STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

miR-125b Is an Adhesion-Regulated microRNA that Protects Mesenchymal Stem Cells from Anoikis

XIANG YU, DANIEL M. COHEN, CHRISTOPHER S. CHEN

Department of Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Key Words. Mesenchymal stem cells • Cell adhesion • miR-125b • Anoikis

ABSTRACT

Mesenchymal stem cells (MSCs) have the capacity for multilineage differentiation and are being explored as a source for stem cell-based therapies. Previous studies have shown that adhesion to extracellular matrix plays a critical role in guiding MSC differentiation to distinct lineages. Here, we conducted a focused screen of microRNAs to reveal one microRNA, miR-125b, whose expression changes as a function of cell adhesion. miR-125b expression was upregulated by limiting cell-matrix adhesion using micropatterned substrates, knocking down beta5 integrin or placing cells in suspension culture. Interestingly, we noted that suspending human MSCs (hMSCs) did not induce substantial apoptosis (anoikis) as is typically observed in adherent cells. Although miR-125b appeared to have some effects on hMSC differentiation, we demonstrated a striking role for miR-125b in protecting hMSCs from anoikis. Knockdown of miR-125b increased anoikis while expressing a mimic protected cells. Mechanistic studies demonstrated that miR-125b protected against anoikis by increasing ERK phosphorylation and by suppressing p53. Lastly, we found that miR-125b expression is quite limited in endothelial cells and mouse embryonic fibroblasts (MEFs). The rapid anoikis normally observed in endothelial cells was antagonized by transfection of a miR-125b mimic, suggesting that miR-125b can confer resistance to anoikis in multiple cell types. We also found that endogenous miR-125b was significantly upregulated during reprogramming of MEFs to induced pluripotent cells, suggesting that miR-125b expression may be associated with stem cell populations. Collectively, these observations demonstrate a novel link between cell-matrix adhesion, miR-125b expression, and a stem cell-specific survival program triggered in adhesion-limited contexts such as might occur in early development and wound healing. STEM CELLS *2012;30:956–964*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into a variety of tissues, including bone, cartilage, fat, and muscle [1]. This multipotential differentiation capacity makes these cells an attractive target for cell-based regenerative therapies, such as bone [2], cartilage [3], and cardiac repair [4]. Differentiation is guided by numerous cues, most notably the presence of soluble factors (growth factors, hormones, and small molecules) as well as insoluble cues that emanate from interactions of cells with the extracellular matrix. The importance of these insoluble cues is highlighted by several studies showing that cell spreading [5], cell shape [5–8], matrix stiffness [7, 9], and integrin engagement and clustering [10] regulate which lineages can be induced by differentiation medium. Although the organization of the actin cytoskeleton and the ability of cells to transduce mechanical forces is a common denominator in these studies, the molecular mechanisms by which these adhesive cues control differentiation remain incompletely understood.

Most mechanistic studies of how cell-matrix adhesion regulates MSC differentiation have focused on well-documented signal transduction pathways such as MAPK signaling [6, 11] or secondary messenger systems, such as cAMP [12]. Although these pathways are indubitably important, there is increasing evidence that MSC differentiation is guided by the activity of microRNAs. First, the expression of several microRNAs has been shown to correlate with specific lineages including miR-140 for chondrocytes [13], miR-138 for osteoblasts [14], and miR-30, −33a, and −17-92 cluster, −143, −103, for adipocytes [15–18]. Moreover, antagonizing the expression of miR-29b and miR 17-92 blocked MSC differentiation to the osteogenesis and adipogenic lineages, respectively [17, 19]. MicroRNAs bind to complementary sequences in the 3′UTR of mRNAs and thereby downregulate gene expression by either attenuated translational efficiency or degradation of the targeted mRNA.

Author contributions: X.Y. and D.M.C.: conception and design, collection of data, data analysis and interpretation, and manuscript writing; C.S.C.: conception and design, financial support, data analysis and interpretation, manuscript writing, and final approval of manuscript.

Correspondence: Christopher S. Chen, M.D., Ph.D., Department of Bioengineering, University of Pennsylvania, 510 Skirkanich Hall, 210 S 33rd St., Philadelphia 19104, Pennsylvania, USA. Telephone: 215-746-1754; Fax: 215-746-1752; e-mail: chrischen@seas.upenn.edu Received September 19, 2011; accepted for publication January 31, 2012; first published online in STEM CELLS *EXPRESS* February 13, 2012. 1066-5099/2012/$30.00/0 doi: 10.1002/stem.1064

MicroRNAs are typically regulated at the transcriptional level [20] and have been well established to respond to chemical cues present in differentiation medium. However, little is known about how cell-matrix adhesion might regulate the expression of microRNAs. Given the prominent roles of both cell-matrix adhesion and microRNAs in controlling stem cell behavior, we hypothesized that cell adhesion might regulate MSC lineage specification through differential expression of microRNAs.

In this study, we performed a focused screen of microRNAs reportedly expressed in human MSCs (hMSCs) or its differentiated lineages [13, 17, 18, 21] to determine whether cell adhesion could regulate microRNA expression. We identified one microRNA, miR-125b, that was specifically induced under conditions of low or absent cell-matrix adhesion. Previous studies have shown that miR-125b is expressed in hematopoietic and epidermal stem cells [22], but this microRNA has not been well characterized in hMSCs. Interesting, we found that miR-125b did not promote cellular differentiation but rather had an unexpected function in promoting cell survival in response to withdrawal of cell-matrix adhesion signals, a process that normally triggers anoikis. This anoikis-resistance phenotype is mediated by the ability of miR-125b to upregulate mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling while suppressing p53 expression. MEK/ERK signaling is known to be critical to survival in many cell types through its downstream effectors such as Myc and Bad [23, 24], while p53 signaling is known to promote apoptosis in response to multiple stresses, including the anoikis response [25]. However, neither ERK nor p53 have previously been linked to miR-125b in the context of anoikis. Moreover, we observe that the upregulation of miR-125b in response to loss of cell-matrix adhesion may be specific to stem cells.

Materials and Methods

Cell Culture and Reagents

hMSCs (Lonza, Walkersville, MD, lonzabio.com) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone, Waltham, MA, thermoscientific.com), 0.3 mg/ml glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin. Experiments were conducted on cells at passage 6 or earlier. For cell seeding density experiments, cells were plated at either low, 5,000 cells per square centimeter, or high, 30,000 cells per square centimeter, densities on tissue culture plastic. Cell suspension studies were carried out on F-127 pluronics-treated polystyrene dishes. Long-term suspension cultures (24 hours or longer) also included 2% methylcellulose reconstituted in DMEM medium (GIBCO, Grand Island, NY, Invitrogen.com) to prevent cell settling and clumping. Examination of cell survival in suspension was performed at day 7. For hMSC differentiation studies, osteogenic media (R&D Systems) and adipogenic media (Lonza) were used. Media were changed every 3 days. Cells were harvested at 2-week time and then assayed for alkaline phosphatase using Fast-Blue and lipid droplets using Oil-red-O staining as previously described [26]. Human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 media (Lonza). Suspension assays of HUVECs were limited to 3 hours incubation on F-127 pluronics-treated polystyrene dishes due to the strong anoikis response in this cell type.

