PROJECT TITLE: MicroRNA-301a Attenuates Collagen Gel Contractility and Promotes Proliferation of Human Bone Marrow Derived Mesenchymal Stem Cells

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SUMMARY:
Previous data in our lab suggests that human mesenchymal stem cells (MSCs) of bone marrow origin display a myofibroblast phenotype *in vitro*. Coincident with the onset of a myofibroblast phenotype, we observed a reduction in miR-301a expression. We hypothesized that miR-301a may govern the differentiation of bone marrow derived MSCs to myofibroblasts. MSCs were isolated from the sternum of patients undergoing open heart surgery and transfected with pre-miR-301a at early passage. We analyzed contractility through plating on flexible collagen gels and cell proliferation. Western blot and qRT-PCR were used to determine mRNA and protein expression of myosin isoforms and myofibroblast markers. Human MSCs were found to express non-muscle myosin (NMM)-IIA and -IIB, other myofibroblast markers such as α-smooth muscle actin (SMA), vimentin, FSP-1, ED-A Fibronectin, and procollagen I and contracted collagen gels in the basal state. Cells cultured in serum rich conditions displayed decreased contractility and increased proliferation whereas cells cultured in serum free conditions showed increased contractility and decreased proliferative capacity. The proliferative phenotype was associated with increased mRNA expression of miR-301a host gene, ska2. Overexpression of miR-301a caused a reduction in collagen gel contraction and an increase in proliferation when compared with a negative-scramble control. This was associated with decreased NMM-IIA and IIB mRNA. Western blot analysis confirmed decreased levels of NMM-IIA and IIB in addition to decreased protein expression of α-SMA, ED-A Fibronectin and procollagen I. In conclusion, MSCs display a dichotomous proliferative versus contractile phenotype *in vitro*. Our results suggest that miR-301a plays a mechanistic role in this dichotomy, such that it suppresses the myofibroblastic, contractile phenotype.

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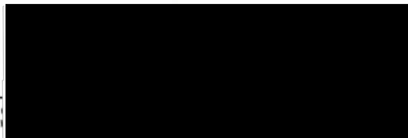

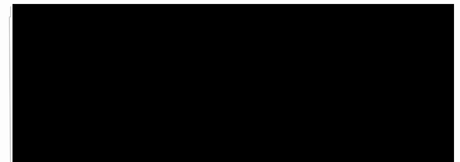

INTRODUCTION

Cardiac Fibrosis

The majority of cardiac injury, specifically that due to myocardial infarction (MI), is associated with fibrosis of the heart. This fibrosis leads to remodeling of tissue architecture and alterations in function of both the injured and undamaged areas (Weber, 2000). Cardiac myocytes are limited in their ability to regenerate therefore wound healing consists primarily of formation of scar tissue and remodeling of both the infarct area and the remainder of cardiac tissue (Soonpaa et al., 1995). Cardiac remodeling ultimately leads to an increase in ventricular stiffness and therefore decreased ventricular relaxation and compliance, and can result in progressive cardiac failure (Vasan & Benjamin, 2001).

Cardiac wound healing occurs in phases and is comparable to wound healing in other organs and tissues. Immediately after injury, damaged cardiomyocytes undergo necrosis and apoptosis (Cleutjens et al., 1999). Necrotic cells release an exudate causing an acute inflammatory response and further cell damage and death in the area (Nahrendorf et al., 2010). The infarct area is then infiltrated by mesenchymal cells and granulation tissue is deposited (Daskalopoulos et al., 2012). These mesenchymal cells consist of fibroblasts and myofibroblasts that surround the damaged area and deposit excess amounts of extracellular matrix (ECM). Myofibroblasts are also involved in maintaining the structural integrity of the tissue and resisting dilatation of the infarct area (van den Borne et al, 2010). Finally, the tissue is remodeled as granulation tissue matures into a stable scar containing high levels of ECM and fibroblast like cells. Remodeling is not restricted to the infarct area but also in areas remote to the initial injury. This remodeling includes hypertrophy of surviving cardiac myocytes, neovascularization and excess deposition of type I collagen with increased crosslinking in the interstitium (Blankesteijn et al., 2001). It is this change in architecture that contributes to ventricular dysfunction and can progress to heart failure.

Myofibroblasts

Myofibroblasts are a hypersecretory, contractile cell population and are the primary drivers of a fibroproliferative response at sites of remodeling after MI (Larsson et al., 2008). Cardiac myofibroblasts are thought to be derived from numerous sources including local fibroblasts that differentiate to myofibroblasts, and epithelial and endothelial cells that, through epithelial to mesenchymal transition (EMT) and endothelial to mesenchymal transition respectively, can adopt a myofibroblast-like phenotype (Kalluri & Neilson, 2003). Circulating mesenchymal precursor cells called fibrocytes are derived from bone marrow stem cells and have been shown to also have features of both fibroblasts and myofibroblasts and contribute to the pool of cardiac myofibroblasts (Bucala et al., 1994).

van Amerongen et al. (2008), extracted bone marrow from transgenic mice expressing green fluorescent protein (eGFP) and injected it into irradiated mice who later underwent coronary artery ligation. They found that 24% of all myofibroblasts present in the post-MI heart of the transplanted mice expressed eGFP, suggesting they were bone marrow derived. Similar

experiments were performed by Mollman and colleagues (2006) using vimentin as a marker of fibroblasts and alpha-smooth muscle actin (α-SMA), vimentin and embryonic smooth muscle myosin heavy chain (SMemb) as markers of a myofibroblast phenotype. They found that 24.7% of all fibroblasts and 57.4% of all myofibroblasts in the infarct area were eGFP positive, indicating bone marrow origin. These studies both indicate that bone marrow derived cells are an important contributor to the myofibroblast and fibroblast population within the infarct area post-MI.

