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Characterization of Differentially Expressed miRNAs by CXCL12/SDF-1 in Human Bone Marrow Stromal Cells

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Abstract: Stromal cell-derived factor 1 (SDF-1) is known to influence bone marrow stromal cell (BMSC) migration, osteogenic differentiation, and fracture healing. We hypothesize that SDF-1 mediates some of its effects on BMSCs through epigenetic regulation, specifically via microRNAs (miRNAs). MiRNAs are small non-coding RNAs that target specific mRNA and prevent their translation. We performed global miRNA analysis and determined several miRNAs were differentially expressed in response to SDF-1 treatment. Gene Expression Omnibus (GEO) dataset analysis showed that these miRNAs play an important role in osteogenic differentiation and fracture healing. KEGG and GO analysis indicated that SDF-1 dependent miRNAs changes affect multiple cellular pathways, including fatty acid biosynthesis, thyroid hormone signaling, and mucin-type O-glycan biosynthesis pathways. Furthermore, bioinformatics analysis showed several miRNAs target genes related to stem cell migration and differentiation. This study's findings indicated that SDF-1 induces some of its effects on BMSCs function through miRNA regulation.

Introduction

Recent developments in regenerative medicine seek to utilize adult stem cells as therapeutics to treat age-related musculoskeletal disease. Gene reprogramming via manipulating the cellular microenvironment is a promising and unique opportunity to direct adult stem cell fate. Bone marrow stromal cells (BMSCs) are mesenchymal lineage progenitors that give rise to multiple cell types, including osteoblasts, chondrocytes, adipocytes, and other connective tissue cells [1, 2]. BMSCs are thought to play an important role in the repair and maintenance of various musculoskeletal tissues. The migration and differentiation of adult stem cells, including BMSCs, is a highly regulated by interaction with the cellular milieu. Molecular factors within the milieu, such as growth factors and chemokines, play a vital role in the extracellular regulation of BMSCs [3].

Chemokines are a class of cytokines that induce cell chemotaxis to areas of inflammation. Stromal cell-derived factor 1 (SDF-1), also known as CXCL12, is a chemokine that plays a unique role in the migration of progenitor cell types [4]. SDF-1 mediates its effect primarily through interaction with CXC chemokine receptor-4 (CXCR4) [5]. SDF-1 is known to mediate cell migration, osteogenic differentiation, and cell survival of BMSCs [6-12]. Our group previously reported that SDF-1 accelerates osteogenic differentiation of BMSCs by regulating osteogenic factors (e.g. RUNX2, BMP2, osteocalcin, collagen alpha 1 type 1) [10]. We also reported that SDF-1 promotes BMSCs migration and osteogenic differentiation in vitro, as well as bone formation in vivo [8, 10]. Similarly, other groups found SDF-1 to be implicit during endochondral ossification and fracture healing, specifically the differentiation of BMSCs into hypertrophic chondrocytes [13-15]. Due to the clinical significance of these findings,

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understanding the molecular mechanisms by which SDF-1 exerts its effects on BMSCs proves highly valuable.

Given the ability of SDF-1 to influence various signaling pathways within BMSCs, we postulate that it likely mediates its control via epigenetic regulation [16]. Epigenetic regulation is a mechanism by which gene expression changes occur without changes in genetic makeup [17]. Epigenetic factors, including DNA methylation and miRNAs, are known to be implicit in the differentiation of BMSCs and musculoskeletal development [18-22]. MiRNAs are small non-coding RNAs that bind mRNA at the 3'-untranslated regions (3'-UTR). Upon binding, the miRNA prevents translation or promotes degradation of the mRNA, thus negatively regulating gene expression at the post-transcriptional level [23, 24]. Several reports suggest that miRNAs regulate almost all cellular events including cell proliferation, differentiation, and development [25-28]. We hypothesize that SDF-1 induces some of its effects via the regulation of miRNAs. In this study we investigate the SDF-1 dependent regulation of miRNAs within human BMSCs and their correlation with respect to survival, proliferation, migration, and differentiation.