Determination of viable MSC cells in suspension cultures (day 7) was conducted using trypan blue exclusion. HUVECs viability assays included an additional step of replating the cells to select for the surviving fraction and 16 hours of culture on tissue culture plates (differential trypan blue staining is not apparent at the early stages on anoikis following 3 hours of suspension). Percent survival was reported as viable cells divided by the number of cells seeded in suspension cultures.

Generation of Induced Pluripotent Stem Cells

Mouse embryonic fibroblasts (MEFs, OCT4-GFP), a gift from Penn iPSC core facility, were maintained in high glucose DMEM containing 10% fetal bovine serum and 0.3 mg/ml glutamine. iPS reprogramming was performed using a lentivirus containing mouse Oct4, Klf4, and Sox2 (Penn iPSC core facility). Following infection, MEFs were cultured on top of Matrigel at a seeding density of ($3 \times 10^5/cm^2$) in DMEM media containing knockout serum replacement (KOSR) (Invitrogen), β-mercaptoethanol (Sigma, St. Louis, MO, sigmaaldrich.com), and leukemia inhibitory factor (LIF) (Millipore, Burlington, MA, millipore.com) for 10 days. Successfully reprogrammed cells expressed green fluorescent protein (GFP) under control of the Oct-4 promoter. GFP-positive foci were identified under epifluorescence and subsequently harvested manually (needle aspirate) for further miRNA analysis.

P53 Knockdown

p53 knockdown lentivirus was made from pLVTH-sip53 plasmid (Addgene plasmid 12239 [27], Cambridge, MA, addgene.org). The knockdown efficiency was tested by comparing p53 expression using immunoblotting (p53 antibody, sc-6243, Santa Cruz, CA, scbt.com) on MSC cells infected with sh-p53 virus or vector-only virus (Supporting Information Fig. A). Viruses were titered based on expression of an internal ribosome entry site (IRES)-enhanced GFP (EGFP) cassette, and infectivity was 80%–90% in all cases.

3′Untranslated Region (UTR) Assay

Seed sequences that may be recognized by mir-125b were cloned using the following primers p53 (sense, AATTCAAGACTTGTT TTATGCTCAGGGTCAACTGCA, antisense, GTTGACCCTGA GCATAAAACAAGTCTTG) and LIN28 (sense, AATTC GGTA CATGAGCAATCTCAGGGATAGCCTGCA, antisense, GGCTA TCCCTGAGATTGCTCATGTACCG). The sequences were cloned into the 3′ of the luciferase in the pGL3-BS construct (gift from Dr. Mitchell Weiss), which already has a strong basal activity. MSCs were transfected with these plasmids and an internal control (Renilla luciferase driven by the thymindine kinase (TK) promoter) using the AMAXA nucleofection system. Following an overnight recovery period, cell were trypsinized and replated at the indicated densities. Luciferase activity was assayed using the dual luciferase kit (Promega) on a GloMAX luminometer. The ratio of firefly to Renilla luciferase was determined and the data normalized to the high density seeding condition.

Caspase-3 Activity

Suspended cells were pelleted by centrifugation and washed in phosphate buffered saline (PBS) for three cycles to remove methylcellulose. Caspase-3 activity, a measure of apoptosis, was determined by EnzChek Caspase-3 Assay Kit according to the manufacturer's instructions (Invitrogen). The fluorescence was assayed on Helios plate reader using 360/460 nm excitation/emission filters. The activity was normalized to total DNA content as determined by CyQUANT Cell Proliferation Assay (Invitrogen) according to the manufacturer's instructions. Caspase activity was assayed at day 3 of suspension for hMSCs, the time point at which maximal caspase-3 activity was present in our experimental system. HUVECs were assayed following 3 hours of suspension culture.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

TUNEL assays were performed to detect apoptosis in situ using TUNEL enzyme and TUNEL reaction mixture (Roche, Nutley, NJ, roche.com). Cells were harvested as described above for the caspase assayed followed by the kit's suggested fixation and

labeling protocol. TUNEL assays are reported for MSCs at days 1 and 10 of suspension (or adherent) culture. TUNEL staining was imaged on a Nikon TE200 microscope using epifluorescence.

Live/Dead Viability Assays

Suspended cells ($\sim 1 \times 10^5$ cells) were washed PBS $\times 3$ to remove excess methylcellulose before seeding on coverslips. Cells were incubated with the live/dead viability solution (calcein AM and ethidium homodimer-1) (Invitrogen) for 30 minutes, per the manufacturer's guidelines imaged on a Nikon TE200 microscope.

Real Time RT-PCR Analysis

Total RNA was isolated using Trizol Kit as specified by the manufacturer (Invitrogen). Three hundred nanograms of total RNA was reverse transcribed by using Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA, appliedbiosystems.com), $\times 5$ Taqman microRNA RT primer, and $\times 5$ Taqman RNU6B RT primer (Applied Biosystems) with reaction conditions (16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes, 4°C end). Real-time PCR was performed using 5 μl of the five times diluted transcribed product, Taqman $\times 2$ universal PCR master mix and $\times 20$ Taqman microRNA real-time primers or $\times 20$ Taqman RNU6B real-time primer, and monitored using an ABI 7300 system (Applied Biosystems). Data analysis was performed using the ABI Prism 7300 Sequence Detection Systems v1.0 software (Applied Biosystems). The RT primer and real-time primers for hsa-miR-19a, hsa-miR-24, hsa-miR-103, hsa-miR-107, hsa-miR-140, hsa-miR-125b, hsa-miR-143, and hsa-miR-320 were purchased from Applied Biosystems. Error bars on the real-time PCR data show the 95% confidence intervals.

Transfection of siRNA or Mimics

Small interfering RNAs targeting miR-125b or a miR-125b mimetic were purchased from Thermo Scientific Dharmacon RNAi Technologies, Lafayette, CO, dharmacon.com (#IH30059505 and #C30059503). The siRNAs were transfected in hMSCs using Lipofectamine RNAiMAX reagent (Invitrogen), with siGLO as a control. siRNA (2.5 μl, 20 μM) and RNAiMAX (6 μl) were used per six well of hMSCs. Transfection efficiency was 90% or higher as assessed by labeling efficiency with the fluorescent siGLO reagent.

Statistical Analysis

Whiskers on all real-time PCR data depict 95% confidence intervals. Error bars on cell counting assays, luciferase activity, or caspase-3 activity denote the standard error of the mean. p values were computed using a two-tailed Student's t test. p values $<.05$ were considered significant (denoted by an asterisk). All reported measurements are the average of at least three independent experiments unless otherwise indicated.