After MI, fibroblasts within the heart are exposed to mechanical stress, transforming growth factor beta (TGF-β) and extra domain A splice variant fibronectin (ED-A fibronectin) which all induce differentiation to myofibroblasts (Serini et al., 1998; Wang et al., 2003). Myofibroblasts produce excess amounts of interstitial collagens (type I and III), fibronectin and laminins contributing to scar formation (Squires et al., 2005). In addition to excess collagen deposition, myofibroblasts are actively involved in the contraction process of wound healing. Myofibroblasts produce a sustained contraction that closely resembles that of smooth muscle cells and is modulated by neurohormones such as angiotensin II. This type of contraction differs from cardiomyocyte contraction in that cardiac muscle cells contract and relax rhythmically in response to electrical stimulation (Tomasek et al., 2002).

Myofibroblasts continue to produce type I and II collagen after scar tissue is formed and may persist within the infarct scar up to 20 years following MI (Willems et al., 1994). The presence of myofibroblasts in the infarct scar may be important in preventing dilatation and maintaining stability of the area (van den Borne et al., 2009). The continued production of collagen after scar resolution becomes problematic when deposition occurs in areas remote to the infarct scar. It has been shown that myofibroblasts contribute to interstitial fibrosis of the heart in areas remote to the source of injury. It is this remote remodeling that can have adverse effects on systolic and diastolic function of cardiac tissue and is associated with progression to heart failure (Katz, 1995; Gaasch, 1994).

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs), or mesenchymal stromal cells, are a non-hematopoietic adult stem cell population that have been primarily isolated from human bone marrow (Kassem & Abdallah, 2008). They can, however, also be found in adipose tissue, tendon, peripheral blood, skeletal muscle, and trabecular bone (Noth et al., 2002; Zuk et al, 2002). The International Society for Cellular Therapy has defined MSCs by the criteria that cells adhere to plastic culture plates in standard culture conditions, express specific surface antigens and have the ability to differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts (Dominici et al., 2006).

Human MSCs (hMSCs) are thought to have therapeutic potential due to the ease with which the population is expanded *in vitro* while maintaining the ability to differentiate into various lineages and their non-immunogenic properties (Chamberlain et al., 2007). In addition to osteoblasts, adipocytes and chondroblasts, there are reports of MSCs being induced to

differentiate *in vitro* into myoblasts that fuse into beating myotubes (Wakitani et al., 1995) and neuron like cells that express markers of mature neurons (Woodbury et al., 2010).

Our laboratory has recently found that primary bone marrow derived hMSCs readily adopt a myofibroblast phenotype over serial passage in standard culture conditions as confirmed by contractility and expression of myofibroblast markers including α-SMA, vimentin, SMemb, ED-A fibronectin, and procollagen type I (sp1.D8) in a similar pattern as human cardiac myofibroblasts. Moreover, hMSCs displayed an increase in collagen gel contraction similar to that of the cardiac myofibroblasts when exposed to TGF-β, which is known to induce a myofibroblast phenotype. We observed that exposure to mitogens, such as fetal bovine serum, induced a proliferative, less contractile phenotype, whereas serum deprivation induced a phenotype of decreased proliferation and increased contraction, suggesting that the myofibroblast phenotype is at the polar opposite of a proliferative phenotype.

Regulation of Myofibroblast Differentiation

Myofibroblast differentiation is governed by a variety of factors including interaction with and rigidity of the ECM, and exposure to ED-A fibronectin and TGF-β**.** Prior to injury, fibroblasts do not express contractile microfilaments or stress fibers and produce little ECM (Tomasek et al., 2002). Differentiation of myofibroblasts, primarily from fibroblasts, occurs in two steps. In the first step, an increase in mechanical stress of the ECM results in fibroblasts adopting a proto-myofibroblast phenotype. Proto-myofibroblasts are characterized by stress fibers linking cells to the ECM, increased expression of ED-A fibronectin and the presence of focal adhesions that contain β-actin and γ-actin microfilaments associated with nonmuscle myosin (Hinz & Gabbiani, 2003). These cells do not express α-SMA (Meran and Steadman, 2011). In the second step, TGF-β exposure drives differentiation of proto-myofibroblasts to myofibroblasts (Serini et al., 1998; Wang et al., 2003) . Myofibroblasts express α-SMA, increased levels of ED-A fibronectin and increased levels and maturity of focal adhesions and stress fibers. (Tomasek et al., 2002). Increased rigidity of the ECM also results in a myofibroblast phenotype resulting in increased expression of α-SMA and integrins (Jones & Ehrlich, 2011).

MicroRNA

MicroRNAs (miRNAs) have recently emerged as a novel intrinsic method of gene regulation. Located in intronic gene sequences, miRNA are 21or 22 nucleotides long and exert their effects post transcriptionally by binding the 3' UTR of mRNA targets and ultimately decreasing mRNA levels (Hobert, 2008). These molecules have been implicated in a variety of cell processes such as differentiation, tumorigenesis and autoimmune disease (Inose et al., 2009; Jiang et al., 2008; Mycko et al., 2012). For example, miR-200 targets Zeb2/SIP1 which is involved in promoting EMT, cellular motility and stem-like properties while preventing apoptosis and senescence (Xia et al., 2010). miR-146a has been shown to be involved in

promoting vascular smooth muscle cell proliferation in vitro and neointimal hyperplasia *in vivo* (Sun et al., 2011). These findings suggest a prominent role for miRNAs in the control of proliferation and differentiation in a variety of cell populations.