Material and Methods

Isolation of human BMSCs and SDF-1 treatment:

Human bone marrow (BM) from young (18–40 years of age) subjects were obtained under sterile conditions from orthopedic surgery patients as per the Institutional Review Board (IRB) of Augusta University. CD271+ MSCs were extracted from the bone marrow using an isolation kit (Miltenyi Biotec Inc., 130-092-283, Sunnyvale, CA, USA) and washed with a standard culture medium composed of DMEM medium (Corning, 10-013-CM, Corning, NY, USA), 1% antibiotics-antimycotics (AA; Invitrogen, 15240-062, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS). Cells were transferred to 100 mm culture dish and incubated at 37°C and 5% carbon dioxide (CO₂) in a humidified environment. After 24 h, the media with non-adherent cells was removed. The adherent cells were washed in phosphate buffer saline (PBS) and further expanded by incubation in the fresh standard culture medium. Culture-expanded CD271+ BMSCs of passage 1 were used for treating with the SDF-1. Human BMSCs were cultured on 24 well plates treated with or without SDF-1 (50 ng/mL concentration) for 72 hrs. Media was changed every day with or without SDF-1. At the end of 72 hrs, miRNAs were isolated using a miRNA isolation kit (SABiosciences Corporation, Frederick, MD, USA).

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the Institutional Review Board (IRB) of Augusta University.

MicroRNA Array and Bioinformatics Analysis:

Total miRNA isolation was performed using a miRNA isolation kit (SABiosciences Corporation, Frederick, MD, USA) that captures small RNAs with lengths less than 200 nucleotides. RNA concentrations were determined using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The quality of RNA samples was characterized by an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) using an RNA6000 Nano Chip (Agilent). miRNA microarrays were performed using an Affymetrix GeneChip® miRNA 4.0 array at the Integrated Genomics Core, Augusta University, GA, USA. The miRNA profile was analyzed for the hierarchical clustering of miRNA to generate heat maps and principal component analysis (PCA). T-tests were conducted to calculate the p-value and determine whether miRNA levels were significantly changed in SDF-1 treatment versus control groups. Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation were performed on differentially expressed miRNAs using DIANA-miRPath v.3. Bioinformatics software (http://www.targetscan.org/vert_72/ and <http://www.mirdb.org/>) were used to predict gene targets of differentially expressed miRNAs considered to be novel.

GEO Database Analysis of Differentially Expressed

MiRNA; To correlate our microarray results, we performed an analysis of existing microarray data in the Gene Expression Omnibus (GEO) public repository. GEO allows researchers to archive and distribute high throughput gene sequencing and microarray data sets [29]. Users can analyze genomic expression profiles of interest from previously performed pre-clinical studies. We searched the GEO database for microarray datasets of miRNA differentially expressed under three unique criteria. 1) GEO was queried for datasets of fracture healing models. This search yielded the study by Hadjiargyrou et al. (GEO accession GSE76197), which profiled miRNA in murine femur fracture across 14 days post-fracture versus intact control. 2) GEO was queried for datasets from osteogenic differentiation of mesenchymal stem cells or bone marrow stromal cells. Only datasets with significant results were included for analysis. The search yielded the following datasets: GSE159508, GSE134946

, GSE72429, GSE115197. 3) GEO was queried for datasets derived from BMP2 osteogenic growth factor treatment of cell lines derived from mesenchymal origin. The search yielded a dataset by Bae *et al.* (GEO accession GSE37036) showcasing miRNA expression in C2C12 myoblasts treated with BMP2 for 72 hours.

GEO2R interactive tool was used to determine differential expression between treatment and control groups by calculating log base 2 of fold change ($\log_2(\text{FC})$). Significance in fold change was determined from the adjusted p -value parameter calculated by the GEO2R interactive tool. The Benjamini & Hochberg false discovery rate method for p -value adjustment was selected as it is the most commonly used adjustment for microarray data. It provides a balance between the discovery of statistically significant genes and the limitation of false positives.

