Reprogramming of Skeletal Myoblasts for Induction of Pluripotency for Tumor-Free Cardiomyogenesis in the Infarcted Heart

Rafaeq P.H. Ahmed, Husnain K. Haider, Stephanie Buccini, Longhu Li, Shujia Jiang, Muhammad Ashraf

Rationale: Skeletal myoblasts (SMs) with inherent myogenic properties are better candidates for reprogramming to pluripotency.

Objective: To reprogram SMs to pluripotency and show that reprogrammed SMs (SiPs) express embryonic gene and microRNA profiles and that transplantation of predifferentiated cardiac progenitors reduce tumor formation.

Methods and Results: The pMXs vector containing mouse cDNAs for Yamanaka’s quartet of stemness factors were used for transduction of SMs purified from male Oct4-GFP+ transgenic mouse. Three weeks later, GFP+ colonies of SiPS were isolated and propagated in vitro. SiPS were positive for alkaline phosphatase, expressed SSEA1, and displayed a panel of embryonic stem (ES) cell–specific pluripotency markers. Embryoid body formation yielded beating cardiomyocyte-like cells, which expressed early and late cardiac-specific markers. SiPS also had an microRNA profile that was altered during their cardiomyogenic differentiation. Noticeable abrogation of let-7 family and significant up-regulation of miR-200a-c and miR-290 to 295 was observed in SiPS and SiPS-derived cardiomyocytes, respectively. In vivo studies in an experimental model of acute myocardial infarction showed extensive survival of SiPS and SiPS-derived cardiomyocytes in mouse heart after transplantation. Our results from 4-week studies in DMEM without cells (group 1), SMs (group-2), SiPS (group-3), and SiPS-derived cardiomyocytes (group 4) showed extensive myogenic integration of the transplanted cells in group 4 with attenuated infarct size and improved cardiac function without tumorigenesis.

Conclusions: Successful reprogramming was achieved in SMs with ES cell-like microRNA profile. Given the tumorigenic nature of SiPS, their predifferentiation into cardiomyocytes would be important for tumor-free cardiogenesis in the heart. (Circ Res. 2011;109:00-00.)

Key Words: heart infarction iPS cells pluripotent tumor formation reprogramming predifferentiation

Cardiac regeneration using cell-based therapy is faced with the dilemma of identifying the ideal type of cells for transplantation into the infarcted heart. Besides suboptimal protocols of cell culture and transplantation, ease and availability of cells in sufficient number, their survival in the infarcted region and differentiation into morpho-functional cardiomyocytes for regeneration are some of the major challenges facing the heart stem cell therapy. Despite progress to clinical application, the use of stem cells has not been without controversies regarding their differentiation potential and ability to integrate with the host myocardium.1–4 Embryonic stem (ES) cells are the prototypical stem cells that unarguably possess near-ideal characteristics in terms of clonality, self-renewal, and multipotentiality.5 However, moral and ethical issues regarding availability and immunologic concerns have hampered their progress to clinical use.

The reprogramming of somatic cells with 4 stemness factors, called induced pluripotent stem cells (iPS cells), has generated alternative sources of stem cells that possess characteristics reminiscent of ES cells in terms of cell biology and pluripotent differentiation characteristics, albeit with availability in large numbers without ethical issues and having better immunologic behavior.6 Although the main focus of the current research in iPS cell technology pertains to refinement of the protocols to enhance the efficiency of reprogramming and to circumvent the safety issues for their human use,7 there are few studies that have shown their regenerative potential in vivo in general and for the infarcted myocardium in particular. Terzic’s group has taken the lead

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Nonstandard Abbreviations and Acronyms

| SMs | skeletal myoblasts |
| ES | embryonic stem |
| IPS | induced pluripotent stem |
| SiPS | skeletal myoblast derived IPS cells |
| EBs | embryoid bodies |
| SiPS-CM | cardiomyocytes derived from the 10-day-old spontaneously beating embryoid bodies obtained from SiPS |
| GFP | green fluorescent protein |
| DMEM | Dulbecco’s modified eagle medium |
| Oct4 | octamer-binding transcription factor 4 |
| Sox2 | sex determining region Y-box 2 |
| Klf4 | Kruppel-like factor 4 |
| c-Myc | cellular-myceloytumatiso proto-oncoprotein gene |
| MEFs | mouse embryonic fibroblasts |
| SSEA-1 | antistage specific embryonic antigen-1 antibody |
| Cx-43 | connexin 43 |
| MyoD | myogenic differentiation 1 |
| Na4SMs | native skeletal myoblasts |
| Q-dots | quantum dots |
| LVFs | left ventricular fractional shortening |
| LVDd | left ventricular chamber dimensions during diastole |
| LVEDd | left ventricular chamber dimensions during systole |
| LVEF | left ventricular ejection fraction |

in this regard to show the reparability of mouse fibroblast-derived IPS cells in an immunocompetent mouse heart model of acute myocardial infarction. The authors have demonstrated that the transplanted undifferentiated IPS cells survived in the infarcted myocardium, and by 4 weeks, global heart function was recovered better than basal DMEM-injected control animals. The authors further reported no observation of teratomas in IPS cell–treated animal hearts. We have successfully generated mouse skeletal myoblast (SM)–derived IPS cells (SiPS), which expressed endogenous markers of stemness similar to mouse ES cells. We report that cardiomyocytes derived from the 10-day-old spontaneously beating embryoid bodies (EBs) obtained from SiPS (SiPS-CM) provide an excellent donor cell source that significantly attenuated infarct size expansion and improved contractile heart function in an experimental mouse model of acute myocardial infarction. Moreover, contrary to previous claims, we observed that SiPS transplanted in the infarcted heart of immunocompetent recipient were teratogenic.