RESULTS

Expression of miR-125b Is Regulated by Cell-Matrix Adhesion

MSCs are typically cultured at different densities to promote specific lineages [5, 28], and we examined whether the expression of a panel of eight microRNAs reportedly expressed in hMSCs or differentiated MSCs [13, 17, 18, 21] was affected by either low or high cell seeding densities. We observed that four miRNAs were induced at least 1.5-fold at high seeding densities (Fig. 1A). Although changes in cell density can impact cell-matrix adhesion, these changes also impact paracrine signaling and cell-cell adhesion. To analyze whether the changes in miRNA levels were a direct consequence of changes in cell-matrix adhesion, we assayed miRNA expression in hMSCs cultured either on continuous fibronectin, which promotes maximal cell adhesion and spreading (spread cells), or on small micropatterns of fibronectin (1,024 μm^2), which restrict cell spreading (round cells) (Fig. 1B). We observed that one miRNA, miR-125b, was elevated twofold in round compared to spread cells (Fig. 1B). To further confirm the specific role of cell-matrix adhesion, we examined whether miR-125b could be induced by specifically antagonizing integrin receptors. Indeed, siRNA-mediated knockdown of beta5 integrin, an adhesion receptor important for MSC spreading, induced miR-125b by twofold (Fig. 1C).

The inverse relationship between levels of cell-matrix adhesion and miR-125b expression suggested that cell-matrix adhesion and/or cell spreading suppressed miR-125b. To test whether cell adhesion per se suppressed miR-125b, we assayed miR-125b levels in hMSCs suspended in 2% methylcellulose. We found that miR-125b was potently induced by the absence of cell-matrix adhesion (Fig. 1D), while the addition of methylcellulose alone had no effect (Supporting Information Fig. B). In fact, the eightfold induction caused by complete loss of adhesion was greater than the effects of cell density, micropatterning, or beta5 integrin knockdown.

miR-125b Is Not Essential for Adipogenic or Osteogenic Differentiation

To investigate a potential role for miR-125b in the differentiation of hMSCs to lineages previously shown to be impacted by high or low seeding densities, we measured miR-125b under conditions that promote either osteogenesis or adipogenesis. miR-125b expression was slightly elevated in either differentiation condition, relative to basal medium; however, miR-125b levels remained below those observed in low cell adhesion contexts (Fig. 1E). To investigate whether changes in miR-125b levels could drive differentiation, hMSCs were transiently transfected with a miR-125b antagonist or mimetic prior to differentiation in a bipotential medium that can promote either osteogenic or adipogenic fates [5]. Interestingly, miR-125b mimetic strongly inhibited the formation of either adipocytes or osteoblasts (Fig. 1F). In contrast, miR-125b knockdown did not appear to enhance hMSC differentiation. The absence of a strong effect of miR-125b knockdown on hMSC differentiation and the relative mild influence of differentiation conditions on miR-125b expression made it unlikely that miR-125b played a role in cell density-dependent control of MSC differentiation.

miR-125b Protects Against Anoikis

The absence of a positive effect of miR-125b on hMSC differentiation left unanswered the functional significance of miR-125b upregulation in response to limited adhesion. Since we observed the most dramatic induction of miR-125b when hMSCs were suspended in methylcellulose, we focused on whether miR-125b might modulate a cellular response to suspension conditions. In most adherent cell types, loss of adhesion triggers cell death in a process known as anoikis [29]. We therefore assayed for survival of hMSCs following suspension for either 24 hours or 7 days. Remarkably, hMSCs were unexpectedly resilient to suspension conditions. While suspension triggers complete cell death in many cells, a sizeable fraction ($\sim$32%) of hMSCs remained viable in the absence of adhesion for 1 week (Fig. 2B).

To test whether miR-125b played a role in this hMSC survival in suspension culture, we assayed the effects of knockdown of miR-125b on cell survival. Transient transfection of miR-125b siRNA inhibitor (si-miR) prevented the induction of miR-125b in response to cell suspension (Fig. 2A). Importantly, we observed that knockdown of miR-125b dramatically reduced hMSC survival in suspension (Fig. 2B, 2C).