Preliminary work in our lab has revealed that microRNA-301a (miR-301a), along with other microRNAs, display altered levels of expression during differentiation of hMSCs to myofibroblasts *in vitro* (Mina Guirgis, BScMed 2009-2010). miR-301a is a relatively unstudied miRNA. The host gene, ska2, is located at 17q22-23 in the human genome and the protein is involved in the onset of anaphase during mitosis and also the maintenance of metaphase (Hanisch et al., 2006). Overexpression of miR-301a has been implicated in pancreatic cancer cell proliferation (Chen et al., 2012) and as being involved in a positive feedback loop mechanism to increase levels of NF-κB in pancreatic cancer cells (Lu et al., 2011). Upregulation was also reported in hepatocellular carcinoma cells promoting proliferation, migration and invasion (Zhou et al., 2012). In human breast cancer cells, miR-301a also promoted cell proliferation, migration and invasion whereas knockdown inhibited cell viability (Shi et al., 2011). Although miR-301a has become a popular microRNA in cancer research, its role in MSC differentiation has not been well studied. However, there is evidence to suggest that miR-301a is heavily involved in proliferation and/or differentiation of human cells.

Hypothesis

We hypothesize that mir-301a expression is altered in response to mitogenic stimulus of hMSCs, and that it plays a mechanistic role in the differentiation of hMSCs to myofibroblasts *in vitro*.

MATERIALS AND METHODS

This protocol received IRB approval for collection of bone marrow from patients undergoing open heart surgery. Written informed consent was obtained from each patient prior to collection of tissue

Tissue Collection

Bone marrow aspirates were obtained from the sternum of patients undergoing open heart surgery and placed in phosphate buffered saline (PBS). Samples were thoroughly mixed and plated on 10cm plastic culture dishes in Dulbecco's modified Eagle's medium (DMEM) F-12 with 20% fetal bovine serum (FBS), 100U/ml penicillin, 100ug/ml streptomycin, and 100mM ascorbic acid. Cells were incubated at 37C with 5% CO2. After 24 hours of incubation, cells were washed twice with PBS and fresh medium was added. Medium was replaced every 2 or 3 days. To obtain cells at later passages, plates were treated with TrypLE Express to detach the cells and replated at lower confluency to expand cultures. Pluripotent properties of human bone marrow-derived MSCs were confirmed through incubation in specific induction media, producing osteoblasts, chondrocytes and adipocytes.

Transfection

Overexpression of miR-301a was obtained by transfection of cells at 60% confluency with pre-miR microRNA (Ambion). Cells were washed thoroughly with PBS and media replaced with 20% FBS DMEM containing no antibiotics one hour prior to transfection. A mixture of Dharmafect transfection reagent (Thermoscientific) and a 50nM concentration of pre-miR-301a or negative-scramble control (Ambion) was prepared as per manufacturers instructions. Cells were incubated with transfection mixture for 3 hours and subsequently washed twice with PBS. Cells were then placed in 20% FBS DMEM F12 without antibiotics.

RNA and qPCR

Passage 0 cells were lysed using TRIzol reagent 48 hours post transfection and RNA was isolated. Total RNA was purified using Ambion's DNA free kit according to manufacturer's instructions and RNA concentration and quality was analyzed with Agilent 2100 Bioanalyzer. Expression of myosin heavy chain (MYH) 9 and 10 were analyzed by quantitative real-time PCR with β-actin used as a reference gene. Expression of miRNA was determined using TaqMan small RNA assay and U6 as a reference gene. PCR was performed using Bio-Rad Mini Opticon detection system and iscript one-step RT-PCR kit.

Western Blot Analysis

Passage 0 cells were lysed 48 hours post transfection and protein was extracted using New RIPA lysis buffer. Protein concentrations of whole cell lysates were measured and equal amounts of protein (10-20ug) were separated on 8% and 15% gels at 130V then transferred to a polyvinylidene difluoride (PDF) membrane. After blocking the membrane with 5% milk powder at room temperature for 1 hour, antibodies against α-SMA (1:5000; Sigma), fibroblast specific protein-1 (FSP-1, 1:500; Abcam), vimentin (1:1000; Santa Cruz), ED-A fibronectin (1:1000; Millipore), procollagen I (1:1000, Developmental Studies Hybridoma Bank), non-muscle myosin IIA (NMMIIA, 1:2000; Abcam), non-muscle myosin IIB (NMMIIB, 1:2000; Abcam) were used to evaluate protein expression with appropriate secondary antibody (goat anti-rabbit IgG, rabbit anti-mouse IgG; Jackson ImmunoResearch Laboratories) conjugated with horseradish peroxidase. β-Tubulin (1:1000, Abcam) was used as a reference protein. Immunoactivity was detected using Pierce ECL Western Blotting System (Thermoscientific) according to manufacturers instructions. Densitometry was performed using GS-800 Calibrated Densitometer (Biorad) and blots were quantified and analyzed using Quantity One software.

Collagen Gel Contraction Assay

To prepare collagen gels, PureCol collagen gel (Advanced Biomatrix) was allowed to set in 24 well culture dishes and cells were plated at a density of 5x10^4 per well. Passage 1 cells were allowed to attach over night and subsequently transfected. Gels were detached from the sides of the wells at 24 hours post transfection and digital photos taken at 24 and 48 hours post transfection to measure the diameter of collagen gel. Each treatment group was repeated in triplicate. Photos were analyzed using IDL based MeasureGel software to determine the change in collagen gel surface area.

