Pharmacological priming of adipose-derived stem cells promotes myocardial repair

Jana S Burchfield,¹ Ashley L Paul,¹ Vishy Lanka,¹ Wei Tan,¹ Yongli Kong,¹ Camille McCallister,¹ Beverly A Rothermel,¹ Jay W Schneider,¹ Thomas G Gillette,¹ Joseph A Hill^{1,2}

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¹Departments of Internal Medicine (Cardiology), University of Texas Southwestern Medical Center, Dallas, Texas, USA
²Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas, USA

Correspondence to

Dr Joseph A Hill, Division of Cardiology, University of Texas Southwestern Medical Center, NB11.200, 6000 Harry Hines Boulevard, Dallas, TX 75390-8573, USA; joseph.hill@utsouthwestern.edu

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ABSTRACT

Adipose-derived stem cells (ADSCs) have myocardial regeneration potential, and transplantation of these cells following myocardial infarction (MI) in animal models leads to modest improvements in cardiac function. We hypothesized that pharmacological priming of pre-transplanted ADSCs would further improve left ventricular functional recovery after MI. We previously identified a compound from a family of 3,5-disubstituted isoxazoles, ISX1, capable of activating an Nkx2-5-driven promoter construct. Here, using ADSCs, we found that ISX1 (20 mM, 4 days) triggered a robust, dose-dependent, fourfold increase in Nkx2-5 expression, an early marker of cardiac myocyte differentiation and increased ADSC viability in vitro. Co-culturing neonatal cardiomyocytes with ISX1-treated ADSCs increased early and late cardiac gene expression. Whereas ISX1 promoted ADSC differentiation toward a cardiogenic lineage, it did not elicit their complete differentiation or their differentiation into mature adipocytes, osteoblasts, or chondrocytes, suggesting that re-programming is cardiomyocyte specific. Cardiac transplantation of ADSCs improved left ventricular functional recovery following MI, a response which was significantly augmented by transplantation of ISX1- pretreated cells. Moreover, ISX1-treated and transplanted ADSCs engrafted and were detectable in the myocardium 3 weeks following MI, albeit at relatively small numbers. ISX1 treatment increased histone acetyltransferase (HAT) activity in ADSCs, which was associated with histone 3 and histone 4 acetylation. Finally, hearts transplanted with ISX1-treated ADSCs manifested significant increases in neovascularization, which may account for the improved cardiac function. These findings suggest that a strategy of drug-facilitated initiation of myocyte differentiation enhances exogenously transplanted ADSC persistence in vivo, and consequent tissue neovascularization, to improve cardiac function.

INTRODUCTION

Globally, millions of people suffer from heart failure stemming from prior myocardial infarction (MI). This syndrome results from an inability to compensate for the loss of myocytes in the infarcted region. Currently, there are no pharmaceutical agents or medical devices that can directly mediate the regeneration of the

Significance of this study

What is already known on this subject?

- Currently, there are no pharmaceutical agents or medical devices that can directly mediate the regeneration of the heart muscle.
- Stem cell therapy has received much attention as a possible approach to mediate myocardial repair and thereby improve cardiac function. However, the efficacy of approaches involving delivery of exogenous cells to the injured myocardium is modest.
- Adipose tissue is a rich source of adult stem cells which can be isolated in large quantities by minimally invasive liposuction.
- Some evidence suggests that it is advantageous to foster differentiation of adult stem cells toward a cardiac lineage at the time of cell delivery to maximize reparative potential.

What are the new findings?

- We tested a strategy in which adipose-derived stem cells (ADSCs) are 'primed' by exposing them to ISX1, a small molecule previously established to promote stem cell differentiation toward a cardiomyocyte phenotype.
- We show that ISX1 treatment in culture promotes ADSC exit from the cell cycle and leads to expression of a variety of markers indicative of a cardiogenic lineage. Importantly, ISX suppressed differentiation into other cellular lineages.
- We observed that delivery of ISX1-primed ADSCs to an infarcted myocardium promoted rescue of the pathological remodeling phenotype above and beyond that afforded by vehicle-treated ADSCs. ISX1-treated ADSCs remained resident within the injured left ventricle for up to 3 weeks, and their administration was associated with enhanced angiogenesis.
- Together, these findings point to a novel strategy that capitalizes on synergies between pharmacological and cell-based modalities.

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Significance of this study

How might it impact on clinical practice in the foreseeable future?

- Current pharmacological therapies to treat cardiovascular diseases, such as ischemic heart disease, heart failure, peripheral vascular disease, do not reverse disease progression. Our study, using pharmacologically treated adipose-derived stem cells, may clinically prevent further tissue damage, enhance healing of damaged tissue, and provide adequate blood flow; thus, stopping or reversing disease progression.
- Few medications are available for the treatment of obesity, and most are FDA-approved for short-term use only. Obesity is a chronic disease, where there is an increase in fat cell (adipocyte) number. In our study, we identified a small molecule that can limit the differentiation of stem cells into mature, fat-containing adipocytes. Therefore, in the future, this molecule may be used to prevent adipogenesis, independent of dietary intake.

heart muscle.^{1 2} Stem cell therapy has received much attention as a possible approach to mediate myocardial repair and thereby improve cardiac function. Unfortunately, the efficacy of approaches involving the delivery of exogenous cells to the injured myocardium is modest. Furthermore, compared with the number of myocytes lost in an MI, only a small number of cells can be administered and only a tiny fraction of those persist within the tissue.

Most clinical trials of myocardial regeneration have employed adult bone marrow stem cells.^{3 4} However, adipose tissue is an even richer source of adult stem cells. Adipose-derived stem cells (ADSCs) can be isolated in large quantities by minimally invasive liposuction producing a higher yield of stem cells per volume.⁵ Cells derived from this tissue include mesenchymal stem cells, endothelial progenitor cells, pericytes, and adipose progenitor cells. Transplantation of these cells can improve cardiac function in animal models.^{6–13} However, cardiogenic conversion in vivo is rare.

