Original Paper

Identification and Characterization of Long Non-Coding RNAs in Osteogenic Differentiation of Human Adipose-Derived Stem Cells

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Key Words

Long noncoding RNAs • Osteogenesis • Adipose-derived stem cells • Microarray

Abstract

Background/Aims: Long noncoding RNAs (lncRNAs) play important roles in stem cell differentiation. However, their role in osteogenesis of human adipose-derived stem cells (ASCs), a promising cell source for bone regeneration, remains unknown. Here, we investigated the expression profile and potential roles of lncRNAs in osteogenic differentiation of human ASCs. **Methods:** Human ASCs were induced to differentiate into osteoblasts *in vitro*, and the expression profiles of lncRNAs and mRNAs in undifferentiated and osteogenic differentiated ASCs were obtained by microarray. Bioinformatics analyses including subgroup analysis, gene ontology analysis, pathway analysis and co-expression network analysis were performed. The function of lncRNA *H19* was determined by *in vitro* knockdown and overexpression. Quantitative reverse transcription polymerase chain reaction was utilized to examine the expression of selected genes. **Results:** We identified 1,460 upregulated and 1,112 downregulated lncRNAs in osteogenic differentiated human ASCs as compared with those of undifferentiated cells (Fold change ≥ 2.0 , $P < 0.05$). Among these, 94 antisense lncRNAs, 85 enhancer-like lncRNAs and 160 lincRNAs were further recognized. We used 12 lncRNAs and 157 mRNAs to comprise a coding-non-coding gene expression network. Additionally, silencing of *H19* caused a significantly increase in expression of osteogenesis-related genes, including *ALPL* and *RUNX2*, while a decrease was observed after *H19* overexpression. **Conclusion:** This study revealed for the first time the global expression profile of lncRNAs involved in osteogenic differentiation of human ASCs and provided a foundation for future investigations of lncRNA regulation of human ASC osteogenesis.

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Introduction

Osteoporosis is a common, age-related skeletal disease characterized by the loss of bone mass and deficits in bone quality, leading to low bone strength and increased risk of fractures [1]. Current treatments for osteoporosis are predominantly focused on pharmacological drugs, although transplantation of mesenchymal stem cells (MSCs) has become an attractive approach during the past decade [2-4]. Bone marrow mesenchymal stem cells (BMSCs) have been considered an ideal cell source for bone regeneration for years; however, increasing evidence suggests that the proliferative ability and osteogenic differentiation capability of BMSCs decrease with age or under other osteoporotic conditions. Meanwhile, this is always accompanied by the generation of additional marrow adipose tissue [5-7]. Recently, adipose-derived stem cells (ASCs) have been studied extensively as an alternative cell source to BMSCs due to their widespread sources, easy access and multipotency [3, 8]. More importantly, ASCs isolated from aged or osteoporotic humans and animals maintain their osteogenic differentiation capability, making them more promising for autologous cell-based therapy for osteoporosis [9-11]. Their osteogenic differentiation is regulated at transcriptional, post-transcriptional and epigenetic levels. However, the precise molecular mechanisms related to osteogenesis require further investigation.

Long noncoding RNAs (lncRNAs) are a novel subset of noncoding RNAs that are over 200 nucleotides in length. Increasing evidence shows that lncRNAs are involved in diverse physiological processes, as well as pathological processes, via different mechanisms [12, 13]. Interestingly, numerous lncRNAs are restricted to specific cell lineages, indicating their potential roles in the determination of cell fate [14]. Previous studies suggested that lncRNAs play important roles in MSC differentiation, including several publications that investigated the expression and potential role of lncRNAs in the osteogenesis of human BMSCs, mouse C3H10T1/2 MSCs and other types of MSCs [15-22]. Most recently, one study demonstrated an inhibitory role of lncRNA-MIR31HG in osteogenic differentiation of human ASCs under an inflammatory microenvironment [23]. However, there remains a lack of data concerning the global expression and potential function of lncRNAs in the differentiation of human ASCs into osteoblasts.