Immunofluorescence Staining

At 72 hours post-transfection, passage 1 cells were fixed with 4% paraformaldehyde for 15 minutes and permeabolized using 0.2% Triton-X-100 in PBS. Cells were washed with PBS and blocked with 5% bovine serum albumin for 30 minutes at room temperature. They were then incubated overnight with primary antibodies (NMMIIA, NMMIIB, ED-A fibronectin) and detected with Alexa fluor 488 secondary antibody (Invitrogen). Finally, cells were incubated with phalloidin (Invitrogen) for dual staining with filamentous actin for 30 minutes at room temperature and mounted on slides using Vectashield mounting medium with DAPI (Vector). Cells were visualized with an epifluorescent microscope with appropriate filters.

Cell proliferation/viability assay (MTT)

Passage 1 cells were plated at a density of 5000 per well in a 96 well plate. Cells were allowed to attach overnight and transfected. At time of transfection, 24 and 48 hours post-transfection, MTT solution (5mg Thiazolyl Blue Tetrazolium Bromide/1 ml PBS) was added and incubated for 3 hours to allow for MTT to be metabolized. Media was aspirated and MTT metabolite formazan was resuspended in dimethyl sulfoxide (DMSO). Optical density was then read at a wavelength of 570nm.

RESULTS

Functional Assessment of hMSCs - Contractility and Proliferation

Collagen gel contraction assays were used to assess the physiologic function of human bone marrow MSCs and to evaluate the effect of miR-301a on the myogenic phenotype. MTT assay was used to assess the proliferative phenotype. We found that hMSCs displayed increased contractility and decreased proliferation in serum free conditions whereas in serum rich conditions, proliferation was increased and contractility decreased (Figure 1). This was associated with altered ska2 and miR-301a expression: ska2 and miR-301a were increased in serum rich conditions compared to serum free conditions (Figure 2).

To assess the mechanistic role of mir-301a on early passage hMSC phenotype, gain of function experiments were conducted. Overexpression of miR-301a in hMSCs resulted in reduced contractility compared with a negative-scramble control (Figure 3). We also observed an increase in cell proliferation at both 24 and 48 hours post transfection (128.9% and 174.1% cell viability respectively) when compared with control (92.1% and 130.4% cell viability respectively) (Figure 4). Therefore, miR-301a overexpression results in a decrease in contractile function and an increase in proliferation of early passage bone marrow derived hMSCs. This implicates mir-301a in the regulation of genes required for differentiation to a myofibroblast phenotype.

Analysis of Myosin Isoforms and Myofibroblast Markers

To determine the cause of decreased contractility of hMSCs and further delineate the phenotype produced as a result of miR-301a overexpression, we evaluated NMM-IIA and -IIB,

and markers suggestive of a myofibroblast phenotype in early passage cells. Myofibroblasts have not been defined by one specific marker so a list of markers including α-SMA, vimentin, ED-A fibronectin, procollagen I and FSP-1 was used to evaluate the resulting phenotype.

Real-time qPCR (RT-qPCR) was performed to evaluate gene expression of myosin isoforms (Figure 5). Early passage hMSCs displayed decreased gene expression of NMMIIA and NMMIIB with overexpression of miR-301a. These results were obtained with multiple experimental repetitions (n=4-5). Western blot analysis (Figure 6) confirmed decreased protein expression of NMMIIA and NMMIIB with overexpression of miR-301a. When tested for myofibroblast markers, we found a decrease in expression of procollagen I, ED-A fibronectin, and α-SMA and an increase in vimentin and FSP-1 expression with miR-301a overexpression. These results were obtained in multiple repetitions (n=3-4), however some level of variability existed in protein expression of myofibroblast markers between patient samples. Finally, immunofluorescent staining was used to examine cell morphology, and evaluate further the expression of NMMIIA and IIB, and ED-A fibronectin (Figure 7). There appeared to be a decrease in immunofluorescent staining of both ED-A fibronectin and NMMIIA with overexpression of miR-301a when compared with control. Immunofluorescent staining of NMMIIB showed little change between treated and control samples.

Therefore, passage 0 hMSCs transfected with miR-301a showed decreased expression in NMMIIA and IIB at both the mRNA and protein level as confirmed by RT-qPCR and Western blot analysis. Expression of myofibroblast markers was also modified in passage 0 cells with overexpression of miR-301a.

DISCUSSION

Bone marrow extracts are initially heterogenous, consisting of both mesenchymal and hematopoietic stem cells with MSCs comprising merely 0.001-0.01% of the total population (Pittenger et al., 1999). Hematopoietic cells, however, do not adhere to plastic culture plates and are washed away as MSCs remain attached (Sekiya et al., 2002). The cells that remain adopt a fibroblastic phenotype when cultured on plastic culture dishes in standard culture conditions. MSCs have often been compared to fibroblasts with respect to appearance as well as gene and protein expression. The myofibroblast phenotype has not previously been well documented. Phenotypic characteristics of myofibroblasts include an ability to contract collagen gels and expression of markers such as α-SMA, collagen, vimentin, NMMIIA and FSP-1, as is seen in cardiac myofibroblasts. Bone marrow derived hMSCs also display decreased proliferative capacity as they are cultured and expanded (Digirolamo et al., 1999). We now show that exposure of hMSCs to mitogens promotes a more proliferative versus a contractile myofibroblastic phenotype, suggesting a dichotomy between the two phenotypes. This concept of a dichotomy between a proliferative versus contractile phenotype has been well documented in the smooth muscle literature, with microRNA playing a mechanistic role. In smooth muscle cells, miR-145 has been implicated in regulating this process. Our study has two primary findings: firstly, hMSCs exhibit a dichotomous phenotype between proliferative versus

contractile function. Secondly, miR-301a likely plays a mechanistic role in suppressing the contractile phenotype while favoring the proliferative phenotype (Figure 8).