Methods

Isolation of Mouse SMs

For our animal experiments, we used the Oct4/GFP transgenic mouse strain (Jackson Laboratories, Bar Harbor, ME) with GFP tagged to the endogenous Oct3/4 gene promoter. For SMs isolation, we followed the standard protocols routinely used in our laboratory as described in the Online Supplement.

Generation and Maintenance of SiPS

Retroviral vectors encoding for Yamanaka’s quartet of pluripotency-determining factors were purchased from Addgene Inc. (Cambridge, MA) (Addgene plasmid #13367; #13366; #13370; #17220 from Takahashi et al.). SMs from Oct4/GFP mice were seeded at a density of 1×10⁵ cells/well in a 6-well plate. Twenty-four hours later, the cells were transduced with infectious supernatants from the respective vectors encoding for Oct4, Sox2, Klf4, and c-Myc for 48-hour transduction. Subsequently, the cells were replated in a 10-cm cell culture dish on mouse embryonic fibroblasts (MEFs) and observed for development of SiPS clones until 3 weeks. The GFP^+ SiPS clones with ES cell-like morphology were mechanically incised, cultured on mouse feeder cells, and expanded individually in ES cell culture medium for use in further experiments. For confirmation of pluripotency induction, SiPS were fixed with 3% paraformaldehyde, permeabilized and stained with antistage-specific embryonic antigen-1 (SSEA-1) antibody. The primary antigen–antibody reaction was detected with goat antirat Alexa Fluor-568 conjugated secondary antibody (1:200; Cell Signaling Tech, Danvers, MA). Nuclei were visualized by 4,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA) staining. The murine SiPS clone Raf1 was expanded on mitotically inactivated murine embryonic fibroblasts (MEFs; 5×10⁴ cells/cm²) as described in the Online Supplement.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Isolation of total RNA, and their subsequent first-strand cDNA synthesis, was performed using an RNasea mini kit (Qiagen, Valencia, CA) and an Omniscript Reverse Transcription kit (Qiagen, Valencia, CA), respectively, per the instructions of the manufacturer and as described earlier. The primer sequences used are given in On-Line Table I.

Alkaline Phosphatase Staining and Immunocytochemistry

Alkaline phosphatase staining was done as per the manufacturer’s instruction using Alkaline Phosphatase Detection kit (Millipore SCRR04). The undifferentiated colonies of IPS and ES cells were immunostained for the expression of the stage-specific embryonic antigen-1 (SSEA-1), Oct3/4, and Sox2 as described in the Online Supplement. The immunostained cells were observed and photographed with a microscope equipped for epifluorescence (Olympus, Tokyo, Japan).

Teratoma Formation and Karyotyping

Teratogenicity of SiPS was assessed in immunodeficient mice (n = 3) as described in the Online Supplement. Karyotyping was carried out with quinacrine-Hoechst staining at the Transgenic Facility of the University of Kansas Medical Center (Kansas City, KS).

Spontaneous Cardiac Differentiation of SiPS

For differentiation into cardiomyocytes, SiPS were grown in suspension culture for 3 days in high-glucose DMEM containing 15% FBS, 5 mmol/L penicillin/streptomycin and 5 mmol/L nonessential amino acids. After 3 days in suspension culture, rounded EBs were formed, which were seeded on gelatin-coated dishes and cultured for 10 days. Adherent spontaneously contracting colonies were mechanically dissected and dissociated into single cardiomyocytes for use in further experiments.

miR Analysis of SiPS and SiPS-CM

Total RNA from SMs, IPS, and SiPS-CM were labeled with Cy3. Samples were hybridized to a mouse miRNA microarray (LC Science, Houston, TX), according to manufacturer’s protocol.
Ultrastructural Studies

Ultrastructural studies were performed on the myocardial tissue samples as described in the Online Supplement using JEOL transmission electron microscope.

Experimental Animal Model and SiPS Engraftment

The present study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85 to 23, revised 1985) and protocol approved by the Institutional Animal Care and Use Committee, University of Cincinnati.

A model of acute myocardial infarction was developed in allogenic 8- to 12-week-old female C57BL/6j immunocompetent mice as described earlier. Briefly, the animals were anesthetized (Ketamine/Xylazine 0.05 mL intraperitoneally), intubated, and mechanically ventilated (Harvard Rodent Ventilator, Model 683). Minimal invasive thoracotomy was performed for permanent ligation of coronary artery with a Prolene #9 to 0 suture. Myocardial ischemia was confirmed by color change of left ventricular wall. The animals were labeled with Q-tracker®-625 (red fluorescence; Invitrogen, Carlsbad, CA). The chest was closed and the animals were allowed to recover. Subsequently, the animals were injected Buprinex (0.05 mL subcutaneously) during the first 24 hours to alleviate pain and were maintained until 4 weeks before euthanasia and recovery of the heart tissue samples.

Transthoracic Echocardiography

The animals (n=8 in groups 1, 2, and 3 each and n=11 in group 4) were anesthetized, and transthoracic echocardiography was performed as detailed in the Online Supplement.

