made between miR combo and negative control miR (negmiR), **P<0.01, *P<0.05, ns: not significant.

Figures 5Bi-5Bviii show neonatal cardiac fibroblasts were transfected with miR combo and either a control siRNA or a siRNA that targeted RelA. After 7 days, chromatin DNA was subjected to ChIP analysis. Primers were designed to target the first 1Kb of the indicated cardiomyocyte sarcomere genes Actn2 (Fig. 5Bi), Myh6 (Fig. 5Bii), Mypn (Fig. 5Biii), Tnni3 (Fig. 5Biv), Ttn (Fig. 5Bv), Myoz2 (Fig. 5Bvi), Tnnc1 (Fig. 5Bvii), and Tnnt2 (Fig. 5Bviii), (represented by -01Kb). Results are presented as the percentage of chromatin input. N=3 independent experiments. Data represented as Mean ± SEM.

Figures 6A-6B show microRNAs activate TLR3.

Figure 6A shows neonatal cardiac fibroblasts were transfected with negmiR or miR combo. A mock transfection where lipid reagent alone was added to the cells was also used. The TLR3 inhibitor CU-CPT-4a, which interferes with RNA binding to TLR3, was added one day post-transfection. IL6 concentration in the media was assessed 4 days post-transfection and values expressed as pg IL6 per µg of total protein. N=4 independent experiments. Data represented as Mean ± SEM. Comparisons are made to the respective mock transfected group (*P<0.05, ns: not significant) and between negmiR and miR combo (†P<0.05).

Figure 6B shows neonatal cardiac fibroblasts were transfected with microRNAs (mock, miR combo) and siRNA (non-targeting control, TLR3, Ikbkb). IL6 concentration in the media was assessed 4 days post-transfection and values expressed as a ratio between miR combo and mock transfected cells. N=3 independent experiments. Data represented as Mean ± SEM.

Comparisons are made to miR combo plus non-targeting control siRNA group, **P<0.01, *P<0.05.

Figures 7Ai-7Dii show TLR3 agonists enhance maturation of miR combo reprogrammed cardiomyocytes. Neonatal cardiac fibroblasts were transfected with negative control miR (negmiR) or miR combo. The day after transfection media was replaced and the cells incubated with vehicle or the TLR3 agonist Poly(I:C) LMW (low molecular weight Poly(I:C)) for a further 4 days. After incubation with the TLR3 agonist, cells were cultured in normal growth media for a further 10 days.

Figures 7Ai-7Aiii show RNA levels of the cardiomyocyte structural proteins Myh6 (αmyosin heavy chain) (Fig. 7Ai), Actn2 (αsarcomeric actinin) (Fig. 7Aii), and Tnni3 (cardiac troponin-I) (Fig. 7Aiii) was determined by qPCR. N=5-14.

Figures 7Bi-7Bvi show cells provided miR combo (Fig. 7Bi, Fig. 7Bii, and 7Biii) and miR combo and TLR3 agonist (Fig. 7Biv, Fig. 7Bv, and 7Bvi) fixed and stained with anti-Actn2 antibodies (red). Nuclei were stained with DAPI (blue). N=6 independent experiments. Scale bar 50 microns. Inset pictures are at 5x magnification to show sarcomere structure.

Figure 7C show quantification of immunostaining. Cells expressing Actn2 were counted and expressed as a percentage of the total cell population.

Figures 7Di-7Dii show neonatal cardiac fibroblasts were transfected with negative control miR (negmiR) or miR combo. The day after transfection media was replaced and the cells incubated with differentiation media (DMEM + 2%FBS + ITS + AA) and the TLR3 agonist Poly(I:C) LMW for the indicated times (Fig. 7Di). Fourteen days after the transfection, the numbers of beating colonies were counted. N=4 independent experiments (Fig. 7Dii). Data represented as Mean ± SEM. *Comparisons made between vehicle and TLR3 agonist for each group ***P<0.001, **P<0.01, *P<0.05. †Comparisons made between miR combo and negmiR for each group †††P<0.001, ††P<0.01, †P<0.05.

Figures 8A-8D show ICR2-activated cardiomyocyte maturation evaluated via qPCR by measuring the expression of Actn2 (Fig. 8A), Myh6 (Fig. 8B), Tnni3 (Fig. 8C), and Cacna1c (Fig. 8D).

Figures 9A-9D shows ICR4-activated cardiomyocyte maturation evaluated via qPCR by measuring the expression of Actn2 (Fig. 9A), Myh6 (Fig. 9B), Tnni3 (Fig. 9C), and Cacna1c (Fig. 9D).

Figure 10 shows high doses of miR combo impair normal cellular functions by measuring the change in cell number.

Figures 11A-11B show miR combo mediated maturation with ICR2 (Fig. 11A) and PolyIC (Fig. 11B) evaluated by antibody staining for a-sarcomeric actinin.

DETAILED DESCRIPTION OF THE INVENTION

Cardiomyocyte maturation may be enhanced or upregulated by agonists of the innate immune system, such as pattern recognition receptor agonists, or inhibited or downregulated by antagonists. As demonstrated in the Examples, cardiomyocyte maturation may be effectively controlled in committed cellular precursors to accelerate or retard maturation via pattern recognition receptors and associated signaling pathways.

"Pattern recognition receptors" or "PRRs" are protein receptors that detect molecules typical of pathogens and/or cellular damage. These proteins are expressed primarily by cells of the innate immune system, such as dendritic cells, macrophages, monocytes, neutrophils and epithelial cells. PRRs are used to identify pathogen-associated molecular patterns (PAMPs), which are associated with microbial pathogens, and damage-associated molecular patterns (DAMPs), which are associated with components of host's cells that are released during cell damage or death. PRRs mediate the immune response to PAMPs and DAMPs and release inflammatory cytokines.

"Pathogen-associated molecular patterns" or "PAMPs" activate immune responses by identifying exogenous molecules. Exemplary PAMPs include, without limitation, nucleic acids, bacterial lipopolysaccharides, endotoxins, bacterial flagellin, lipoteichoic acid, peptidoglycan, and unmethylated CpG motifs. Induction of the immune response to one or more exogenous molecules assists with the prevention or recovery from infection. "Damage-associated molecular patterns" or "DAMPs" activate immune responses by identifying host molecules. Exemplary DAMPs include, without limitation, nuclear or cytosolic proteins released outside the cell and nucleic acids.

Toll-like receptors are a subset of PRRs. "Toll-like receptors" or "TLRs" are a class of extracellular, membrane-bound PPRs that share a common structural motif of a leucine-rich repeat. TLRs interacting with PAMPs or DAMPs trigger signaling through NFκB resulting in the increase of inflammatory cytokines. The TLRs include TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12, and TLR13. "Cytokines" include broad category of proteins, typically between about 5 to about 20 kDa, that are involved with cell signaling, such as chemokines, interferons, interleukins, lymphokines, and tumour necrosis factors.

"NFκB" or "nuclear factor kappa-light-chain-enhancer of activated B cells" is a protein complex that controls transcription of DNA, cytokine production, and cell survival. NFκB is important in regulating cellular responses because it belongs to the category of "rapid-acting" primary transcription factors, i.e., transcription factors that are present in cells in an inactive state and do not require new protein synthesis in order to become activated. Proteins of the NFκB family share a Rel homology domain in their N-terminus. A subfamily of NF-κB proteins, including RelA, RelB, and c-Rel, have a transactivation domain in their C-termini. In contrast, the NF-κB1 and NF-κB2 proteins are synthesized as large precursors, p105, and p100, which undergo processing to generate the mature NF-κB subunits, p50 and p52, respectively.

NFκB may be activated by PAMPs and DAMPs as well as other heterologous compounds, including heterologous nucleic acids. An "activator" is a substance that increases the activity of an enzyme. An "activator of NFκB" is a substance that increase the activity of NFκB. Activators of NFκB may interact with cells to increase the activity of NFκB through various mechanisms, including, but limited to interaction with various PPRs such as TLRs or, more specifically, TLR3. "TLR3" or "Toll-like receptor 3" is a transmembrane protein encoded by the *TLR3* gene that is a member of the toll-like receptor family of PRRs of the innate immune system. TLR3 recognizes nucleic acids, such as dsRNA associated with viral infections, and induces the activation of NFκB. As demonstrated in the Examples that follow, NFκB activation or inhibition may be effectively used to accelerate or retard cardiomyocyte maturation.

Enhancement or upregulation of cardiomyocyte maturation.

A first aspect of the invention is compositions and methods for enhancing or upregulating cardiomyocyte maturation via the direct reprogramming of precursor cells. Reprogramming compositions for enhancing or upregulating cardiomyocyte maturation comprise (i) one or more miRs comprising a nucleotide sequence having at least 80% sequence identity to miR-1, miR-126, miR-133, miR-133a, mir-206, miR-208, miR-499, mir-4995p, and combinations thereof and (ii) an activator of NFκB. The activator of NFκB may be a TLR-pathway agonist, suitably a TLR3-pathway agonist. A "TLR-pathway agonist" is a composition or substance capable of interacting a TLR receptor or a substance associated with a TLR pathway that induces a biological response. A "TLR3-pathway agonist" is a TLR-pathway agonist that is capable of interacting with TLR3 or a substance associated with a TLR3 pathway that induces a biological response.

A "miR", also known as "miRNA" or "microRNA", is a small non-coding RNA typically comprising RNA having between about 15 to about 25 nucleotides. Some miRs are capable of folding back onto themselves to resemble dsRNA. miR-1, miR-126, miR-133, miR-133a, mir-206, miR-208, miR-499, mir-499-5p may be capable activating NFκB. Although miR-1, miR-126, miR-133, miR-133a, mir-206, miR-208, miR-499, mir-499-5p, or combinations thereof may be suitable for use activating NFκB, as used herein "activator of NFκB" excludes miR-1, miR-126, miR-133, miR-133a, mir-206, miR-208, miR-499, mir-499-5p, or any combination thereof. The use of miRs for direct reprogramming of cells to cardiomyoctes and cardiomyocytic tissue is described in US Patent Pub. No. 2014/0011281, published Jan. 9, 2014, and US Patent Pub. No. 2018/0042969, the contents of which are incorporated herein by reference in its entirety.

Nucleotide sequences of these preferred oligonucleotide constructs or combinations of constructs (and their corresponding mature forms) are listed below. Exemplary oligomeric compounds (stem-loop precursors) range in size from 50-90 nucleotides in length (or any length within that range, with an average length of approximately 70 nucleotides), and exemplary mature oligonucleotide compounds are 17 to 25 subunits in length, e.g., oligomeric compounds are 17, 18, 19, 20, 21, 22, 23, 24 or 25 subunits in length. For example, a stem-loop precursor is approximately 70 nucleotides and the mature nucleotide product is approximately 22 nucleotides in length. The uncapitalized "mir-" refers to the pre-miRNA, while a capitalized "miR-" refers to the mature form. A pre-microRNA comprises a stem-loop secondary structure.

TABLE 1: miRs

Mmu-miR-1

STEM-LOOP (SEQ ID NO: 1)
GCUUGGGACACAUACUUCUUUAUAUGCCCAUAUGAACCUGCUAAGCUAUG
GAAUGUAAAGAAGUAUGUAUUUCAGGC

MATURE (SEQ ID NO: 2)
UGGAAUGUAAAGAAGUAUGUAU

Mmu-miR-133a

STEM-LOOP (SEQ ID NO: 3)

GCUAAAGCUGGUAAAAUGGAACCAAAUCGCCUCUUCAAUGGAUUUGGUCC
CCUUCAACCAGCUGUAGC

MATURE (SEQ ID NO: 4)

UUUGGUCCCCUUCAACCAGCUG

Mmu-miR-206

STEM-LOOP (SEQ ID NO: 5)

CCAGGCCACAUGCUUCUUUAUAUCCUCAUAGAUAUCUCAGCACUAUGGAA
UGUAAGGAAGUGUGUGGUUUUGG

MATURE (SEQ ID NO: 6)

UGGAAUGUAAGGAAGUGUGUGG

Mmu-miR-208a

STEM-LOOP (SEQ ID NO: 7)

UUCCUUUGACGGGUGAGCUUUUGGCCCGGGUUAUACCUGACACUCACGUA
UAAGACGAGCAAAAAGCUUGUUGGUCAGAGGAG

MATURE (SEQ ID NO: 8)

AUAAGACGAGCAAAAAGCUUGU

Human miR-1-1

STEM-LOOP (SEQ ID NO: 9)

UGGGAAACAUACUUCUUUAUAUGCCCAUAUGGACCUGCUAAGCUAUGGAA
UGUAAAGAAGUAUGUAUCUCA

Human miR-1-2

STEM-LOOP (SEQ ID NO: 10)
ACCUACUCAGAGUACAUACUUCUUUAUGUACCCAUAUGAACAUACAAUGC
UAUGGAAUGUAAAGAAGUAUGUAUUUUUGGUAGGC

MATURE SEQUENCE FOR BOTH miR1 STEM-LOOPS: (SEQ ID NO: 11)
UGGAAUGUAAAGAAGUAUGUAU

Human miR-133a
Human miR-133a-1
STEM-LOOP (SEQ ID NO: 12)
ACAAUGCUUUGCUAGAGCUGGUAAAAUGGAACCAAAUCGCCUCUUCAAUG
GAUUUGGUCCCCUUCAACCAGCUGUAGCUAUGCAUUGA

Human miR-133a-2
STEM-LOOP (SEQ ID NO: 13)
GGGAGCCAAAUGCUUUGCUAGAGCUGGUAAAAUGGAACCAAAUCGACUGU
CCAAUGGAUUUGGUCCCCUUCAACCAGCUGUAGCUGUGCAUUGAUGGCGC
CG
MATURE SEQUENCE FOR BOTH miR133a STEM LOOPS
(SEQ ID NO: 14)
UUUGGUCCCCUUCAACCAGCUG

Human miR-206
STEM-LOOP (SEQ ID NO: 15)
UGCUUCCCGAGGCCACAUGCUUCUUUAUAUCCCCAUAUGGAUUACUUUGC
UAUGGAAUGUAAGGAAGUGUGUGGUUUCGGCAAGUG

MATURE SEQUENCE FOR miR-206 (SEQ ID NO: 16)
UGGAAUGUAAGGAAGUGUGUGG

Human miR-208a

STEM-LOOP (SEQ ID NO: 17)
UGACGGGCGAGCUUUUGGCCCGGGUUAUACCUGAUGCUCACGUAUAAGAC
GAGCAAAAAGCUUGUUGGUCA

MATURE SEQUENCE FOR miR-208 (SEQ ID NO:18)
AUAAGACGAGCAAAAAGCUUGU

Human miR-138-1
STEM-LOOP (SEQ ID NO: 19)
CCCUGGCAUGGUGUGGUGGGGCAGCUGGUGUUGUGAAUCAGGCCGUUGCC
AAUCAGAGAACGGCUACUUCACAACACCAGGGCCACACCACACUACAGG

Human miR-138-2
STEM-LOOP (SEQ ID NO: 20)
CGUUGCUGCAGCUGGUGUUGUGAAUCAGGCCGACGAGCAGCGCAUCCUCU
UACCCGGCUAUUUCACGACACCAGGGUUGCAUCA

MATURE SEQUENCE FOR BOTH miR-138-1 and miR-138-2 (SEQ ID NO: 21)
AGCUGGUGUUGUGAAUCAGGCCG

Human miR-499-5p
STEM-LOOP (MMu-miR-499) (SEQ ID NO: 22)
GGGUGGGCAGCUGUUAAGACUUGCAGUGAUGUUUAGCUCCUCUGCAUGUG
AACAUCACAGCAAGUCUGUGCUGCUGCCU

MATURE (Mmu-miR-499/Hsa-miR-499-5p; sequence is conserved) (SEQ ID NO: 23)
UUAAGACUUGCAGUGAUGUUU

Human miR-126

STEM-LOOP (Hsa-miR-126) (SEQ ID NO: 42)
CGCUGGCGACGGGACAUUAUUACUUUUGGUACGCGCUGUGACACUUCAAACUCGU
ACCGUGAGUAAUAAUGCGCCGUCCACGGCA

MATURE SEQUENCE FOR miR-126 (SEQ ID NO: 43)
UCGUACCGUGAGUAAUAAUGCG

Mature Sequence for miR-126-5p (SEQ ID NO: 44)
CAUUAUUACUUUUGGUACGCG

As demonstrated in the Examples that follow, high doses of miRs may impair normal cellular function. As a result, it was surprisingly found that miRs in combination with a distinct activator of NFκB may enhance or upregulate cardiomyocyte maturation without the deleterious effects of high doses of miRs. Suitably, the one or more miRs contacting cells are present in an amount less than about 0.30 mM, suitably less than or equal to about 0.28 mM, 0.26 mM, 0.24 nM, 0.22 mM, 0.20 mM, 1.8 mM, 1.6 mM, 0.14 mM, 0.12 mM, or 0.10 mM.

The one or more miRs may be suitably selected from a variety of miRs, including one or more nucleotide sequences having at least 80% sequence identity to miR-1, miR126, miR-133, miR-133a, mir206, miR-208, miR-499, and mir-499-5p. Suitably the one or more MiRs may comprise a nucleotide sequence having at least 85%, 90%, 95% or more sequence identity to miR-1, miR126, miR-133, miR-133a, mir206, miR-208, miR-499, and mir-499-5p. Suitably a combination more than one miR may include any two, any three, or any four miRs having a nucleotide sequences having at least 80%, 85%, 90%, 95% or more sequence identity to miR-1, miR126, miR-133, miR-133a, mir206, miR-208, miR-499, and mir-499-5p. Suitably the combination may include four miRs having at least 80%, 85%, 90%, 95% or more sequence identity to miR-1, miR-133a, miR208, and mir-499-5p. Suitably, the combination may include four miRs consisting essentially of miR-1, miR-133a, miR208, and mir-499-5p.