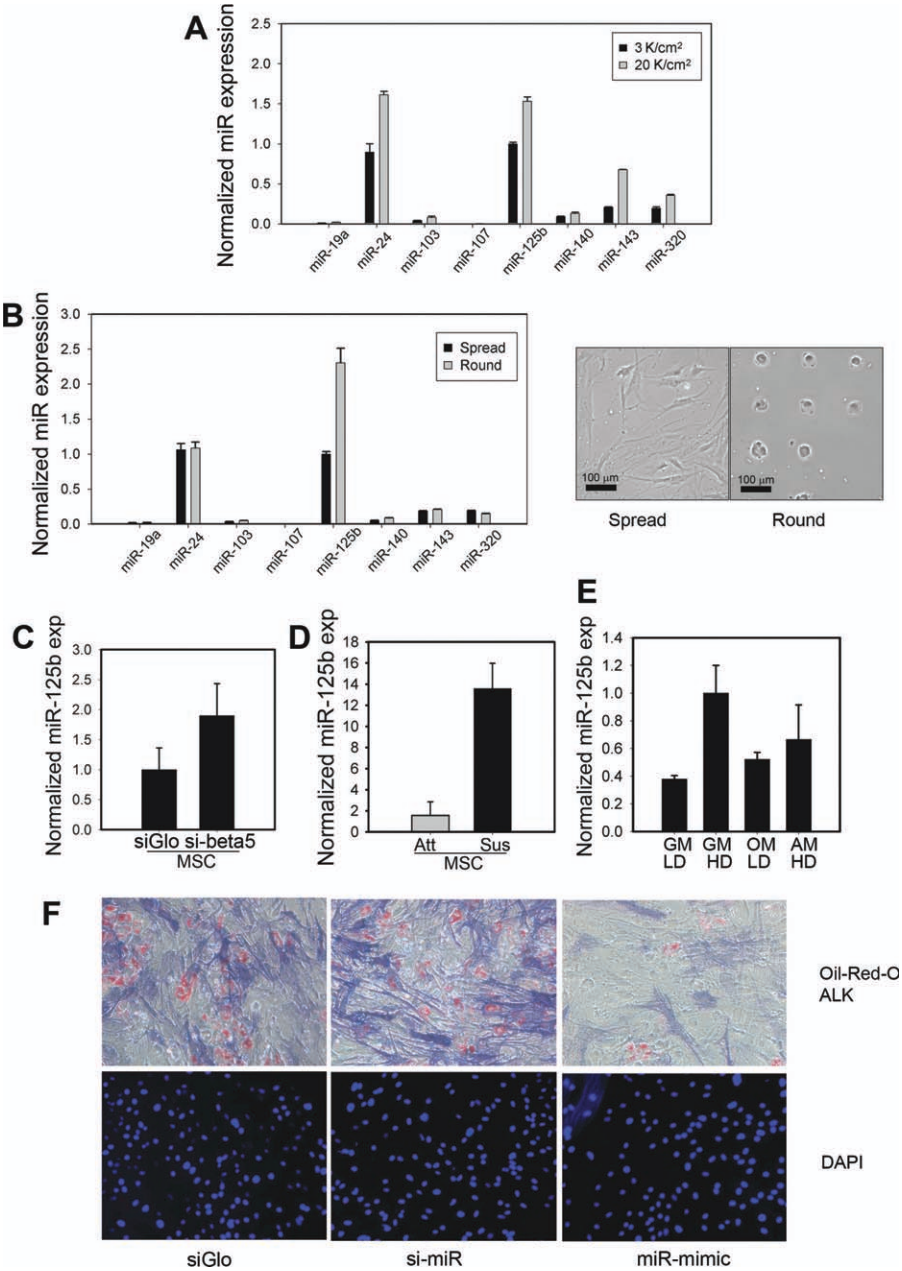

Figure 1. miR-125b is an adhesion-regulated microRNA whose expression can suppress differentiation. **(A):** A panel of miRNAs was assayed by real-time PCR for expression at low (3 K/cm^2) or high (20 K/cm^2) seeding densities. **(B):** miRNAs expression as a function of cell spreading; spread (continuous fibronectin) or round (32 × 32 μm^2 micropatterns). **(C):** miR-125b expression was induced by knockdown of beta5 integrin. **(D):** miR-125b expression was strongly induced by cell suspension, suspended (Sus) versus attached (Att) cells. **(E):** miR-125b levels as a function of differentiation, GM: growth media, OM: osteogenic media, AM: adipogenic media, LD: low density, 3 K/cm^2, HD: high density, 20 K/cm^2. **(F):** miR-125b mimetic (miR mimic) suppressed adipogenic and osteogenic differentiation of hMSCs compared to a transfection control (siGLO) or to miR-125b knockdown cells (si-miR). Oil-red-O staining used to visualize lipid droplets and Fast Blue staining to visualize alkaline phosphatase activity following 1 week of differentiation. All real-time data show the mean ± 95% confidence intervals. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; MSC, mesenchymal stem cells.

Moreover, this effect was specific to suspended hMSCs since knockdown of miR-125b had no effect on the viability of adherent hMSCs (Fig. 2D). To confirm that this change in viability was associated with apoptosis, we performed TUNEL staining on the suspended cells (Supporting Information Fig. C) and measured the activity level of caspase-3, a terminal effector of apoptosis [30]. Consistent with a requirement for upregulation of miR-125b to promote survival in suspended hMSCs, we observed 1.5-fold higher levels of caspase-3 activity in miR-125b knockdown cells compared to controls (Fig. 2E). Together, these data show that a subpopulation of hMSCs are refractory to the anoikis pathway triggered by cell suspension, and this resistance to anoikis appears to be miR-125b dependent.

miR-125b Targets p53 in hMSCs

The repertoire of genes targeted by mir-125b in hMSCs is not yet known; however, p53 and lin28 have been identified as

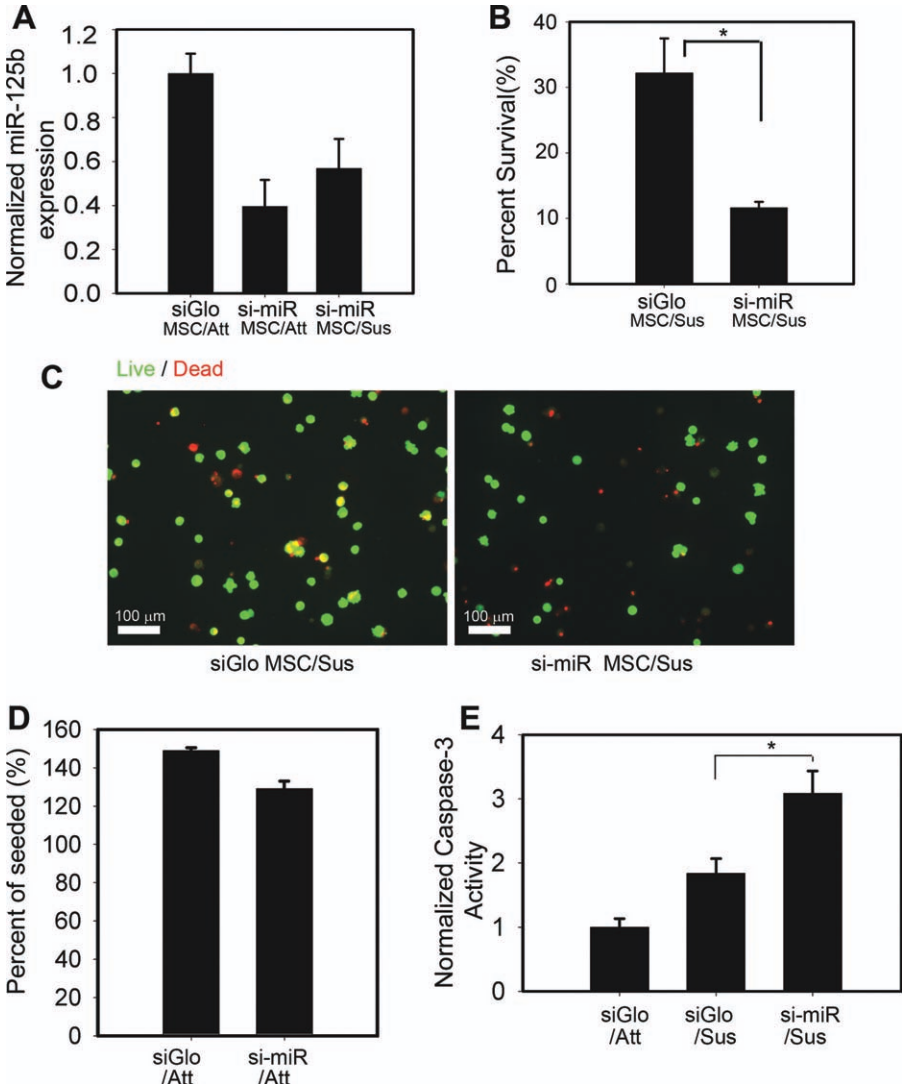

Figure 2. miR-125b protects human MSCs against anoikis. **(A):** miR-125b expression levels in adherent (Att) or suspended (Sus) hMSCs transfected with miR-125b antagonist (si-miR). **(B):** Knockdown of miR-125b decreased viability of hMSCs held in suspension. Viability assayed by trypan blue exclusion and reported as percent of cells versus total number seeded. **(C):** Imaging hMSC survival by live (Green cytosolic) versus dead (punctate red nuclear) staining. Note that red fluorescence of siGLO causes some viable cells to be double labeled (yellow). These are easily distinguished from dead cells that exhibit punctate nuclear staining. **(D):** Knockdown of miR-125b had no effect on cell survival under adherent culture conditions. Percent of seeded exceeds 100% due to cell proliferation. **(E):** Knockdown of miR-125b promoted apoptosis (caspase-3 activity) in suspended hMSCs. All real-time data show the mean ± 95% confidence intervals. Error bars in (B), (D), and (E) ± SEM. *, $p < .05$ for Student's *t* test. Abbreviation: MSC, mesenchymal stem cell.

miR-125b targets in several cell types [31, 32]. To begin to examine whether miR-125b protected cells from anoikis through its reported effects on these genes, we cloned the 3′UTR sequences of these genes into a luciferase plasmid to generate putative reporters for miR-125b activity. We then characterized these reporters under different conditions of cell-matrix adhesion that we had previously observed to trigger elevated miR-125b expression. In this assay, the p53 3′UTR luciferase reporter showed decreased expression at high seeding density, whereas no change occurred in the lin28 reporter (Fig. 3A). These results suggested that p53 may be a target for miR-125b regulation in hMSCs.