miR-301a is a relatively unstudied microRNA. Research thus far has implicated miR-301a in promoting proliferation, invasion and motility in multiple types of cancer cells, illustrating its role as a potential oncogene. Our results indicate that miR-301a overexpression antagonizes the myogenic phenotype of early passage hMSCs, as evidenced by decreased collagen gel contractility. This decrease in contractility could be mediated by the observed decrease in expression of NMMIIA, NMMIIB and α-SMA. We also observed a decrease in expression of the myofibroblast markers α-SMA, ED-A fibronectin, and procollagen I, and an increase in cell proliferation. These results suggest that miR-301a plays a mechanistic role in increasing proliferative potential of hMSCs while attenuating expression of a myofibroblast phenotype. This correlates with the findings that miR-301a promotes proliferation in various types of malignant cells. Like all microRNAs, it is unlikely that mir-301a has a direct effect on hMSC phenotype, but rather that it suppresses protagonists of the myogenic phenotype. Computational modeling suggests that miR-301a may target members of the TGF-β signaling pathway, as well as genes known to influence differentiation of a variety of cells, such as the SOX and klf molecules. Further work is required to determine if mir-301a exerts its effect through targeting these genes in hMSCs, and if these effects translate into altered fibrosis *in vivo*. The use of microRNA has been suggested as a therapeutic intervention, and it may be that miR-301a could be used to treat established fibrosis.

Limitations

Experiments performed on human samples can exhibit some level of variability. In collection of bone marrow samples from a human population, there are many factors that can contribute to this kind of variability such as age, sex, medications, diet, and disease states. All of these can have effect on the composition and characteristics of bone marrow. Characterization of a myofibroblast phenotype has also been variable between studies as there is no definitive marker of a myofibroblast cell but rather a panel of markers that together are suggestive of this phenotype.

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FIGURES

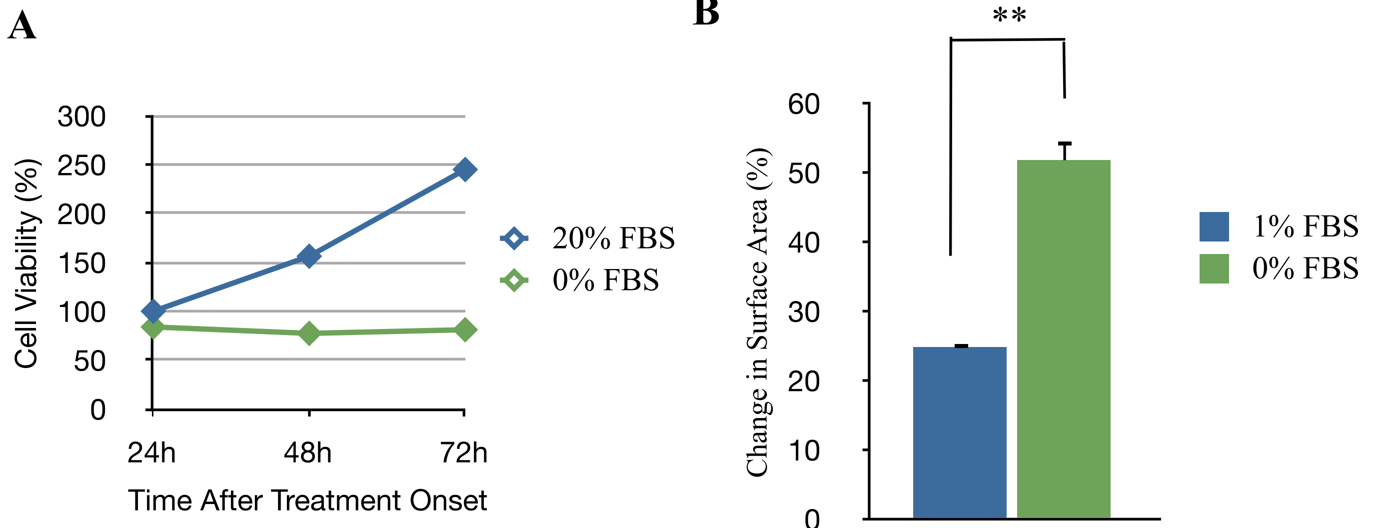


Figure 1: High serum conditions increase cell proliferation and decrease collagen gel contraction of human bone marrow MSCs.
Passage 1 bone marrow MSCs display increased proliferation when cultured in 20% FBS compared with those cultured in 0% FBS (A). Samples were analyzed in sets of 6 for a single patient (n=1). Passage 1 MSCs cultured in 1% FBS show decreased contractility when compared with those in serum free conditions (B). Collagen gel experiments were performed in triplicate on 3 patient samples (n=3). Results are displayed as averages ± SEM. *$^{**}p < 0.01$ vs. serum free control*. Abbreviations: mesenchymal stem cells (MSCs), fetal bovine serum (FBS).