Results

MiRNA Differentially Expressed Following SDF-1 Treatment: miRNA microarray analysis was conducted to compare miRNA profiles of human BMSCs with and without SDF-1 treatment. We found 104 miRNAs to be differentially regulated ($p < 0.05$) with an absolute fold change of 1.5 or greater following SDF-1 treatment. Out of these 104 miRNAs, 49 miRNAs were downregulated, and 55 miRNAs were upregulated. **Table 1** presents the top 50 most differentially expressed miRNAs. A heat map with hierarchical clustering visually depicts the unique profiles of miRNA from SDF-1 treated and control groups (**Figure 1**).

Principal Component Analysis; Principle component analysis (PCA) was performed to distinguish the miRNA profile in SDF-1 treated versus control BMSCs. The PCA depicts unique clusters for treated samples versus control samples highlighting the clear distinction between these two groups (**Figure 2**).

Novel miRNAs Target Key Genes in Migration and Differentiation; In silico analysis was performed on novel miRNAs with lesser-known functions scarcely reported in the existing literature. To elucidate their roles, miRNAs underwent bioinformatic analysis utilizing targets.org and mirdb.org. Criteria for target genes include complementary binding between miRNA seed sequence and the mRNA transcript for the gene of interest. Several target genes were identified that are known to modulate stem cell migration and differentiation into musculoskeletal tissues. **Table 2** presents these novel miRNAs and their respective gene targets.

Table 1: Select (top 50) differentially expressed miRNAs in response to SDF-1 treatment in human BMSCs.

MicroRNA ID/Probeset ID	Fold-Change	p-Value
hsa-miR-654-5p	-2.5901	0.02547
hsa-miR-339-3p	-2.25636	0.02332
hsa-miR-1226-5p	-2.14647	0.00692
hsa-miR-23b-5p	-2.11548	0.02960
hsa-miR-330-3p	-2.07703	0.01226
hsa-miR-668-5p	-2.06023	0.00677
hsa-miR-3162-5p	-1.99216	0.02709
hsa-miR-3911	-1.94415	0.02799
hsa-miR-6831-5p	-1.8794	0.01496
hsa-miR-92b-5p	-1.87062	0.00466
hsa-miR-4673	-1.83306	0.02000
hsa-miR-493-3p	-1.83031	0.01104
hsa-miR-1233-5p	-1.81938	0.00178
hsa-miR-27a-5p	-1.78655	0.00742
hsa-miR-34c-3p	-1.77045	0.02972
hsa-miR-1275	-1.76887	0.00589
hsa-miR-1268b	-1.70929	0.01389
hsa-miR-4484	-1.69334	0.00523
hsa-miR-4640-5p	-1.67491	0.01385
hsa-miR-423-5p	-1.66952	0.00188
hsa-miR-23a-5p	-1.63963	0.00454
hsa-miR-134-5p	-1.6315	0.00105
hsa-miR-550a-3-5p	-1.58899	0.01087
hsa-miR-324-5p	-1.58876	0.01565
hsa-miR-6850-5p	-1.58559	0.00621
hsa-miR-376a-3p	1.76442	0.01016
hsa-miR-146b-5p	1.77157	0.01273
hsa-miR-548q	1.77686	0.02127
hsa-miR-940	1.8313	0.01507
hsa-miR-505-5p	1.88502	0.03045
hsa-miR-4688	1.88885	0.00777
hsa-miR-6769a-5p	1.92262	0.00585
hsa-miR-151b	2.00276	0.01503
hsa-miR-4284	2.02122	0.04587
hsa-miR-148a-3p	2.04888	0.01323
hsa-let-7f-5p	2.05026	0.00014
hsa-miR-7845-5p	2.12776	0.03009
hsa-miR-6891-5p	2.14821	0.00195
hsa-miR-493-5p	2.17137	0.00349
hsa-let-7d-3p	2.19535	0.04102
hsa-miR-503-5p	2.20447	0.01386
hsa-miR-3121-3p	2.27425	0.00499
hsa-miR-495-3p	2.36848	0.00776
hsa-miR-30b-5p	2.43776	0.03964
hsa-miR-376c-3p	2.47408	0.00670
hsa-miR-140-5p	2.48671	0.00463
hsa-miR-196a-5p	2.68803	0.02805
hsa-miR-1246	2.74544	0.01874
hsa-miR-4706	2.96761	0.01040
hsa-miR-24-2-5p	3.12261	0.00626

Figure 1: Hierarchical clustered heat map depicting miRNA expression in SDF-1 treated human BMSCs versus control ($n = 3$ each group). Differential expression pattern is observed between the miRNAs of the two groups.