In this study, we compared the expression profiles of lncRNAs between osteogenic differentiated human ASCs and undifferentiated cells by microarray analysis, followed by bioinformatics analyses and *in vitro* experiments used to explore the potential roles of these differentially expressed lncRNAs in osteogenesis. These findings provided a primary foundation for future investigations of lncRNA regulation of ASC osteogenesis.

Materials and Methods

Cell culture

The use of human cells was approved by the Ethics Committee of The First Affiliated Hospital of Sun Yat-sen University, China ([2013]C-260). Human ASCs derived from the fat tissue of different healthy donors (aged 18-45-years, $n = 4$) were purchased from Cyagen (Suzhou, China). ASCs were maintained in OriCell™ Human MSC Growth Medium containing 10% MSCs-Qualified Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin and 1% Glutamine (Cyagen, China) under 37°C, 5% CO₂ conditions. The medium was replaced every 3 days, and when cells reached 80% confluence, they were passaged using 0.25% trypsin/EDTA (Gibco, USA).

Osteogenic differentiation of ASCs

Third passage human ASCs were seeded into six-well plates at a density of 2×10^4 cells/cm², and cells were cultured in standard growth medium until they reached 50% to 70% confluence. The medium was then changed to OriCell™ human ASC Osteogenic Differentiation Medium (Cyagen) containing 10% FBS, 1% Penicillin-Streptomycin, 10 mM L-glutamine, 50 μM L-ascorbic acid, 10 mM beta-glycerophosphate and 100 nM dexamethasone. The differentiation medium was carefully replaced every 3 days. After a 14-

day induction, cells were harvested for RNA analysis and meanwhile stained with alizarin red (Cyagen) to measure mineralization by microscopy according to manufacturer's instructions. Undifferentiated human ASCs were used as controls. For detection of dynamic expression of lncRNA *H19*, human ASC cultures were induced to osteogenic differentiation for 21 days, and RNA was isolated from the cells at days 0, 7, 14, and 21 for quantitative real-time reverse transcription PCR (qRT-PCR).

Transient transfection

The *H19* siRNA and scramble negative control siRNA were purchased from RiboBio (Guangzhou, China). The target sequences of the three siRNAs against *H19* are as follows: si-H19-1, GACACCATCGGAACAGCAG; si-H19-2, CCCGTCCCTTCTGAATTGA; si-H19-3, CGTGACAAGCAGGACATGA. The *H19* overexpression plasmid pEX-3-H19 was constructed by cloning the entire *H19* coding fragment into the XhoI/EcoRI site of the pEX-3 vector. The pEX-3-H19 plasmid and pEX-3 empty plasmid were designed and synthesized by GenePharma (Shanghai, China). Human ASCs cultured in antibiotic-free growth medium were allowed to grow to 60% to 70% confluence prior to transfection with 50 nM siRNA or 50 nM plasmid using Lipofectamine® 2000 Transfection Reagent (Invitrogen, USA) according to manufacturer's instructions. After 48 hours, cells were induced to osteogenic differentiation for 3 days as above and total RNA was extracted for qRT-PCR analysis.

RNA isolation and quality control

Total RNA from human ASCs was extracted using Trizol reagent (Invitrogen) according to the instructions of manufacturer. A NanoDrop ND-1000 spectrophotometer was used for measuring RNA quantity and quality, and agarose gel electrophoresis was used to examine RNA integrity.

Microarray hybridization

Human lncRNA Microarray V3.0 (Arraystar, USA) used in this study is a commercial array (Agilent Design ID: Agilent-045997) designed for the global profiling of human lncRNAs and mRNAs. Approximately 30,586 lncRNAs and 26,109 mRNAs can be detected using this microarray. According to the most highly-respected public transcriptome databases, including Refseq, UCSC knowngenes, Genecode and landmark publications, lncRNAs were carefully constructed on the microarray. Each individual transcript was identified by a specific exon or splice junction probe. Negative probes and positive probes were also included in the array for hybridization quality control.