Histological and Immunohistological Studies

The animals were euthanized at 7 days and 4 weeks after heart function studies. The heart tissue samples were removed and used for histological and immunohistological studies as described earlier.

Results

Overexpression of Stemness Factors and SMs Reprogramming

The SMs were characterized for purity by flow cytometry for desmin expression (On-Line Figure II, A and B) and by immunochemistry for desmin and vimentin expression (Online Figure II, D) and RT-PCR for MyoD and vimentin expression (Online Figure II, E). We successfully reprogrammed NaSMs to achieve pluripotency subsequent to overexpression of 4 stemness factors Oct3/4, Klf4, Sox2, and c-Myc (Figure 1A, B1–B3). Culture of the undifferentiated SiPS on MEFs or on gelatin-coated dishes without feeder cells exhibited a typical ES-like cell morphology that appeared as compact, opaque round clusters with well-defined margins in the undifferentiated state and expressed Oct3/4 promoter driven GFP (Figure 1, B1–B3). On the other hand, the nonreprogrammed NaSMs with elongated spindle shape retained their morphological characteristics in culture for 20 days and did not express Oct3/4 promoter-driven GFP, which indicated their nontransformed status (Figure 1C and 1D). Subcellular characteristics included a large nucleus/cytoplasmic ratio and scanty cytoplasmic contents in SiPS in comparison with the NaSMs (Figure 1E through 1G). Unlike NaSMs, SiPS endogenously overexpressed Oct4, Sox2, cMyc, Klf4, and Nanog besides a panel of other pluripotency gene markers (Figure 1H, lanes 3 to 4), which was comparable with mouse ES cells (Figure 1H, lane 5). RT-PCR was performed to observe changes in exogenous and endogenous gene expression of stemness factors in SMs and SiPS (Online Figure III). Fluorescence immunostaining revealed that reprogramming induced strong positive expression of the undifferentiated ES cell markers, including SSEA-1 and higher alkaline phosphatase, which were absent in NaSMs (Online Figure I, A and B). Karyotyping revealed that more than 85% of the cells were euploid and without any chromosomal abnormalities, indicating that the clones used in the study possessed normal genetic makeup. Subcutaneous injection of SiPS formed teratomas in all the 3 immunodeficient hosts, which showed typical 3 germ layer characteristics (Online Figure I, C).

In Vitro Pread differentiation of Cardiac Progenitors From SiPS for Transplantation

Of the 2 murine SiPS clones, ie, Raf1 and Raf2, characterized in vitro for pluripotency, Raf1 was differentiated by a standard EB-based differentiation protocol. Between days 3 and 5 in differentiation culture medium, the floating EBs were transferred to adherent culture conditions in which the cells were cultured for 5 to 6 days. By day 10 after initiation of differentiation, few spontaneously beating areas were observed that continued to increase and expand with longer time maintenance of the culture (Online Movie I). Such synchronous contractile activity in SiPS-CMs was observed for up to 3 to 4 weeks. Profiling of cardiac specific genes was performed for characterization of the spontaneously beating EBs (Figure 2). We observed multifold increase in cardiac-specific genes expression in 10-day-old beating EBs, including maturation markers α-MHC, β-MHC, and cardiac troponin-I in comparison with SiPS and NaSMs (Figure 2A and Online Figure IV). Mouse heart was used as a positive control. The cardiac-specific molecular changes during differentiation of SiPS were confirmed by fluorescence immunostaining of SiPS and SiPS-CMs (Figure 2B). A higher propensity of SiPS-CMs positive for Nkx2.5, myosin heavy chain, cardiac troponin-I, cardiac actin, and gap-junction proteins N-cadherin and connexin-43. Incidentally, the level of pluripotency markers Oct3/4, Sox2, and Nanog concomitantly declined in SiPS during differentiation to become SiPS-CMs (Figure 2C, Online Figure V). RT-PCR was also performed to observe gene expression changes in endogenous and exogenous Oct3/4 and Sox2 in SiPS-CMs in comparison with SiPS (Online Figure VI).
Transmission electron microscopy revealed ultrastructural features of the developing cardiomyocytes in SiPS-CMs with fully developed sarcomeres and ribosomes (Figure 3, A through C). Tight junctions between adjacent SiPS-CMs were also observed in the spontaneous beating regions in the culture (indicated by red arrows; Figure 3C).

Prevention/Reduction of Tumorgenicity by Predifferentiated Cardiac Progenitors

As published in our first report on teratogenicity of SiPS in which 6 of the 16 SiPS-transplanted animals developed cardiac tumors,13 no tumor formation was observed in any other group included in the present study. Figure 4C is a representative echocardiograph of an animal heart that developed cardiac tumor in the LV 4 weeks after SiPS transplantation. Histological studies confirmed that these tumors consisted of cells from the 3 germ layers.13 Transmission electron microscopy revealed regions with myogenic differentiation identified on the basis of their typical striated muscle fiberlike structure with sarcromeric organization (Figure 4D). Moreover, comparative miRNA profile revealed that cardiomyocytes isolated from SiPS expressed tumor suppressive miRs 125b, 16, 199a, 214, 26a, 200b, and 200c in abundance when compared with SiPS (Figure 5). These results suggest that directed differentiation of iPS cells prior to transplantation is highly desirable to prevent tumor formation.