In the great majority of studies, exogenous cells delivered to the injured myocardium do not differentiate into significant numbers of cardiomyocytes, nor do they persist in the tissue beyond a few days. Rather, their passage through the tissue appears to trigger a response which is poorly characterized, but which may involve neovascularization, re-entry of some cell types into the cell cycle, or other events. In any case, effect sizes observed to date remain modest.

Some evidence suggests that it is advantageous to foster differentiation of adult stem cells toward a cardiac lineage at the time of cell delivery to maximize reparative potential. This strategy has been accomplished by transfecting cells with small interfering RNA (siRNA) or expression constructs^{14–16} or by co-transplantation with cells engineered to express proteins capable of directing stem cell differentiation.^{17–21} Another strategy entails use of small molecules or proteins to promote cardiogenic conversion.²²

We previously screened a large chemical library and identified small molecules capable of activating the transcription factor, *Nkx2.5*, one of the earliest lineage-restricted genes to be expressed in cardiac progenitor cells.²³ This screen was engineered using a firefly *luciferase* (*luc*) gene inserted into the *Nkx2.5* locus on a 180-kb mouse bacterial artificial chromosome harboring all of the transcriptional and epigenetic regulatory elements necessary for the cardiac-specific expression of *Nkx2.5*. This gene was then stably integrated into P19 carcinoma cells (subclone CL6; P19CL6) for high throughput screening.^{22 24} Small molecules identified in this screen offer significant advantages over previous screens for cardiogenic small molecules that targeted late cardiac differentiation markers such as α -myosin heavy chain (α -MHC) or atrial natriuretic factor (ANF).^{25 26} Transplantation of human peripheral blood mononuclear cells treated with the compounds we isolated in our *Nkx2.5* screen improved cell engraftment and functional recovery in cryoinjured rat hearts.²² In addition, these molecules activated muscle-specific transcriptional programs in multipotent Notch-activated epicardium-derived cells (NECs),²⁷ directed neuronal cell fate determination,^{24 28} induced myofibroblast differentiation,²⁹ and increased insulin production in pancreatic β cells.³⁰ We set out to test whether one of the most promising of these small molecules, a 3,5-disubstituted isoxazole (ISX1), could direct ADSCs into a cardiac lineage and foster myocardial repair.

METHODS**ADSC isolation and gene expression analysis**

The mice from which ADSC isolation was performed were euthanized with a lethal dose (120 mg/kg) of sodium pentobarbital. White adipose tissue from posterior subcutaneous (inguinal), perigonadal, visceral retroperitoneal, anterior subcutaneous (interscapular) of C57BL6/J or ubiquitin- green fluorescent protein (GFP) transgenic (GFP expressed from the ubiquitin C promoter) mice (The Jackson Laboratory, Bar Harbor, Maine, USA) was digested in adipose isolation buffer (100 mM HEPES, 120 mM NaCl, 50 mM KCl, 1 mM CaCl₂, pH 7.4) with collagenase I for 1 h 40 min at 37°C with shaking at 100 RPM. Digested adipose was filtered through a 210 μ m filter, and mature adipocytes were discarded. Red blood cells within the stromal vascular fraction (SVF) were lysed using ammonium chloride, and the SVF was filtered through a 30 μ m filter, washed in 1×phosphate-buffered saline (PBS), and plated at 80,000 cells/cm². After 24 h, the cell culture medium was changed, and cells were exposed, at pre-confluency, to various concentrations of ISX1 (0.2, 2, 20, 80 μ M) (n=4), or subsequently at 20 μ M ISX1 or vehicle (Dimethyl sulfoxide [DMSO]) for variable lengths of time (1, 4, 7 days) (n=3–7). The empirically determined effective dose of 20 μ M ISX1 and exposure time of 4 days were used in all experiments.

Isolated RNA was reverse transcribed with subsequent quantitative real-time PCR analysis using TaqMan *Nkx2-5* specific primers (n=4). ADSCs were co-cultured with neonatal rat cardiomyocytes (NRVMs, 1:10), treated with diluent or 20 μ M ISX1, and RNA was isolated after 3 weeks of co-culturing (n=4–7). Mouse gene expression was measured using quantitative real-time PCR and

TaqMan mouse-specific primers: *Troponin C*, *Atrial natriuretic factor (Anf)*, *Brain natriuretic peptide (Bnp)*.

Primary culture of NRVMs

Neonatal rat pups were separated from the dam on the morning immediately following parturition. They were placed in a 1500 mm Petri dish chilled to 4°C and resting on crushed ice. Following a 10 min period of low temperature acclimation, the pups were euthanized by decapitation and their hearts harvested for further processing.

Cardiomyocytes were isolated and plated as previously described.³¹ Briefly, left ventricles of Sprague-Dawley rats aged 1–2 days were collected and digested with collagenase. The resulting suspension was pre-plated to remove fibroblasts. Myocytes were plated at a density of 1250 cells/mm² in cell culture medium containing 10% fetal calf serum and 100 µmol/L of bromodeoxyuridine. Cells were 95% pure cardiomyocytes, which were co-cultured with ADSCs at a ratio of 10:1.

Immunocytochemistry for Nkx2-5 in cells

After 4 days with ISX1 or DMSO, ADSCs were washed with PBS, fixed with 4% paraformaldehyde for 30 min, and incubated for 3 h at 30°C with a mixture of MeOH:DMSO (4:1). Immunocytochemistry was performed using rabbit anti-Nkx2-5, IgG antibody (1:200) (Gene Tex) overnight with subsequent secondary antibody incubation for 30 min (goat anti-rabbit Cy3 1:200), and counterstaining was performed using 2 µg/mL Hoechst 33342 dye (Molecular Probes).

Flow cytometry

Cells were incubated with antibodies for markers of mesenchymal stem cells (CD105, CD44, CD90.1, CD73, CD166), adipose progenitor cells (CD29, CD34, Sca1, CD24), endothelial cells (CD31), hematopoietic stem cells (CD45, CD117), and monocytes (CD11b, CD14) along with their respective isotype control antibodies (eBiosciences, San Diego, CA) listed in online supplementary methods. Cells were analyzed using FACS Caliber (BD) (n=2).