Sample preparation and array hybridization were modified from the protocol for Agilent One-Color Microarray-Based Gene Expression Analysis (Agilent Technology, USA) and utilized the Arraystar Flash RNA Labeling Kit (Arraystar, USA). Briefly, purified RNA was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts by utilizing a mixture of oligo dT and random primers to avoid 3' bias. After measuring concentration and specific activity, the fluorescent cRNAs were hybridized onto the lncRNA array slide, followed by the arrays being washed, fixed and scanned using a DNA microarray scanner (Agilent Technology). Feature Extraction Software v11.0.1.1 (Agilent Technology) was used to extract raw data from the acquired array images.

qRT-PCR

For detection of mRNAs and lncRNAs, 1 µg of total RNA was reversely transcribed to cDNA using random hexamer primer and SuperScript III Reverse Transcriptase (Invitrogen). Then, qRT-PCR was performed on a Bio-Rad CFX96 System (Bio-Rad, USA) using SYBR Premix Ex Taq™ II (Takara, Japan) according to manufacturer's instructions. The reaction volume contained 10 µL SYBR Premix Ex Taq II (2x), 0.8 µL of forward primer and reverse primer (10 µM), 2 µL template cDNA and 6.4 µL dH₂O to a final volume of 20 µL. The reaction conditions were as follows: denaturation at 95°C for 5 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Melting curve analysis was performed from 65°C to 95°C with an increment of 0.5°C. All qRT-PCR reactions were performed in triplicate. *GAPDH* expression was used to normalize expression of lncRNAs and mRNAs. The 2^{-ΔΔCt} method was used to calculate relative gene expression. Primers for qRT-PCR were designed with Primer 5.0 and BLAST was used to determine individual specificity. Amplification efficiency was determined by standard curve for each primer. The templates for standard curve were the PCR products which were synthesized from cDNA of undifferentiated human ASCs and purified via agarose gel electrophoresis. Ten 10-fold serial dilutions of templates were prepared and 1/ (dilution factor) was used as a concentration. Standard curve was obtained by plotting Ct value on the X-axis

and log(template concentration) on the Y-axis. The amplification efficiency was calculated as $10^{-1/\text{slope}-1}$ (for all online suppl. material, see www.karger.com/doi/10.1159/000478751, Table S1).

Bioinformatics analysis and statistical analysis

After quantile normalization of the raw data, lncRNAs and mRNAs flagged as “Present” or “Marginal” in at least four out of eight samples were used for subsequent data analysis with GeneSpring GX v12.1 software package (Agilent Technology). Differentially expressed lncRNAs and mRNAs between two samples in each pair of cells were identified through Fold Change filtering (Fold Change ≥ 2.0). The significance of differences in expression levels between the two groups was evaluated by paired *t*-test. And *P*-value filtering ($P < 0.05$) was used to identify significant differentially expressed transcripts. Hierarchical Clustering and combined analysis were performed using homemade scripts. Euclidean distance and average linkage rule were used for clustering. To explore the potential functions of the dysregulated lncRNAs, classification and subgroup analysis was performed. Both gene ontology (GO) analysis and pathway analysis were carried out to determine the roles of the differentially expressed mRNAs. Fisher’s exact test was used to test the significance of GO terms and Pathway identifiers enrichment in the differentially expressed gene list. Pearson’s product-moment correlation coefficient was used to assess correlations between the expression of lncRNAs and co-expressed coding transcripts. Those dysregulated lncRNAs and mRNAs exhibiting Pearson’s coefficients >0.99 were selected to construct a coding-non-coding gene co-expression network (CNC network) using Cytoscape. Microarray and bioinformatics analyses were performed by KangChen Bio-tech (Shanghai, China). For qRT-PCR validation experiment, Spearman’s correlation coefficients were used to correlate the fold changes measured by qPCR and microarray. In cases of multiple-group analysis, one-way analysis of variance (ANOVA) followed by Dunnett-*t* test was conducted. A value of $P < 0.05$ was considered statistically significant.