Figure 2: Primary component analysis mappings of SDF-1 treated human BMSCs versus control. SDF-1 treatment and control group show a unique miRNA clustering pattern for each group. Differential expression pattern confirms that seen in miRNA heat map.

Table 2: Target genes of differentially expressed miRNA in response to SDF-1 treatment in human BMSCs.

microRNA	No of Targets		Stem Cell Related Genes
	Target Scan	miRDB Scan	Common Targets
hsa-miR-1226-5p	3601	304	BMPER, BMP2, COLGALT2, QSOX1, FOXN3, FOXD4L3, TGFA, IL18, ITGA10, ITGAL, ITGB2, DLX3, WNT7A, SMURF1
hsa-miR-3911	3759	496	BMP4, COL8A1, COLGALT1, FOXP3, IL1RAP, IL17RA, IL6ST, ITGB3, CCR3, WNT7A, RHOG, FGF11
hsa-miR-4640-5p	4689	727	COL26A1, COL4A3BP, FOXR2, FOXA3, FOXL2, TNFRSF12A, TNFRSF18, C1QTNF8, TGFB3, IL10RA, IL15, IL2RG, ILF3, ITGA2, ITGA5, TRAF4, WNT5B, WNT7B, RHOA, RHOF, MAPK4, MAPKAPK3, MAPK14, MAPK15, MAPK8IP3, MAPKBP1
hsa-miR-4706	1615	86	SOX11, FOXRED2, FOXE1, HOXC10, TNFRSF8, IL17REL, CXCR3
hsa-miR-7845-5p	3726	387	SOX4, SOX12, FOXJ2, HOXC4, TNFAIP3, IL9R, ITGA6, TAB2
hsa-miR-6769a-5p	4673	431	BMP6, COL4A5, COL6A5, FOXP4, HOXB9, HOXA11, HOXB13, TNFRSF21, ILF3, ITGB2, WNT7B, WNT4, RHOF

Table 3: KEGG annotation of cellular pathways potentially affected by downregulated miRNAs (a) and upregulated miRNAs (b) due to SDF-1 treatment in human BMSCs.

(a)			
KEGG Pathway	p-Value	No. of Genes Involved	No. of miRNAs Involved
Fatty acid biosynthesis	6.50E-09	2	2
Tyrosine metabolism	0.04460	10	7
Other types of O-glycan biosynthesis	0.04460	11	10
Thyroid hormone signaling pathway	0.04460	34	13
(b)			
KEGG Pathway	p-Value	No. of Genes Involved	No. of miRNAs Involved
Prion diseases	8.70E-19	3	3
GABAergic synapse	4.30E-09	16	8
Nicotine addiction	9.79E-06	11	6
Biotin metabolism	9.37E-05	1	2
Morphine addiction	0.00067	16	7
Mucin type O-Glycan biosynthesis	0.00146	6	5
Choline metabolism in cancer	0.00296	21	13
Sulfur metabolism	0.02884	3	3

KEGG Pathway Annotation and GO Enrichment Analysis of Differentially Expressed MiRNAs; The collective effect of differentially expressed miRNA was characterized with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation and Gene Ontology (GO) enrichment analysis. KEGG annotation identified several cellular pathways that are affected by these miRNAs (**Table 3**). Notable pathways include fatty acid

biosynthesis, thyroid hormone signaling, and mucin-type O-glycan biosynthesis pathways. GO analysis determined 102 biological processes associated with the differentially expressed miRNAs (**Table 4**). Common processes affected by both upregulated and downregulated miRNAs include gene expression, nucleic acid binding transcription factor activity, cellular protein modification process, enzyme binding, and small molecule metabolic process.

Table 4: GO analysis of biological processes (top 50) potentially affected by downregulated miRNAs (a) and upregulated miRNAs (b) due to SDF-1 treatment in human BMSCs.