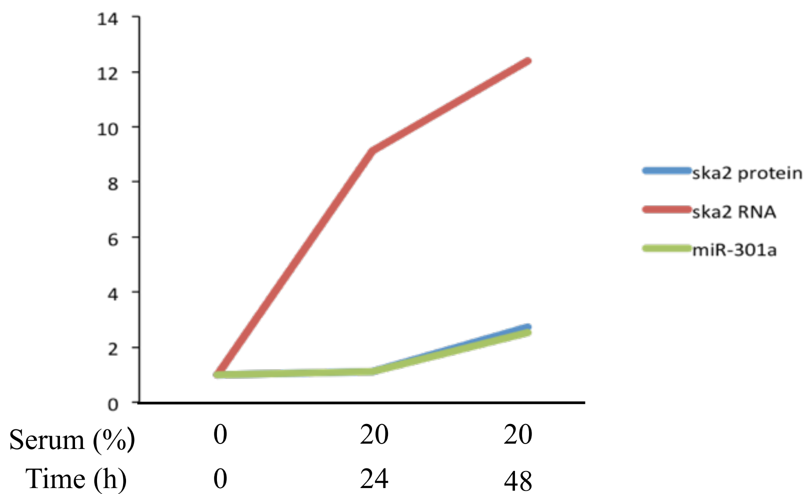


Figure 2: Expression of miR-301a and its host gene in response to reintroduction of serum.
Protein and mRNA expression of ska2 and mRNA expression of miR-301a are increased in high serum conditions. This is representative data from human bone marrow derived MSCs from a single patient.

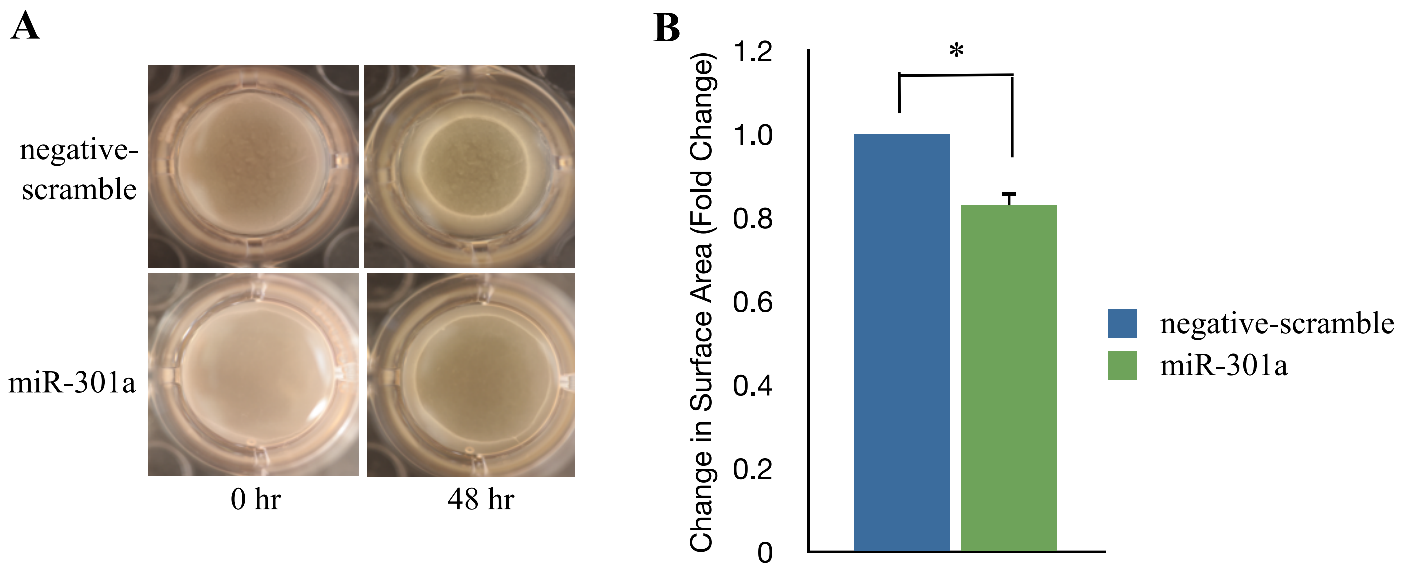
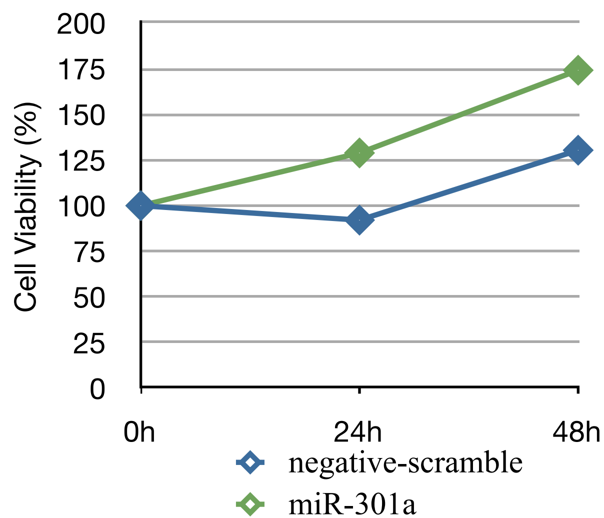


Figure 3: Overexpression of miR-301a decreases hMSCs ability to contract collagen gels. Representative images of P1 hMSCs from a single patient showing increased gel surface area with overexpression of miR-301a (A). Compared with a negative-scramble control, hMSCs contracted collagen gels less when transfected with miR-301 (B). Samples were run in triplicate and comparable results obtained in multiple repetitions (n=3). Results are displayed as mean ± SEM. **p* < *0.05 vs. negative-scramble control.* Abbreviations: human mesenchymal stem cells (hMSCs).

Figure 4: Increased cell proliferation is observed with miR-301a overexpression Single patient data showing that miR-301a increases hMSC cell viability in P1 cells over 48 hours compared with a negative-scramble control. Samples were analyzed in sets of 6 for a single patient (n=1). Abbreviations: human mesenchymal stem cells (hMSCs).