Histological Benefits of SiPS and Their Derivatives

Histochemical studies on the animal hearts from various treatment groups (n=4 per group), 4 weeks after their respective treatment, were performed on at least 2 middle slices of the heart. We observed extensive myocyte damage in the LV, which was replaced by scar tissue and thinning of the LV wall was reduced in all the treatment groups (Figure 4). However, all the cell treatment groups had significantly attenuated infarct size in comparison with DMEM-injected controls (41.3%±1.3%). However, infarct size between cell treatment groups did not show significant difference despite the fact that SiPS-CMs–treated animal hearts had the smallest infarct size (23.9%±3.1%) in comparison with SMs (25.9±1.8) and SiPS treated (26±3.3). Architectural analysis of hematoxylin-eosin–stained cardiac tissues had extensive presence of islands of myofibers in the infarcted hearts of the cell-treated groups (Figure 4). Four weeks after cell transplantation, Q-dot-labeled cells were observed in the infarct and peri-infarct areas in the LV (Figure 6A).
Immunohistochemistry for cardiac troponin-I and Cx-43 was performed to determine the fate and integration of the transplanted cells in the heart. Colocalization of Q-dots (red fluorescence) with cardiac troponin-I (green fluorescence) in the infarct and peri-infarct regions in the SiPS-CMs–transplanted animal hearts indicated their myogenic differentiation (Figure 6, B through D), which formed gap junctions with the host myocytes (Figure 6, E and F).

Heart Function Studies
Permanent LAD coronary artery ligation led to significant deterioration in the indices of LV contractile function, including LVEF and LVFS in comparison with the baseline values (n=4; 74.2±2.6; 36.7±2.2%, respectively). Transplantation of NaSMs, SiPS, and SiPS-CMs significantly preserved the global pump function of the infarcted myocardium (Figure 7). In comparison with the DMEM-treated controls (36.3%±1.5%; 15.35±1, P<0.001), LVEF and LVFS were significantly higher in NaSMs-transplanted animal hearts (48.04%±1.5%; 20.4±1.6), SiPS-transplanted hearts (46.7±1.7; 19.9±1.3) and SiPS-CMs-transplanted hearts (54.7±2; 24.1±2), respectively. Intergroup analysis showed that in comparison with DMEM treatment, both LVEF and LVFS significantly improved in cell-transplanted groups (DMEM- versus cell-transplanted groups, P<0.05). However, LVEF and LVFS showed significant improvement in SiPS-CMs-treated group in comparison with other cell-transplanted hearts. The pathological remodeling of LV in the cell-transplanted groups was also significantly reduced as shown by LV chamber dimensions during systole (LVDs) and diastole (LVDd). In comparison with the chamber dimensions during systole (4.4±0.2) and diastole (5.69±0.3) in the DMEM-treated group, both LVDs and LVDd were significantly preserved following intervention with NaSMs (3.7±0.1 and 4.7±0.1), SiPS (3.9±0.2 and 4.9±0.24), and SiPS-CMs (3.4±0.2 and 4.4±0.1), respectively.

Figure 2. Spontaneous cardiomyogenic differentiation of skeletal myoblast–derived induced pluripotent stem cells (SiPS). A, reverse transcription polymerase chain reaction (RT-PCR) for cardiomyocyte specific gene expression changes in SiPS cardiomyocytes (CM), which developed by spontaneous differentiation of SiPS on day 10 after embryoid bodies formation (lane 3) in comparison with NaSMs (lane 1), undifferentiated SiPS (lane 2), and ES cells (lane 5). The heart was used as a positive control (lane 4). B, Fluorescence immunostaining of SiPS-CM from a single beating cluster for detection of cardiac-specific marker proteins, including myosin heavy chain (red), cardiac troponin-I (green), Nkx2.5 (red), N-Cadherin (red), Cardiac actin (green), and Cx-43 (red). DAPI staining was used for visualization of nuclei (original magnification=100×). C, RT-PCR for pluripotency gene expression in SiPS-CM showed down-regulation of Oct3/4, Sox2, and Nanog with the appearance cardiac-specific markers as shown in Figure 2C (lane 1 = SiPS; lane 2 = SiPS-CM; lane 3 = heart [positive control]; lane 4 = ES cells).
Discussion

The breakthrough discovery that somatic cells could be reprogrammed to pluripotent status has generated immense interest in pluripotent stem cells because of their vast therapeutic applications. Thus, the prospect of using iPS cells has added new dimensions to stem cell–based therapy. We report reprogramming of mouse SMs and their application for cardiac repair following myocardial infarction in comparison with SiPs and NatSMs. Our results avidly showed the superiority of allogenic SiPS-CMs in terms of safety and regenerative capacity in an immunocompetent mouse model of acute myocardial infarction. The major findings of the study are that (1) SMs can be easily reprogrammed to develop iPS cells; (2) undifferentiated iPS cells are tumorigenic in immunocompetent hosts; (3) directed differentiation of iPS cells to derive cardiac progenitors circumvent their tumorigenicity following transplantation in immunocompetent recipients; and (4) we provide a comparative miR expression profile in NatSMs, SiPS, and SiPS-CMs that essentially determined the pluripotent status of SiPS during reprogramming of SMs and showed an upregulated expression of tumor-suppressive miRs in differentiated cardiac cells.