Suitably the one or more miRs comprise mirl; mirl, mir133a, and mir208; mirl, mir133a, and mir206; mirl, mir133a, mir208, and mir499-Sp; mirl, mir133a, mir206, and mir499-Sp; mirl and mir133; mirl and mir138; mirl and mir206; mirl and mir208; mir133 and mir138; mir133 and mir206; mir133 and mir208; mir138 and mir206; mir138 and mir208; mir206 and mir208; mirl, mir138, and mir208; mirl, mir206, and mir208; mir138, mir206, and mir208; mirl, mir133, and mir206; mirl, mir133, and mir208; mirl, mir138, and mir206; mir133, mir138, and mir208; and mir133, mir138, and mir206. In certain embodiments, the one or more miRs consist essentially of mirl; mirl, mir133a, and mir208; mirl, mir133a, and mir206; mirl, mir133a, mir208, and mir499-Sp; mirl, mir133a, mir206, and mir499-Sp; mirl and mir133; mirl and mir138; mirl and mir206; mirl and mir208; mir133 and mir138; mir133 and mir206; mir133 and mir208; mir138 and mir206; mir138 and mir208; mir206 and mir208; mirl, mir138, and mir208; mirl, mir206, and mir208; mir138, mir206, and mir208; mirl, mir133, and mir206; mirl, mir133, and mir208; mirl, mir138, and mir206; mir133, mir138, and mir208; and mir133, mir138, and mir206.

In the Examples that follow, “miR combo” is a combination of mirl, mir133a, mir208, and mir499-5p while “negmiR” is a miRNA that does not target TLR3 and used as a negative control.

Suitably, the composition comprises an activator of NFκB such as a TLR agonist or TLR3 agonist. The TLR3 agonist may comprise an RNA composition such as a 5’-triphospate, 2’-fluoro modified non-linear RNA. The 5’-triphospate, 2’-fluoro modified non-linear RNA comprises 2’-fluoro modified pyrimidines or 2’-fluoro modified purines. The 2’-fluoro modification may be present on at least one pyrimidine or purine, and may be present on any number of pyrimidines or purines, including all of the pyrimidines, all of the purines, or all of the pyrimidines and purines. Suitably the 2’-fluoro-modification is present in 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the pyrimidines and/or purines or any range therebetween. The 2-fluoro modification may be present on a uridine, a cytidine, a guanine, an adenine, or any combination thereof. In some embodiments, only uridines are 2’-fluoro modified. In an embodiment, all of the uridines in the RNA are 2’-fluoro-modified, all of the cytidines in the RNA are 2’-fluoro-modified, all of the guanines in the RNA are 2’-fluoro-modified, all of the adenines in the RNA are 2’-fluoro-modified, or any combination thereof. 5’-triphospate, 2’-fluoro modified non-linear RNA is described in International Pub. No. 2018/187328, published Oct. 11, 2013, the contents of which are incorporated herein by reference in its entirety.

The RNA compositions may comprise phosphorothioate modified nucleotides where a sulfur atom is substituted for a non-bridging oxygen of the phosphate. Suitably the phosphorothioate modification is present in 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the nucleotides or any range therebetween. In certain embodiments, the last 3 to 5 nucleotides at the 5'- and/or 3'-end of the oligonucleotide are phosphorothioate modified. In other embodiments, all of the nucleotides of the oligonucleotide are phosphorothioate modified.

The RNA compositions may comprise a blunt-end stem loop, a stem-loop having a 5’-overhang, a stem-loop having a 3’-overhang, or both a 5’-overhang and a 3’-overhang. Blunt-end stem loops comprise a 5’-terminal nucleotide and its 3’-terminal complement that are capable of hybridizing with each other, forming the stem-loop. Stem-loops having only a 5’-overhang comprise a 3’-terminal nucleotide capable of hybridizing with its complement to form the stem loop. Stem-loops having only a 3’-overhang comprise a 5’-terminal nucleotide capable of hybridizing with its complement to form the stem loop. For stem-loops having both a 5’-overhang and a 3’-overhang, neither the 5’-terminal nucleotide nor the 3’-terminal nucleotide form a part of the stem-loop.

A 5’- or 3’-overhang may be any length that allows for the RNA composition to inhibit cell growth or induce cell death. Suitably, the 5’- and/or 3’-overhang may be about 1 to about 50 nucleotides in length. In some embodiments, the 5’- and/or 3’-overhang is about 1 to about 10 nucleotides in length, including lengths of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides or any range of lengths therebetween. In other cases, the 5’- and/or 3’-overhang is about 10 to about 50 nucleotides in length, including lengths of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides or any range of lengths therebetween.

In certain embodiments, the RNA composition comprises multiple stem-loops. RNA compositions having multiple stem-loops minimally comprise a first stem-loop, a second stem-loop, and a spacer between the stem-loops.

The RNA composition may comprise a nucleotide sequence allowing for a terminal nucleotide to hybridize with it complement to form either the first stem-loop, the second stem-loop, or both. In some embodiments, the RNA composition comprises a 5’-triphosphate modified terminal nucleotide capable of hybridizing with its complementary nucleotide to form either the first or second stem-loop. In some embodiments, the RNA composition comprises a 3’-terminal nucleotide capable of hybridizing with its complementary nucleotide to form either stem-loop.

The RNA composition may comprise a 5’- or 3’-overhang associated with either or both of the first stem-loop and the second stem-loop. The 5’- or 3’-overhang associated with either the first stem-loop or the second stem-loop may be any length that allows for the RNA composition to inhibit cell growth or induce cell death. Suitably, the 5’- and/or 3’-overhang may be about 1 to about 50 nucleotides in length. In some embodiments, the 5’- and/or 3’-overhang is about 1 to about 10 nucleotides in length, including lengths of 1, 2, 3, 4, 5, 6, 7, 8, 9, of 10 nucleotides or any range of lengths therebetween. In other cases, the 5’- and/or 3’-overhang is about 10 to about 50 nucleotides in length, including lengths of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides or any range of lengths therebetween.

The spacer connects the stem loops in a multi-stem loop composition. In some embodiments, the spacer comprises a segment of ssRNA, a segment of dsRNA, or a combination thereof. A dsRNA segment may comprise a completely or partially hybridized segment of a segment of a first nucleotide sequence with a second nucleotide sequence. Spacers having only partial hybridization may have any number of nucleotide-pair mismatches that prevent nucleotide pairing between complementary nucleotides along the spacer. Preferably, the spacer remains thermodynamically or kinetically stable under physiological conditions. In some cases, the stem-loop has 1, 2, 3, 4, 5, or more nucleotide-pair mismatches.

The spacer may be any suitable length to provide the benefit of cytotoxicity without substantially inducing IFN production. Suitably, the length of the spacer may include between about 5 to about 100 nucleotides along a ssRNA segment, about 5 to about 100 hybridized or mismatched nucleotide pairs along a dsRNA segment, or a combination thereof. In some embodiments, the length of the spacer is about 5 to about 50 nucleotides, including lengths of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides or any range of lengths therebetween.

In some embodiments, the spacer is not associated with secondary structure. In other embodiments, the spacer is associated with secondary structure. Structured spacers may comprise a stem-loop, resulting in RNA compositions comprising at least a third stem-loop. The third stem-loops may be formed from the complete or partial hybridization of nucleotides and result in a hair-pin structural motif. The stem-loop may be formed from any suitable number of nucleotide pairings, including any number of nucleotide pairings between about 5 and about 30 or about 8 to about 25. In certain embodiments, the stem-loop comprises 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotide pairings or any number of nucleotide pairings therebetween. Stem-loops having only partial hybridization may have any number of nucleotide-pair mismatches that prevent nucleotide pairing between complementary nucleotides along the stem so long as the stem-loop remains stable under physiological conditions. In some cases, the stem-loop has 1, 2, 3, 4, or 5 nucleotide-pair mismatches or any range of nucleotide-pair mismatches therebetween.

Exemplary RNA oligonucleotides are provided in **Table 2**. The RNA compositions, referred to as Immunogenic Cancer cell-killing RNAs (ICRs), comprising 2'F pyrimidine-incorporated 5'ppp RNAs were designed and generated to contain 5'ppp and various predicted secondary structures including 3'-overhanged hairpin (ICR1, ICR1A, ICR1B, ICR1C), blunt-ended hairpin (ICR2-3, ICR2, ICR2A, ICR2B), 5'overhanged hairpin (ICR3, ICR3A, ICR3B, ICR3C), ssRNA comprising multiple stem-loops (ICR4, ICR4A) and dsRNA comprising multiple stem-loops (ICR5, which is formed from the hybridization of ICR5X and ICR5Y) at various lengths. Linear 5'ppp ssRNA (ICR-L) and long dsRNA (pIC) were also generated for comparison. As will be apparent to those of skill in the art, each of ICR1, ICR1A, ICR1B, ICR1C, ICR2A, ICR2B, ICR3, ICR3A, ICR3B, ICR3C, ICR4, ICR4A, ICR5X, and ICR5Y comprise the oligonucleotide sequence of ICR2.

TABLE 2: Single-stranded RNA

RNA	Sequence (5'→3')	SEQ ID NO
ICR1	ggaug cggua ccuga cagca uccua	SEQ ID NO: 45
ICR1A	ggaug cggua ccuga cagca uccua aagug	SEQ ID NO: 24
ICR1B	ggaug cggua ccuga cagca uccua aagug gugga aguga g	SEQ ID NO: 25
ICR1C	ggaug cggua ccuga cagca uccua aagug gugga aguga gugag ugaaa uaaaa a	SEQ ID NO: 26
ICR2-3	ggacg uaccu gacgu cc	SEQ ID NO: 27
ICR2-2	ggauc guacc ugacg aucc	SEQ ID NO: 28

ICR2-1	ggauc gguac cugac agauc c	SEQ ID NO: 29
ICR2	ggaug cggua ccuga cagca ucc	SEQ ID NO: 30
ICR2A	ggacg augcg guacc ugaca gcauc gucc	SEQ ID NO: 31
ICR2B	ggaug cggua ccuga cagca uccac cuggg augcu gucag guacc gcauc c	SEQ ID NO: 32
ICR3	ggagc ggaug cggua ccuga cagca ucc	SEQ ID NO: 33
ICR3A	gggga ggaca gcgga ugcgg uaccu gacag caucc	SEQ ID NO: 34
ICR3B	ggaau gaggg gagga cagcg gaugc gguac cugac agcau cc	SEQ ID NO: 35
ICR3C	gggua aguga augag gggag gacag cggau gcggu accug acagc aucc	SEQ ID NO: 36
ICR4	ggaug cggua ccuga cagca uccua aacuc auggu ccaug *uuugu c*caug gacca	SEQ ID NO: 37
ICR4A	ggaug cggua ccuga cagca uccua aacuc auggu ccaug *uuugu c*caug gacca acuac cgaca uugua u*gugu ug*aua uaaug u	SEQ ID NO: 38
ICR5X	ggaug cggua ccuga cagca uccug aguuu aguug uugu	SEQ ID NO: 39
ICR5Y	ggaug cggua ccuga cagca uccac aacaa cuaaa cuca	SEQ ID NO: 40
ICR-L	gguuu uuuuu uuuuu uuuuu uuu	SEQ ID NO: 41

In some embodiments, the RNA composition comprises an oligonucleotide capable of forming a stem-loop. In some embodiments, the RNA composition comprises one or more stem-loops formed from the complete or partial hybridization of an oligonucleotide having at least 50% sequence identity to ICR2. In particular embodiments, the RNA composition comprises one or more stem-loops formed from the complete or partial hybridization of an oligonucleotide having at least 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92,%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to ICR2. The RNA composition may also consist essentially of one or more stem-loops formed from the complete or partial hybridization of an oligonucleotide having at least 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92,%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to ICR2.

In some embodiments, the RNA composition comprises one or more oligonucleotides having at least 50% sequence identity to ICR1, ICR1A, ICR1B, ICR1C, ICR2A, ICR2B, ICR3, ICR3A, ICR3B, ICR3C, ICR4, ICR4A, ICR5X, or ICR5Y. In particular embodiments, the RNA composition comprises one or more oligonucleotides having at least 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92,%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to any ICR1, ICR1A, ICR1B, ICR1C, ICR2A, ICR2B, ICR3, ICR3A, ICR3B, ICR3C, ICR4, ICR4A, ICR5X, or ICR5Y. The RNA composition may also consist essentially of one or more oligonucleotides having at least 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92,%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to any of ICR1, ICR1A, ICR1B, ICR1C, ICR2A, ICR2B, ICR3, ICR3A, ICR3B, ICR3C, ICR4, ICR4A, ICR5X, or ICR5Y.

In some embodiments, the activator of NFκB may be a substance other than an RNA product. Suitably, the activator of NFκB may be a microbe (e.g., a bacteria or a virus), a microbial product (e.g., a bacterial product or a viral product), an cytokine, a oxidative stressor, a physical stressor, a therapeutically used drug, a modified protein, an overexpressed protein, an overexpressed ligand, a apoptic mediator, a mitogen, a growth factor, a hormone, a physiological mediator, a chemical agent. Exemplary activators of NFκB are described in Pahl, H. L., Oncogene (1999) 18, 6853-6866, which is incorporated herein by reference in its entirety.

The TLR-pathway agonist may further comprise reprogramming media. Reprogramming media comprises a base tissue culture media, insulin-transferrin-selenium (ITS) or ascorbic acid in a somatic cell-reprogramming, e.g., fibroblast-to-cardiomyocyte-reprogramming, amount. The media may further comprise bovine serum albumin (BSA) or L-glutamine. A somatic cell reprogramming amount of insulin-transferrin-selenium is characterized by insulin being present in an amount of 10 nanomolar to 10 micromolar (e.g., 100 nM), transferrin being present in an amount of 0.002 to l gram per liter (e.g., 0.055 g/1), and selenium being present in an amount of 1-100 μg per liter (e.g., 6.7 μg per liter). Optionally, the media comprises 0.2 mM to 20 mM L-glutamine (e.g., 2 mM). The media may also optionally include 50 μM to 50 millimolar ascorbic acid such as 100-500 μM, e.g, 250 μM, of ascorbic acid. The use of reprogramming media for direct reprogramming of cells to cardiomyoctes and cardiomyocytic tissue is described in US

Patent Pub. No. 2018/0042969, the contents of which are incorporated herein by reference in its entirety.

The reprogramming composition may comprise one or more reprogramming efficiency-enhancing molecules. “Reprogramming efficiency-enhancing molecules” are molecules suitable for increasing the efficiency of conversion to cardiac myocytes. Exemplary reprogramming efficiency-enhancing molecules include valproic acid, bone morphogenetic protein 4 (BMP4), Janus protein tyrosine kinase (JAK) inhibitor 1, RG108, R(+) Bay K 8644, PS48, and A83-01. These agents are delivered (e.g., infused or injected) to the subject before, after, or together with the TLR-pathway agonist.

The reprogramming composition may comprise a cytoplasmic delivery agent. A “cytoplasmic delivery agent” is an agent that transport of molecules, suitably nucleic acids, across membranes. Exemplary cytoplasmic delivery agents include, without limitation, transfection agents such as DharmaFECT, liposomes, synthetic polymers, cell-penetrating peptides, nanoparticles, viral particles, electroporation buffers, nucleofection reagents, or any combination thereof

Methods of enhancing or upregulating cardiomyocyte maturation comprise contacting a cell with an effective amount of any of the compositions described for a sufficient time such that the cell is reprogrammed into a cardiomyocyte. Suitably the cell is a fibroblast, e.g., a cardiofibroblast or a dermal fibroblast, and/or comprises cardiac fibrotic tissue.

Methods of enhancing or upregulating cardiomyocyte maturation in a subject comprising administering an effective amount of any of the compositions described or any of the pharmaceutical compositions described. Suitably the subject is in need of enhancing or upregulating cardiomyocyte maturation to alleviate symptoms, eliminate the causation of resultant symptoms either on a temporary or permanent basis, and/or to prevent or slow the appearance or to reverse the progression or severity of resultant symptoms of cardiac fibrotic tissue. Suitably the cell is a fibroblast, e.g., a cardiofibroblast or a dermal fibroblast, and/or comprises cardiac fibrotic tissue.

Inhibition or downregulation of cardiomyocyte maturation

Another aspect of the invention is compositions and methods for inhibiting or downregulating cardiomyocyte maturation. Compositions for inhibiting or downregulating cardiomyocyte maturation comprise a TLR inhibitor, a NFκB inhibitor, a ikbkb inhibitor, or any combination thereof.

A “TLR inhibitor” is a composition or substance capable of interacting specifically with a TLR receptor that blocks or dampens a biological response. The TLR inhibitor may be a TLR3 inhibit. Suitably a TLR inhibitor may be a TLR antagonist. A “TLR-pathway antagonist” is a composition or substance capable of interacting a TLR receptor or a substance associated with a TLR pathway that blocks or dampens a biological response. A “TLR3-pathway antagonist” is a TLR-pathway antagonist that is capable of interacting with TLR3 or a substance associated with a TLR3 pathway that blocks or dampens a biological response. Exemplary TLR or TLR3 inhibitors include, without limitation, CU-CPT-4a, or a siRNA that interferes with the translation of the TLR protein.

A “NFκB inhibitor” is a composition or substance capable of interacting specifically with NFκB that blocks or dampens a biological response. Exemplary NFκB inhibitors include, without limitation, Bay 11-7085, or a siRNA that interferes with the translation of a NFκB protein such as RelA.

A “ikbkb inhibitor” or “inhibitor of NFκB kinase subunit beta” is a composition or substance capable of interacting specifically with ikbkb that blocks or dampens a biological response or a siRNA that interferes with the translation of the ikbkb protein.

Suitably the TLR inhibitor is a TLR3 inhibitor. e.g., an antibody, shRNA small molecule or other competitive inhibitor capable of blocking TLR3 activation and/or signaling. Suitably, the TLR inhibitor is a TLR3 inhibitor such as CU-CPT-4a used in the Examples.

Suitably the composition may further comprise a cytoplasmic delivery agent, cellular media, or any combination thereof.

Methods of inhibiting or downregulating cardiomyocyte maturation comprise contacting a cell with an effective amount of any of the compositions described for a sufficient time such that cardiomyocyte maturation is inhibited or down-regulated in the cell. Suitably the cell is a fibroblast, e.g., a cardiofibroblast or a dermal fibroblast, and/or comprises cardiac fibrotic tissue.