Results

Differentially expressed lncRNAs in osteogenic differentiated human ASCs

After a 14-day osteogenic induction, alizarin red staining indicated that calcium deposition was positive, and the expression of osteogenesis-related genes, including collagen type 1 alpha 1 chain (*COL1A1*), alkaline phosphatase liver/bone/kidney (*ALPL*) and runt-related transcription factor 2 (*RUNX2*) was significantly increased in differentiated cells as compared with undifferentiated human ASCs (Fig. 1). These results confirmed osteogenic differentiation.

We identified a total of 17,246 lncRNAs expressed in human ASCs according to microarray analysis. Hierarchical clustering analysis showed homogeneous lncRNA expression profiles within the same group and distinct expression profiles between the two groups (Fig. 2A). Comparing the osteogenic differentiated ASCs to undifferentiated cells, 1,460 lncRNAs were upregulated and 1,112 lncRNAs were downregulated (Fold change ≥ 2.0 , $P < 0.05$) (Fig. 2B).

Fig. 1. Analysis of osteogenic differentiation of human ASCs. Alizarin red staining was performed for undifferentiated human ASCs (A) and differentiated cells after a 14-day osteogenic induction (B) (100x). (C) Osteogenesis-related genes expression was determined by qRT-PCR. Gene expression is presented as relative fold change by normalized to *GAPDH* expression and to undifferentiated cells control values using the $2^{-\Delta\Delta Ct}$ method. Values represent the mean $\pm$ SD of four different pairs of samples. * $P < 0.05$, ** $P < 0.01$ vs. undifferentiated ASCs as determined by paired *t*-test.

Fig. 2. LncRNA and mRNA expression profiles based on microarray data. Hierarchical Clustering results show a distinguishable lncRNA (A) and mRNA (C) expression profile between the two groups and homogeneity within the same group. “Value” stands for the gene expression. The number “5” represents a relatively low expression level and is represented by the color green, whereas the number “9” represents a relatively high expression level and is represented by the color red in cluster maps. “Count” represents the number of genes that exhibited the same expression level. A total of 17,246 lncRNAs and 21,591 mRNAs were detected in human ASCs. Volcano Plots of lncRNAs (B) and mRNAs (D) in differentiated cells versus undifferentiated ASCs. Red squares in the plots represent the differentially expressed transcripts with statistical significance (Fold change ≥ 2.0 , $P < 0.05$). Compared to undifferentiated ASCs, 2,572 lncRNAs and 2,586 mRNAs were significantly dysregulated.

Fig. 3. Classification analysis of dysregulated lncRNAs. (A) LncRNAs were classified into six categories including intergenic, natural antisense, intronic antisense, intron sense-overlapping, exon sense-overlapping and bidirectional according to the relative position between lncRNAs and their nearby genes. The graph showed the

numbers of differentially expressed lncRNAs in each category. (B) Chromosome distribution analysis shows the numbers of both upregulated and downregulated lncRNAs in different chromosomes.

Among these, the expression of 88 lncRNAs were altered >10 -fold in each pair of samples, including 55 upregulated and 33 downregulated genes. Specifically, the most upregulated lncRNA was *uc002lbc.1* (Fold change: 84.37) and the most downregulated was *uc.247+* (Fold change: 82.20) (Table 1). The microarray data was submitted to the Gene Expression Omnibus (Accession number: GSE89330).

Differentially expressed mRNAs in osteogenic differentiated human ASCs

A total of 21,591 mRNAs were identified by microarray analysis, with the differentially expressed mRNA profile revealing that 946 mRNAs were upregulated and 1,640 mRNAs were downregulated during osteogenic differentiation (Fold change ≥ 2.0 , $P < 0.05$) (Fig.