Cell viability and cell proliferation

ADSC viability was measured using the CellTiterBlue Assay (viable cells reduce resazurin into fluorescent resorufin) (n=2). Briefly, 25,700 cells were plated on a 96-well plate, ISX1 or DMSO was added for 4 days, and CellTiterBlue was added for 4 h at 37°C. ADSC proliferation was measured using a colorimetric Cell Proliferation ELISA, BrdU (Roche). For analysis of BrdU incorporation, cells were plated at a density of 12,750 cells/well on a 96 well plate, ISX1 or DMSO was added for 4 days, and cells were exposed to the BrdU antibody (n=2).

MI and cell transplantation

Mice were anesthetized by intraperitoneal injection of ketamine/midazolam (100 mg/kg and 1 mg/kg, respectively) and maintained under inhalation of 1.5% isoflurane during the surgical procedure. Left anterior descending (LAD) coronary artery ligation of female C57BL6/J mice aged 9–12 weeks (The Jackson Laboratory) was performed as previously described.³² ADSCs from male ubiquitin-GFP mice

were treated for 4 days with DMSO or 20 µM ISX1 and 0.3×10^6 to 0.4×10^6 cells in 50 µL by intramyocardial injection into the peri-infarct region of female C57BL6/J mice immediately after LAD ligation (5 injection sites) in a blinded manner. Sham-operated mice were subjected to thoracotomy, suturing around the LAD without occlusion, followed by surgical closure. No cells were transplanted into sham-operated control hearts.

Echocardiography

Cardiac function was measured using high-resolution two-dimensional targeted M-mode echocardiography on anesthetized mice (Vevo 2100 small-animal microultrasound system, VisualSonics, Toronto, Canada) (n=10–15). Data were analyzed in a blinded manner.

Measurement of infarct size

In a separate series of mice, infarct sizes at 1 day after MI were measured. Mice were subjected to MI in which the LAD coronary artery is surgically ligated. Animals were maintained on 0.8% isoflurane as anesthesia throughout the surgical procedure. Toe pinch was employed at random intervals to ensure that an adequate plane was maintained. Immediately postoperative and prior to regaining consciousness, the mice were treated with 0.5 mg/kg Buprenorphine Lab SR and monitored for signs of pain or distress twice daily for 72 h and daily thereafter.

Five to six 1 mm sections of 1-day post-MI hearts were incubated for 20 min at 37°C with 1% 2,3,5-triphenyltetrazolium chloride (TTC), and percent infarct was calculated by measuring the area of infarct and weight of each section. The weight of the infarction = $(A1 \times W1) + (A2 \times W2) + (A3 \times W3) + (A4 \times W4) + (A5 \times W5)$ and percent infarct = $(\text{weight of the infarction} / \text{weight of left ventricle (LV)}) \times 100$ (n=5–6).

Immunohistochemistry

Transplanted ubiquitin-GFP cells were detected in frozen sections of 3-week post-MI hearts using rabbit anti-GFP antibody (1:200) (Invitrogen) and goat Cy3-anti-rabbit (1:200). In addition, tissue sections from the heart, kidney, lung, skeletal muscle, spleen, liver, and white adipose tissue were assessed using rabbit anti-GFP antibody (1:200) (Invitrogen) and streptavidin HRP (1:800) with TACS Blue Label (R&D Systems). Photographs were taken at 400× and 630× magnification. The presence of transplanted cells was verified using confocal microscopy. Frozen sections of hearts 3 weeks post-MI and 3 weeks post-cell transplant were stained with Fluorescein isothiocyanate (FITC) conjugated BS-I Isolectin B4 from *Bandeiraea simplicifolia* (Sigma, St. Louis, Missouri, USA) at a 1:75 dilution for vascular membrane staining. Fifteen photos in the border zones of each heart at 200× magnification were quantified for vessel density and calculated as number of vessels/field (n=7–11).

ADSC differentiation

ADSCs were grown in an Methylisobutylxanthine, Dexamethasone, Insulin (MDI) induction medium (1×Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 160 nM insulin, 250 nM dexamethasone (Sigma)) for 2 days, then in an insulin-containing medium

(160 nM insulin) for 2 days followed by culturing in 1×DMEM for 3 weeks. Mature adipocytes were stained with 0.12% Oil Red O, and photographs were taken at ×400 magnification. Oil Red O stain was eluted using 100% isopropanol and measured at 500 nm (n=3). ADSCs were differentiated into osteoblasts for 3 weeks using osteogenic differentiation medium: 1×DMEM, 10% FBS, 0.1 μM dexamethasone, 50 μM 2-phospho-L-ascorbic acid trisodium salt, 10 mM glycerol-2-phosphate disodium salt hydrate. Cells were stained with 2% alizarin red for 45 min, photos were taken at ×200 magnification, and the stain was eluted with 10% cetylpyridinium and measured at 540 nm (n=4). Von Kossa staining was performed using 5% silver nitrate for 30 min in the dark with subsequent exposure to ultraviolet light for 1 h, with photos taken at 200× magnification. For chondrogenic differentiation, ADSCs were pelleted in a 15 mL tube and differentiated into chondrocytes using 1×DMEM, 1% FBS, 10 ng/mL TGFβ1, 155 μM 2-phospho-L-ascorbic acid trisodium salt, 1% solution of insulin, transferrin, selenium, and 0.1 μM dexamethasone. At the end of 3 weeks, pellets were digested with papain and sulfated glycosaminoglycans were measured using dimethyl-methylene blue reagent at 525 nm. Data were quantified on the basis of a standard curve using whale chondroitin 4-sulfate (n=4).

Histone acetyltransferase and deacetylase activity

Histones were isolated using the EpiQuik Total Histone Extraction Kit according to the manufacturer's instructions with the exception of no addition of dithiothreitol (Epigentek). Histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities were measured in nuclear extracts of ADSCs exposed to 20 μM ISX1 or DMSO for 4 days using a colorimetric assay (440 nm) according to the manufacturer's instructions (BioVision) (n=4 and 3, respectively). Positive controls included HeLa cell nuclear

extracts and HDAC inhibition using 5 μM trichostatin A (TSA, Sigma). HDAC inhibitors, valproic acid (Sigma) and TSA, were added daily with a fresh medium containing either DMSO or ISX1. Treated cells were subsequently analyzed for *Nkx2-5* gene expression (n=4–8 and n=5–7, respectively).