The rationale to reprogram SMs was based on our results that SMs endogenously expressed 3 (ie, Sox2, cMyc, and Klf4) of the Yamanaka’s quartet of transcription factors required for reprogramming, thus making these cells an easier choice for reprogramming than were the terminally differentiated fibroblasts. It is highly likely that reprogramming SMs would require fewer factors. Second, it is anticipated that iPS cells carry forward the epigenetic memory of the somatic cells of their origin. Therefore, SMs that possess inherent myogenic potential would be a better choice for reprogramming than would the nonmyogenic fibroblasts. Despite the presence of 3 of the 4 required stemness factors, the use of Oct4 alone did not induce pluripotency in SMs. It appears that there is an optimum threshold level of expression for each of these factors that is required to ensure reprogramming of SMs. A recent study has shown that SMs can be reprogrammed using Yamanaka’s quartet of stemness factors with simultaneous suppression of MyoD in the reprogrammed cells, which substantiates our results. However, the authors did not show their cardiac differentiation capability in vitro as well as in an experimental animal model. In an interesting study, neurospheres were obtained from mouse iPS cell lines derived from embryonic fibroblasts, adult tail-tip fibroblasts, hepatocytes, and stomach epithelial cells and evaluated the teratogenicity of the secondary neurospheres. Interestingly, iPS cells of different tissue origins differed in their teratogenicity, which also correlated well with the presence of undifferentiated iPS cells in the neurospheres. These data support our notion that certain somatic...
cell types may be superior choices for reprogramming and would also influence the subsequent differentiation potential of their derived iPS cells. Another important consideration in the selection of SMs for reprogramming was that the choice of cell may impact the tumorgenicity of their derived iPS cells. Moreover, SMs have been extensively studied for myocardial repair in both experimental animal models and human patients. However, their lack of functional integration postengraftment in the heart and the issues of arrhythmogenicity have marred their clinical applicability. We believe that reprogrammed SMs would provide an excellent autologous source of cells for transplantation.

Since inception of the iPS cell technology, in vivo cardiomyogenic potential of iPS cells has been mostly studied after their blastocyst injection. The injected iPS cells differentiated into mature cardiomyocytes in the chimeric heart. These data were well supported by in vitro experimental studies that examined the cardiomyogenic potential of ES cells. Molecular studies demonstrated upregulation of mesodermal gene and protein markers of cardiac differentiation, which was confirmed by fluorescence immunostaining and ultrastructural studies. Similar to ES cells, iPS cells can differentiate into functionally competent cardiomyocytes, however, very few studies showed that iPS cells regenerated the infarcted myocardium after transplantation. Terzic et al have reported improved global cardiac function in iPS cell–treated mice, however, without substantiating cardiogenesis in the infarcted mice heart with immunohistological evi-
Given the tumorigenic nature of pluripotent stem cells, it is important to ensure that the transplantation of SiPS is free of undifferentiated cells, which are potential contributors of tumorigenicity of iPSCs. Even the use of isogenic animals for transplantation of SiPS failed to curtail their teratogenic characteristics in the heart. Isolation of specific subtypes of SiPS with no oncogenic tendency will also help to curtail tumorigenicity of SiPS.

In view of difficulties in using iPSC–based cell therapy, we hypothesized that predifferentiation or guided differentiation of SiPS would be a safer and effective approach for transplantation. We therefore opted to transplant 8- to 10-day-old EBs that contained developing and beating myocytes. We observed increased myogenesis in SiPS-CMs transplanted hearts and, more important, without incidence of tumor formation as against significantly higher number of animals (n = 4) developing tumors in SiPS transplanted hearts. Because the number of cells transplanted in group 3 and group 4 were similar, contribution of residual SiPS population toward improved cardiac function would be marginal. Strategies to enhance the purity of SiPS-CMs and to exclude the presence of undifferentiated SiPS are therefore warranted. Directed differentiation protocols will require optimal pretreatment of SiPS with specific cytokines, growth factors, or small molecules rather than the use of spontaneous differentiation, which may lack reproducibility.

Our study provides the first ever miR expression profiling of SMs during the process of reprogramming and differentiation. The miRs are global regulators of stem cell functions, including their differentiation capacity. Significant changes were noticed in the expression pattern of miR let-7, miR-200, and miR-290 families that largely varied with the differentiation status of the cells. Upregulation of let-7 family of miRs is associated with differentiation status rather than type of the
We observed that let family of miRs including let7 a-g, i, and miR-98 were down-regulated in SiPS in comparison with NatSMs. Nevertheless, there was an obvious increase in their expression during myogenic differentiation of SiPS. The 5-member miR-200 family (miR-200a-c, miR-141, and miR-429) also showed a similar trend. Of these members, miR-200a-c were not detected in either NatSMs or SiPS; however, their expression increased in SiPS-CMs, which indicated the incomplete differentiation status of SMs. Besides these 2 important miR families, SiPS also expressed other signature ES cell miRs—including miR-290, 292, and 293—that are not only regulated by pluripotency genes Oct4, Nanog and Sox2 but also target these genes in “incoherent feed-forward loops.” These data reflected similarity of miR expression profiles between SiPS and ES cells. Similarly, some other miRs with significantly altered expression during transition of SMs to SiPS and then onward differentiation into cardiomyocytes included miR-23a and 23b (regulated by cMyc), miR-214 (part of regulatory circuit controlling differentiation and cell fate decision), and miR-21, a known suppressor of Nanog, Sox2, and Oct4, that showed substantial increase following cardiomyogenic differentiation of SiPS. The miR-24 a ubiquitously expressed miRNA, has an antiproliferative effect independent of p53 function and was inhibited...