(a)			
GO Category	p-Value	Genes	miRNAs
organelle	3.09E-80	2037	20
ion binding	2.08E-59	1318	20
cellular nitrogen compound metabolic process	1.72E-44	1000	20
biosynthetic process	2.83E-30	842	20
small molecule metabolic process	4.38E-25	519	20
blood coagulation	1.23E-15	120	19
cellular protein modification process	1.23E-15	475	20
catabolic process	2.19E-15	413	20
gene expression	2.23E-15	133	19
neurotrophin TRK receptor signaling pathway	1.22E-14	70	15
symbiosis, encompassing mutualism through parasitism	1.38E-12	121	18
cellular protein metabolic process	2.43E-12	108	16
molecular function	7.74E-12	3342	20
cytoskeletal protein binding	1.13E-11	186	20
enzyme binding	1.68E-11	280	19
cellular component	3.94E-11	3380	20
viral process	8.06E-11	104	17
nucleobase-containing compound catabolic process	3.91E-10	197	20
membrane organization	5.65E-09	132	19
protein complex	2.67E-08	735	20
glycosaminoglycan metabolic process	4.57E-08	34	14
protein binding transcription factor activity	5.32E-08	110	18
transmembrane transporter activity	2.99E-07	231	20
Fc-epsilon receptor signaling pathway	4.34E-07	38	13
vesicle-mediated transport	1.53E-06	229	18
nucleic acid binding transcription factor activity	1.99E-06	198	19
activation of signaling protein activity involved in unfolded protein response	3.04E-06	22	13
platelet degranulation	3.04E-06	24	13
platelet activation	1.25E-05	49	14
cell-cell signaling	2.48E-05	139	19
cytosol	3.63E-05	520	20
synaptic transmission	5.02E-05	91	18
toll-like receptor TLR1:TLR2 signaling pathway	5.11E-05	19	8
toll-like receptor TLR6:TLR2 signaling pathway	5.11E-05	19	8
cellular component assembly	6.83E-05	244	20
macromolecular complex assembly	0.0001746	168	19
vitamin metabolic process	0.0002525	21	12
toll-like receptor 10 signaling pathway	0.0003075	17	6
extracellular matrix disassembly	0.0003484	28	14
post-translational protein modification	0.0003484	35	14
mRNA metabolic process	0.0003484	44	16
enzyme regulator activity	0.0003484	164	19
response to stress	0.0003484	414	20
leukocyte migration	0.0003586	31	12
extracellular matrix organization	0.0003644	84	18
water-soluble vitamin metabolic process	0.0003887	19	12
platelet alpha granule lumen	0.0004625	14	8
inositol phosphate metabolic process	0.0007525	14	8
cell death	0.0007856	175	19
chondroitin sulfate metabolic process	0.0007939	15	10

Table 4: GO analysis of biological processes (top 50) potentially affected by downregulated miRNAs (a) and upregulated miRNAs (b) due to SDF-1 treatment in human BMSCs.