Western blotting

Histones were isolated using the EpiQuik Total Histone Extraction kit (Epigentek) according to the manufacturer's instructions. Lysates (2.5 μg) were separated using 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the Polyvinylidene difluoride (PDVF) membrane. Membranes were incubated with acetylated histone H3K9 polyclonal, acetylated histone H4K8 polyclonal antibodies (Epigentek), histone 3, trimethylated histone H3K9, trimethylated histone H3K4 polyclonal antibodies (Abcam), and histone 4 antibody (Santa Cruz). Band densities on the western blots were quantified using Image J software (n=3).

Protein array

Paracrine factors released into cell culture media were measured using a RayBio Mouse Angiogenesis Array according to the manufacturer's instructions (RayBiotech, Inc). Individual dot intensities were quantified by densitometry and internal positive controls were used to normalize between different membranes (n=2).

Approvals

The study was approved by the UT Southwestern Institutional Animal Care and Use Committee, and all animal procedures conform to NIH guidelines (Guide for the Care and Use of Laboratory Animals).

Figure 1 Adipose-derived stem cells (ADSCs) express *Nkx2-5* in response to ISX1. (A) Increased *Nkx2-5* gene expression in ADSCs treated for 4 days with ISX1 compared with vehicle (DMSO). Data include n=4–5 in each group and are presented as mean±SEM, *p<0.05. (B) Immunocytochemistry of nuclear *Nkx2-5* protein in vehicle-treated and ISX1-treated ADSCs. *Nkx2.5* (red) and Hoechst 33342 dye (blue). Bar=40 μm.

Figure 2 ISX1 augments cardiac gene expression in adipose-derived stem cells (ADSCs) co-cultured with neonatal cardiomyocytes (NRVMs). Increased cardiac gene expression (ANF, BNP, Troponin C) in cells co-cultured with NRVMs for 3 weeks and exposed to ISX1 compared with vehicle (DMSO). Data include $n=4-7$ in each group and are presented as mean $\pm$ SEM, * $p<0.05$.

Statistical analysis

Statistical analyses were performed using StatView software. Error bars in graphs of three or more independent cell isolations indicate $\pm$ SE of the mean (SEM). Error bars in graphs of two independent cell isolations indicate $\pm$ SD. Comparisons between two groups of three or more independent isolations were performed using a two-tailed unpaired Student's *t* test (figures 1–3, 4A,D and 5B,D). Comparisons between three or more groups were performed using one-way analysis of variance, followed by the Student-Neumann-Keuls post hoc test (figures 4B,C, 6B–D and 7C).

RESULTS

Characterization of ADSCs

Cells from the SVF of mouse white adipose tissue and SVF maintained in culture for 4 days, denoted as passage 0 (P0), were characterized by flow cytometry. Flow cytometry data are shown from one representative experiment for P0 and SVF (see online supplementary figure S1A,B, respectively) and quantitated data isolations are shown in online supplementary figure S2. The SVF and the P0 cells expressed markers of adipose progenitors (CD29, CD34, Sca1, CD24) and mesenchymal stem cell markers (CD105, CD73) (table 1). Inflammatory cells were detected in the SVF and not the P0 cells, which was the passage used for all subsequent experiments. Importantly, we noted the presence of CD34, a marker which distinguishes activated early progenitors from quiescent cells³³ and indicates increased differentiation potential.³⁴ CD34 levels decline with long-term maintenance in culture.³⁵ Therefore, subsequent experiments were performed using cells with limited time and passaging in culture.

Pharmacological priming of ADSCs

Treatment of ADSCs with 20 μ M ISX1 resulted in a robust increase in *Nkx2-5* gene expression as measured by qPCR (figure 1A). Data were derived from four to five different cell isolations in each group, performed in triplicate per experiment. This increase was both dose ($n=4$ at each concentration of ISX1) and time dependent ($n=3-7$) (see online supplementary figure S3A,B). ISX1-elicited increases in *Nkx2.5* protein levels, which was observed by immunostaining (figure 1B). ISX1 was not toxic to ADSCs at any concentration for up to 4 days (see online supplementary material 4A). However, long-term treatment (3 weeks) with a dose of 80 μ M caused cell death as assessed by visualization of hematoxylin-stained cells (see online supplementary figure 4B) ($n=2$). It is well known that on differentiation, stem cells exit the cell cycle and cease to divide. Consistent with ISX1 promoting differentiation of ADSCs, there was a dose-dependent decrease in cell proliferation (see online supplementary figure 4C) ($n=2$).

To examine the ability of ISX1 to promote differentiation toward a cardiomyocyte-like phenotype, we co-cultured mouse ADSCs with NRVMs (1:10) and measured early and late cardiac marker gene expression, including ANF, brain natriuretic peptide (BNP), and troponin C by qPCR using mouse-specific primers. ISX1-treated cultures manifested significant increases in all three of these markers as compared with vehicle-treated controls (figure 2) ($n=4-7$). These data suggest that ISX1-treated ADSCs adopt a phenotype mimicking that of cardiac myocytes.