**Figure 6.** Immunohistological evidence that transplanted skeletal myoblast–derived induced pluripotent stem cell cardiomyocytes (SiPS-CMs) adopted cardiomyogenic fate in vivo. A, Epifluorescence observation of the histological section showing extensive presence of the transplanted SiPS-CMs in the infarcted myocardium (indicated by red fluorescence) 4 weeks after transplantation. B, Immunostaining of the histological sections from SiPS-CM transplanted animal heart for cardiac actin expression (green fluorescence), which colocalized with red fluorescence to show myogenic differentiation of the transplanted cells. D, Represents the magnified image of an area selected in C, (Red box) to show colocalization of red and green fluorescence. E and F, Fluorescence immunostaining of histological sections for Cx-43 expression (green fluorescence) to show integration of SiPS-CM (red fluorescence) in the center of the infarct. (Original magnifications: A–10×; B–20×; C–40×.)

**Figure 7.** Skeletal myoblast–derived induced pluripotent stem cell cardiomyocytes (SiPS-CM) transplantation better preserved the cardiac function. A and B, Whereas LVEF and LVFS were significantly deteriorated in DMEM-injected control animal hearts, cell therapy, irrespective of the type of the cell injected, preserved the contractile function of the heart (P<0.05 versus DMEM injected control). However, significant improvement in LVES and LVFS was observed in SiPS-CM treatment group. Similarly, LV chamber dimensions during diastole and systole were better preserved in the cell transplanted animal hearts than in the controls, thus indicating prevention of pathological remodeling.
in SiPS in comparison with SMs and SiPS-CMs. Moreover, miR-26a is a muscle-specific miR that is up-regulated during myogenesis30 and was up-regulated both in SMs and in SiPS-CMs in comparison with the SiPS. Let-7, miR-125b, miR-16, miR-199a, miR-214, miR-26a, miR-200b, and miR-200c are among the tumor-suppressive miRs that are significantly up-regulated in SiPS-CM.31–33 All these data clearly reflect not only the similarity between miR expression profile of ES cells and SiPS during reprogramming of SMs but also up-regulation of tumor-suppressive miRs in SiPS-CMs in comparison with SiPS.

In conclusion, the study results indicate SMs as better candidates for reprogramming to pluripotency because of their inherent expression of 3 of the 4 stemness-determining factors. Although the study used conventional retroviral vector-based ectopic expression of four stemness factors, intrinsic expression of 3 of the required 4 stemness factors raises the possibility of their reprogramming with a smaller number of factors or even by treatment with nonviral methods for clinical applications. Transplantation of cardiac progenitors predifferentiated from SiPS was optimal for tumor-free cardiogenesis with resultant structural and functional recovery in an immunocompetent animal model of acute myocardial infarction. Given that tumorigenesis is a time-dependent process, it would be prudent to carry out longer-term studies using an immunocompetent animal model of acute myocardial infarction. Given that tumorgenesis is a time-dependent process, it would be prudent to carry out longer-term studies. 

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Disclosures
Nothing to disclose.

References


**Novelty and Significance**

**What Is known?**

- Committed skeletal muscle progenitor cells (myoblasts) can be reprogrammed into pluripotent stem cells that are similar to embryonic stem cells.
- Skeletal myoblast–derived induced pluripotent stem cells (SiPS) are potential candidates for the regeneration of ischemic myocardium.
- SiPS transplanted into the ischemic myocardium have been reported to differentiate into cells of all 3 germ layers, leading to tumor formation in the heart.

**What New Information Does This Article Contribute?**

- Predifferentiation of SiPS into developing cardiomyocytes before transplantation is important for regeneration of infarcted myocardium with minimal risk of myocardial tumor formation.
- Cardiac progenitors derived from SiPS are an excellent source of cells for cardiac regeneration. Transplantation of these cells was found to reduce infarct size and improve heart function in a mouse model of acute myocardial infarction.
- In addition to known pluripotency-related miRs such as Let-7 family miRs and 290 cluster miRs, this study also reveals that other miRs—such as 125b, 16, 199, 214, 26a, and 200a/b, which are tumor-suppressive miRs—are expressed in differentiated cardiomyocytes. These miRs can be used as tumorigenic biomarkers of iPSC cells–derived progenitors.

A major goal of cardiac stem cell research is to identify the ideal type of cell for cardiac regeneration. Skeletal myoblasts have been used in clinical trials involving cardiac ischemia, albeit myoblast transplantation is reported to cause arrhythmias. We have recently reported that myoblasts can be reprogrammed to induce pluripotent stem cells, and their direct transplantation is associated with a high risk of tumor formation even in an immunocompetent host. In this study we differentiated myoblast-derived IPS into cardiomyocytes (SiPS-CM) prior to transplantation to assess their potential to regenerate the infarcted myocardium. Our in vivo results in a mouse model of myocardial injury indicate that differentiation before transplantation reduces the risk of tumor formation while simultaneously improving heart function and reducing infarct size. Our results suggest that skeletal myoblasts may be better candidates for reprogramming using novel nonviral approaches because they show a modest expression of 3 of the 4 stemness genes at baseline. By comparing the miRNA profile of committed myoblasts and cardiomyocytes with the miRNA profile of SiPS, this study highlights the miRs that are specifically regulated under pluripotent state. These pluripotency-specific miRs are ideal candidates for nonviral miRNA reprogramming of differentiated cells. Moreover, as pluripotency and tumorigenicity are closely regulated, this study could be helpful in identifying potential miRNA biomarkers for tumor-free cardiac regeneration.
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Supplementary Methods