To investigate whether endogenous p53 is specifically targeted by miR-125b, we measured levels of p53 expression in response to direct manipulations of miR-125b levels. In adherent cells, transfection of a miR-125b antagonist upregulated p53 expression, while a miR-125b mimetic suppressed p53 (Fig. 3B). Moreover, suspending cells, which upregulated miR-125b, decreased p53 levels. In contrast, miR-125b-knockdown cells showed persistent p53 expression in suspension culture (Fig. 3C). Together these results show that p53 is a target of miR-125b in hMSCs.

To address whether inhibition of p53 expression by miR-125b may be part of the mechanism by which miR-125b prevents anoikis, we investigated the effects of p53 knockdown in suspended hMSCs. Using a lentivirally encoded p53 shRNA [27], we observed that reduction of p53 was associated with an 80% decrease in caspase-3 activity in suspended hMSCs (Fig. 3D). This decreased level of apoptosis in p53 knockdown cells suggests that p53 suppression by miR-125b may be an effective mechanism to protect hMSCs against anoikis.

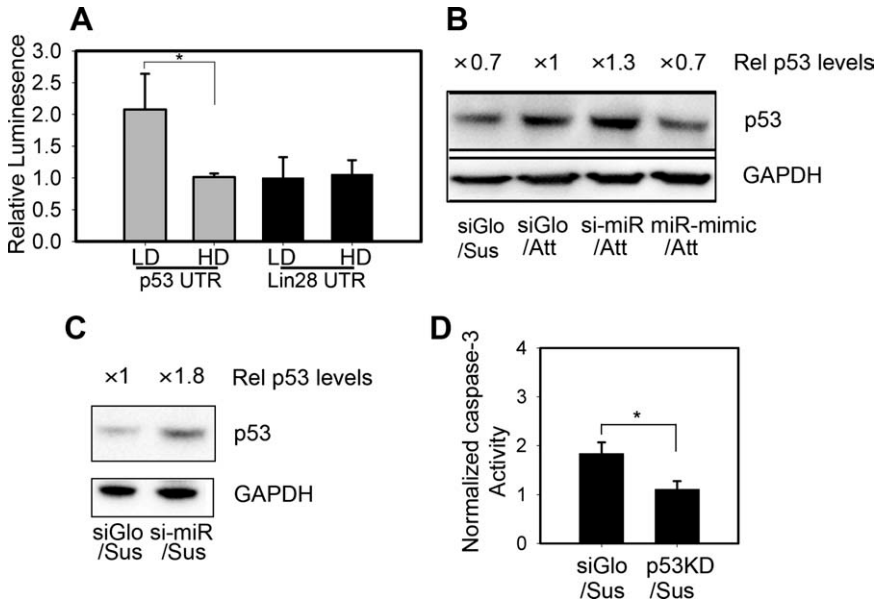

Figure 3. miR-125b targets p53 to regulate human mesenchymal stem cells (hMSCs) survival. **(A):** miR-125b targets p53 and LIN28 were analyzed for density-dependent expression (HD, high density; LD, low density) in a 3′UTR luciferase reporter assay in hMSCs. **(B):** p53 levels in adherent hMSCs transfected with transfection control (siGLO), miR-125b antagonist (si-miR), or mimetic (miR mimic). **(C):** Rescue of p53 expression in suspended hMSCs by miR-125b antagonist. **(D):** p53-knockdown (p53 KD) protected hMSCs from anoikis. Error bars in (A) and (D) ± SEM. *, $p < .05$ for Student's t test. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; UTR, untranslated region.

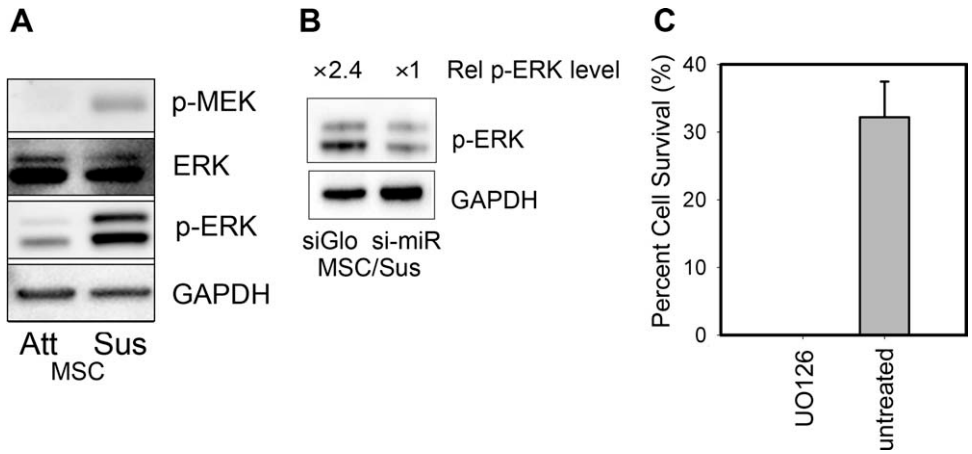

Figure 4. miR-125b expression and human MSC (hMSC) survival are associated with elevated phospho-ERK. **(A):** Suspended hMSCs showed elevated levels of active MEK and phospho-ERK. **(B):** miR-125b knockdown reduced levels of phospho-ERK in suspended hMSCs. **(C):** ERK signaling was absolutely required for survival of hMSCs in suspension, UO126 (MEK inhibitor). Error bar in C shows SEM. Abbreviations: ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MSC, mesenchymal stem cell.

miR-125b Regulates Levels of Phospho-ERK in Suspended hMSCs

To further characterize how miR-125b protects against anoikis, we examined how signal transduction pathways associated with cell survival changed in suspended hMSCs. As expected, hMSCs showed a dramatic downregulation of phospho-focal adhesion kinase (FAK) in response to loss of cell adhesion (data not shown). In contrast, suspended hMSCs showed unexpectedly elevated levels of phospho-MEK and phospho-ERK (Fig. 4A). Since ERK signaling has been implicated in p53-mediated survival [33] and hMSC viability [34], we examined more closely whether miR-125b was involved with the observed changes in ERK phosphorylation.

We observed that knockdown of mir-125b strongly attenuated phospho-ERK levels in suspended hMSCs (Fig. 4B). To confirm that activation of ERK is functionally relevant to an anoikis protection mechanism in hMSCs, we treated suspended cells with a pharmacological inhibitor of MEK. In the presence of the inhibitor UO126, no cells were able to survive suspension conditions revealing an absolute requirement for MEK-ERK signaling in promoting hMSC survival in suspension (Fig. 4C).