Inhibition of other ADSC differentiation pathways

Since ADSCs have the capacity to differentiate into a variety of tissues of mesenchymal origin, including adipose tissue, bone, and cartilage, we determined whether ISX1 promoted ADSCs differentiation into adipocytes, osteoblasts, and chondrocytes. To examine adipocyte differentiation, we cultured ADSCs for 21 days using adipogenic differentiation medium in the presence or absence of ISX1. Using Oil Red O staining as a marker of adipocyte differentiation, we found that ISX1 suppressed adipogenesis as compared with

Figure 3 Adipose-derived stem cells (ADSCs) manifest diminished capacity to undergo adipogenesis, osteogenesis, and chondrogenesis in response to ISX1. (A) Representative images of Oil Red O staining in ADSCs differentiated (3 weeks) into mature adipocytes and treated with vehicle (DMSO) or ISX1. Bar=20 μ m. Black arrows indicate Oil Red O lipid droplets. (B) Quantification of eluted Oil Red O stain. (C) Representative images of Alizarin Red staining in ADSCs differentiated (3 weeks) into mature osteoblasts and treated with vehicle (DMSO) or ISX1. Bar=40 μ m. (D) Quantification of eluted Alizarin Red stain. (E) Representative images of von Kossa staining in ADSCs differentiated (3 weeks) into mature osteoblasts and treated with vehicle (DMSO) or ISX1. Bar=40 μ m. (F) Quantification of sulfated glycosaminoglycans after 3 weeks in chondrogenesis differentiation medium and exposed to vehicle (DMSO) or ISX1. Data include n=3–4 per group and are presented as mean $\pm$ SEM (* p <0.05).

vehicle-treated controls (figure 3A), a finding which is not due to a decrease in cell number as visualized by hematoxylin staining (see online supplementary figure 3C). Lipid accumulation was quantified by eluting intracellular Oil Red O stain using isopropanol and measuring amounts by spectrophotometry (figure 3A,B) (n=3).

To determine whether ISX1 provoked osteogenic differentiation, we exposed ISX1-treated and vehicle-treated ADSCs to an osteogenic medium for 21 days. ISX1 led to a decrease in the osteogenic capacity of ADSCs as shown by Alizarin Red and von Kossa staining (figure 3C–E) (n=4). We also measured sulfated glycosaminoglycans after 21 days of exposure to a chondrogenic differentiation protocol and found that ISX1 inhibited ADSC differentiation into chondrocytes (figure 3F) (n=4). In aggregate, these results suggest that ISX1 can specify the differentiation pathway of ADSCs, limiting differentiation into adipose, bone, or cartilage lineages while promoting differentiation toward a cardiac lineage.

ISX1-elicited chromatin remodeling

Stem cell differentiation involves a tightly regulated program of gene expression governed by both genetic and epigenetic mechanisms. Histone acetylation states

(regulated by HATs and HDACs) are a major determinant of stem cell differentiation.³⁶ To test for a role of epigenetic mechanisms in ADSC-dependent ISX1-primed myocardial repair, we first evaluated HDAC activity, observing that ISX1 did not change HDAC activity in ADSCs (figure 4A) (n=3). Further, the effects of ISX1 on *Nkx2-5* expression could not be mimicked by the HDAC inhibitors valproic acid (VPA) (n=4–8) or TSA (n=5–7) (figure 4B, C). However, ISX1 did provoke an increase in HAT activity (n=4), culminating in an increase in the acetylation of histones 3 and 4, key molecules involved in cell differentiation (n=3)^{37–38} (figures 4D and 5A–D).

Acetylation of specific lysine residues on histones 3 and 4 help determine whether a gene is transcriptionally active versus repressed.³⁹ Pertinent to this, we found that ISX1 provoked an increase in the acetylation of lysine 9 (histone 3) and lysine 8 (histone 4) (figure 5A–D). ISX1 treatment did not lead to a decrease in trimethylated histone 3 lysine 9, a ‘repressive mark’,⁴⁰ or to an increase in the ‘active mark’, trimethylated histone 3 lysine 4^{41–42} (see online supplementary figure 5). These results suggest that ISX1-elicited alterations in gene expression involve changes in histone acetylation via an increase in HAT activity rather than changes in histone methylation.

Figure 4 ISX1 increased histone acetyltransferase (HAT) activity but not histone deacetylase (HDAC) activity in adipose-derived stem cells (ADSCs), and HDAC inhibition alone is insufficient to induce *Nkx2-5* gene expression. (A) HAT activity of nuclear extracts of ADSCs treated with ISX1 or vehicle (DMSO). Data include n=4 per group and are presented as mean $\pm$ SEM ($p < 0.05$). (B) HDAC activity in nuclear extracts of ADSCs treated with ISX1 or vehicle (DMSO). Data include n=3 per group (NS, non-significant). (C) *Nkx2.5* gene expression after addition of valproic acid (VPA) alone or in combination with ISX1. Data include n=4–8 in each group. (D) *Nkx2.5* gene expression after addition of trichostatin A (TSA) alone or in combination with ISX1. Data include n=5–7 in each group.

Transplantation of ISX1-primed ADSCs after MI

A number of studies have demonstrated efficacy of exogenously derived stem cells in promoting myocardial regeneration in the setting of MI.^{3 43} Our results suggest that ISX1-treated ADSCs may be ‘primed’ toward a cardiomyocyte fate, which we hypothesize facilitates paracrine signaling events that promote repair. To test this, we studied a mouse model of MI in which the LAD coronary artery is surgically ligated and ADSCs treated with either vehicle or ISX1 are transplanted into the myocardium at the time of surgery. Sham-operated and mock-transplanted MI-injured animals served as controls. Representative echocardiograms are shown in figure 6A. Transplantation of ADSCs (0.3×10^6 to 0.4×10^6 cells) immediately following MI led to an improvement in contractile performance ($p < 0.05$) measured by echocardiography and quantified as percent fractional shortening (%FS) 3 weeks post-MI ($21.1 \pm 1.4\%$)

as compared with mock-transplanted hearts ($15.9 \pm 1.7\%$) ($n = 10-15$) (figure 6B). Treatment of ADSCs with ISX1 for 4 days prior to transplantation did not lead to significant changes in left ventricular diastolic dimension (figure 6C). Treatment of ADSCs with ISX1 for 4 days prior to transplantation was associated with yet further protection of systolic function ($27.1 \pm 1.1\%$ FS), as well as a decrease in left ventricular end systolic dimension (LVESD) (figure 6D).