Pharmaceutical compositions

The compositions utilized in the methods disclosed herein may be formulated as pharmaceutical compositions that include: (a) a therapeutically effective amount of one or more compounds as disclosed herein; and (b) one or more pharmaceutically acceptable carriers, excipients, or diluents. The pharmaceutical composition may include one or more compounds as disclosed herein in a range of about 0.1 to 2000 mg, including about 0.5 to 500 mg or about 1 to 100 mg. The pharmaceutical composition may be administered to provide the compound at a daily dose of about 0.1 to 100 mg/kg body weight, including about 0.5 to 20 mg/kg body weight or about 0.1 to 10 mg/kg body weight. In some embodiments, after the pharmaceutical composition is administered to a patient (*e.g.*, after about 1, 2, 3, 4, 5, or 6 hours post-administration). The concentration of the compound at the site of action is an effective amount of a composition if at least some of the cells at the site of action have or will mature into a cardiomyocyte.

The compounds utilized in the methods disclosed herein may be formulated as a pharmaceutical composition that includes a carrier. For example, the carrier may be selected from the group consisting of proteins, carbohydrates, sugar, talc, magnesium stearate, cellulose, calcium carbonate, and starch-gelatin paste.

The compounds utilized in the methods disclosed herein may be formulated as a pharmaceutical composition that includes one or more binding agents, filling agents, lubricating agents, suspending agents, sweeteners, flavoring agents, preservatives, buffers, wetting agents, disintegrants, and effervescent agents.

Suitable diluents may include pharmaceutically acceptable inert fillers, such as microcrystalline cellulose, lactose, dibasic calcium phosphate, saccharides, and mixtures of any of the foregoing.

The compounds utilized in the methods disclosed herein may be formulated as a pharmaceutical composition for delivery via any suitable route. For example, the pharmaceutical composition may be administered via oral, intravenous, intramuscular, subcutaneous, topical, and pulmonary route.

The compounds utilized in the methods disclosed herein may be administered in conventional dosage forms prepared by combining the active ingredient with standard pharmaceutical carriers or diluents according to conventional procedures well known in the art.

Pharmaceutical compositions comprising the compounds may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) route. Such formulations may be prepared by any method known in the art of pharmacy, for example by bringing into association the active ingredient with the carrier(s) or excipient(s).

Pharmaceutical compositions adapted for oral administration may be presented as discrete units such as capsules or tablets; powders or granules; solutions or suspensions in aqueous or non-aqueous liquids; edible foams or whips; or oil-in-water liquid emulsions or water-in-oil liquid emulsions.

Pharmaceutical compositions adapted for transdermal administration may be presented as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be delivered from the patch by iontophoresis.

Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

The compositions and methods disclosed herein may be administered as pharmaceutical compositions and, therefore, pharmaceutical compositions incorporating the compounds are considered to be embodiments of the compositions disclosed herein. Such compositions may take any physical form which is pharmaceutically acceptable; illustratively, they can be orally administered pharmaceutical compositions. Such pharmaceutical compositions contain an effective amount of a disclosed compound, which effective amount is related to the daily dose of the compound to be administered. Each dosage unit may contain the daily dose of a given compound or each dosage unit may contain a fraction of the daily dose, such as one-half or one-third of the dose. The amount of each compound to be contained in each dosage unit can depend, in part, on the identity of the particular compound chosen for the therapy and other factors, such as the indication for which it is given. The pharmaceutical compositions disclosed herein may be formulated so as to provide quick, sustained, or delayed release of the active ingredient after administration to the patient by employing well known procedures.

The compounds for use according to the methods of disclosed herein may be administered as a single composition or a combination of compounds. For example, a composition for cardiomyocyte maturation may be administered as a single compound or in combination with another compound for cardiomyocyte maturation or that has a different pharmacological activity.

As indicated above, pharmaceutically acceptable salts of the compounds are contemplated and also may be utilized in the disclosed methods. The term "pharmaceutically acceptable salt" as used herein, refers to salts of the compounds which are substantially non-toxic to living organisms. Typical pharmaceutically acceptable salts include those salts prepared by reaction of the compounds as disclosed herein with a pharmaceutically acceptable mineral or organic acid or an organic or inorganic base. Such salts are known as acid addition and base addition salts. It will be appreciated by the skilled reader that most or all of the compounds as disclosed herein are capable of forming salts and that the salt forms of pharmaceuticals are commonly used, often because they are more readily crystallized and purified than are the free acids or bases.

Acids commonly employed to form acid addition salts may include inorganic acids such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, phosphoric acid, and the like, and organic acids such as p-toluenesulfonic, methanesulfonic acid, oxalic acid, p-bromophenylsulfonic acid, carbonic acid, succinic acid, citric acid, benzoic acid, acetic acid, and the like. Examples of suitable pharmaceutically acceptable salts may include the sulfate, pyrosulfate, bisulfate, sulfite, bisulfate, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, bromide, iodide, acetate, propionate, decanoate, caprylate, acrylate, formate, hydrochloride, dihydrochloride, isobutyrate, caproate, heptanoate, propiolate, oxalate, malonate, succinate, suberate, sebacate, fumarate, maleat-, butyne-.1,4-dioate, hexyne-1,6-dioate, benzoate, chlorobenzoate, methylbenzoate, hydroxybenzoate, methoxybenzoate, phthalate, xylenesulfonate, phenylacetate, phenylpropionate, phenylbutyrate, citrate, lactate, alpha-hydroxybutyrate, glycolate, tartrate, methanesulfonate, propanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, mandelate, and the like.

Base addition salts include those derived from inorganic bases, such as ammonium or alkali or alkaline earth metal hydroxides, carbonates, bicarbonates, and the like. Bases useful in preparing such salts include sodium hydroxide, potassium hydroxide, ammonium hydroxide, potassium carbonate, sodium carbonate, sodium bicarbonate, potassium bicarbonate, calcium hydroxide, calcium carbonate, and the like.

The particular counter-ion forming a part of any salt of a compound disclosed herein is may not be critical to the activity of the compound, so long as the salt as a whole is pharmacologically acceptable and as long as the counterion does not contribute undesired qualities to the salt as a whole. Undesired qualities may include undesirably solubility or toxicity.

Pharmaceutically acceptable esters and amides of the compounds can also be employed in the compositions and methods disclosed herein. Examples of suitable esters include alkyl, aryl, and aralkyl esters, such as methyl esters, ethyl esters, propyl esters, dodecyl esters, benzyl esters, and the like. Examples of suitable amides include unsubstituted amides, monosubstituted amides, and disubstituted amides, such as methyl amide, dimethyl amide, methyl ethyl amide, and the like.

In addition, the methods disclosed herein may be practiced using solvate forms of the compounds or salts, esters, and/or amides, thereof. Solvate forms may include ethanol solvates, hydrates, and the like.

Subjects

As used herein, a “subject” may be interchangeable with “patient” or “individual” and means an animal, which may be a human or non-human animal, in need of treatment. A “subject in need of treatment” may include a subject having a disease, disorder, or condition that is responsive to therapy with the compositions disclosed herein. For example, a “subject in need of treatment” may include a subject having a cardiovascular disease, such as an atherosclerotic disease, or having suffered a myocardial infarction.

Methods of treatment

As used herein, the terms “treating” or “to treat” each mean to alleviate symptoms, eliminate the causation of resultant symptoms either on a temporary or permanent basis, and/or to prevent or slow the appearance or to reverse the progression or severity of resultant symptoms of the named disease or disorder. As such, the methods disclosed herein encompass both therapeutic and prophylactic administration.

As used herein the term “effective amount” refers to the amount or dose of the compound, upon single or multiple dose administration to the subject, which provides the desired effect in the subject under diagnosis or treatment. The disclosed methods may include administering an effective amount of the disclosed compositions (*e.g.*, as present in a pharmaceutical composition) for inducing cardiomyocyte maturation or inhibiting cardiomyocyte maturation.

An effective amount can be readily determined by the attending diagnostician, as one skilled in the art, by the use of known techniques and by observing results obtained under analogous circumstances. In determining the effective amount or dose of compound administered, a number of factors can be considered by the attending diagnostician, such as: the species of the subject; its size, age, and general health; the degree of involvement or the severity of the disease or disorder involved; the response of the individual subject; the particular compound administered; the mode of administration; the bioavailability characteristics of the preparation administered; the dose regimen selected; the use of concomitant medication; and other relevant circumstances.

A typical daily dose may contain from about 0.01 mg/kg to about 100 mg/kg (such as from about 0.05 mg/kg to about 50 mg/kg and/or from about 0.1 mg/kg to about 25 mg/kg) of each compound used in the present method of treatment.

Compositions can be formulated in a unit dosage form, each dosage containing from about 1 to about 500 mg of each compound individually or in a single unit dosage form, such as from about 5 to about 300 mg, from about 10 to about 100 mg, and/or about 25 mg. The term “unit dosage form” refers to a physically discrete unit suitable as unitary dosages for a patient, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical carrier, diluent, or excipient.

EXAMPLES

EXAMPLE: Cardiomyocyte maturation requires TLR3 activated NFκB

We demonstrate that TLR3 inhibition blocked cardiomyocyte maturation; precursor cells committed to the cardiomyocyte lineage failed to express maturation genes and sarcomeres did not develop. We establish that the effects of TLR3 upon cardiomyocyte maturation were dependent upon the RelA subunit of NFκB. Importantly, under conditions that promote the development of mature cardiomyocytes NFκB became significantly enriched at the promoters of cardiomyocyte maturation genes. Furthermore, activation of the TLR3-NFκB pathway enhanced cardiomyocyte maturation. This study therefore demonstrates that the TLR3-NFκB pathway is necessary for the maturation of committed precursors into mature cardiomyocytes.

Introduction

Cardiomyocytes are an essential component of the heart. Genetic manipulation can cause abnormal cardiac morphogenesis; typically leading to embryonic lethality. This embryonic lethality is difficult to diagnose prenatally and limits our understanding of the process by which precursors commit to the cardiac lineage and mature into fully functional cardiomyocytes. Due to this hurdle, many researchers have taken to replicating cardiomyocyte development in the culture dish. Various methods have been employed including reprogramming strategies [1-8] [9-14].

In vitro modelling suggests that cardiomyocyte development has two phases; Initiation and Maturation [15-18]. In the Initiation phase of cardiomyocyte development, the precursor cell initially expresses a number of so-called pioneer transcription factors. These pioneer transcription factors induce significant epigenetic remodeling; the precursor phenotype is silenced and various genes that are necessary for commitment to the cardiomyocyte lineage are activated. The pioneer transcription factors have been identified: combined expression of Gata4, Tbx5, Mef2C and Hand1 is necessary for the initial commitment to the cardiomyocyte lineage [16]. The key epigenetic mechanism in the Initiation phase of cardiomyocyte development is histone methylation. H3K4 becomes methylated [19] whereas H3K27 is de-methylated [14]. These epigenetic modifications work in concert to activate and repress numerous genes that are necessary to stabilize commitment to the cardiomyocyte lineage. Whereas the steps in the Initiation phase of cardiomyocyte development are well known, the process of cardiomyocyte maturation has not been studied in detail.

Two recent studies have shown that TLR3 is important for reprogramming fibroblasts to iPS [20] and endothelial cells [21]. Specifically, activation of TLR3 causes global changes in the expression and activity of epigenetic modifiers that favor DNA accessibility, and phenotypic fluidity. Interestingly, TLR3 plays a role in the inflammatory response and it is known that inflammation plays a major role in the cardiac response to injury [22, 23]. We show herein that cardiomyocyte maturation requires TLR3 activated NFκB. Precursor cells that had committed to the cardiomyocyte lineage were prevented from maturing into cardiomyocytes by TLR3 inhibitors or TLR3 knockdown. Further experiments demonstrate that TLR3 controlled cardiomyocyte maturation via NFκB. Pharmacological inhibition of NFκB, as well as knockdown of Ikbkb (Inhibitor of Nuclear Factor Kappa B Kinase) which activates NFκB, prevented cardiomyocyte maturation. Moreover, conditions that induce cardiomyocyte formation induced NFκB binding to the promoters of cardiomyocyte maturation genes. Moreover, we found that microRNAs activate TLR3.

Materials & Methods

Chemicals: TLR agonists were purchased from Invivogen (Mouse TLR1-9 agonist kit, tlrl-kit1mw). The TLR3 antagonist CU-CPT-4a, NFκB antagonist Bay 11-7085 and the AP1 antagonist SR 11302 were purchased from Tocris.

MicroRNA transfection: Mouse (C57BL/6) neonatal cardiac fibroblasts were isolated from 2 day old mouse neonates according to the method outlined in Jayawardena et al [10]. Following isolation fibroblasts were cultured in growth media containing DMEM (ATCC, Catalogue number 30-2002) supplemented with 15%v/v FBS (Thermo Scientific Hyclone Fetal bovine serum, Catalogue number SH30071.03, Lot number AXK49952) and 1%v/v penicillin/streptomycin (Gibco, Catalogue number 15140-122, 100units Penicillin, 100ug/ml Streptomycin). Fibroblasts were passaged once the cells had reached 70-80% confluence using 0.05% w/v trypsin (Gibco, Catalogue number 25300-054). Freshly isolated fibroblasts were labelled as Passage 0. Experiments were conducted with cells at passage 2. For all experiments, cells were seeded at 5000 cells/cm^2 in growth media. After 24 hours, the cells were transfected with transfection reagent alone (Dharmafect-I, ThermoScientific), with transfection reagent plus non-targeting microRNAs (negmiR), or with transfection reagent plus our previously reported combination of cardiac reprogramming microRNAs[9] (miR combo, miR-1, miR-133, miR-208, miR-499).

qPCR: Total RNA was extracted using Quick-RNA MiniPrep Kit according to the manufacturer's instructions (Zymo Research). Total RNA (50ng-100ng) was converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). cDNA was used in a standard qPCR reaction involving FAM conjugated gene specific primers and TaqMan Gene Expression Master Mix (Applied Biosystems). The following primers were used for qPCR:

Gapdh (Mm99999915_m1), Tnni3 (Mm00437164_m1), Actn2 (Mm00473657_m1), Myh6 (Mm00440359_m1), Cacna1c (Mm00437917_m1), Mef2C (Mm01340482_m1), Tbx5 (Mm00803518_m1), Gata4 (Mm00484689_m1) and Hand2 (Mm00439247_m1).

Immunofluorescence: Cells were fixed with 2%v/v paraformaldehyde (EMS) as described previously [24]. Fixed cells were blocked in antibody buffer (5%w/v BSA, 0.1%v/v Tween-20, in PBS) for 1 hr at room temperature. Following blocking, cells were incubated overnight at 4°C with α-sarcomeric actinin antibody (Sigma, A7811, 1:100) in antibody buffer. After the overnight incubation, cells were washed three times in antibody buffer. Following washing, cells were incubated with Alexa-Fluor conjugated secondary antibodies (Invitrogen, Goat Anti-mouse 594nm) at a 1:500 dilution in antibody buffer for 1hr at room temperature. Nuclei were stained by DAPI at 1μg/ml for 30 minutes at room temperature in antibody buffer. Following washing in PBS to remove unbound complexes, immunofluorescence was measured using a Zeiss Axiovert 200 inverted microscope.

siRNA knockdown: siRNA pools (four siRNAs targeting the gene) and a negative control siRNA were purchased from Dharmacon. siRNAs were made to 20μM in nuclease free water, aliquoted, and stored -80°C until use. Fibroblasts were seeded into 12 well plates at 20,000 cells per well one day prior to transfection. On the day of transfection siRNAs were diluted to 5μM in nuclease free water. For each well, 5μl of the working siRNA solution was diluted with 95μl Optimem-Serum Free media. In a separate tube 5μl of Dharmafect-I (Dharmacon) was diluted with 95μl Optimem-Serum Free media. After 5 minute incubation the two solutions were combined. After 20 minutes complete media lacking antibiotics was added (800μl) and the transfection complexes added to the cells.

ChIP assays: ChIP assays were performed according the manufacturer's instructions (Cell Signaling, SimpleChIP Enzymatic Chromatin IP kit #9003). Neonatal cardiac fibroblast nuclei were digested with 0.1ul Micrococcal nuclease per 4x10^6 cells (amount of Micrococcal nuclease was empirically determined according the manufacturer's instructions). Immunoprecipitation was performed with ChIP validated antibodies: (1) rabbit IgG control (Cell Signaling, #2729); (2) Histone H3 (Cell Signaling, #4620); and (3) RelA (Cell Signaling, #8242). Immunoprecipitated DNA was quantified by qPCR (ThermoFisher, Power SYBR Green PCR Master Mix, #4367659) with primers for the promoters of Myh6 (Qiagen, EpiTect ChIP qPCR Primer Assay For Mouse Myh6, NM_010856.3 (-)08Kb #GPM1045733(-)08A and EpiTect ChIP qPCR Primer Assay For Mouse Myh6, NM_010856.3 (-)01Kb #GPM1045733(-)01A), Actn2 (Qiagen, EpiTect ChIP qPCR Primer Assay For Mouse Actn2, NM_033268.3 (-)01Kb, #GPM1044781(-)01A) and Tnni3 (Qiagen, EpiTect ChIP qPCR Primer Assay For Mouse Tnni3, NM_009406.3 (-)01Kb, #GPM1052593(-)01A). PCR reactions included the positive control Histone H3 sample and the negative control rabbit IgG sample. A serial dilution of the 2% input chromatin DNA (undiluted, 1:5, 1:25, 1:125) was used to create a standard curve and determine efficiency of amplification. Percent input was calculated and negative control IgG values subtracted. Data is presented as the fold change of percent input between miR combo and negmiR treated samples.

IL6 ELISA: IL6 ELISA kits were from R&D Systems. Fresh media (1ml) was added to the cells one day prior to assaying for IL6. Per manufacturer's instructions 50ul of media was assayed and the amount of IL6 in pg/ml in the culture media was determined via a standard curve. The IL6 pg/ml value was then adjusted for the total volume of the media (1ml) and the total cellular protein in each well to correct for differences in cell number[25].

Generating beating reprogrammed cardiomyocytes: Isolated mouse (C57BL/6) neonatal cardiac fibroblasts (passage 2) were seeded into 12-well dishes at 15000 cells/cm^2 in growth media. Twenty-four hours later growth media was removed and the cells transfected with negmiR or miR combo as described above. One day later, the transfection complexes were removed and media was replaced with a chemically defined reprogramming media[12] that contained 1ug/ml Poly(I:C) (LMW). For the next four days, cells received fresh chemically defined reprogramming media [12] containing 1ug/ml Poly(I:C) (LMW) daily. After this period, the cells received chemically defined reprogramming media [12] without Poly(I:C) (LMW) for a further 10 days. Media was replaced every other day. Beating colonies were identified with a Zeiss Axiovert 200 inverted microscope.