Isolation of mouse SMs

For our animal experiments, we used the Oct4/GFP transgenic mouse strain (Jackson Laboratories, Maine, USA) with GFP tagged to the endogenous Oct3/4 gene promoter which helped to monitor the reprogramming process as the reprogrammed cells showed green fluorescence. For SMs isolation, we followed the standard protocols routinely used in our laboratory. Briefly, young (4-6 weeks old) male Oct4/GFP transgenic mice were sacrificed and skeletal muscle samples from the hind limb were harvested immediately and kept in ice cold basal Medium-199 (M-199; Mediatech, VA, USA) before digestion. The muscle samples were minced into coarse slurry and enzymatically digested using Collagenase-I/Dispase (Roche Diagnostics) for 30-minutes at 37°C. The tissue slurry thus obtained was washed with low serum containing Medium-199 (2% fetal calf serum), centrifuged at 1000rpm for 5-minutes and filtered through 90mm nylon mesh to remove any tissue debris. The muscle extract was pre-plated 3-times at an interval of 1-hour each and twice additionally at 8 and 16 hours to remove the debris and contaminating cell populations. After the last pre-plate, 0.1 mmol/L 5-bromodeoxyuridine (BD-Pharmingen, San Diego, CA) was added to the cell culture for 3-days to inhibit fibroblast growth followed by 3-days of treatment with 15ng/mL basic fibroblast growth factor (bFGF) (Sigma Aldrich, MO). The cells were later propagated in M-199 supplemented with 20%fetal bovine serum (FBS) at 37°C/5% CO2 atmosphere, and purity of the culture was determined by desmin-specific immunostaining. The purified SMs were repeatedly passaged at regular time intervals to prevent their premature differentiation in vitro.

Propagation of SiPS in vitro

The murine SiPS clone Raf1 was expanded on mitotically inactivated murine embryonic fibroblasts (MEFs; 5x10^4 cells/cm^2). The cells were cultured in ES cell culture medium composed of KnockoutTM Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 15% KnockoutTM serum replacement (Invitrogen, Carlsbad, CA), 0.2mmol/L L-glutamine (Invitrogen, Carlsbad, CA), 0.1mmol/L mercaptoethanol (Invitrogen, Carlsbad, CA), 0.1mmol/L non-essential amino acid stock (Invitrogen, Carlsbad, CA), and 0.1% human leukemia inhibitory factor (LIF; Millipore, CA). The colonies thus generated were detached regularly at an interval of 2-3 days with 0.2% collagenase-IV (Invitrogen, Carlsbad, CA), dissociated into single cells with 0.025% trypsin (Sigma Aldrich, MO) and re-plated onto MEFs for propagation.

Reverse transcription polymerase chain reaction (RT-PCR)

Isolation of total RNA, and their subsequent first-strand cDNA synthesis, was performed using an RNAeasy mini kit (Qiagen, Valencia, CA) and an Omniscript Reverse Transcription kit (Qiagen, Valencia, CA), respectively, per the instructions of manufacturer. For PCR amplification, 300ng of the cDNA from the reverse-transcription reaction was then added to a PCR mix containing the suggested quantity of QIAGEN PCR buffer, Q-Solution, dNTP mix, reverse and forward primers, Taq DNA polymerase and distilled water. The cycling conditions were set at 4-minutes at 95°C for initial denaturation, 32 cycles of denaturation at 95°C for 1-minute, annealing at 55°C for 1-minute and extension at 72°C for 1-minute. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualized and photographed on a UV-transluminator (Alpha InnoTech, USA). The primer sequences used are given in Supplementary Table-I.
**Immunocytological studies**

The undifferentiated colonies of iPS and ES cells were immunostained for the expression of the stage-specific embryonic antigen-1 (SSEA-1), Oct3/4 and Sox2 using specific primary antibodies. The EBs on day-10 of their differentiation were immunostained for the detection of cardiomyocyte specific marker proteins. The cells from the spontaneously beating areas were mechanically dissected and dissociated to obtain small clusters of contracting cells that were seeded again onto 0.1% gelatin-coated four chambered slides. The cells were fixed with 4% paraformaldehyde for 15-minutes at room temperature and subsequently blocked with Cas-block (Invitrogen, Carlsbad, CA, USA) 1-hour at room temperature. The primary antibodies used for detection of antigen of interest included anti-SSEA-1, mouse monoclonal anti-sarcomeric actinin antibody, mouse monoclonal IgG1 anti-cardiac troponin-I, mouse monoclonal anti-myosin heavy chain, anti N-cadherin, and anti connexin-43 antibodies as detailed in Supplementary Table-II. The cells were incubated for overnight at 4°C with the respective primary antibody diluted in blocking solution. The primary antibody-antigen reaction was detected with fluorescently conjugated specific secondary antibodies. Nuclei were visualized with 5μg/ml of 4'-6-diamidino-2-phenylindole (DAPI) and the immunostained cells were observed and photographed with a microscope equipped for epifluorescence (Olympus, Tokyo, Japan).

**Teratoma formation in nude mice**

Immunodeficient nude mice were purchased from Jackson Laboratories, USA. Undifferentiated SiPS (1x10^5 cells) were injected intramuscular into 2-3 month old immunodeficient mice. Two weeks after injection, teratomas were fixed with 10 % formalin and used for hematoxylin and eosin staining as well as immunostaining for three germ layer differentiation evaluation.