To ensure that these differences in cardiac function were not a consequence of differences in initial infarct size, we performed 2,3,5-triphenyltetrazolium chloride (TTC) staining 24 h after ligation of the LAD coronary artery in a separate series of mice. There were no statistically significant differences in infarct size between hearts transplanted with cells or those injected with the diluent of cells or between vehicle-treated cells and ISX1-treated cells at day 1 (figure 7A) ($n = 5-6$) or scar size (see online supplementary

Figure 5 ISX1 increased histone 3 and 4 acetylation and histone 4 lysine 8 acetylation. (A) Representative western blot illustrating increased acetylation of histone 3 lysine 9 (H3K9) in response to ISX1. (B) Quantification of acetylated H3K9 compared with total histone 3. Data include n=3 in each group. Data are presented as mean $\pm$ SEM (* $p < 0.05$). (C) Representative western blot illustrating increased acetylation of histone 4 lysine 8 (H4K8) in response to ISX1. (D) Quantification of acetylated H4K8 compared to total histone 4. Data include n=3 per group.

Figure 6 Transplantation of adipose-derived stem cells (ADSCs) pretreated with ISX1 in myocardial infarction (MI)-injured hearts leads to improved functional recovery. (A) Representative M-mode echocardiograms. (B) Ventricular systolic function depicted as percent fractional shortening (%FS) in sham-operated mice, in hearts treated with cell diluent (phosphate-buffered saline, PBS), or in hearts transplanted with ADSCs treated with vehicle (DMSO) or ISX1. (C) Left ventricular end-diastolic dimension (LVEDD) in millimeters. (D) Left ventricular end-systolic dimension (LVESD) in millimeters. Data include $n=10$ in the sham-operated control group, $n=12$ in diluent of the cells-treated group, $n=15$ in (PBS), $n=15$ in the vehicle-treated ADSC-transplanted group, $n=13$ in the ISX1-treated ADSC-transplanted group. Data are presented as mean $\pm$ SEM. * $p<0.05$, Sham versus diluent, vehicle-treated cells, ISX1-treated cells, ** $p<0.05$, ISX1-treated cells versus diluent, vehicle-treated cells.

figure S6). This suggests that the long-term (3 weeks) improvement in cardiac function afforded by ISX1-primed ADSCs was not due to differences in initial infarct size.

To explore possible mechanisms whereby ISX1-treated ADSCs might improve cardiac function post-MI, we examined the extent of neovascularization in the MI-injured hearts. Isolectin B4 staining was used to mark endothelial cells lining blood vessels. Transplanted vehicle-treated ADSCs increased vessel density by approximately 20% over that of mock-transfected hearts (figure 7B, C) ($n=7-11$). This effect was doubled in hearts transplanted with ISX1-treated ADSCs with vessel densities increasing nearly 40% as compared with diluent-injected control hearts

(figure 7B, C). This suggests that enhanced vascularization contributes to the improvement in cardiac function in the ISX1-treatment arm.

Paracrine factors are believed to promote the protective effects of ADSCs on ischemic injury. To examine whether ISX treatment altered paracrine factor secretion by ADSCs, we performed an angiogenesis protein array on the cell culture medium from ADSCs treated with vehicle or ISX1. No dramatic differences were observed in the levels of paracrine factors tested between the two sample conditions. However, we did observe small differences in select cytokines and growth factors including an increase in granulocyte-colony stimulated factor (G-CSF) and

Figure 7 Enhanced neovascularization after adipose-derived stem cells (ADSC) transplantation but no difference in the original infarct size. (A) Percent infarct size between all groups (myocardial infarction (MI)+cell diluent (phosphate-buffered saline, PBS), MI+vehicle-treated cells, MI+ISX1-treated cells) as assessed by TTC (2,3,5-triphenyltetrazolium chloride) staining (n=5–6). (B) Representative images of isolectin B4 staining (green) of blood vessels. Bar=200 μ m. (C) Quantification of vessel abundance normalized to microscopic field in hearts transplanted with ADSCs treated with cell diluent (PBS), vehicle (DMSO), or ISX1. Data are presented as mean $\pm$ SEM; (n=7–11); *p<0.05.

interferon (IFN) γ (n=2) (see online supplementary figure S7). Thus, we believe it is unlikely that alterations in paracrine factor expression alone could account for the functional changes elicited when comparing hearts treated with the ISX1-treated ADSCs versus vehicle-treated ADSCs.

We next tested whether the transplanted cells persist in the infarcted myocardium, potentially acting more directly to improve cardiac function after MI. We isolated ADSCs from adipose tissue of mice that ubiquitously express GFP. At 3 weeks post-MI, no GFP⁺ cells were detected in vehicle-treated ADSC-transplanted hearts. In contrast, we detected GFP⁺ cells in the ISX1-treated ADSC-transplanted hearts (see online supplementary figure S8, figure 8A). These GFP⁺ cells co-localized with troponin T-stained cardiac myocytes, suggesting that these cells manifest myocyte-like

characteristics. Using confocal microscopy, we confirmed the presence of GFP⁺ transplanted cells, and their co-localization to troponin T-stained cardiac myocytes (figure 8B). However, these transplanted cells did not appear to fully differentiate into cardiac myocytes.

An inventory of other tissues (approximately 15 histological sections each), including the kidney, lung, skeletal muscle, spleen, liver, and white adipose tissue, failed to reveal GFP⁺ cells in either ISX1-treated or vehicle-treated ADSC transplanted hearts (see online supplementary figure S9), suggesting that cells transplanted into the myocardium did not home to other organs. Whereas no vehicle-treated ADSCs were detected at 3 weeks post-MI (figure 8A), increased neovascularization has been linked to paracrine factors released from these cells.⁴⁴ However, the clear

Table 1 Cell marker expression

	SVF (%)	P0 (%)
CD29	79	44
CD34	52	17
Sca1	60	38
CD24	40	7
CD105	30	17
CD117	2	0
CD44	0	2
CD90	9	0
CD73	36	7
CD166	15	0
CD31	2	0
CD45	0	0
CD11b	14	1
CD14	26	0
Ter119	0	0

Cell surface marker expression on freshly isolated stromal vascular fraction (SVF) and cells cultured for 4 days (P0). Both SVF and P0 cells expressed no endothelial cell or inflammatory cell markers but rather expressed markers of adipose progenitor cells, endothelial progenitor cells, and mesenchymal stem cells.

presence of ISX1-treated ADSCs at 3 weeks post-MI raises the prospect that paracrine actions elicited by these cells may be sustained and persistent relative to the more transient passage of vehicle-treated ADSCs. Together, these data suggest that the increase in neovascularization is due to localization of the ISX1-treated ADSCs to the myocardium, resulting in a more sustained paracrine-elicited effect.