Images: Images were processed with CorelDraw and Zeiss software (Axiovision Rel4.8 and Zen Blue).

Statistics: All statistical analysis was performed using GraphPad. Experiments containing two conditions a t-test was performed. ANOVA was used for experiments with three or more conditions followed by Bonferroni post-hoc tests for comparisons between individual groups. A P-value of less than 0.05 was considered significant.

Results

TLR3 inhibition blocks the maturation phase of cardiac reprogramming. The mechanisms by which committed cells mature into cardiomyocytes are unclear. Two recent studies have shown that TLR3 is important for reprogramming fibroblasts to iPS [20] and endothelial cells [21]. Moreover, TLR3 induces inflammation and inflammation is known to be important in injury. Consequently, we asked ourselves if TLR3 played a hitherto unknown role in the development of mature cardiomyocytes. We were interested in TLRs as these receptors are key mediators of the inflammatory responses in the heart.

In the first instance, we tested our hypothesis with the specific TLR3 pharmacological inhibitor CU-CPT-4a [26, 27]. We were specifically interested in the maturation phase of cardiac reprogramming. To that end, we carried out an initial screen for the mRNA levels for components of that are involved in cardiomyocyte sarcomere function. We used our previously described miR combo to induce cardiac reprogramming. MiR combo is a combination of four microRNAs (miR-1, -133, -208, -499) that robustly induces cardiac reprogramming both in vitro and in vivo[9, 11-14]. As shown in Figure 1A miR combo significantly induced the expression of 13 components of the cardiomyocyte sarcomere. The effect of miR combo upon cardiomyocyte sarcomere gene expression was completely abolished by the TLR3 pharmacological inhibitor CU-CPT-4a (Figure 1).

We verified our initial screen by measuring the mRNA levels of three components of the cardiomyocyte sarcomere: Myh6 (αmyosin heavy chain), Actn2 (αsarcomeric actinin) and Tnni3 (cardiac troponin-I). As we observed in our initial screen, pharmacological inhibition of TLR3 completely inhibited miR combo reprogramming with respect to the expression of Myh6, Actn2, and Tnni3 (Figures 1Bi-1Biii). We then assessed the effects of TLR3 inhibition upon the maturation of reprogrammed fibroblasts at the cellular level. The ability of miR combo to generate mature cardiomyocytes with organized sarcomeres was completely inhibited by CU-CPT-4a (Figures 1Ci-1Ciii). These results were then verified by siRNA mediated knockdown of TLR3. Knockdown of TLR3 by siRNA was robust (Figure 1D) and completely abrogated miR combo reprogramming with respect to the expression of Myh6, Actn2, and Tnni3 (Figures 1Ei-1Eiii).

Neither TLR3 inhibition nor TLR3 activation affects the initiation phase of cardiac reprogramming. Following these results, we wanted to investigate the mechanism by which

TLR3 influenced the maturation of reprogrammed cells in more detail. During heart development, in the initial phase of differentiation of precursors into cardiomyocytes epigenetic processes act to turn on expression of the cardiomyocyte-lineage commitment factors Gata4, Hand2, Tbx5 and Mef2C are expressed[28]. Similarly, increased expression of these cardiomyocyte-lineage commitment factors in fibroblasts represents the initial phase of cardiac reprogramming [1-6, 9, 12, 14]. We found that the expression of the cardiomyocyte-lineage commitment factors Gata4, Hand2, Tbx5 and Mef2C, that was induced by miR combo, was not affected by either TLR3 knockdown (Figures 2Ai-2Aiv) or by TLR3 activation (Figures 2Bi-2Biv). This data indicates that the effects of TLR3 upon the cardiac reprogramming were not due to changes in the initiation phase of cardiac reprogramming.

TLR3 controls the maturation phase of cardiac reprogramming via the RelA subunit of NFκB. TLR3 mediates the activation of a number of transcription factors[22]. Of these transcription factors, two mediate the vast majority of the effects of TLR3: AP1 and NFκB[22]. Consequently, we hypothesized that TLR3 would influence maturation of reprogrammed cells via AP1 and/or NFκB. Pharmacological inhibition of AP1 had no effect on the ability of miR combo to reprogram fibroblasts (data not shown). In contrast, the pharmacological inhibition of NFκB completely inhibited miR combo reprogramming at both the RNA (Figures 3Ai-3Aiii) and protein (Figures 3Bi-3Biii) level.

We further verified a role for NFκB in the maturation of reprogrammed cells by targeting Ikbkb; a kinase that is necessary for NFκB activation[29]. Knockdown of Ikbkb was robust (Figure 3C). Importantly, knockdown of Ikbkb completely inhibited miR combo reprogramming with respect to the expression of the cardiomyocyte maturation markers Myh6, Actn2, and Tnni3 (Figures 3Di-3Diii). In agreement with the studies described above, Ikbkb knockdown did not influence the initiation phase of cardiac reprogramming (data not shown). Moreover; Ikbkb knockdown did not affect miR combo mediated suppression of endodermal, ectodermal and vascular markers (data not shown).

There are five NFκB proteins: NF-κB1 (p105/p50); NF-κB2 (p100/p52); RelA (p65); RelB; and c-Rel. Only RelA, RelB and c-Rel induce transcription. We focused on RelA as it is the most highly expressed Rel protein. Knockdown of RelA, which was found to be robust (Figure 4A), prevented the appearance of Actn2(+) cells in miR combo transfected fibroblasts

(Figures 4Bi-4Biii). Effects at the protein level were also observed at the mRNA level; targeting RelA with siRNA completely inhibited miR combo reprogramming with respect to sarcomere-related gene expression (Figures 4Ci-4Ciii). We also noted that RelA knockdown had no effect on the expression of the endodermal marker Gata6 or the general differentiation marker Tgfb2 (Figures 4Di-4Dii).

Finally, we wanted to determine how RelA controlled the expression of cardiomyocyte maturation genes. Consequently, we used ChIP assays to determine if miR combo induced RelA binding to the promoters of components of the cardiomyocyte sarcomere. Significant enrichment of RelA was observed at the *Actn2*, *Myh6*, *Mypn* and *Tnni3* promoters (Figures 5Ai-5Aiv) following miR combo treatment. Similar enrichment was also observed for the *Ttn*, *Myoz2*, *Tnnc1* and *Tnnt2* promoters; however, this failed to reach $P<0.05$ significance (Figures 5Av-5Aviii). There was no enrichment in the unrelated gene RPL30 (data not shown). Targeted knockdown of RelA completely removed the ChIP signal. This result verified that RelA was indeed binding to the promoters of the cardiomyocyte sarcomere genes (Figures 5Bi-5Bviii).

MicroRNAs activate TLR3 The pharmacological inhibitor and siRNA mediated knockdown experiments suggested that miR combo activated TLR3. To test this further we transfected cells with microRNAs and assessed TLR3 activity by measuring IL6 secretion into the media. IL6 secretion is an accepted measurement of the activity of TLRs, including TLR3 [30-37]. When compared to mock transfected fibroblasts both the control non-targeting miRNA (negmiR) and miR combo significantly induced IL6 secretion (Figure 6A). Comparisons between negmiR and miR combo indicated that miR combo had the stronger effect. The induction of IL6 secretion by microRNAs was TLR3 dependent; the addition of the TLR3 inhibitor CU-CPT-4a, which inhibits interaction between RNA and TLR3, completely ablated the effect of negmiR and miR combo upon IL6 secretion (Figure 6A). Targeted knockdown of TLR3 or Ikbkb inhibited miR combo induced IL6 secretion; further validating that miR combo activated the TLR3-NFκB pathway (Figure 6B).

Pharmacological activation of TLR3 enhances maturation of reprogrammed fibroblasts. Following the identification of the mechanism by which TLR3 controlled miR combo reprogramming, we next examined if stimulation of TLR3 could enhance the efficiency of miR combo. As expected, miR combo increased RNA levels of Myh6, Actn2 and Tnni3 (Figures 7Ai-7Aiii). The effect of miR combo upon Myh6, Actn2 and Tnni3 expression was significantly enhanced by the addition of the TLR3 agonist Poly(I:C) (Figures 7Ai-7Aiii). Intriguingly, Poly(I:C) also induced expression of Myh6, Actn2 and Tnni3 in the control negmiR samples (Figures 7Ai-7Aiii). This effect, considering that Poly(I:C) had no effect on the expression of cardiomyocyte-commitment factors, is further evidence that the TLR3 pathway controls the maturation phase of cardiac reprogramming. We then performed immunostaining to determine if the effects at the RNA level were also observed at the protein level. Indeed, we found that the number of Actn2(+) cells that formed in response to miR combo treatment was increased by the TLR3 agonist Poly(I:C) (Figures 7Bi-7Bvi, with quantification provided in Figure 7C). We also noted that TLR3 activation enhanced sarcomere maturation (see figure inserts in Figures 7Bi-7Bvi).

In accordance with our previous study [9], we found that transfecting fibroblasts with miR combo led to the appearance of spontaneously beating colonies (Figures 7Di-7Dii). The ability of miR combo to form spontaneously beating colonies was increased 3-fold by the addition of the TLR3 agonist Poly(I:C) (Figures 7Di-7Dii). Importantly, mature beating colonies were observed within one week of transfection.

Discussion

In this study we demonstrate that TLR3 activated NFκB is an important mechanism for the maturation of committed precursors into cardiomyocytes.

Our study clearly identified a role for TLR3 activated NFκB specifically in the maturation phase of cardiac reprogramming. This differs from previous studies which have linked TLR3 activated NKκB to the reprogramming to iPS [20] or endothelial cells [21]. These previous studies demonstrate that TLR3-NFκB causes global changes in the expression and activity of epigenetic modifiers that favors increased DNA accessibility. In this open chromatin configuration, the activation of the pluripotency program by the Yamanaka factors[20], or the induction of endothelial lineage by trans-differentiation factors[21], is facilitated. In these studies, the epigenetic plasticity that is induced by TLR3 activation is largely mediated by NFκB, as shown using pharmacological or molecular antagonists of NFκB.

We have extended this work by examining the role of TLR in maturation of cardiomyocyte precursors. We found that TLR3 activation increased the binding of NFκB directly to cardiomyocyte sarcomere genes. By contrast, we found that TLR3 played no role in the commitment of precursors into the cardiomyocyte lineage. TLR3 inhibition or knockdown did not influence the expression of various transcription factors that are necessary for commitment into the cardiomyocyte lineage.

As mentioned above, we found that miR combo induced RelA binding to the promoters of various components of the cardiomyocyte sarcomere. Canonical RelA binding sites are present in the Myh6 promoter but are absent in the promoters of the Actn2, Mypn and Tnni3 genes. Non-canonical RelA binding sites have been identified in other genes [43], and they are present in in the Actn2, Mypn and Tnni3 gene promoters. However, it is also possible that RelA influences cardiac gene expression through an indirect mechanism. RelA, and NFκB, have been shown to modulate gene expression through binding to other proteins [44-46] as well as by modulating the activity of the epigenetic machinery. It is possible that the RelA subunit of NFκB plays a similar role in cardiomyocyte maturation.

Our study suggests that microRNAs directly activate TLR3. Several TLRs are known to bind to nucleic acids: TLR3; TLR7; TLR8; and TLR9 [47]. TLR3 recognizes double-stranded (ds) RNA; whereas TLR7 and TLR8 bind to single-stranded RNA. In contrast, TLR9 is activated by unmethylated CpG sequences in DNA molecules [47]. Only a limited number of reports have demonstrated that microRNAs bind to TLRs. Even though microRNAs are dsRNA molecules, the microRNAs miR-21, miR-29a, and Let-7b bind, and activate, TLR7 and TLR8 [48, 49]. With respect to TLR3, it was originally suggested that microRNAs might be too small to induce efficient dimerization, and thus activation, of TLR3 [50]. However, this assumption is likely to need revision both in light of our results as well as the recent report that the plant derived microRNA FvmiR168 binds to dendritic cell TLR3[51]. We found that miR combo more strongly induced TLR3 than the negative control microRNA used in our studies. This may suggest that TLR3 activation by microRNAs is sequence dependent. In support of this notion, siRNA mediated activation of TLRs has been shown to be sequence dependent [52].

EXAMPLE: TLR3 activation enhances cardiomyocyte maturation

Cardiac fibroblasts were transfected with miR combo or negmiR (control) and incubated with various concentrations of ICR2 (Figs. 8A-8D) or ICR4 (Figs. 9A-9D) for 4 days. Reprograming was evaluated via qPCR, where we measure the expression of genes that are necessary for sarcomere function (e.g. Actn2, Myh6, Tnni3) as well as cardiac ion channels

(Cacna1c). Gene expression was evaluated at day 14. To further verify that ICR2 induced maturation, cardiac fibroblasts were incubated with miR combo in addition to PolyIC or ICR2 for 14 days. Sarcomeres were visualized by antibody staining for a-sarcomeric actinin (Figs 11A and 11B).

EXAMPLE: High doses of miR combo impair normal cellular functions

Neonatal cardiac fibroblasts were transfected with either 0.1mM or 0.3mM miR combo. Equivalent concentrations of a non-targeting miRNA were used as a control. Cell number is represented as a fold change derived from GAPDH expression at day 0 and day 14 (Fig. 10). The dotted line indicates the cell number at day 0. Cell number increased significantly in both concentrations of control miR and the standard concentration of miR combo. High concentrations of miR combo impaired normal cell number growth either by inhibiting cell proliferation or by increasing rate of cell death. N=2.

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CLAIMS

We claim:

1. A reprogramming composition comprising:

(a) one or more miRs comprising a nucleotide sequence having at least 80% sequence identity tomiR-1 (SEQ ID NO: 11), miR-133a (SEQ ID NO: 14), miR-208 (SEQ ID NO: 18), and mir-499-5p (SEQ ID NO: 23), and combinations thereof; and

(b) an activator of NFκB.

2. The composition of claim 1, wherein the activator of NFκB comprises a TLR3 agonist.

3. The composition of claim 1 or 2, wherein the activator of NFκB comprises a modified 5'-triphospate, 2'-fluoro modified non-linear RNA, the RNA comprising a stem-loop formed from the complete or partial hybridization of at least 8 nucleotide pairings.

4. The composition of claim 3, wherein the non-linear RNA comprises an oligonucleotide having at least 80% sequence identity to ICR2 (SEQ ID NO: 30), ICR4 (SEQ ID NO: 37), ICR4A (SEQ ID NO: 38), ICR5 X (SEQ ID NO: 39), or ICR5Y (SEQ ID NO: 40).

5. The composition of claim 4, wherein the non-linear RNA comprises an oligonucleotide having at least 80% sequence identity to ICR2 (SEQ ID NO: 30).

6. The composition of claim 4, wherein the non-linear RNA comprises an oligonucleotide having at least 80% sequence identity to ICR4 (SEQ ID NO: 37).

7. The composition of any one of claims 1-8 further comprising a cytoplasmic delivery agent, reprogramming media, a reprogramming efficiency-enhancing molecule, or any combination thereof.

8. The composition of any one of claims 1-7, wherein the one or more miRs consists essentially of: miR-1 (SEQ ID NO: 11), miR-133a (SEQ ID NO: 14), miR208 (SEQ ID NO: 18), and mir-499-5p (SEQ ID NO: 23).

9. The composition of any one of claims 1-8, wherein the one or more miRs comprise a portion of a pre-miRNA.

10. A pharmaceutical composition comprising an effective amount of the composition of any of claims 1-9 and one or more pharmaceutically acceptable carriers, excipients, or diluents.

11. A method for enhancing or upregulating cardiomyocyte maturation in a cell comprising contacting the cell with an effective amount of the reprogramming composition of claim 1 for a sufficient time such that the cell is reprogrammed into a cardiomyocyte.

12. The method of claim 11, wherein the cell is a fibroblast.

13. The method of claim 11, wherein the cell comprises cardiac fibrotic tissue.

14. The method of any one of claims 11-13, wherein the activator of NFκB comprises a TLR3 agonist.

15. The method of claim 14, wherein the activator of NFκB comprises a modified 5'-triphospate, 2'-fluoro modified non-linear RNA, the RNA comprising a stem-loop formed from the complete or partial hybridization of at least 8 nucleotide pairings.

16. The method of claim 15, wherein the non-linear RNA comprises an oligonucleotide having at least 80% sequence identity to ICR2 (SEQ ID NO: 30), ICR4 (SEQ ID NO: 37), ICR4A (SEQ ID NO: 38), ICR5X (SEQ ID NO: 39), or ICR5Y (SEQ ID NO: 40).

17. The method of claim 16, wherein the non-linear RNA comprises an oligonucleotide having at least 80% sequence identity to ICR2 (SEQ ID NO: 30).

18. The method of claim 16, wherein the non-linear RNA comprises an oligonucleotide having at least 80% sequence identity to ICR4 (SEQ ID NO: 37).

19. The method of any one of claims 11-18, wherein the composition further comprising a cytoplasmic delivery agent, reprogramming media, a reprogramming efficiency-enhancing molecule, or any combination thereof.

20. The method of any one of claims 11-19, wherein the one or more miRs consists essentially of: miR-1 (SEQ ID NO: 11), miR-133a (SEQ ID NO: 14), miR208 (SEQ ID NO: 18), and mir-499-5p (SEQ ID NO: 23).

21. The method of any one of claims 11-20, wherein the one or more miRs comprise a portion of a pre-miRNA.

22. A method of enhancing or upregulating cardiomyocyte maturation in a subject comprising administering (i) an effective amount of the of the composition of claim 1 or (ii) a pharmaceutical composition comprising the effective amount of the composition of claim 1 and one or more pharmaceutically acceptable carriers, excipients, or diluents.

23. The method of claim 22, wherein the subject has a cardiovascular disease or has suffered a myocardial infarction.

24. The method of claim 22 or 23, wherein the activator of NFκB comprises a TLR3 agonist.

25. The method of claim 24, wherein the activator of NFκB comprises a modified 5'-triphospate, 2'-fluoro modified non-linear RNA, the RNA comprising a stem-loop formed from the complete or partial hybridization of at least 8 nucleotide pairings.