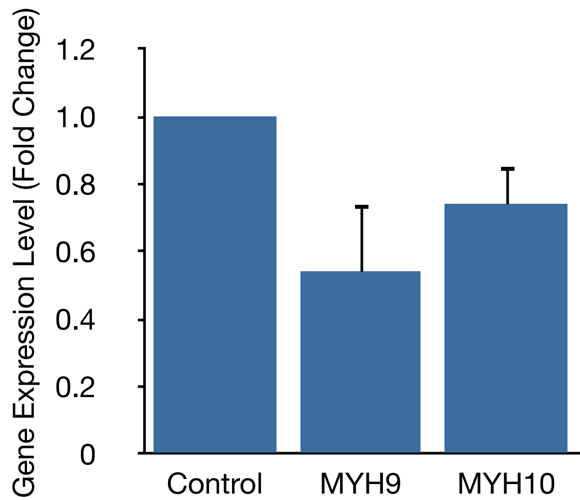


Figure 5: mRNA expression of myosin isoforms is decreased with overexpression of miR-301a in hMSCs.
Passsage 0 cells exhibit reduced MYH9 and MYH10 expression with transfection of miR-301a compared with a negative-scramble control. Samples were analyzed in triplicate and similar results obtained in multiple repetitions (n=3-5). Results are expressed as mean ± SEM. Abbreviations: human mesenchymal stem cells (hMSCs), myosin heavy chain (MYH).

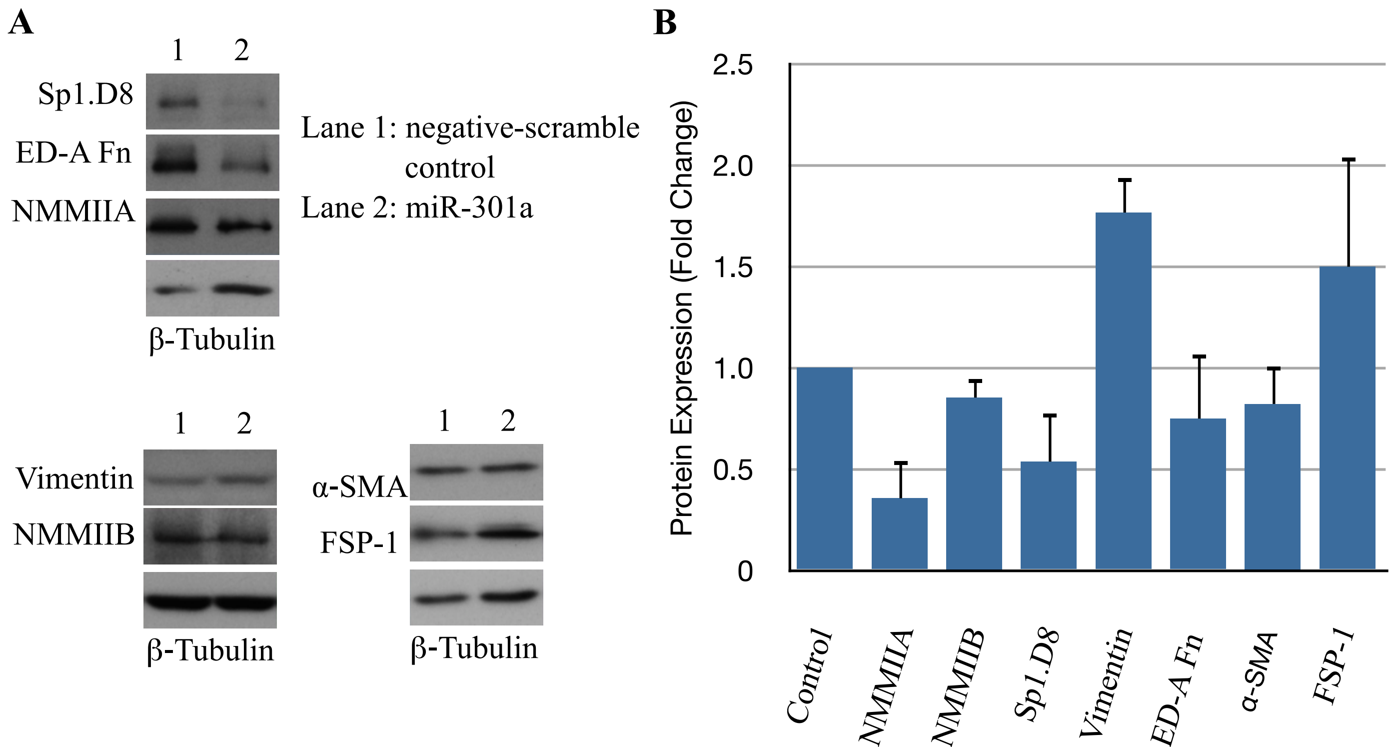


Figure 6: Transfection of P0 hMSCs with miR-301a alters protein expression of non-muscle myosin isoforms and myofibroblast markers.
Passage 0 hMSCs transfected with miR-301a showed decreased protein expression of NMMIIA, NMMIIB, SP1D8, ED-A Fn and α-SMA compared with a negative-scramble control 48 hours post transfection. Increased expression of vimentin and FSP1 was also observed. Representative Western blots (A) and a corresponding histogram with results from multiple (n=3-4) patients (B). Results are displayed as averages ± SEM. Abbreviations: human mesenchymal stem cells (hMSCs), non muscle myosin II (NMMII), ED-A Fibronectin (ED-A Fn), and alpha-smooth muscle actin (α-SMA), procollagen I (Sp1.D8), fibroblast specific protein 1 (FSP-1).