Expression of miR-125b Is Cell-Type Restricted

We were interested in exploring whether the upregulation of miR-125b in low adhesion contexts could be observed in other cell types, particularly ones that are known to be susceptible to anoikis. To begin to address this question, we measured miR-125b levels in adherent versus suspended HUVECs, a cell type known to be highly dependent on adhesion for survival. We observed that miR-125b is poorly expressed in HUVECs

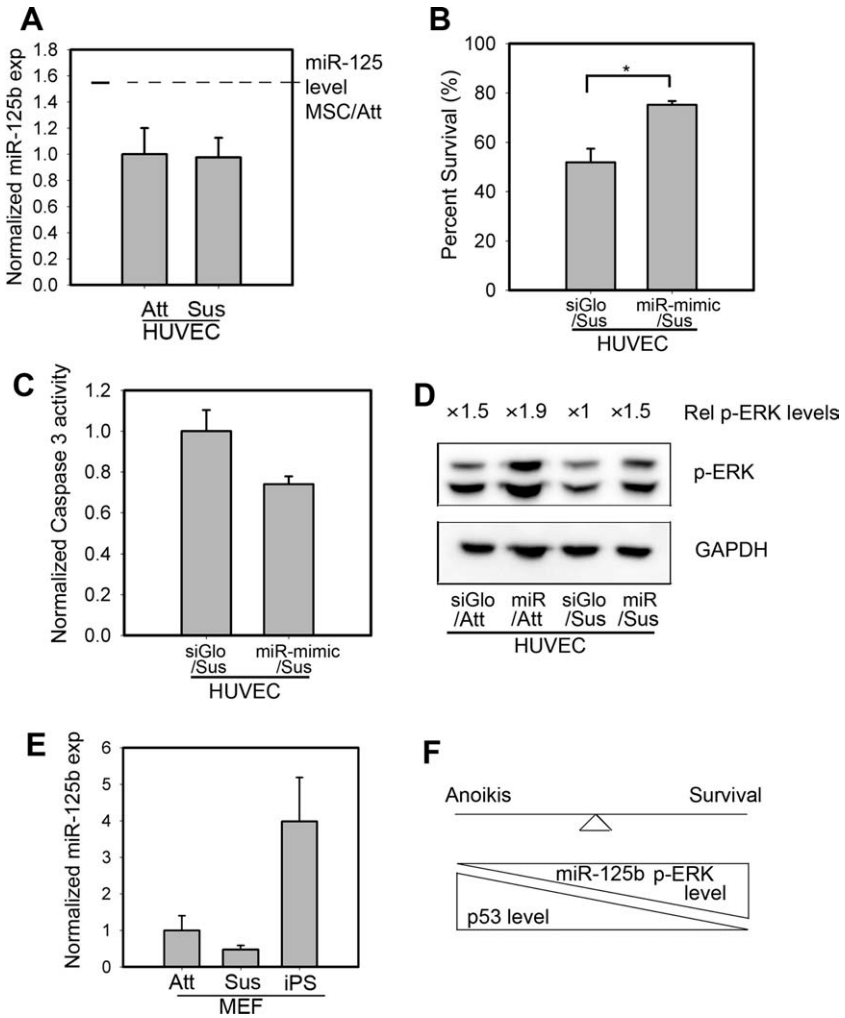

Figure 5. Upregulation of miR-125b in response to suspension is a stem cell-specific phenomenon. **(A):** miR-125b was expressed at low levels in HUVECs and could not be induced by cell suspension. Dotted line shows basal level of miR-125b expression in attached human MSCs. **(B):** Exogenous miR-125b improved HUVEC survival in suspension conditions (75.2% viability compared to 51.9% of control cells following 3 hours of suspension and replating). **(C):** Exogenous miR-125b decreased caspase-3 activity in suspended HUVECs. **(D):** HUVECs did not sustain ERK activation in response to cell suspension; however, exogenous miR-125b elevated phospho-ERK levels. **(E):** miR-125b expression was upregulated upon reprogramming of MEFs to an iPS-like state but not by cell suspension. **(F):** Qualitative model of how miR-125b regulates stem cell survival in low adhesion contexts. Data in (A) and (E) show mean ± 95% confidence intervals. (B) and (C) show mean ± SEM. *, $p < .05$ for Student's *t* test. Abbreviations: ERK, extracellular signal-regulated kinases; HUVEC, human umbilical vein endothelial cell; iPS, induced pluripotent stem; MEF, mouse embryonic fibroblast; MSC, mesenchymal stem cell.

compared to hMSCs; moreover, these cells were unable to upregulate miR-125b in response to suspension (Fig. 5A). Despite the absence of miR-125b upregulation in suspended HUVECs, we hypothesized that the ability of miR-125b to protect against anoikis may be conserved across cell types. Indeed, HUVECs transfected with miR-125b mimetic inhibited suspension-induced death by nearly half (Fig. 5B). In accordance with this improved level of survival, miR-125b mimetic reduced caspase-3 activity in suspended HUVECs (Fig. 5C). Since we had previously observed a positive correlation between miR-125b, cell survival, and ERK signaling in hMSCs, we tested whether miR-125b mimetic could regulate ERK phosphorylation in HUVECs. Indeed, we observed that miR-125b mimetic was able to upregulate levels of phospho-ERK in both adherent and, more importantly, in suspended HUVECs (Fig. 5D).

Given that HUVECs could not upregulate miR-125b in response to loss of cell adhesion, we wondered whether the regulation of miR-125b by changes in cell-matrix adhesion is a stem cell-specific response. To investigate this possibility, we measured miR-125b levels in MEFs, in adherent or suspension culture as well as following a reprogramming protocol to generate induced pluripotent cells. While MEFs failed to induce mir-125b in response to suspension conditions (Fig. 5E), miR-125b was enriched in MEFs following induction to pluripotency by approximately threefold compared to the parental fibroblasts. These results suggest that robust expression of miR-125b is preferentially associated with stem cells.

Discussion

In vivo, MSCs are resident in a number of different tissues [35] but also transiently circulate in the bloodstream in inflammatory settings perhaps to home to sites of injury [36]. Here, we provide the first demonstration that MSCs can resist anoikis, perhaps explaining how MSCs are able to transit

through the circulatory system to populate distant sites without undergoing apoptosis. We also show that this response is mediated by upregulation of miR-125b.

Interestingly, miR-125b is reportedly enriched in several stem cells, most notably hematopoietic stem cells (HSCs) as well as skin stem cells [37]. miR-125b appears to influence multiple aspects of HSC biology [22], and one study has shown that miR-125b affects proliferation of these cells [38]. In skin stem cells, miR-125b has been shown to control the balance between stemness and differentiation [37]. One of the hallmarks of HSCs and skin stem cells is that they exist in a specialized niche with minimal cell-matrix interactions [39, 40]. Here, we show in hMSCs that changing cell adhesion itself is a key regulator in upregulating miR-125b. Although the environment of circulating MSC is quite distinct from these stem cell niches, the coincidence of low or absent cell-matrix adhesion with high miR-125b expression is all these setting is quite striking. This strong association of miR-125b expression, stem cell specificity, and low adhesion is further strengthened by our observation that miR-125b is upregulated in induced pluripotent stem cells (iPSCs), which grow as aggregates and are relatively deficient in cell-matrix contact. In contrast, adhesion-dependent cell types, such as HUVECs and MEFs, show low miR-125b expression that is unresponsive to changes in cell-matrix adhesion. Taken together, these observations suggest that elevated miR-125b may be a general mechanism used specifically by stem cells to enable their survival, and perhaps maintain their stemness, in the context of low or no signaling from cell-matrix adhesion.