26. The method of claim 25, wherein the non-linear RNA comprises an oligonucleotide having at least 80% sequence identity to ICR2 (SEQ ID NO: 30), ICR4 (SEQ ID NO: 37), ICR4A (SEQ ID NO: 38), ICR5X (SEQ ID NO: 39), or ICR5Y (SEQ ID NO: 40).

27. The method of claim 26, wherein the non-linear RNA comprises an oligonucleotide having at least 80% sequence identity to ICR2 (SEQ ID NO: 30).

28. The method of claim 26, wherein the non-linear RNA comprises an oligonucleotide having at least 80% sequence identity to ICR4(SEQ ID NO: 37).

29. The method of any one of claims 22-28, wherein the composition further comprising a cytoplasmic delivery agent, reprogramming media, a reprogramming efficiency-enhancing molecule or any combination thereof.

30. The method of any one of claims 22-29, wherein the one or more miRs consists essentially of: miR-1 (SEQ ID NO: 11), miR-133a (SEQ ID NO: 14), miR208 (SEQ ID NO: 18), and mir-499-5p (SEQ ID NO: 23)p.

31. The method of any one of claims 22-30, wherein the one or more miRs comprise a portion of a pre-miRNA.

32. A method for inhibiting or downregulating cardiomyocyte maturation in a cell comprising contacting the cell with an effective amount of a composition comprising a TLR3 inhibitor, a NFκB inhibitor, a ikbkb inhibitor, or a combination thereof for a sufficient time such that cardiomyocyte maturation is inhibited or down-regulated in the cell.

33. The method of claim 32, wherein the cell is a cardiomyocyte-committed precursor.

34. The method of claim 33, wherein the cell is a fibroblast.

35. The method of any one of claims 32-34, wherein the composition comprises the TLR3 inhibitor.

36. The method of claim 35, wherein the TLR3 inhibitor is a TLR3 siRNA or CU-CPT-4a.

37. The method of any one of claims 32-34, wherein the composition comprises the NFκB inhibitor.

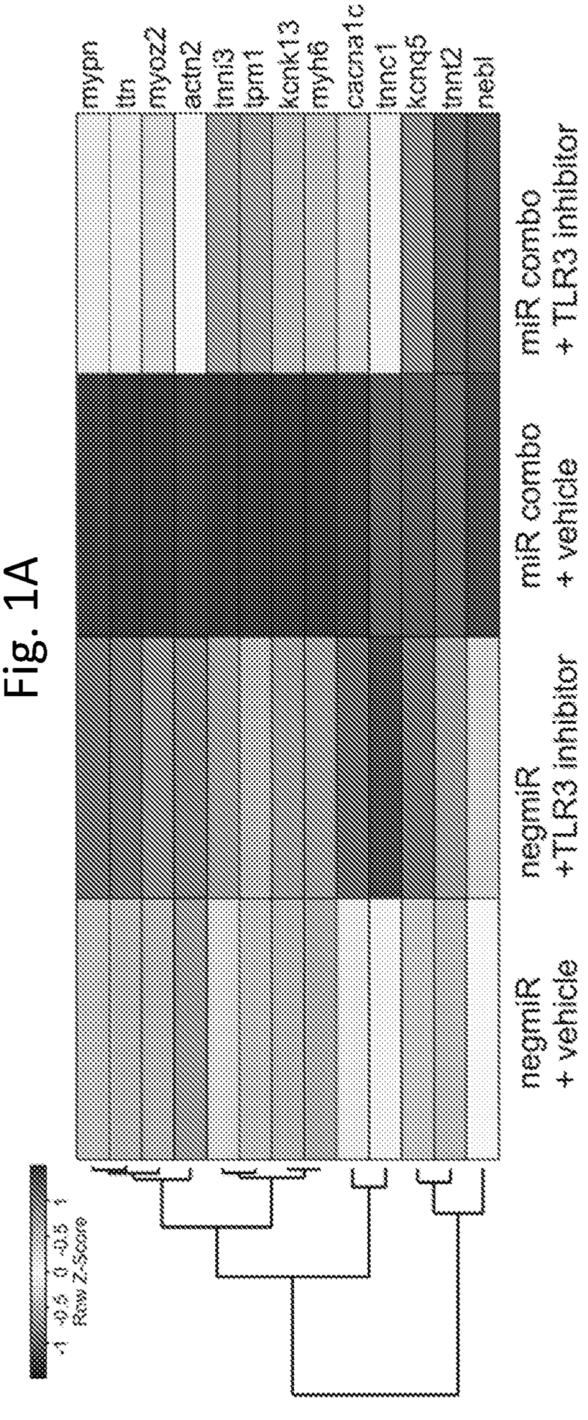
38. The method of claim 37, wherein the NFκB inhibitor is a NFκB siRNA, a RelA siRNA, or Bay 11-7085.

39. The method of any one of claims 32-34, wherein the composition comprises the ikbkb inhibitor.

40. The method of claim 39, wherein the ikbkb inhibitor is a ikbkb siRNA.

41. The method of any one of claims 32-40, wherein the composition further comprises a cytoplasmic delivery agent, cellular media, or any combination thereof.

Fig. 1A



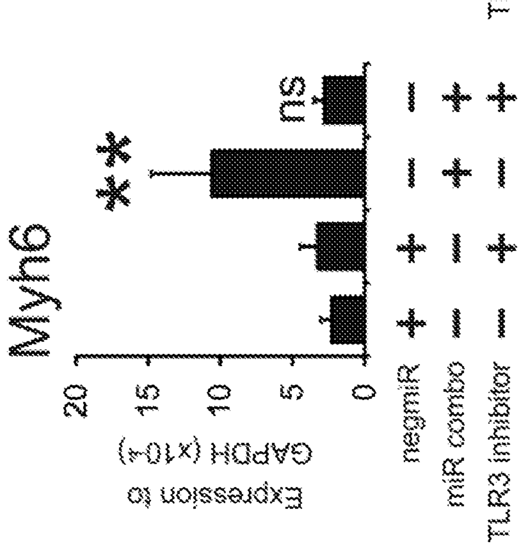


Fig. 1Bi

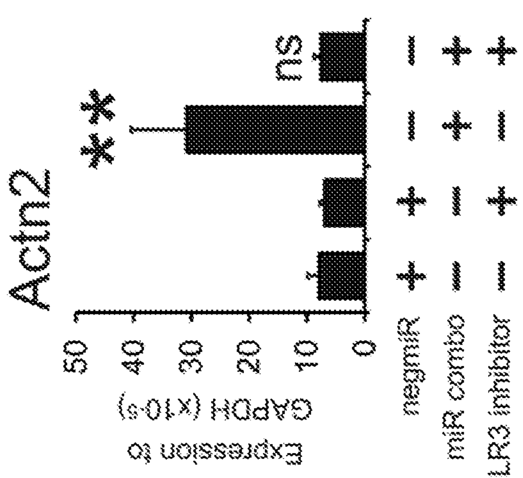


Fig. 1Bii

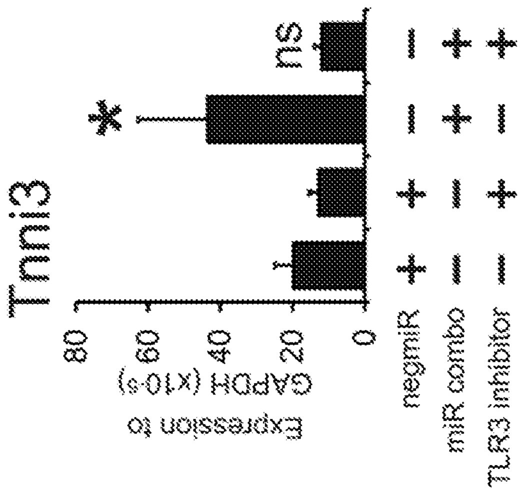


Fig. 1Biii

Fig. 1Ci

Fig. 1Cii

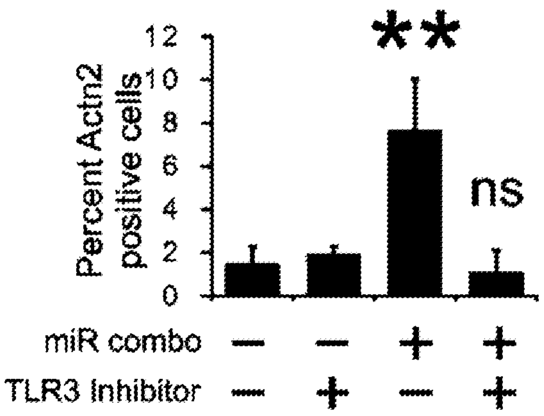


Fig. 1Ciii

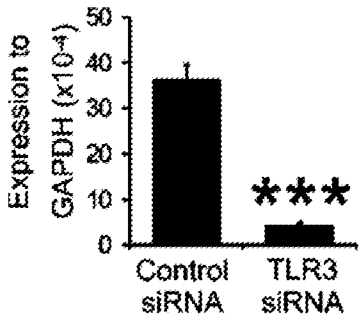


Fig. 1D

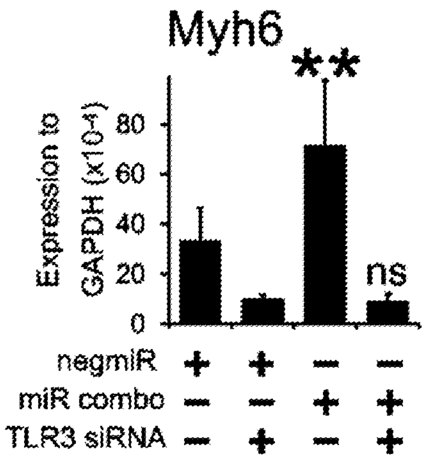


Fig. 1Ei

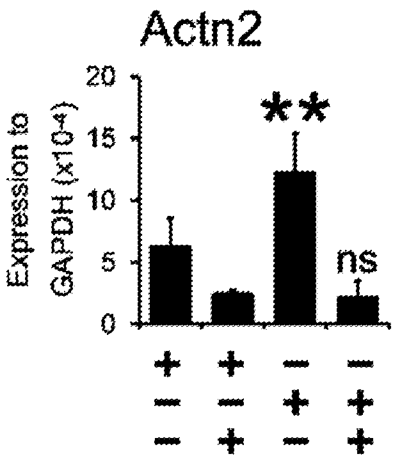


Fig. 1Eii

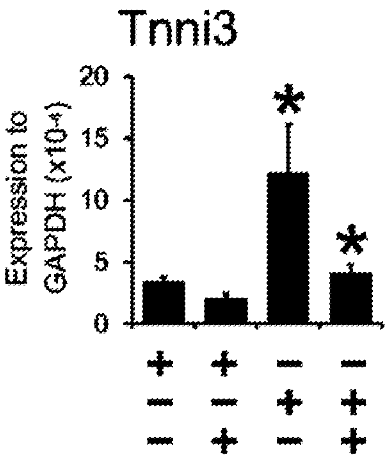
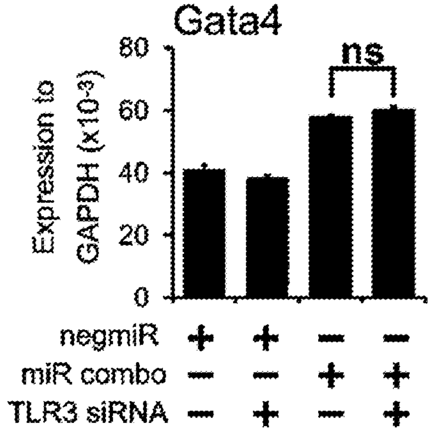


Fig. 1Eiii

Fig. 2Ai



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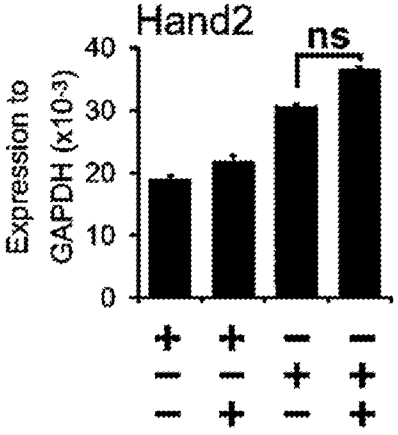


Fig. 2Aiii

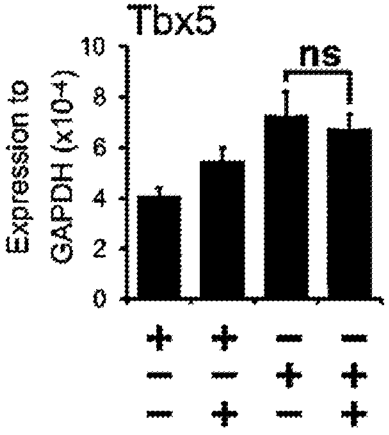
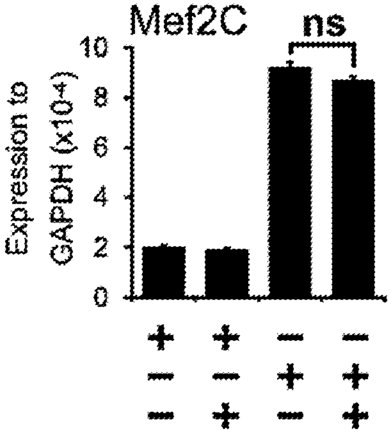


Fig. 2Aiv



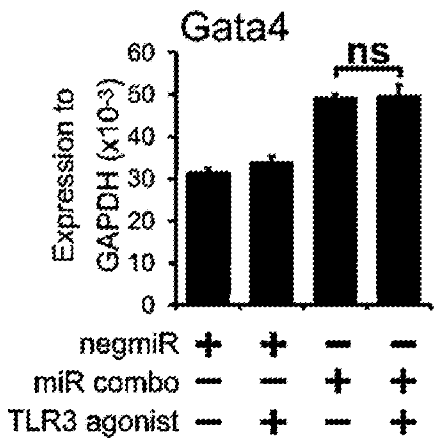


Fig. 2Bi

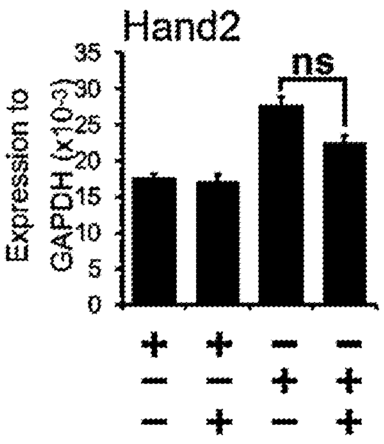
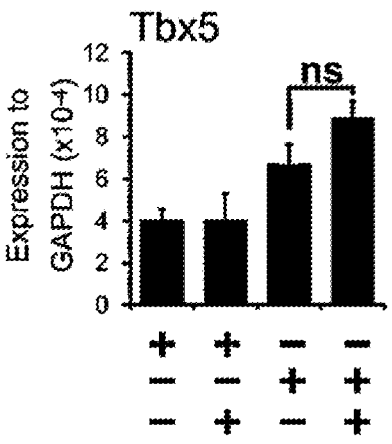


Fig. 2Bii



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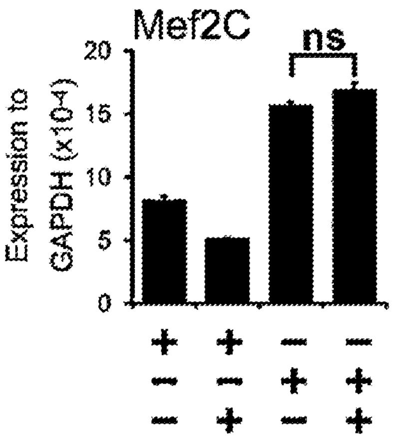