**Ultra-structural studies**

Conventional transmission electron microscopy was performed on the myocardial tissue samples which were cut into 1-mm³ cubes and fixed in 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) at 4°C for 24-hours. The tissue samples were then post-fixed in 1% OsO4 in the same buffer followed by uranyl acetate staining for 30-minutes at room temperature. The tissue samples were then dehydrated in a series of 50%, 70%, 90%, and 100% ethanol solutions and embedded in Epon-812. Thin sections (~60nm) were cut with an ultra-microtome, placed on grid and after staining with lead acetate, viewed in a JEOL transmission electron microscope.

**Transthoracic echocardiography**

The animals (n=8/group) were anesthetized and lightly secured in the supine position on a warm pad. After the chest was shaved, Acoustic gel was applied and transthoracic echocardiography was performed using HDI-5000 SONOS-CT (HP) ultrasound machine with a 7-MHz transducer. The heart was imaged in the two-dimensional mode in the parasternal long-axis and/or parasternal short-axis views which were subsequently used to position the M-mode cursor perpendicular to the ventricular septum and LV posterior wall, after which M-mode images were obtained. For each animal, measurements were obtained from 4-5 consecutive heart cycles. Measurements of ventricular septal thickness (VST), LV internal dimension (LVID), and LV posterior wall thickness (LVPW) were made from two-dimensionally directed M-mode images of the LV in both systole and diastole. The average value from all measurements in an animal were used to determine the indices of LV contractile function, i.e., LV fractional shortening (LVFS) and LV ejection fraction (LVEF) using the following relations \( \text{LVFS} = \frac{(\text{LVEDD} - \text{LVESD})}{\text{LVEDD}} \times 100 \) and \( \text{LVEF} = \frac{[(\text{LVEDD}^3 - \text{LVESD}^3)]}{\text{LVEDD}^3} \times 100 \) and expressed as percentages.
Supplementary Video-I: Spontaneously beating SiPS derived cardiomyocytes in cell culture. The EBs were cultured for 10 days in differentiation medium without LIF. (A) Spontaneously beating region in 10 days EBs in cell culture (B) SiPS derived single beating cardiomyocyte in cell culture.

Legends to the Supplementary Figures

Supplementary Figure-I: Reprogramming of mouse SMs using 4-factor transduction protocol. (A) Fluorescence immunostaining of GFP-Oct3/4 expressing SiPS (green) for pluripotency markers including Oct3/4, Sox2 and SSEA-1 (red fluorescence). The nuclei were visualized by DAPI staining (blue). (B) Staining of SiPS for alkaline phosphatase activity (dark brown) in vitro. (C) Teratogenicity of SiPS in immunodeficient mice at 2-weeks after injection of 500,000 SiPS/animal. Histological studies of teratoma showed typical three germ layer characteristics.

Supplementary Figure-II: Characterization of purified SMs cell culture. The purity of SMs culture was assessed by: (A-B) Flow cytometry for desmin expression which showed 93% cells in the culture were desmin positive, (C-D) Immunocytochemistry for desmin expression which showed more than 98% myoblasts were positive for desmin expression and scanty presence of fibroblasts identified by vimentin expression (D). (E) RT-PCR for MyoD expression in purified myoblast culture which was very low in vimentin expression.

Supplementary Figure IIIA-D: Exogenous and endogenous gene expression changes of the stemness factors in SMs and SiPS. RT-PCR showing changes in exogenous and endogenous gene expression of Oct4, Sox2, Klf4 and cMyc in native SMs (lane-1), and SiPS (lanes-2 & 3).

Supplementary Figure-IV: Changes in cardiac marker expression in SiPS derived cardiomyocytes. Densitometry showing changes in cardiomyocytes specific transcription factors and protein markers in 10 days beating SiPS derived cardiomyocytes. Significantly higher expression changes for Nkx2.5, MEF2c, \(\alpha\)-myosin heavy chain, \(\beta\)-myosin heavy chain, and cardiac troponin-T were observed in SiPS derived cardiomyocytes as compared to the SiPS.

Supplementary Figure-V: Changes in pluripotency marker expression in SiPS derived cardiomyocytes. Real time PCR showing significantly downregulated expression of pluripotency markers in spontaneously differentiated SiPS derived cardiomyocytes in 10 days EBs which was associated with simultaneous increase in cardiac specific markers.

Supplementary Figure VIA-B: Exogenous and endogenous gene expression changes of the stemness factors in SiPS and SiPS-CMs. RT-PCR showing changes in exogenous and endogenous gene expression of (A) Oct4 and (B) Sox2 in native SMs (lane-1), SiPS-CM (lane-2) and the heart (lane-3). The graphs represent an average of two experiments.
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Gapdh for 5’-tggcc ttccgtgttctacc (300bp)  
rev5’-tgtaggccatgaggtccaccac

mus-Endogenous Oct4 for 5’-tctttccaccaggeccccgcctc (224 bp)  
mus-Endogenous Oct4 rev 5’-tgccgggagctgagggagatcc

mus-Endogenous Sox2 for 5’-tagagctagactccgggcgatga (297 bp)  
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mus-Total Klf4 for 5’-caccatggacccgggcgtggcagaa (739 bp)  
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Table-II: Antibodies used for immunological studies

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Rafeeq et al. 2010 Supplementary Figure-1

**A**

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<th>SSEA-1</th>
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**B**

Endodermal epithelium

**C**

Mesoderm

Desmin

Tubulin

Endoderm

α-fetoprotein
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