2C-D). Of these, SLIT and NTRK like family member 1 (*SLITRK1*) was the most up-regulated mRNA and Matrix metalloproteinase 3 (*MMP3*) was most downregulated (Table 2).

GO analysis showed that the upregulated mRNAs were highly enriched in extracellular matrix organization (ontology: biological process), extracellular space (ontology: cellular component) and peptidase regulator activity (ontology: molecular function), and that the downregulated mRNAs were highly enriched in system development (ontology: biological process), cell periphery (ontology: cellular component) and protein binding (ontology: molecular function) (see supplementary material, Fig. S1). Pathway analysis revealed that the most highly enriched pathway for upregulated mRNAs was the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway and the most highly enriched pathway for downregulated mRNAs was the steroid biosynthesis network (see supplementary material, Fig. S2). Notably, there is mounting evidence suggesting that the PI3K/Akt pathway is involved in osteogenesis [24-26].

Classification and subgroup analysis of differentially expressed lncRNAs

To obtain general signatures of dysregulated lncRNAs, we analyzed up-regulated and downregulated lncRNAs according to classification and chromosome distribution. Based on the relative position of lncRNAs and the nearby coding genes on chromosomes, lncRNAs were divided into six categories including intergenic, natural antisense, intronic antisense, intron sense-overlapping, exon sense-overlapping and bidirectional. In our microarray data, the majority (60.34%) of the differentially expressed

Table 1. Top 15 differentially expressed lncRNAs (differentiated vs. undifferentiated)

Gene Symbol	Genome Relationship	Fold change	P-value
AK126075	natural antisense	+84.37	0.000031
MACC1-AS1	intronic antisense	+63.27	0.000032
XLOC_013994	intergenic	+53.65	0.00000087
XLOC_001314	intergenic	+47.14	0.000012
SAA3P	pseudogene	+36.57	0.000000052
AC012065.7	intergenic	+36.49	0.000011
BC040860	intergenic	+35.69	0.00021
XLOC_009537	intergenic	+34.39	0.000068
chr4:72215925-72232750	intergenic	+29.89	0.000015
XLOC_001453	intergenic	+28.29	0.000029
AK055386	intergenic	+28.13	0.000052
RP11-46309.5	natural antisense	+26.52	0.0000026
PTCSC3	intergenic	+26.42	0.0000026
RP11-93L9.1	intergenic	+26.23	0.00019
AC226119.4	pseudogene	+25.54	0.0000039
uc.247	intergenic	-82.20	0.00052
CTD-2514K5.2	bidirectional	-54.85	0.0000062
XLOC_007489	intergenic	-53.13	0.00000011
XLOC_008619	intergenic	-41.20	0.0000092
RP11-43118.1	exon sense-overlapping	-34.12	0.00027
RP11-101C21.1	intergenic	-26.62	0.0000024
RP3-399L15.3	intronic antisense	-26.15	0.000022
RP11-64D22.2	pseudogene	-23.54	0.00087
HLA-DQB	intergenic	-21.67	0.000074
WI2-237311.2	exon sense-overlapping	-19.27	0.0000058
RP11-432B10.1	intergenic	-18.24	0.000012
LINC00565	intergenic	-17.73	0.000013
chr13:52409200-52419600	intergenic	-17.55	0.00039
RP11-91I20.3	intergenic	-16.30	0.000086
XLOC_010397	intergenic	-15.67	0.0000027

Table 2. Top 15 differentially expressed mRNAs (differentiated vs. undifferentiated)