DISCUSSION

Stem cell therapy for treatment of MI has been the subject of great hope and interest. However, the benefit afforded by this strategy is currently of modest impact, and therefore there is great interest in identifying means of enhancing it. Most evidence points to paracrine signaling events—which remain elusive—that promote repair.

We tested a strategy in which ADSCs, cells that are readily harvested from a rich and prevalent depot, are ‘primed’ by exposing them to ISX1, a small molecule previously established to promote stem cell differentiation toward a cardiomyocyte phenotype.^{22 27} We show that ISX1 treatment in culture promotes ADSC exit from the cell cycle and leads to expression of a variety of markers indicative of a cardiogenic lineage. Importantly, ISX suppressed differentiation into other cellular lineages. Mechanistically, ISX1 provoked increased HAT activity, an established mechanism governing stem cell differentiation and increased acetylation of specific histone residues. Finally, we observed that delivery of ISX1-primed ADSCs to the infarcted myocardium promoted rescue of the pathological remodeling phenotype above and beyond that afforded by vehicle-treated ADSCs. ISX1-treated ADSCs remained resident within the injured LV for up to 3 weeks, and their administration was associated with enhanced angiogenesis. Together, these findings point to a novel strategy that capitalizes on synergies between pharmacological and cell-based modalities. As a result, findings reported here raise the prospect that limitations which have plagued exogenous stem cell delivery to the injured myocardium may respond to pharmacological manipulation.

Figure 8 Transplanted GFP⁺ adipose-derived stem cells (ADSCs) pretreated with ISX1 detected in hearts 3 weeks postmyocardial infarction (MI) and co-localized with cardiac myocytes. (A) Representative images of heart sections immunostained for green fluorescent protein (GFP) (red) and Troponin T (green). Bar=40 μm. (B) Confocal images of hearts transplanted with ISX1-treated ADSCs. GFP (red), Troponin T (green), overlap (yellow). Bar=20 μm.

Table 2 PCR primers and antibodies used in analyses

<i>Real-time PCR TaqMan primers used in gene expression analyses. Antibodies and isotype control antibodies used to detect cell surface markers by flow cytometry</i>	
TaqMan Nkx2-5 primers	Mm00657783_m1
TaqMan Nppa primers (ANF)	Mm01255748_g1
TaqMan Nppb primers (BNP)	Mm 01255770_g1
TaqMan TnnC primers (Troponin C)	Mm00437111_m1
List of antibodies used for flow cytometry	
Flow cytometry antibody	Isotype control
PE anti-mouse CD29	PE Armenian Hamster IgG
eFluor660 CD34	eFluor660 rat IgG2a, κ
PE-Cy7 anti-mouse Ly-6A/E (Sca1)	PE-Cy7 rat IgG2a, κ
PE anti-mouse CD24	PE rat IgG2c, κ
PE anti-mouse CD105	PE rat IgG2a, κ
PE-Cy7 anti-mouse CD117 (c-Kit)	PE-Cy7 rat IgG2a, κ
PE-Cy7 anti-mouse CD44	PE-Cy7 rat IgG2b, κ
PE anti-mouse CD90.1 (Thy1.1)	PE mouse IgG2a, κ
PE anti-mouse CD73	PE rat IgG1
PE anti-mouse CD166 (ALCAM)	PE rat IgG2a, κ
PE-Cy7 anti-mouse CD31	PE-Cy7 rat IgG2a, κ
PerCP-Cy5.5 anti-mouse CD45	PerCP-Cy5.5 rat IgG2b, κ
PE-Cy7 anti-mouse CD11b	PE-Cy7 rat IgG2b, κ
PE anti-mouse CD14	PE rat IgG2a, κ
PerCP-Cy5.5 anti-mouse Ter119 (Ly-76)	PerCP-Cy5.5 rat IgG2b, κ

Myocardial regeneration using exogenously administered ADSCs

We report that ADSCs improve cardiac function, a finding consistent with some previous reports.^{6–13} However, not all studies demonstrated improvements in LV functional recovery after ADSC or mesenchymal stem cell (MSC) transplantation. Failure to observe cardiac protection in some studies may be attributed to a relative lack of sensitivity of measures of ventricular function in large animals, such as porcine models.⁴⁵ Assessment of regional myocardial function may provide a more sensitive metric of changes in myocardial function of modest magnitude.

The passage number of the cells used in transplants may also be critical for determining efficacy of treatment. Studies using ADSCs cultured for long periods of time and transplanted at passages 8–10 demonstrated no benefit.⁴⁶ Indeed, the protective effects of MSCs are lost between passages 5 and 10, but retained until passage 3.⁴⁷ Furthermore, in the setting of long-term culture (passage >5), chromosomal instability is observed in mouse ADSCs.⁴⁸ Even in cells from adipose tissue selected for cardiomyocyte-like properties by culture on a semisolid methylcellulose medium, the cardiac phenotype was not efficiently maintained with extended time in culture.⁴⁹

Another parameter that may determine whether ADSC transplantation is efficacious is timing of injection after infarction. Injecting cardiac pre-differentiated ADSCs at 1 month post MI was not associated with improvement in cardiac function,⁷ and these cells did not survive in a chronic infarction model.⁴⁹ Even in an acute model, the number of transplanted mononuclear or mesenchymal cells declines with time after transplantation.⁵⁰ Specifically, the percentage of donor cells retained in the heart decreased

rapidly from 34–80% of injected cells (0 h) to 0–3.5% (6 weeks) independent of cell type, number, and application time. In our hands, we could not detect any vehicle-treated ADSCs retained in the heart 3 weeks post transplantation, whereas ISX1-treated ACSCs were still observed in the myocardium at this point, suggesting increased residence time of these transplanted cells. Importantly, a survey of other organs uncovered no transplanted cells, either ISX1-treated or untreated, at 3 weeks.