Fig. 2Biv

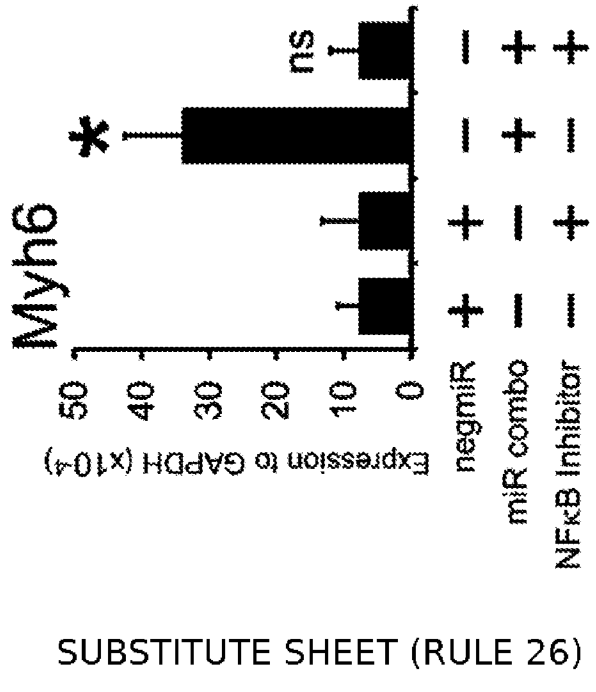


Fig. 3Ai

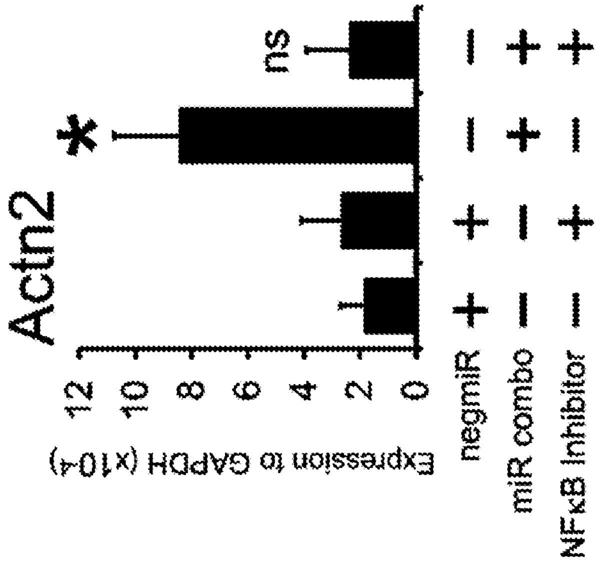


Fig. 3Aii

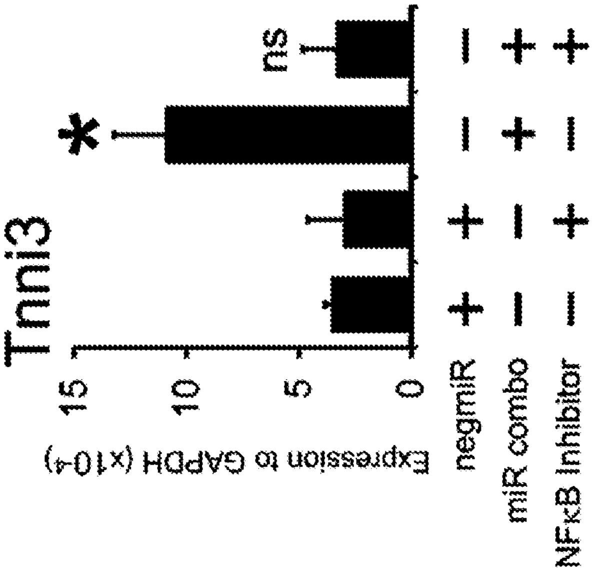


Fig. 3Aiii

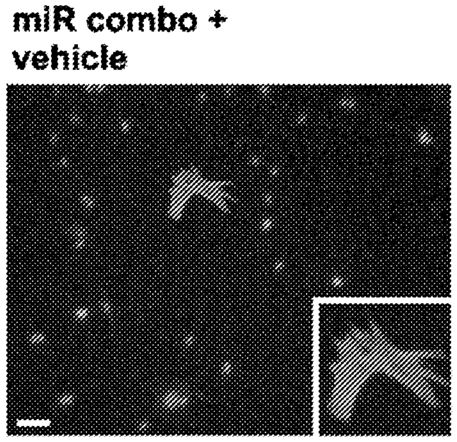
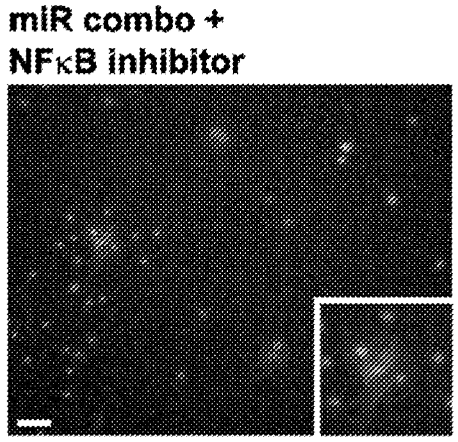


Fig. 3Bi



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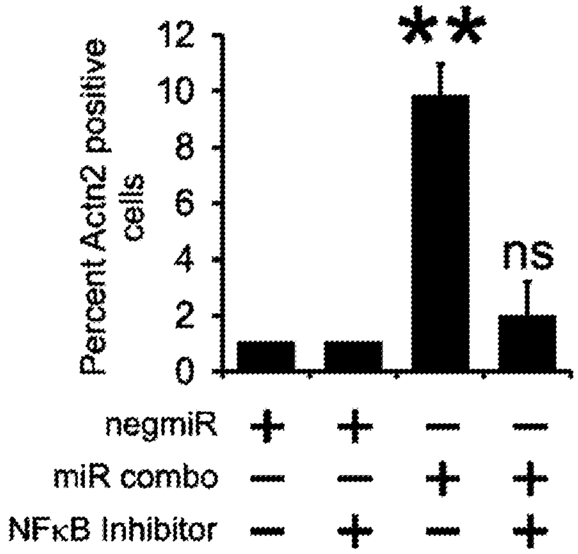


Fig. 3Biii

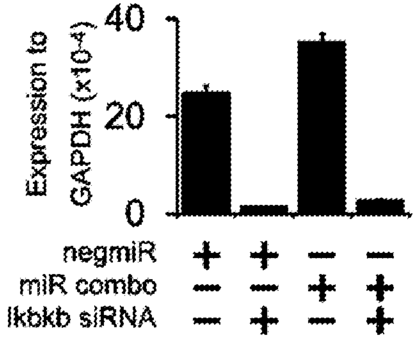


Fig. 3C

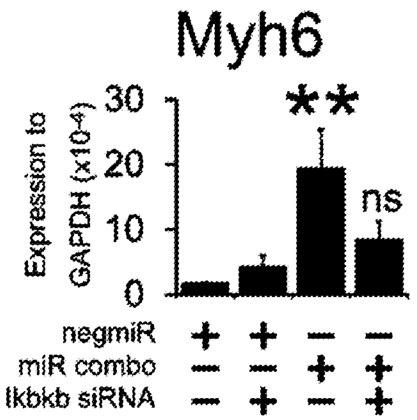


Fig. 3Di

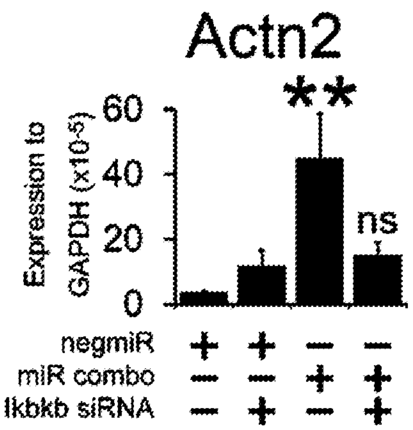


Fig. 3Dii

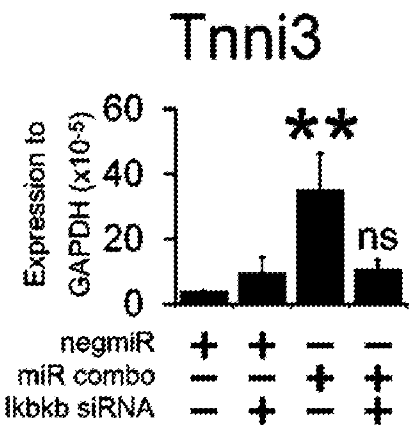


Fig. 3Diii

Fig. 4A

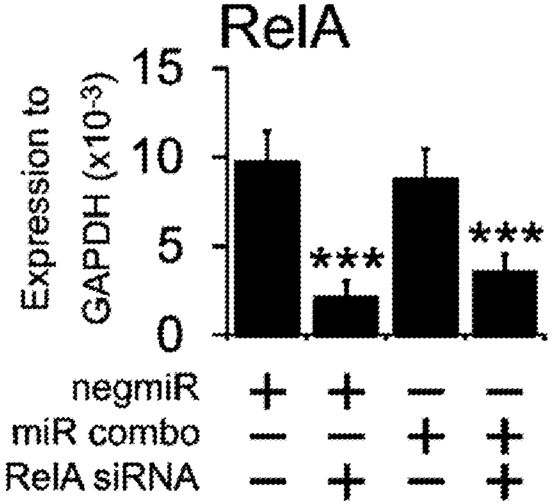
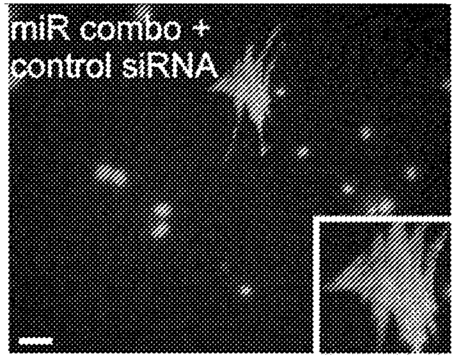


Fig. 4Bi



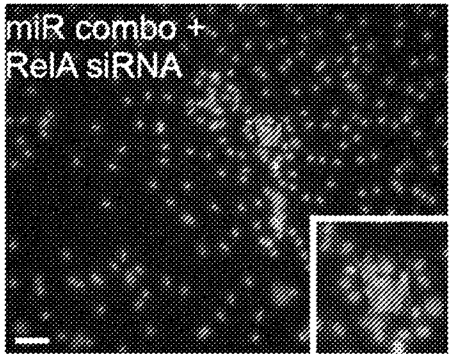


Fig. 4Bii

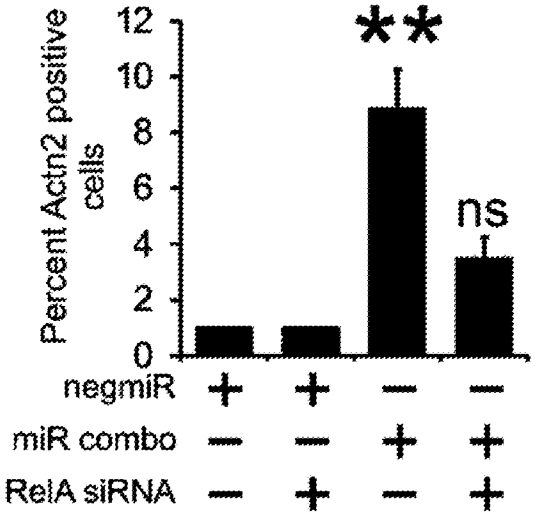
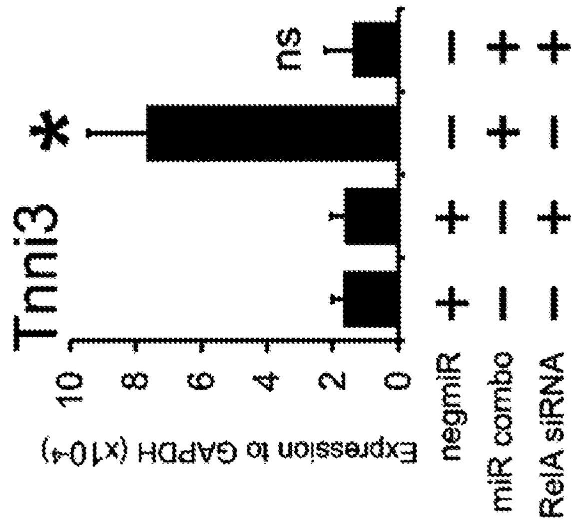


Fig. 4Biii

Fig. 4Ciii



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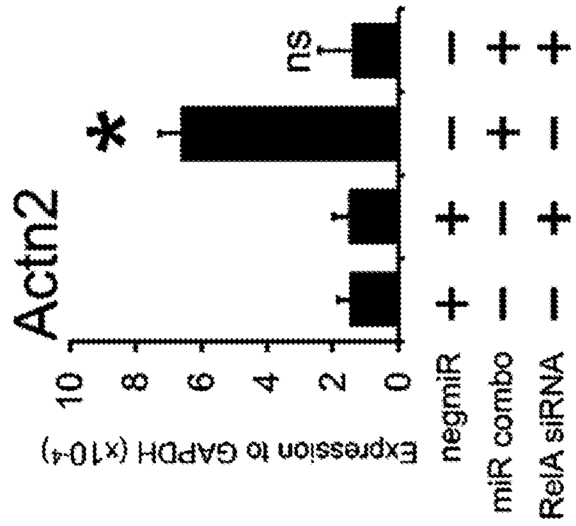
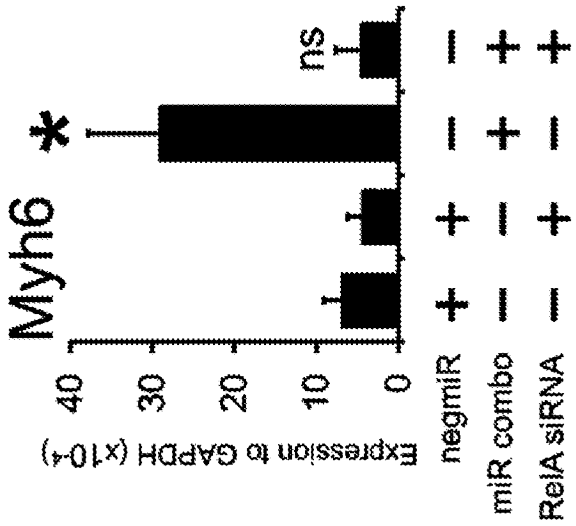


Fig. 4Ci



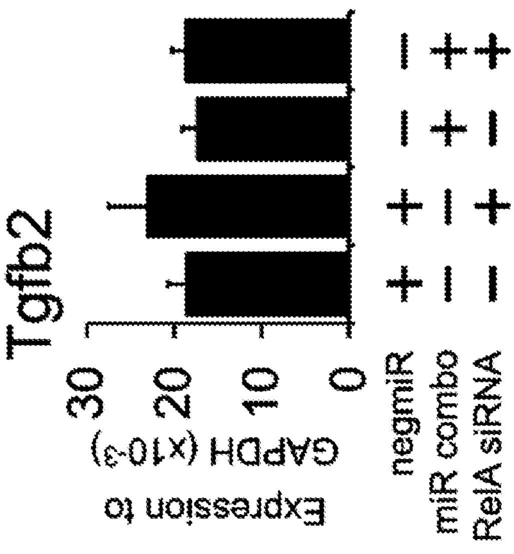


Fig. 4Dii

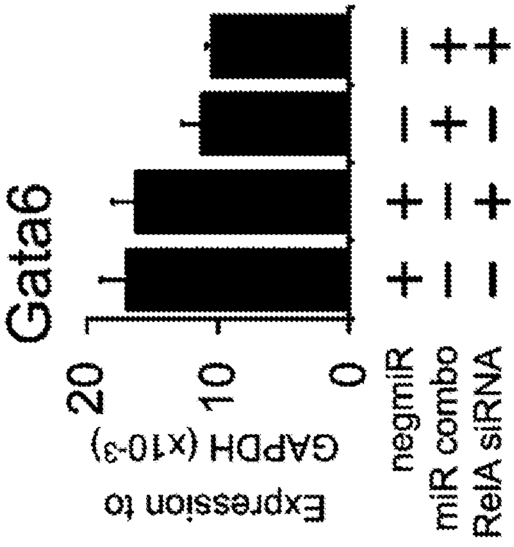


Fig. 4Di

Fig. 5Ai

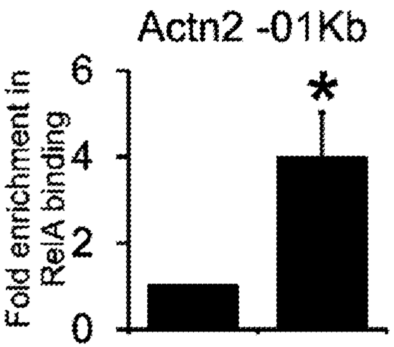


Fig. 5Aii

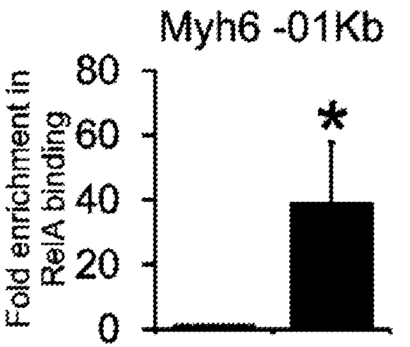


Fig. 5Aiii

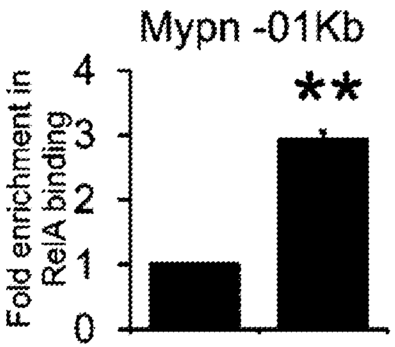
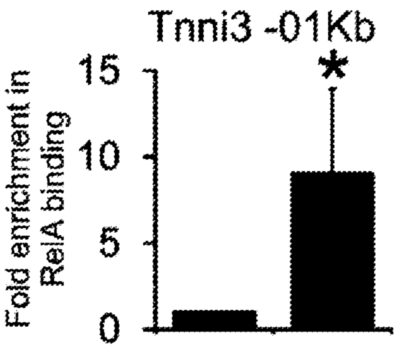


Fig. 5Aiv



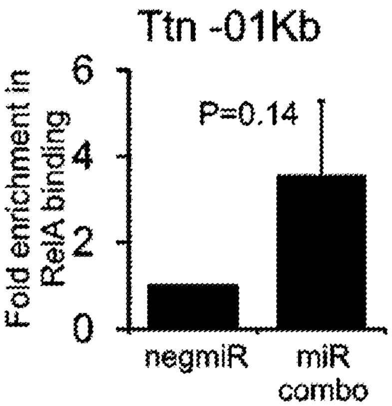


Fig. 5Av

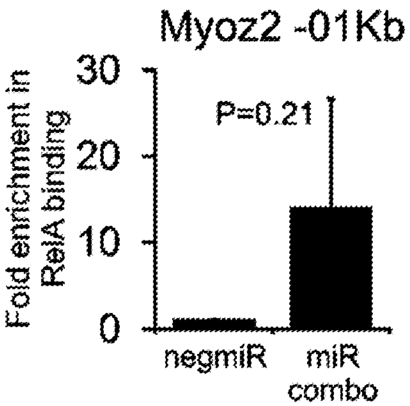


Fig. 5Avi

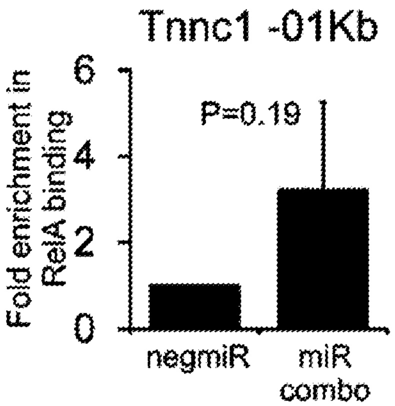


Fig. 5Avii

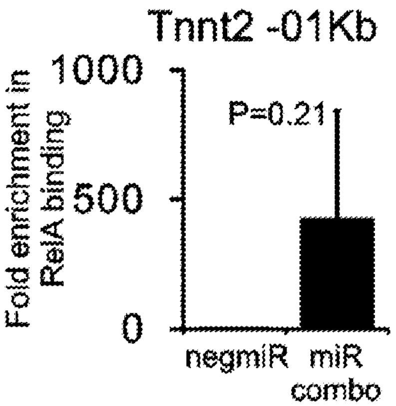


Fig. 5Aviii

Fig. 5Bi

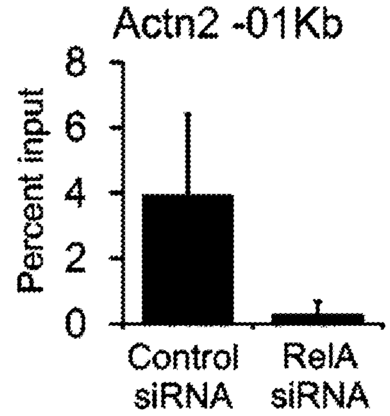


Fig. 5Bii

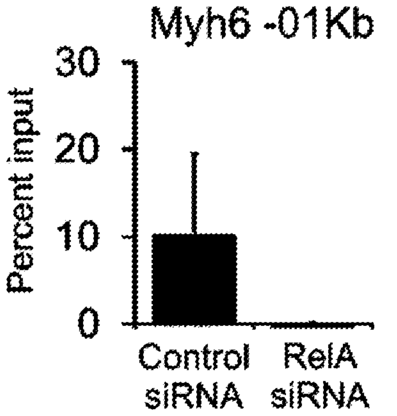


Fig. 5Biii

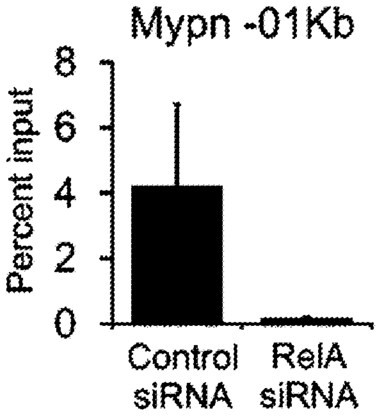
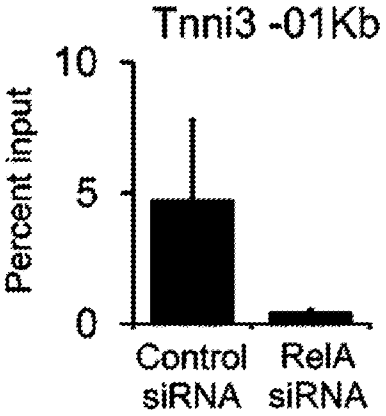