Gene Symbol	Seqname	Fold change	P-value
SLITRK1	NM_052910	+628.32	0.00000026
CORIN	NM_006587	+181.07	0.0000042
BZW2	NM_014038	+169.84	0.0000027
FAM153B	ENST00000253490	+125.82	0.000017
C3orf58	NM_173552	+89.94	0.0000020
MMP7	NM_002423	+72.25	0.000084
STEAP4	NM_024636	+57.91	0.00000028
CIDEA	NM_022094	+57.56	0.00000090
MRPS18B	NM_014046	+54.39	0.00022
FRZB	NM_001463	+53.18	0.0000016
ADH1A	NM_000667	+48.80	0.00000048
OR51A2	ENST00000380371	+48.12	0.00000023
GPR110	NM_025048	+43.57	0.000068
LPL	NM_000237	+42.37	0.000011
SAA2	NM_030754	+39.70	0.0000052
MMP3	NM_002422	-139.40	0.0000042
PKIB	NM_032471	-96.58	0.0000028
FAM181A	NM_138344	-86.68	0.00046
KLK8	ENST00000320838	-84.958	0.00000084
PI16	ENST00000373674	-70.058	0.000012
F7	NM_000131	-66.79	0.000014
MMP10	NM_002425	-50.63	0.00000097
SPP2	NM_006944	-50.30	0.0000027
FCN2	ENST00000291744	-49.22	0.00022
GAGE2B	ENST00000381725	-43.81	0.0000012
OR7A10	ENST00000248058	-43.52	0.0000025
SLPI	NM_003064	-42.37	0.000038
MFN2	NM_001127660	-41.49	0.0000013
SLC39A13	NM_152264	-40.05	0.00000030
LRSAM1	NM_001005374	-39.15	0.000042

Fig. 4. Comparison of the expression of selected lncRNAs between microarray and qRT-PCR results. Ten lncRNAs (five upregulated and five downregulated) from microarray data were selected for validation by qRT-PCR in four paired undifferentiated and osteogenic differentiated human ASCs. For microarray analysis, the raw intensity of genes was quantile normalized. For qRT-PCR analysis, gene expression was normalized to *GAPDH* expression and calculated using the $2^{-\Delta\Delta Ct}$ method. The heights of the columns in the graph represent the mean fold changes (differentiated/undifferentiated) of expression. Paired *t*-test was used for comparison between undifferentiated and differentiated conditions. The expression of these lncRNAs as measured by microarray was significantly different between undifferentiated and differentiated cells ($P < 0.001$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicated differentiated vs. undifferentiated samples in qRT-PCR results.

Table 3. Top 15 differentially expressed antisense lncRNAs and their nearby mRNAs

Seqname	Fold change	Genome Relationship	Nearby Gene	Fold change
ENST00000563280	+26.52	natural antisense	FOXC2	+8.74
NR_037595	+11.36	natural antisense	HHIP	+3.82
AA321097	-10.089	intronic antisense	FOXP1	-5.10
ENST00000438380	-10.01	intronic antisense	NR6A1	-3.74
ENST00000444873	-9.43	intronic antisense	BCL11A	-23.11
ENST00000503242	-9.02	intronic antisense	NREP	-3.18
ENST00000439592	+8.84	intronic antisense	DMD	+3.19
NR_026882	-8.48	intronic antisense	C11orf9	-2.07
ENST00000435932	-8.39	natural antisense	ELN	-2.28
NR_037884	+8.05	natural antisense	ADH1A	+48.80
ENST00000418335	-7.96	natural antisense	DPP4	-3.26
ENST00000534431	-7.19	natural antisense	KIAA1549L	-3.03
ENST00000455081	-7.02	intronic antisense	MAPRE3	-3.46
ENST00000509939	+6.98	natural antisense	ADH1A	+48.80
ENST00000444923	+6.63	intronic antisense	VWA5B1	-2.74

Table 4. Top 15 differentially expressed enhancer-like lncRNAs and their nearby mRNAs