(b)			
GO Category	p-Value	Genes	miRNAs
organelle	1.132E-41	1034	20
cellular nitrogen compound metabolic process	4.30E-27	526	19
ion binding	1.465E-25	650	19
biosynthetic process	2.089E-12	414	19
small molecule metabolic process	1.419E-08	247	20
cellular protein modification process	7.94E-08	242	19
gene expression	1.065E-07	69	16
molecular function	2.308E-07	1688	20
cellular component	2.40E-07	1709	20
neurotrophin TRK receptor signaling pathway	1.807E-06	35	14
catabolic process	1.807E-06	204	18
blood coagulation	2.374E-06	58	17
protein complex	2.374E-06	389	19
toll-like receptor TLR1:TLR2 signaling pathway	0.000331	13	8
toll-like receptor TLR6:TLR2 signaling pathway	0.000331	13	8
nucleobase-containing compound catabolic process	0.000415	97	17
cell-cell signaling	0.000424	78	19
enzyme binding	0.000559	134	18
nucleoplasm	0.000570	126	18
cellular protein metabolic process	0.000677	49	14
platelet activation	0.001002	28	13
mitotic cell cycle	0.001150	43	14
viral process	0.001360	48	13
nucleic acid binding transcription factor activity	0.001360	103	17
post-translational protein modification	0.001411	22	11
response to stress	0.001583	222	19
toll-like receptor signaling pathway	0.002292	18	9
synaptic transmission	0.002292	50	15
pyrimidine nucleoside biosynthetic process	0.002340	4	2
toll-like receptor 4 signaling pathway	0.002396	16	8
toll-like receptor 10 signaling pathway	0.002454	11	7
apoptotic signaling pathway	0.002901	22	12
symbiosis, encompassing mutualism through parasitism	0.002901	52	13
Fc-epsilon receptor signaling pathway	0.003500	19	11
cell death	0.004487	96	14
toll-like receptor 2 signaling pathway	0.004505	13	8
toll-like receptor 5 signaling pathway	0.007802	11	7
TRIF-dependent toll-like receptor signaling pathway	0.009640	11	7
Fc-gamma receptor signaling pathway involved in phagocytosis	0.010326	11	6
epidermal growth factor receptor signaling pathway	0.010326	26	12
membrane organization	0.010326	60	14
glycosaminoglycan metabolic process	0.012232	15	11
transmembrane transporter activity	0.012232	111	18
anatomical structure morphogenesis	0.014649	17	9
toll-like receptor 9 signaling pathway	0.015862	11	6
carnitine shuttle	0.018310	4	4
MyD88-independent toll-like receptor signaling pathway	0.022856	11	7
transcription initiation from RNA polymerase II promoter	0.027497	28	12
MyD88-dependent toll-like receptor signaling pathway	0.027508	14	9
regulation of transcription from RNA polymerase II promoter in response to hypoxia	0.030148	6	5

Figure 3: Common miRNAs regulated in presence of SDF-1 treatment (at 72 hours) and during murine femur fracture healing (across multiple time points ranging from day 1 through day 14). Fracture healing data was retrieved from GEO dataset uploaded by Hadjiargyrou *et al.* (GEO accession GSE76197) in which murine bone fractures were generated and miRNA-enriched RNA was isolated from calluses at post-fracture days 1, 3, 5, 7, 11, and 14 and compared to intact bone (control) ($n = 3$). Microarray analysis revealed 306 and 374 up- and down-regulated miRNAs, respectively, of which 14 were similar to our study and presented in this figure with their respective fold changes. Significance in fold change (*) of GEO data was determined by GEO2R adjusted p -value < 0.05 .

SDF-1 Versus Fracture Healing and Osteogenic Induced MiRNA Expression Profile; GEO dataset archived by Hadjiargyrou *et al.* (GEO accession GSE76197) was analyzed to determine the differential expression of miRNA in murine femur fracture model across 14 days, and results were compared to that of SDF-1. The analysis determined 12 common miRNAs were significantly upregulated in our study and fracture healing [Hadjiargyrou *et al.*, 2016] at one or more time points across the 14-day study. Conversely, two common miRNAs were significantly downregulated across both studies. These specific miRNAs are presented in **Figure 3**. Subsequent GEO search yielded several more datasets depicting differently regulated miRNAs during osteogenic differentiation of human mesenchymal stem/stromal cells (GSE159508, GSE134946, GSE72429, GSE115197). The analysis determined that several common miRNAs were differentially expressed across these studies and with SDF-1 treatment. These results are presented in **Table 5**.

SDF-1 Versus BMP2 Induced MiRNA Expression Profile; GEO dataset archived by Bae *et al.* (GEO accession

GSE37036) was analyzed to determine the miRNA expression profile with BMP2 treatment, and results were compared to that of SDF-1. The analysis revealed several common miRNAs were differentially expressed in both studies. Specifically, let-7f-5p, miR-140-5p, miR-196a-5p, and miR-24-2-5p were upregulated in both SDF-1 and BMP2 treatment, as presented in **Figure 4**. miR-330-3p was downregulated with the treatment of either growth factor. This suggests that both BMP2 and SDF-1 induce osteogenic differentiation by regulating the same downstream osteogenic miRNAs. Furthermore, SDF-1 is known to have potential BMP2 induced bone formation, a phenomenon that could be explained in part by shared downstream osteogenic miRNA.