Fig. 5Biv



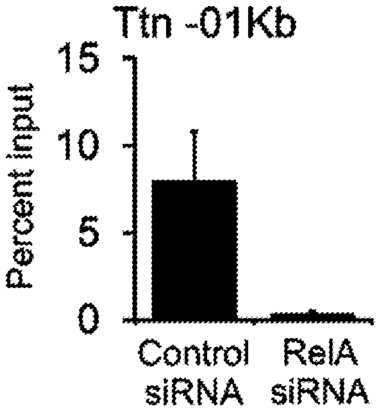


Fig. 5Bv

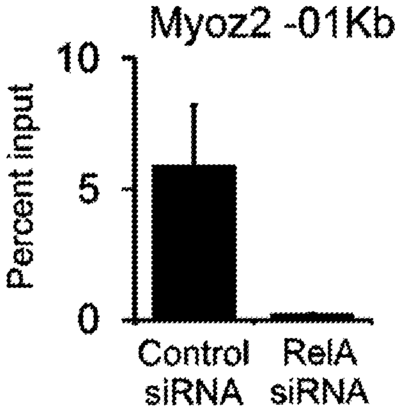


Fig. 5Bvi

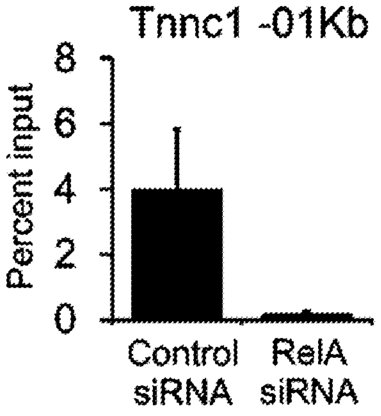


Fig. 5Bvii

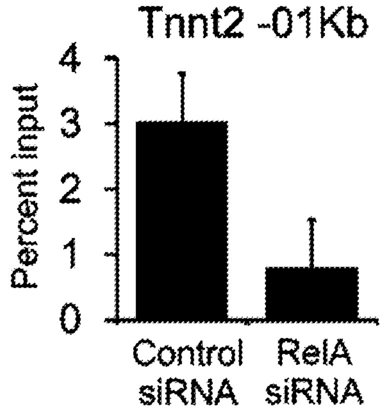


Fig. 5Bviii

Fig. 6A

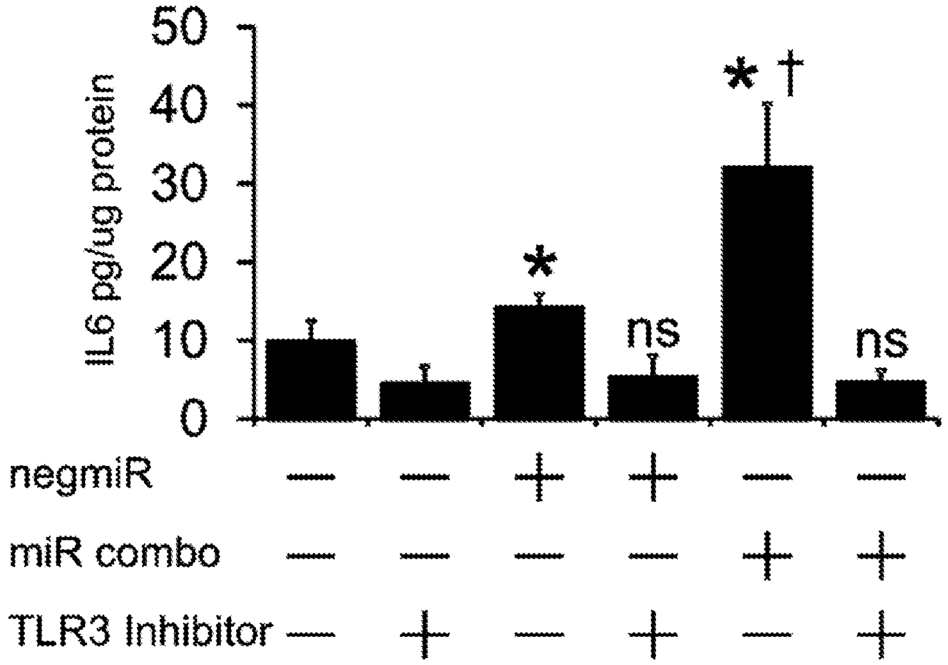

IL6 pg/ug protein
50
40
30
20
10
0
*
ns
* †
ns
negmiR — — + + — —
miR combo — — — — + +
TLR3 Inhibitor — + — + — +

Fig. 6B

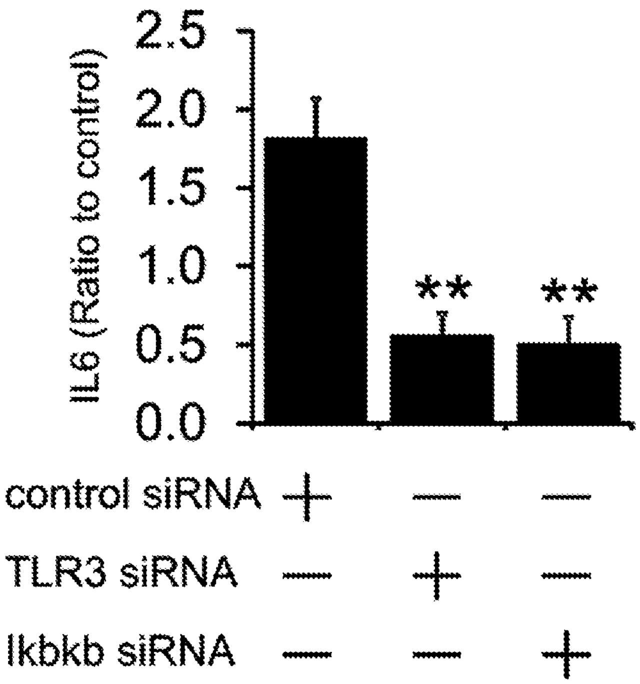

IL6 (Ratio to control)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
control siRNA + — —
TLR3 siRNA — + —
Ikbkb siRNA — — +

Fig. 7Ai

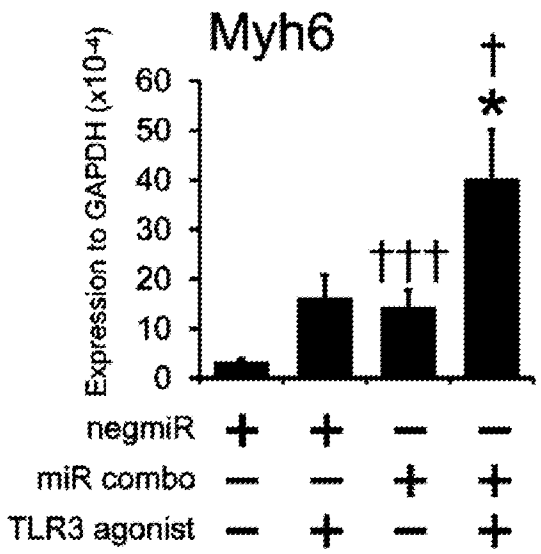


Fig. 7Aii

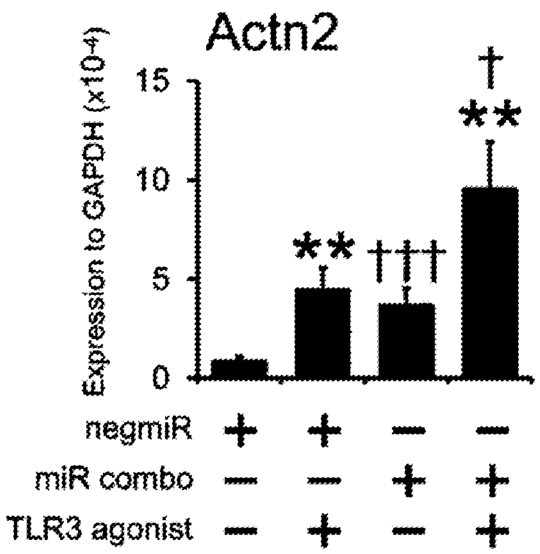


Fig. 7Aiii

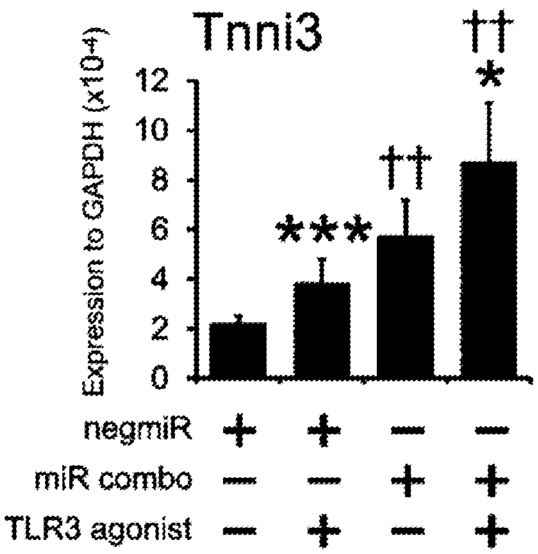
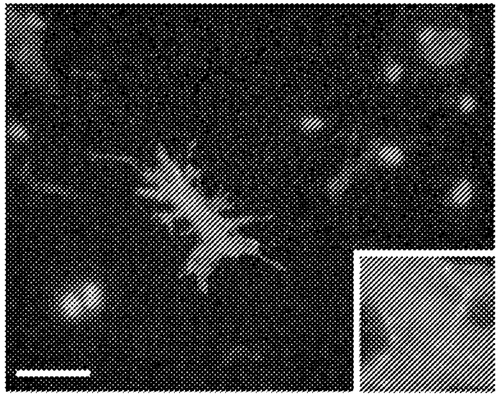
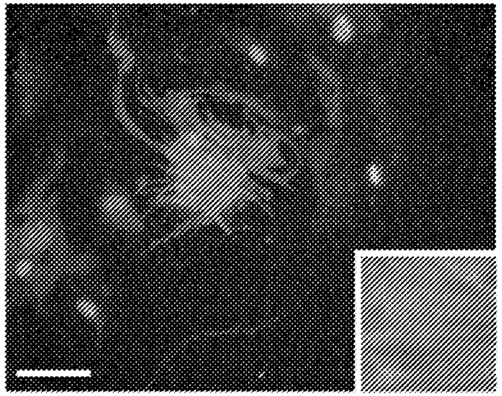