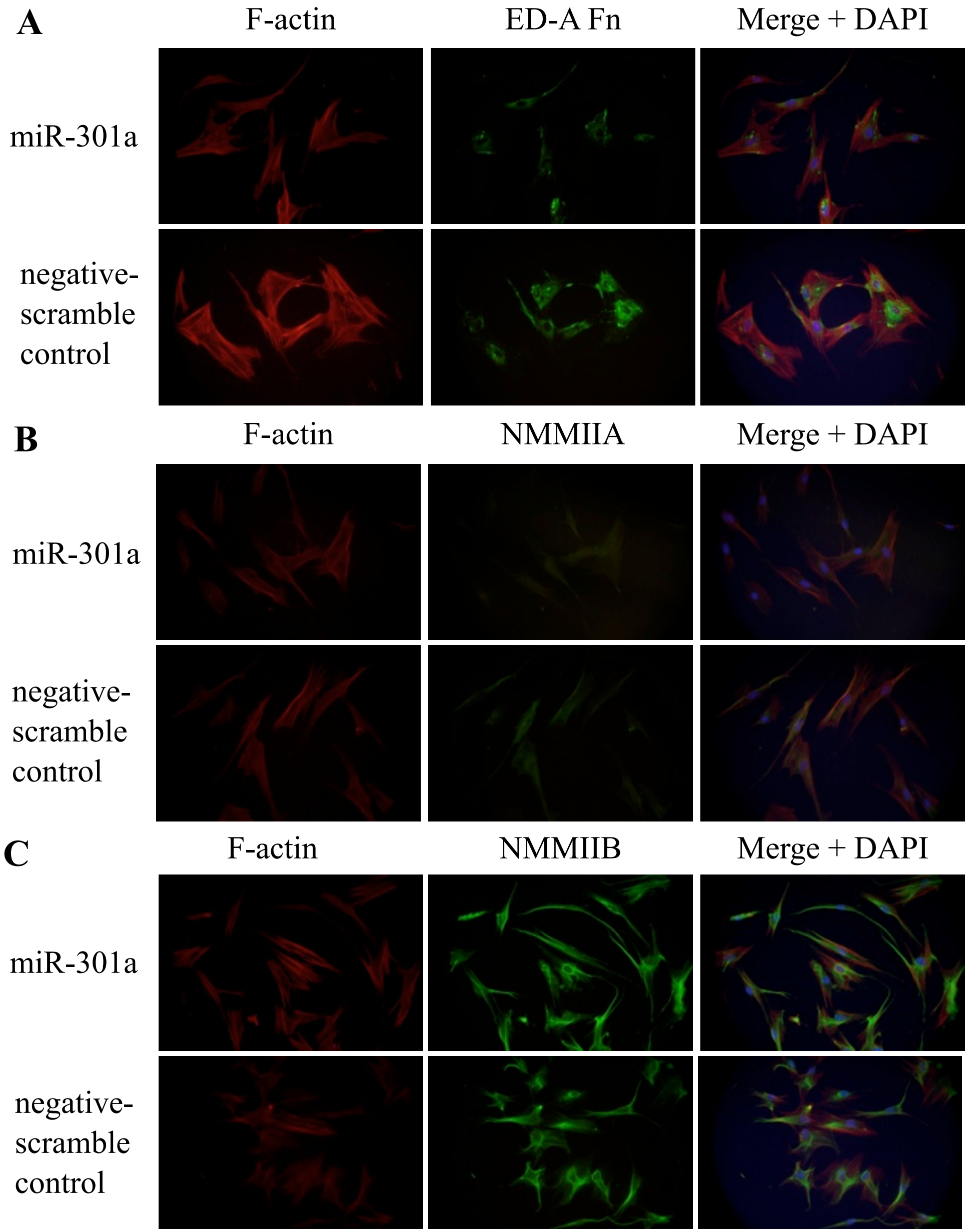


Figure 7: Overexpression of miR-301a alters immunofluoresent staining of NMMIIA and ED-A Fn. Overexpression of miR-301a in human mesenchymal stem cells results in reduced immunofluorescent staining with ED-A Fn (A) and an absence of NMMIIA (B). There is little visible change in expression of NMMIIB (C) on immunofluorescence. F-actin fibers are stained red and nuclear material stained with DAPI (blue). Abbreviations: non-muscle myosin (NMM), extra domain A fibronectin (EDA-Fn).

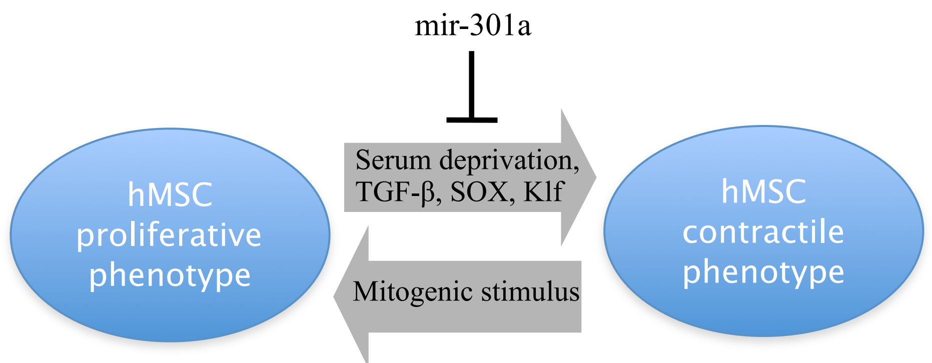


Figure 8: Proposed schema for action of miR-301a in regulating the proliferative vs contractile phenotype. The contractile phenotype is induced by serum deprivation as well as signaling molecules such as TGF-β, the SOX and klf proteins. miR-301a, expressed in response to mitogenic stimuli, likely suppresses the expression of these molecules and therefore favors the proliferative phenotype.