Discussion

Adult bone marrow stromal cells are the primary source of stem cells in the field of regenerative medicine. As mentioned previously, the extracellular milieu plays

Figure 4: Common miRNAs regulated in the presence of SDF-1 treatment (at 72 hours) and BMP2 treatment (at 72 hours). BMP2 data was determined from GEO database analysis of dataset by Bae *et al.* (GEO accession GSE37036) in which C2C12 cells were treated with BMP2 (300 ng/mL) or vehicle (control) for 72 hours (n = 2). Afterwards miRNA was isolated and Exiqon miRNA microarray was performed yielding a total of 34 differentially expressed miRNAs, of which five common miRNAs showed similar trend in the presence of SDF-1 and BMP2 treatment. Reported *p*-values are adjusted values calculated by GEO2R analyzer that indicate significance in the fold change (differential expression) for BMP2 treatment versus control.

a paramount role in controlling the migration and differentiation of BMSCs. Manipulating this environment offers a unique opportunity to control cell fate and achieve enhanced therapeutic results. Based on previous findings from our group and others, extracellular SDF-1 is beneficial for osteogenesis and fracture healing [8-10, 13-15]. We hypothesized that extracellular SDF-1 mediates its effects on BMSCs via miRNA-dependent gene regulation.

While various studies have shown the pervasive role of miRNAs regulating cellular events, no study has previously identified SDF-1 regulation of miRNAs in human BMSCs. Our identification of these miRNAs provides an important link in understanding the mechanism by which SDF-1 exerts its osteogenic effect on BMSCs. Herein we identified a list of miRNAs that were differentially expressed in BMSCs following SDF-1 treatment. Several of these miRNAs are also regulated in osteogenic differentiation and fracture healing. Comparing our findings with the GEO dataset by Hadjiargyrou *et al.* (in which they analyzed miRNA expression in murine femur fracture) 14 common miRNAs were expressed similarly across both studies (**Figure 3**). Furthermore, we identified several common miRNAs regulated in the presence of SDF-1 and during osteogenic differentiation of mesenchymal stem/stromal cells (**Table 5**). We speculate that SDF-1 partially enhances

fracture healing and osteogenic differentiation through the regulation of these miRNAs.

Several groups have demonstrated SDF-1 potentiates BMP2 induced bone formation [10, 30, 31]. BMP2 is known to enhance the proliferation and osteogenic differentiation of BMSCs [32-34]. Comparing our findings with the GEO dataset by Bae *et al.* (in which miRNA expression was analyzed in cells treated with BMP2) several common miRNAs were differentially expressed across both studies (**Figure 4**). These findings suggest SDF-1 and BMP2 could act synergistically via shared downstream osteogenic miRNA. Additionally, our results show SDF-1 downregulates miR-654-5p, a miRNA known to directly target BMP2 within BMSCs (verified by reporter assay) [35]. MiR-654-5p is also observed to be persistently decreased in patients during osteoblast differentiation [36]. Taken together, this is consistent with our prior finding that SDF-1 increases BMP2 mRNA levels in vitro [10]. BMP2 is approved by the FDA for lumbar fusion surgery and is used off-label in many other applications, despite concerns of pro-oncogenic effects [37]. Our findings support the use of lower BMP2 dosage in conjunction with synergistic molecules to achieve clinical outcomes with decreased oncogenic risk.

Table 5: List of common miRNAs differently expressed after SDF-1 treatment and after various osteogenic differentiation protocols (retrieved via GEO database analysis). GSE159508 presents human periodontal ligament stem cells (hPDLSCs) cultured in osteogenic medium for 14 days compared to hPDLSCs cultured in normal medium (10% FBS in DMEM) for 14 days. GSE134946 and GSE115197 presents human BMSCs cultured in osteogenic medium for 7 days compared to non-induced BMSCs from day 0. GSE72429 presents human synovial membrane MSCs and adipose derived stem cells (ADSCs) osteogenically differentiated compared to undifferentiated controls. Differential expression is reported as miRNA fold change after treatment compared to respective control. All reported fold changes are statistically significant based upon GEO2R adjusted *p*-value.