How does elevated miR-125b promote cell survival in the absence of adhesion? Upon cell suspension, cells lose integrin-dependent signals. Typically, this loss of integrin signaling causes marked reduction of phospho-Akt and phospho-ERK, and hence a reduction in prosurvival signaling [41, 42]. Surprisingly, while losing integrin-dependent signaling such as FAK phosphorylation, hMSCs show robust ERK phosphorylation and ultimately increased cell survival. Moreover, pharmacological inhibition of MEK completely ablates the ability of hMSCs to survive in suspension. These observations suggest that miR-125b may regulate survival through MEK/ERK signaling. Although the mechanistic link between miR-125b and MEK/ERK signaling remains to be elucidated, it is possible that miR-125b can inhibit the expression of suppressors of this pathway. Indeed, miR-125b is predicted to target SMEK1, a MEK1 phosphatase (targetscan.org). In addition, it is interesting to note that ERK signaling can be downregulated by dual specificity phosphatases in response to p53 signaling [33]. Thus, suppression of p53 by miR-125b may contribute to sustained ERK signaling. Alternatively, miR-125b and upregulation of ERK may be parallel pathways that coordinately promote resistance to anoikis.

In addition to prominent roles for miR-125b and ERK in supporting anoikis resistance, our experiments show that the downregulation of p53 in suspended hMSCs is critical to their survival. Since p53 is a direct target of miR-125b, it is likely that the ability of miR-125b to suppress p53 plays a major role in promoting resistance to anoikis. In fact, direct knockdown of p53 was also observed to protect hMSCs against anoikis. These results are consistent with several studies showing that loss of p53 confers resistance to anoikis [43, 44]. Interestingly, loss of p53 in response to suspension has been attributed to mouse double minute 2 (MDM2)-based degradation in MEFs and several cancer cell lines [45]. The observation that miR-125b can also target p53 suggests an additional level of regulation that may enable long-term suppression of p53 in the absence of cell adhesion. Whether and how miR-125b mediated suppression and MDM2-based p53 degradation cooperate to regulate p53 levels in normal and cancer cells is an interesting question for future studies.

Because p53 is implicated in a diverse set of cellular processes including cell cycle control, apoptosis, and regulating genomic stability, it may seem somewhat surprising that stem cells might adopt a survival mechanism contingent on reducing the levels of this critical protein. Indeed, loss of p53 function can have deleterious effects on MSC functionality. For example, mutations in p53 have been implicated in age-related transformation of MSCs [46], and the proliferation of MSCs is particularly sensitive to p53 levels [47]. Moreover, p53 appears to have a role in MSC differentiation, in particular during osteogenic commitment [48]. Nonetheless, it is important to point out that MSCs are nonproliferative in cell suspension conditions, and these cells may only exist in this state during quiescence or when transiently circulating. Given that we do not know how miR-125b and p53 are regulated upon tissue engraftment, it is premature to speculate on how miR-125b would affect tissue repair. However, in pilot studies, we have observed that miR-125b is rapidly downregulated upon reattachment, and MSCs regained their sensitivity to anoikis after a short period of adherent cultivation (Supporting Information Fig. D), suggesting the possibility that homeostatic levels of miR-125b and its effectors are rapidly re-established upon cell attachment.

Although miR-125b is gaining much attention as a critical regulator of stem cell behavior [37, 22], the transcriptional mechanisms controlling its expression have received limited attention. Two transcription factors, nuclear factor (NF)-kappaB-p65 [49] and caudal-type homeobox protein 2 (CDX2) [50], have been shown to bind the promoter region of miR-125b-1 and regulate its expression in response to soluble factors stimuli, but there are no links to insoluble or adhesive cues. Further study will be needed to determine whether miR-125b responds to cell suspension at the transcriptional level, and whether this is associated with the miR-125b-1, b-2, or both loci.

Conclusions

This study demonstrates that miR-125b is an adhesion-regulated microRNA that functions to protect stem cells against anoikis. miR-125b appears to confer resistance to anoikis by upregulating phospho-ERK while suppressing p53 levels. These findings have important implications for how stem cells survive in the unique developmental, wounding, and circulatory contexts of reduced or absent cell-matrix adhesion signaling.

Acknowledgments

We thank Jennifer Leight for technical assistance with the caspase activity assays, Michael Yang and Duc Nguyen for HUVEC cells, and Yang-Kao Wang for help with the micropatterning. We also thank Lin Gao, Wenli Yang, Frederick Anokye-Danso, and Ed Morrisey for help with iPSCs and Jeroen Eyckmans and Mark Breckenridge for reading the manuscript. This work was supported by National Institutes of Health (GM74048), Penn Center for Engineering Cells and Regeneration, and American Heart Association (10POST4220014).

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflict of interests.