Differentiation of ADSCs into cardiac progenitors

We did not detect increased expression of late cardiac markers in ADSCs treated with ISX1 without co-culture with neonatal cardiac myocytes, nor did we observe full differentiation in vivo. This suggests that ISX1 alone is not sufficient to induce the full cardiac differentiation program, but rather promotes differentiation of cardiomyocyte progenitors. Alternatively, the heterogeneous population of cells we employed includes cell types that may harbor pre-defined epigenetic marks, which are unresponsive to ISX1, and therefore mask the effects of ISX1 in the responsive cell types. Additionally, it is possible that one or more cell types within such a heterogeneous population may inhibit the differentiation of other subpopulations. Pertinent to this, a novel population of multipotent stem cells from the SVF of adipose tissue has been identified as an early population of stem cells (Lin[−]:CD29⁺:CD34⁺:Sca1⁺:CD24⁺) which can be induced to undergo adipogenesis, osteogenesis, and skeletal myogenesis.^{34 51} However, populations negative for CD34 or unfractionated SVF populations were incapable of forming myotubes when seeded with C2C12 cells, suggesting the presence of cells within unfractionated SVF or CD34[−] populations that repress myocyte

differentiation. In accordance with this, we observed a trend toward enhanced responsiveness to ISX1 in subpopulations of cells expressing CD34 as measured by increased *Troponin I* expression (data not shown).

At the outset, we did not expect that a single cue could trigger full and complete cardiomyocyte differentiation, a process which is widely accepted to involve multiple steps.⁵² Also, the lack of full cardiac myocyte differentiation in our study could result from sustained expression of *Nkx2-5*. Although *Nkx2-5* expression is necessary for heart development, cell differentiation involves a dynamic process whereby genes are expressed early in differentiation and subsequently repressed. The precise timing of expression may be necessary for correct and full differentiation. In addition, the point at which a stem cell is positioned along a progression of differentiation may also determine which transcription factors are necessary for re-programming. Indeed, a recent study reported that elimination of *Nkx2-5* in a transcription factor cocktail augmented re-programming of adult fibroblasts,⁵³ suggesting that (1) *Nkx2-5* was unable to induce transcription of the myosin heavy chain promoter or (2) adult cells employ transcription factors other than those used by embryonic stem cells in heart development. Whether activation of other transcription factors in ADSCs is more efficacious to induce their differentiation into cardiac myocytes is not known.

Mechanisms of repair

Although we did observe retention of cells in the ISX1 treatment arms at 3 weeks post-MI, their small numbers and incomplete differentiation suggest that the functional benefits to the heart are unlikely due to engraftment of functional myocytes. This was expected. Rather, our findings suggest that transplanted stem cells pre-differentiated pharmacologically are better able to trigger events in the neighboring tissue that promote the healing process.

In fact, previous studies have shown that ADSCs improve cardiac function by increasing both capillary and arteriole densities despite low absolute rates of engraftment.^{8, 54} Consistent with this, we found increased neovascularization in response to transplantation of ADSCs, which was augmented by ISX1. It is possible that this increase in neovascularization is due to the localization of ISX1-treated ADSCs to the myocardium resulting in a more sustained paracrine-elicited effect.

Chromatin remodeling

Chromatin remodeling involves changes in the methylation state of DNA and post-translational modifications of histone proteins that regulate chromatin conformation. DNA methylation of a gene promoter typically leads to transcriptional silencing. However, a recent report suggests that there is no correlation between DNA methylation state and gene expression in ADSCs.⁵⁵ Specifically, ADSCs can differentiate toward myogenic and endothelial lineages despite hypermethylation of the non-adipogenic lineage-specific promoters *MYOG* and *CD31*.⁵⁵ In addition, during P19CL6 differentiation into cardiac myocytes, the DNA methylation status at the *Nkx2-5* promoter did not change, suggesting that DNA methylation does not play a major role in ADSC-induced cardiomyogenesis in our study.⁵⁶ However, many studies have shown that treatment of

ADSCs with the DNA methylation inhibitor, 5-azacytidine, promotes cardiomyogenesis.⁵⁷ This may be due to the effects of 5-azacytidine on histone modifications.³⁷

Histone methylation and acetylation states are major determinants of chromatin structure and transcriptional activity, including the transcriptional processes of stem cell differentiation. We did not observe an ISX1-elicited decrease in trimethylated histone 3 lysine 9 (H3K9me3), which is enriched in heterochromatin,⁵⁸ nor an increase in the active mark trimethylated histone 3 lysine 4 (H3K4me3), suggesting that histone methylation may not be a major underlying mechanism.

Alternatively, histones can be acetylated and deacetylated by HATs and HDACs, respectively. We did not observe a difference in HDAC activity in the nuclei of ADSCs exposed to ISX1 or vehicle. Further, we observed no additive or synergistic effects using HDAC inhibitors. However, we did observe an increase in HAT activity in response to ISX1. Congruent with this, we observed an increase in global histone acetylation. Specifically, we detected an increase in the acetylation of histone 4 lysine 8 (H4K8) and histone 3 lysine 9 (H3K9). H4K8 acetylation occurs in neonatal cardiomyocytes in response to 5-azacytidine³⁷ and may be important in cardiac myocyte differentiation. In addition, our findings that ISX1 decreased osteogenesis and increased H3K9 acetylation are in alignment with a decrease in global H3K9 acetylation in MSC-induced osteogenic differentiation.³⁸

Summary and perspective

Cell-based strategies of myocardial repair hold considerable promise in ischemic heart disease, heart failure, and other cardiovascular pathologies. However, efficacy in preclinical and clinical trials remains modest, highlighting the need to identify novel means of enhancing the treatment effect. The findings reported here suggest that an approach which couples thoughtful selection of multipotent cells receptive to exogenous differentiation cues along with novel triggers of differentiation holds promise in the development and exploitation of this important treatment strategy.

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