MicroRNA ID	SDF-1 Treatment (<i>n</i> = 3)	GEO Accession ID				
		GSE159508 (<i>n</i> = 3)	GSE134946 (<i>n</i> = 3)	GSE72429* (<i>n</i> = 3)	GSE72429** (<i>n</i> = 2)	GSE115197 (<i>n</i> = 2)
miR-654-5p	-2.59	-	-2.95	-	-	-
miR-339-3p	-2.26	-	-3.03	-	-2.10	-
miR-493-3p	-1.83	-	-3.95	-	-	-
miR-27a-5p	-1.79	-1.26	-	-	-	-
miR-146b-5p	1.77	2.44	-	-	9.25	0.01
mir-548q	1.78	1.39	-	7.29	-	-
miR-940	1.83	1.59	1.32	0.86	-	-
miR-505-5p	1.89	0.70	-	-	-	-
miR-4284	2.02	-	-	1.14	-	-
miR-148a-3p	2.05	1.06	-	-	-	-
let-7d-3p	2.20	1.28	-	-	-	-
miR-24-2-5p	3.12	-	-	0.25	-	-

* Synovial membrane MSC samples, osteogenic differentiation versus undifferentiated control

** Adipose derived stem cell samples, osteogenic differentiation versus adipogenic differentiation (control)

Existing literature indicates that several differentially expressed miRNAs by SDF1 play an important role in osteogenic differentiation of BMSCs. MiR-339 has been found to inhibit the osteogenic differentiation of BMSC via the direct targeting of DLX5, a known factor in osteogenic differentiation [38]. We speculate that SDF-1 likely enhances BMSC osteogenesis via the downregulation of miR-339-3p. MiR-503-5p has been found to promote bone formation by targeting the negative osteogenic regulator SMURF1 within BMSCs [39]. Our results suggest SDF-1 decreases SMURF1 by upregulating miR-503-3p. Let-7f-5p has been found to promote cell survival in various cell lines by targeting caspase-3 and caspase-9 while increasing levels of anti-apoptotic factors Bcl-2 and Bcl-xL [40, 41]. Taken with our results, this indicates an increase in cell survival of BMSCs mediated by SDF-1, consistent with previous reports [9]. We also identified several novel miRNAs with no prior known functions. Bioinformatic analysis revealed a considerable number of these miRNAs are involved in stem cell homing and commitment to musculoskeletal lineage (Table 2).

To better understand the collective effect of differentially expressed miRNA, KEGG pathway annotation and GO enrichment analysis was conducted. KEGG annotation identified several cellular pathways potentially affected, with fatty acid biosynthesis, thyroid hormone signaling, and mucin-type O-glycan biosynthesis pathways most relevant. Fatty acids and their metabolites have been well linked to stem cell proliferation and differentiation [42]. Both saturated and unsaturated fatty acids have differential effects on BMSC survival [43]. Furthermore, polyunsaturated fatty acids have been found to modulate proliferation, migration, and differentiation of various tissue-specific sources of MSCs [44-46]. GO analysis identified several cellular processes (TLR signaling, mitotic cell cycle, post-translation protein modification, extracellular matrix disassembly, apoptotic signaling pathway) that are vital in BMSC survival, proliferation, migration, and differentiation.

SDF-1 signaling has been recognized for its chemotactic and osteogenic function in BMSCs. Our findings further solidify its expanded role in BMSC biology.

We have elucidated several miRNAs that are upregulated and downregulated in response to SDF-1. Among these miRNAs, we correlated some of their functions with roles identified in existing literature, and we characterized novel miRNAs concerning their potential targets in BMSCs (Table 2). We also determined key signaling pathways that are collectively influenced by the differentially expressed miRNAs. Limitations to our study do exist. Our study only utilized BMSCs in tissue culture and thus needs to be validated *in vivo*. Moreover, our study only utilized one concentration and one time period for SDF-1 treatment. It is valuable to understand the dose and time-dependent effects of SDF-1 *in vitro* and *in vivo* models. Overall, our study indicated that SDF-1 induces some of its osteogenic and chemotactic effects by regulating miRNAs in human BMSCs.

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