References

1 Pittenger MF, Mosca JD, McIntosh KR. Human mesenchymal stem cells: Progenitor cells for cartilage, bone, fat and stroma. Curr Top Microbiol Immunol 2000;251:3–11.
2 Jones E, Yang X. Mesenchymal stem cells and bone regeneration: Current status. Injury 2011;42:562–568.
3 Galle J, Bader A, Hepp P et al. Mesenchymal stem cells in cartilage repair: State of the art and methods to monitor cell growth, differentiation and cartilage regeneration. Curr Med Chem 2011;17:2274–2291.
4 Boyle AJ, McNiece IK, Hare JM. Mesenchymal stem cell therapy for cardiac repair. Methods Mol Biol 2010;660:65–84.
5 McBeath R, Pirone DM, Nelson CM et al. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 2004;6:483–495.
6 Kilian KA, Bugarija B, Lahn BT et al. Geometric cues for directing the differentiation of mesenchymal stem cells. Proc Natl Acad Sci USA 2010;107:4872–4877.
7 Fu J, Wang YK, Yang MT et al. Mechanical regulation of cell function with geometrically modulated elastomeric substrates. Nat Methods 2010;7:733–736.
8 Gao L, McBeath R, Chen CS. Stem cell shape regulates a chondrogenic versus myogenic fate through Rac1 and N-cadherin. Stem Cells 2010;28:564–572.
9 Engler AJ, Sen S, Sweeney HL et al. Matrix elasticity directs stem cell lineage specification. Cell 2006;126:677–689.
10 Huebsch N, Arany PR, Mao AS et al. Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate. Nat Mater 2010;9:518–526.
11 Meyers VE, Zayzafoon M, Gonda SR et al. Modeled microgravity disrupts collagen I/integrin signaling during osteoblastic differentiation of human mesenchymal stem cells. J Cell Biochem 2004;93:697–707.
12 Siddappa R, Martens A, Doorn J et al. cAMP/PKA pathway activation in human mesenchymal stem cells in vitro results in robust bone formation in vivo. Proc Natl Acad Sci USA 2008;105:7281–7286.
13 Miyaki S, Nakasa T, Otsuki S et al. MicroRNA-140 is expressed in differentiated human articular chondrocytes and modulates interleukin-1 responses. Arthritis Rheum 2009;60:2723–2730.
14 Eskildsen T, Taipaleenmaki H, Stenvang J et al. MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells in vivo. Proc Natl Acad Sci USA 2011;108:6139–6144.
15 Zaragosi LE, Wdziekonski B, Brigand KL et al. Small RNA sequencing reveals miR-642a-3p as a novel adipocyte-specific microRNA and miR-30 as a key regulator of human adipogenesis. Genome Biol 2011;12:R64.
16 Davalos A, Goedeke L, Smibert P et al. miR-33a/b contribute to the regulation of fatty acid metabolism and insulin signaling. Proc Natl Acad Sci USA 2011;108:9232–9237.
17 Wang Q, Li YC, Wang J et al. miR-17–92 cluster accelerates adipocyte differentiation by negatively regulating tumor-suppressor Rb2/p130. Proc Natl Acad Sci USA 2008;105:2889–2894.
18 Xie H, Lim B, Lodish HF. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. Diabetes 2009;58:1050–1057.
19 Kapinas K, Kessler C, Ricks T et al. miR-29 modulates Wnt signaling in human osteoblasts through a positive feedback loop. J Biol Chem 2010;285:25221–25231.
20 He L, Hannon GJ. MicroRNAs: Small RNAs with a big role in gene regulation. Nat Rev Genet 2004;5:522–531.
21 Goff LA, Boucher S, Ricupero CL et al. Differentiating human multipotent mesenchymal stromal cells regulate microRNAs: Prediction of microRNA regulation by PDGF during osteogenesis. Exp Hematol 2008;36:1354–1369.
22 O'Connell RM, Chaudhuri AA, Rao DS et al. MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. Proc Natl Acad Sci USA 2010;107:14235–14240.
23 Krysov S, Dias S, Paterson A et al. Surface IgM stimulation induces MEK1/2-dependent MYC expression in chronic lymphocytic leukemia cells. Blood 2012;119:170–179.
24 Scheid MP, Schubert KM, Duronio V. Regulation of bad phosphorylation and association with Bcl-x(L) by the MAPK/Erk kinase. J Biol Chem 1999;274:31108–31113.
25 Grossmann J. Molecular mechanisms of "detachment-induced apoptosis–Anoikis." Apoptosis 2002;7:247–260.
26 Wang YK, Yu X, Cohen DM et al. Bone morphogenetic protein-2-induced signaling and osteogenesis is regulated by cell shape, RhoA/ROCK, and cytoskeletal tension. Stem Cells Dev 2011 [Epub ahead of print].
27 Wiznerowicz M, Trono D. Conditional suppression of cellular genes: Lentivirus vector-mediated drug-inducible RNA interference. J Virol 2003;77:8957–8961.
28 Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.
29 Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994;124:619–626.
30 Salvesen GS. Caspases: Opening the boxes and interpreting the arrows. Cell Death Differ 2002;9:3–5.
31 Wu L, Belasco JG. Micro-RNA regulation of the mammalian lin-28 gene during neuronal differentiation of embryonal carcinoma cells. Mol Cell Biol 2005;25:9198–9208.
32 Le MT, Teh C, Shyh-Chang N et al. MicroRNA-125b is a novel negative regulator of p53. Genes Dev 2009;23:862–876.
33 Yin Y, Liu YX, Jin YJ et al. PAC1 phosphatase is a transcription target of p53 in signalling apoptosis and growth suppression. Nature 2003;422:527–531.
34 Pricola KL, Kuhn NZ, Haleem-Smith H et al. Interleukin-6 maintains bone marrow-derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism. J Cell Biochem 2009;108:577–588.
35 Sacchetti B, Funari A, Michienzi S et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 2007;131:324–336.
36 Karp JM, Leng Teo GS. Mesenchymal stem cell homing: The devil is in the details. Cell Stem Cell 2009;4:206–216.
37 Zhang L, Stokes N, Polak L et al. Specific microRNAs are preferentially expressed by skin stem cells to balance self-renewal and early lineage commitment. Cell Stem Cell 2011;8:294–308.
38 Bousquet M, Harris MH, Zhou B et al. MicroRNA miR-125b causes leukemia. Proc Natl Acad Sci USA 2010;107:21558–21563.
39 Bianco P. Minireview: The stem cell next door: Skeletal and hematopoietic stem cell "niches" in bone. Endocrinology 2011;152:2957–2962.
40 Tumbar T, Guasch G, Greco V et al. Defining the epithelial stem cell niche in skin. Science 2004;303:359–363.
41 Khwaja A, Rodriguez-Viciana P, Wennstrom S et al. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J 1997;16:2783–2793.
42 Schlaepfer DD, Hanks SK, Hunter T et al. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature 1994;372:786–791.
43 Vitale M, Di Matola T, Bifulco M et al. Apoptosis induced by denied adhesion to extracellular matrix (anoikis) in thyroid epithelial cells is p53 dependent but fails to correlate with modulation of p53 expression. FEBS Lett 1999;462:57–60.
44 Nikiforov MA, Hagen K, Ossovskaya VS et al. p53 modulation of anchorage independent growth and experimental metastasis. Oncogene 1996;13:1709–1719.
45 Lewis JM, Truong TN, Schwartz MA. Integrins regulate the apoptotic response to DNA damage through modulation of p53. Proc Natl Acad Sci USA 2002;99:3627–3632.
46 Li H, Fan X, Kovi RC et al. Spontaneous expression of embryonic factors and p53 point mutations in aged mesenchymal stem cells: A model of age-related tumorigenesis in mice. Cancer Res 2007;67:10889–10898.
47 Armesilla-Diaz A, Elvira G, Silva A. p53 regulates the proliferation, differentiation and spontaneous transformation of mesenchymal stem cells. Exp Cell Res 2009;315:3598–3610.
48 Molchadsky A, Shats I, Goldfinger N et al. p53 plays a role in mesenchymal differentiation programs, in a cell fate dependent manner. PLoS One 2008;3:e3707.
49 Zhou R, Hu G, Gong AY et al. Binding of NF-kappaB p65 subunit to the promoter elements is involved in LPS-induced transactivation of miRNA genes in human biliary epithelial cells. Nucleic Acids Res 2010;38:3222–3232.
50 Lin KY, Zhang XJ, Feng DD et al. miR-125b, a target of CDX2, regulates cell differentiation through repression of the core binding factor in hematopoietic malignancies. J Biol Chem 2011;286:38253–38263.


See www.StemCells.com for supporting information available online.