Seqname	Fold change	Genome Relationship	Nearby Gene	Fold change
TCONS_00018338	-41.20	downstream	TCF7L2	-2.05
NR_034119	+18.98	downstream	EFNB2	+8.19
ENST00000447028	-11.81	upstream	SLC22A12	-2.64
ENST00000432961	-10.84	upstream	HES4	+2.15
ENST00000417705	-9.98	upstream	HES4	+2.15
ENST00000450938	+9.21	downstream	TLR4	+2.88
ENST00000457833	-8.85	downstream	THBS2	-3.61
ENST00000447481	-8.51	upstream	ENPP1	+3.91
ENST00000421509	+7.05	upstream	TLR4	+2.88
ENST00000436510	-6.32	downstream	IER5L	-2.56
ENST00000443783	-6.09	upstream	ARHGAP8	-2.35
ENST00000427208	-5.68	upstream	SULT1C2	-5.86
ENST00000414039	-5.63	upstream	F3	-2.42
ENST00000449119	+5.42	upstream	FAM150B	-3.11
ENST00000425666	-5.40	upstream	DIRAS2	-2.05

lncRNAs were intergenic lncRNAs (Fig. 3A). The upregulated and downregulated lncRNAs were dispersed in various chromosomes (Fig. 3B).

Table 5. Top 15 differentially expressed lincRNAs and their nearby mRNAs

Seqname	Fold change	Genome Relationship	Nearby Gene	Fold change
TCONS_00016098	-53.13	upstream	NR4A3	+3.38
TCONS_00018338	-41.20	downstream	TCF7L2	-2.05
TCONS_00003663	+28.29	upstream	SIX2	+2.41
TCONS_00024702	+19.66	upstream	BEAN1	-31.45
TCONS_00021794	-15.67	upstream	PCDH8	-4.18
TCONS_00005994	-14.50	upstream	CX3CR1	+5.96
TCONS_00025773	-12.70	upstream	TIMP2	-2.07
TCONS_00020439	+11.61	downstream	NCKAP1L	+2.35
TCONS_00013698	-8.66	downstream	SCIN	-2.53
TCONS_00008419	-8.58	downstream	ABLIM2	-2.53
TCONS_00008650	+8.39	downstream	SAP30	+2.29
TCONS_00018417	+7.79	upstream	AKR1E2	-2.15
TCONS_00011165	+7.78	upstream	BTN3A1	-2.02
TCONS_00011825	-7.73	upstream	OPN5	-6.17
TCONS_00001811	+7.53	upstream	BATF3	+2.12

Fig. 5. Construction of CNC network. Triangular nodes represent lncRNAs and circular nodes represent mRNAs. The sizes of nodes represent degrees of connectivity, with larger sizes indicating greater degrees. Solid lines represent positive correlations and dashed lines represent negative correlations between lncRNAs and mRNAs.

Differentially expressed lncRNAs were further classified into subgroups including antisense lncRNAs, enhancer-like lncRNAs and long intergenic non-coding RNAs (lincRNAs). The associated mRNAs were also identified to help predict the potential function of these dysregulated lncRNAs. Over 30% of human transcripts have corresponding antisense lncRNAs and these lncRNAs exert biological functions by regulating their sense mRNAs [27]. Therefore, we identified 94 dysregulated antisense lncRNAs, as well as differentially expressed sense mRNAs, from our microarray data (Table 3). Additionally, according to a previous study [28], 85 enhancer-like lncRNAs were found to be differentially expressed during osteogenesis of human ASCs and the associated mRNAs were identified to explore the putative targets of these lncRNAs (Table 4). Moreover, based on Cabili's work [29], expression of 160 lincRNAs changed significantly at 14 days after osteogenic induction and the differentially expressed nearby coding genes were subsequently identified (Table 5).