Fig. 7Bi



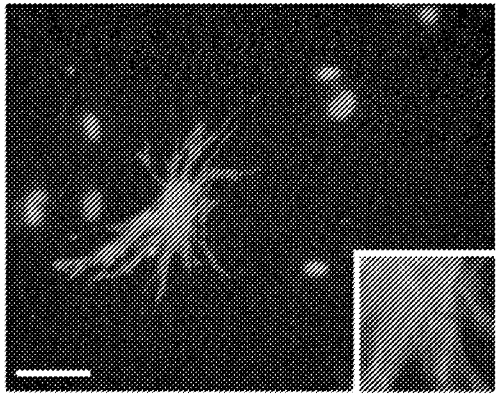
miR combo + vehicle

Fig. 7Bii



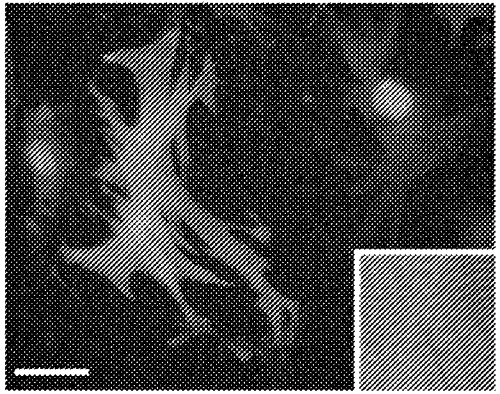
miR combo + vehicle

Fig. 7Biii



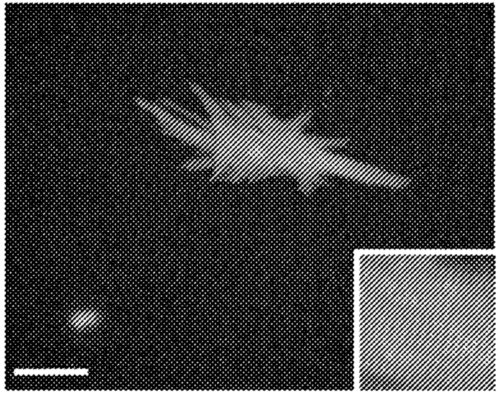
miR combo + vehicle

Fig. 7Biv



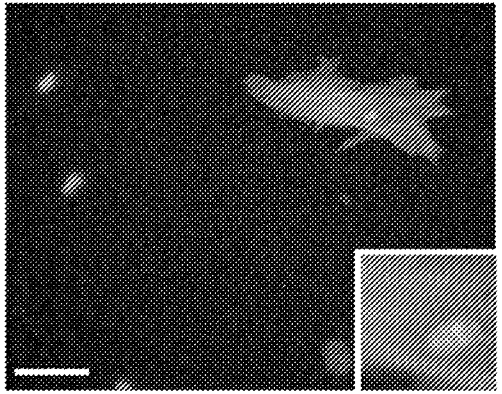
miR combo + TLR3 agonist

Fig. 7Bv



miR combo + TLR3 agonist

Fig. 7Bvi



miR combo + TLR3 agonist

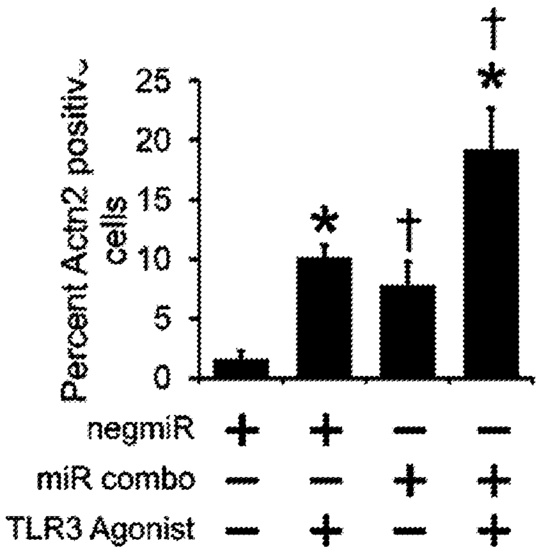


Fig. 7C

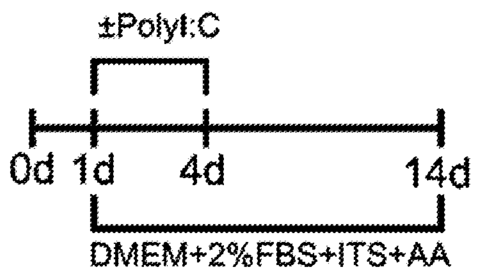


Fig. 7Di

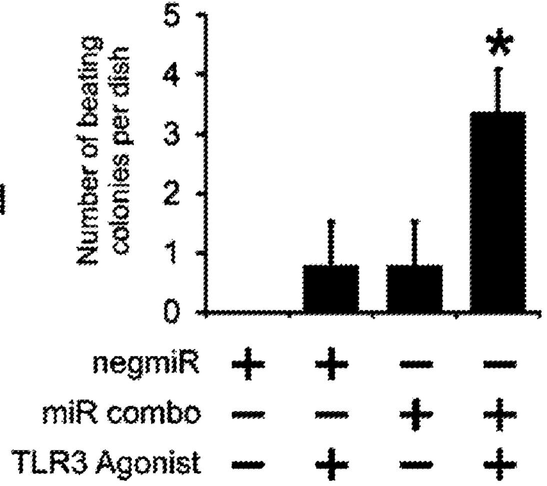


Fig. 7Dii

ICR2

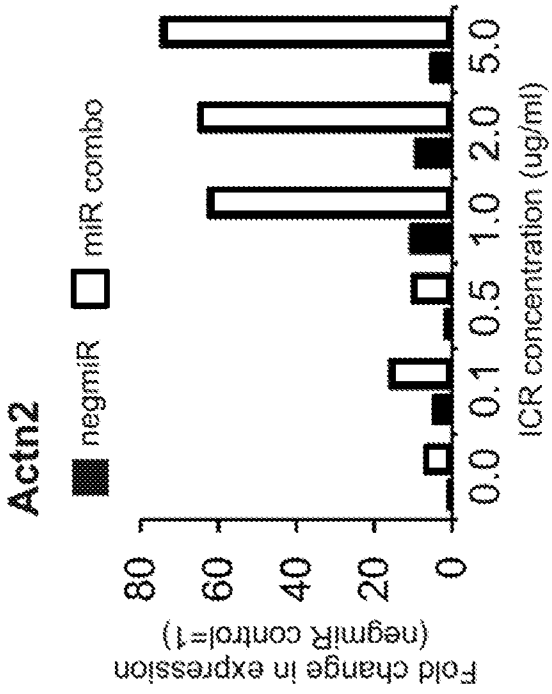


Fig. 8A

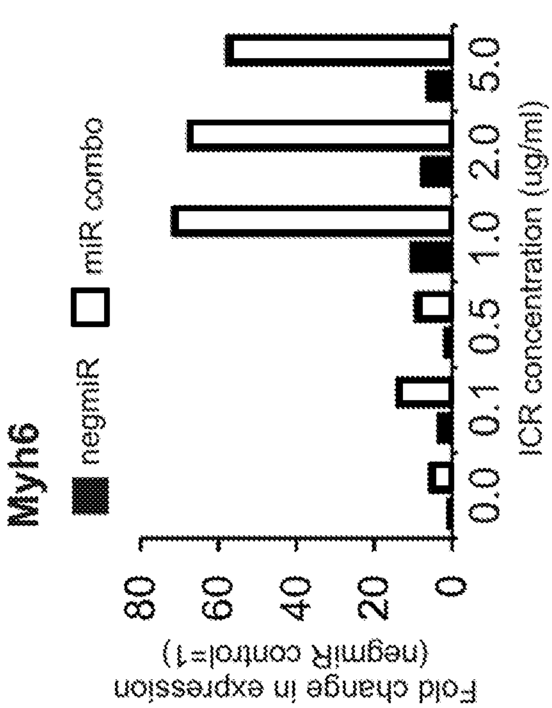


Fig. 8B

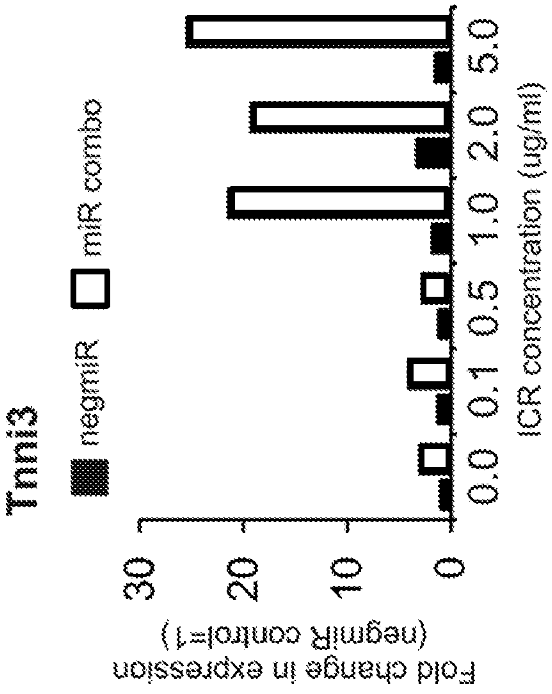


Fig. 8C

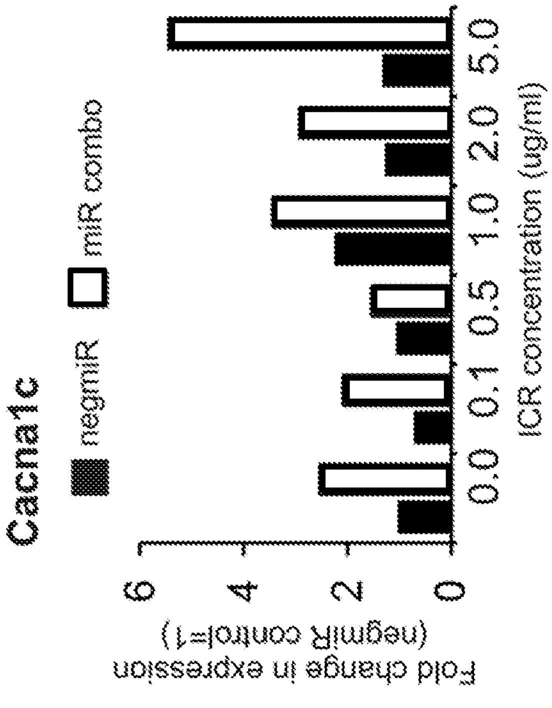


Fig. 8D

ICR4

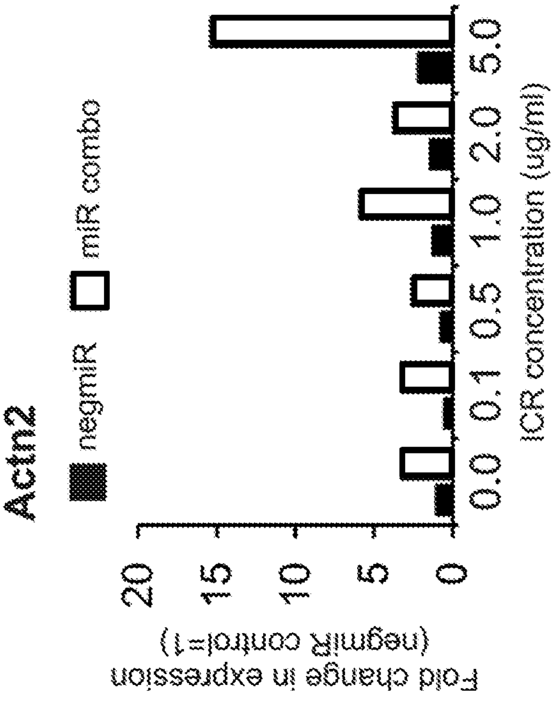


Fig. 9A

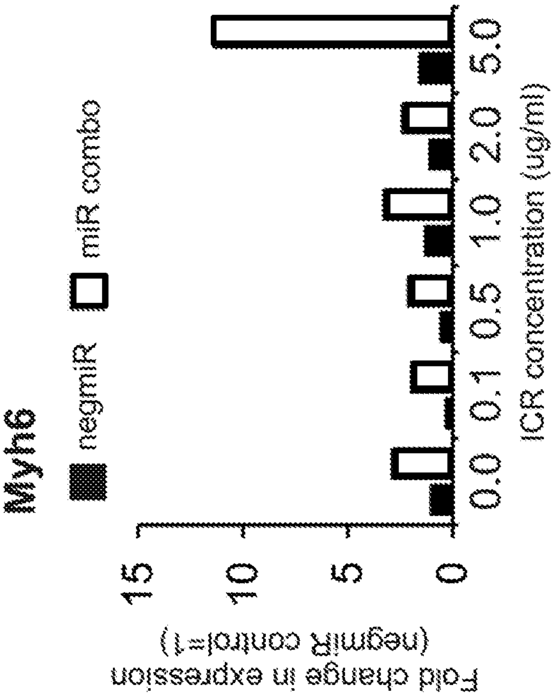


Fig. 9B

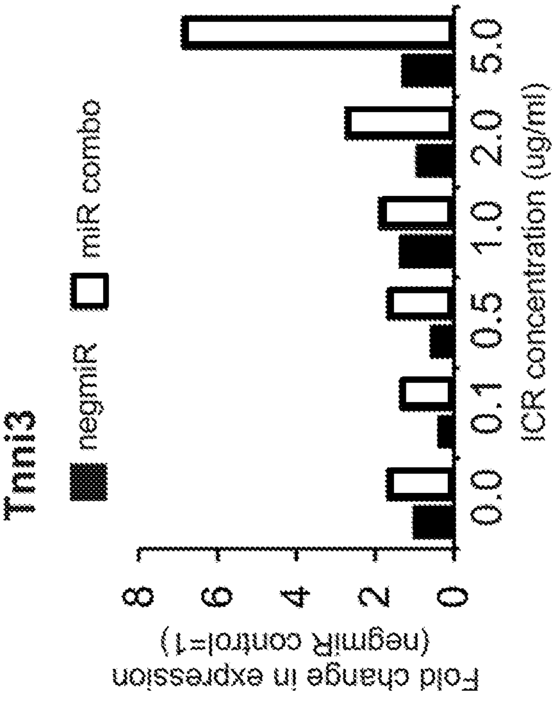


Fig. 9C

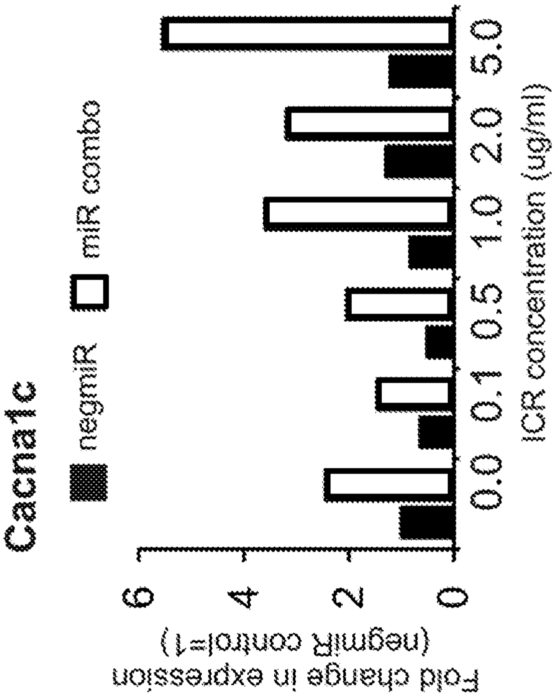


Fig. 9D

Fig. 10

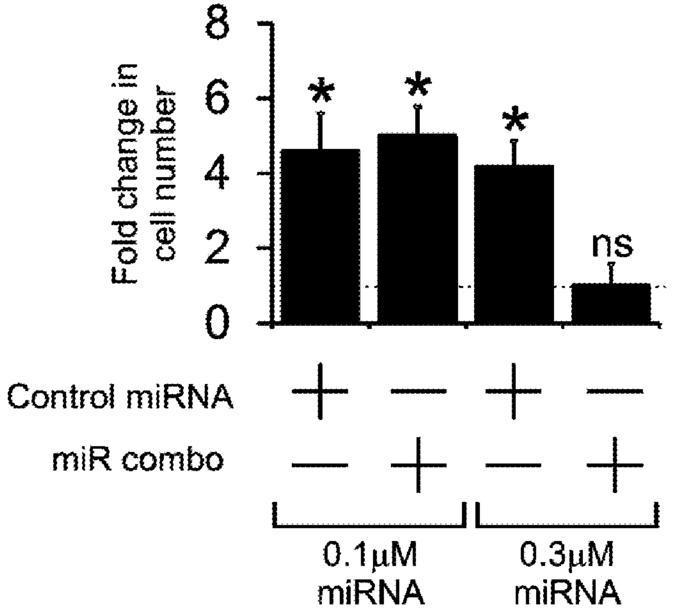


Fig. 11A

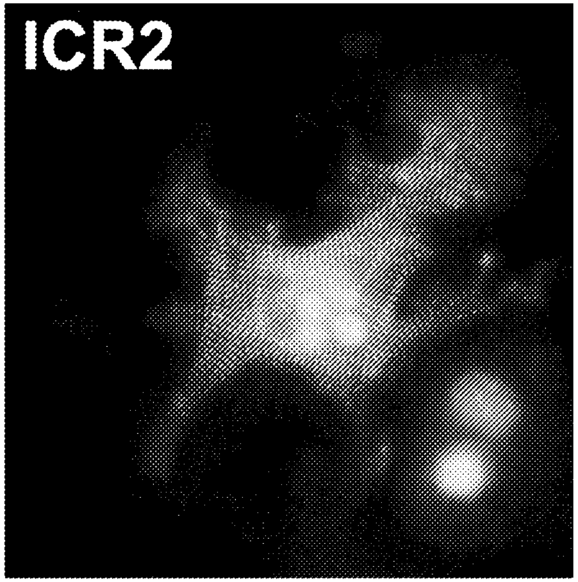
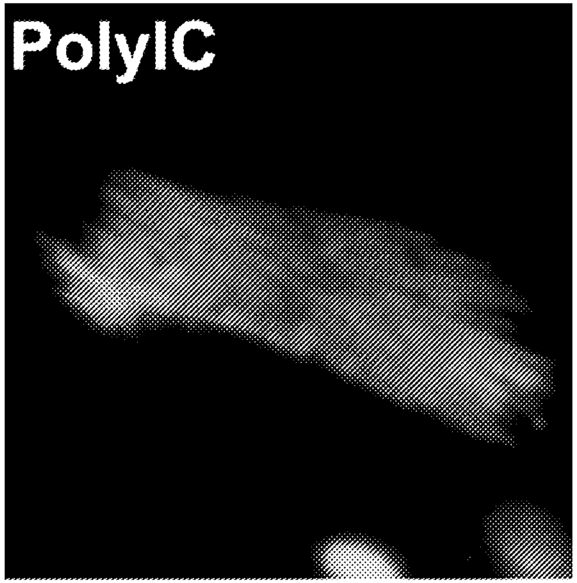


Fig. 11B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/23461

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 35/34, A61K 35/12, C12N 5/074 (2019.01)
CPC - A61K 35/34, A61K 35/12, A61P 9/00, C12N 5/00, C12N 5/0696, C12N 2506/09

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2014/0011281 A1 (DZAU et al.) 9 January 2014 (09.01.2014); abstract; para [0008]; SEQ ID NO: 5	1-5
Y	WO 2013/109763 A2 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 25 July 2013 (25.07.2013); Abstract; para [0048], [00160]	1-5
Y	US 2016/0030332 A1 (DUKE UNIVERSITY) 4 February 2016 (04.02.2016) abstract; para [0009]; SEQ ID NO: 16	3-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search 27 June 2019	Date of mailing of the international search report 25 JUL 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/23461

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-10, 19-21, 29-31, 41
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Groups I+: Claims 1-6, drawn to a reprogramming composition comprising one or more of miRs and an activator of NFkB. The composition will be searched to the extent that the miR encompasses miR-1 (SEQ ID NO: 11) and the activator of NFkB encompasses ICR2 (SEQ ID NO: 30). It is believed that claims 1-5 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass SEQ ID NOs: 11 and 30. Additional miR(s) and activator(s) of NFkB will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected miR(s) and activator(s) of NFkB. Applicants must further indicate, if applicable, the claims which encompass the first named.invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be miR-133a (SEQ ID NO: 14) and an activator of NFkB (ICR4, SEQ ID NO: 37) (Claims 1-4, 6).
*****Continued in Supplemental Box*****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5 limited to SEQ ID NOs: 11, 30

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box No. III (Observations where unity of invention is lacking):

Group II: Claims 11-18, 22-28, 32-40, drawn to a method for modulating cardiomyocyte maturation in a cell.

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I+ include the special technical feature of a composition which differs from the special technical feature of a method as disclosed by Groups II.

No technical features are shared between the nucleotide sequences of miRs or activators of NFkB of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ and II were considered to share the technical features of including: a miR and an activators of NFkB. These shared technical features are previously disclosed by US 2014/0011281 A1 to Dzau et al. (hereinafter "Dzau") and WO 2013/109763 A2 to the Board of Trustees of the Leland Stanford Junior University (hereinafter "Stanford").

Dzau teaches (instant claim 1) a reprogramming composition (Abstract "A method for promoting conversion of cells into cardiomyocytic tissue is carried out by contacting fibrotic tissue (e.g., scar tissue) with a microRNA oligonucleotide or combination of microRNA oligonucleotides. The methods lead to direct reprogramming of fibroblasts to cardiomyocytes or cardiomyoblasts.") comprising:
(a) one or more miRs comprising a nucleotide sequence having at least 80% sequence identity to miR-1 (SEQ ID NO: 11) (nucleotides 1 -22 of SEQ ID NO: 2 are 100% homologous to SEQ ID NO: 11).

Dzau does not specifically teach (b) an activator of NFKB. Stanford teaches wherein an activator of NFKB accelerates reprogramming of cells (Abstract "The nuclear reprogramming of somatic cells with non-integrating factors is shown to be greatly accelerated by activation of innate immune responses in the somatic cell. Methods of activating innate immunity include activation of toll-like receptors, e.g. TLR3. Somatic cells with activated innate immune responses can be reprogrammed to induced pluripotent cells or to transdifferentiated cells."; *[00160] " This effect of Poly I:C to enhance transdifferentiation (Fig. 23E), was markedly reduced by the addition of p65 decoy* suggesting that TLR3-induced activation of NFkB is involved in direct reprogramming."). Given that a TLR3 agonist has been shown to activate NFKB and accelerate cell reprogramming, it would have been obvious to an artisan of ordinary skill in the art to include an activator of NFKB in the reprogramming composition taught by Dzau.

Dzau further teaches a method for enhancing or upregulating cardiomyocyte maturation in a cell (para [0011], The methods lead to fibrotic tissue or other tissue types or cells being directly reprogrammed into cardiomyocytic tissue without a stem cell intermediary state. *The treated tissue is characterized by an increased expression of a cardiomyocyte marker protein after having been contacted with the* compositions........compared to the level of the cardiomyocyte marker protein before the contacting step........Exemplary marker proteins include cardiac troponin, sarcomeric actinin, L-type calcium channel, brachyury, Flk1, Islet1, Mesp1, Gata4, Mef2c, Hand2, and TroponinT2.).

The inventions of Groups I+ further share the technical features of a modified 5' - triphospate, 2' -fluoro modified non-linear RNA, the RNA comprising a stem-loop formed from the complete or partial hybridization of at least 8 nucleotide pairings, which is disclosed by US 2016/0030332 A1 to Duke University *(hereinafter 'Duke Univ') (abstract, a composition including a 5' triphosphate, 2' fluoro-modified* pyrimidine non-linear single stranded RNA at least 17 nucleotides long with a least 3 base pairings.).

As said technical features were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the groups.

Groups I+ and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Item 4 (continued):
Claims 7-10, 19-21, 29-31, 41 are improper multiple dependent claims because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).