Fig. 6. Expression and function of H19 during osteogenic differentiation of human ASCs. A. Human ASCs were induced towards osteoblast differentiation for 7, 14 and 21 days, and expression of H19 was determined by qRT-PCR and compared to that at day 0. B-E. Human ASCs were transfected with scramble siRNA (NC) or H19 siRNA (si-H19-1, si-H19-2 and si-H19-3) or pEX-3 empty plasmid (pEX-3) or pEX-3-H19 for 48 hours, followed by induction of osteogenic differentiation for 3 days. Gene expression of H19 (B and C), and COL1A1, ALPL and RUNX2 (D and E) were measured by qRT-PCR and compared to that of NC or pEX-3. Gene expression was normalized to GAPDH expression and calculated by the $2^{-\Delta\Delta Ct}$ method. Data was presented as mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ represent significant differences relative to control groups as determined by one-way ANOVA followed by Dunnett-t test or paired t-test.

qRT-PCR validation

In order to validate our microarray results, the expression of five upregulated lncRNAs (SAA3P, LOC284080, XLOC_001453, XLOC_011980 and HHIP-AS1) and five downregulated lncRNAs (XLOC_007489, CTC-564N23.3, XLOC_010397, XLOC_002619 and LINC00638) with fold changes >10.0 in the microarray profile were examined by qRT-PCR in four osteogenic differentiated human ASC cultures and paired undifferentiated cultures. Although the qRT-PCR results failed to confirm the expression pattern of XLOC_001453 and XLOC_002619, results for the other eight lncRNAs exhibited similar trends as those observed in the microarray data. Furthermore, the changes determined by qRT-PCR were significant ($P < 0.05$) and the fold changes measured by qRT-PCR correlated with that from microarray ($r = 0.6121$, $P = 0.0334$), demonstrating consistency between the qRT-PCR and microarray results (Fig. 4).

Table 6. List of core regulatory lncRNAs in CNC network

Seq name	Chromosome	Str-and	Start	End	Fold change	Regulation	P-value
TCONS_00016098	chr9	+	102648727	102657226	-53.13	down	0.00000011
TCONS_00021794	chr13	+	53631290	53650848	-15.67	down	0.0000026
ENST00000512213	chr5	+	76382625	76401633	-15.11	down	0.00000090
TCONS_00022189	chr13	+	64201007	64209643	-11.50	down	0.0000031
ENST00000533267	chr11	-	18134172	18137625	+36.57	up	0.00000052
ENST00000531617	chr17	-	72208812	72209447	-54.85	down	0.0000062
ENST00000563280	chr16	-	86598750	86601367	+26.52	up	0.0000026
NR_024396	chr14	+	105287537	105290055	-15.24	down	0.0000059
ENST00000581971	chr18	-	73556879	73580207	+12.40	up	0.000018
TCONS_00029072	chr21	-	9826937	9827584	+53.65	up	0.00000087
TCONS_00025773	chr17	-	76613497	76614757	-12.70	down	0.00000082
ENST00000561386	chr15	+	62022048	62023588	+23.36	up	0.0000055

Construction of CNC network

In order to explore interactions among differentially expressed lncRNAs and mRNAs, a CNC network was constructed based on 55 upregulated and 33 downregulated lncRNAs exhibiting > 10-fold changes in all four pairs of samples. Pearson's correlation for each differentially expressed lncRNA and its paired mRNA was calculated and those transcripts with coefficients > 0.999 were chosen to build the network. In the CNC network, one lncRNA was able to correlate with different mRNAs, and vice versa. Transcripts exhibiting correlations with larger numbers of genes displayed greater degrees of connectivity and were considered as core regulatory factors. The lncRNAs with degrees ≥ 40 were further selected to construct a more visible network. The final CNC network comprised 12 lncRNAs and 157 mRNAs, with 645 network pairs (Fig. 5). Both positive and negative correlations between lncRNAs and mRNAs were present, with detailed information regarding core regulatory lncRNAs shown in Table 6.

Effects of lncRNA H19 on human ASCs osteogenesis

The expression of *H19*, which is upregulated and exhibits functions involved in osteogenic differentiation of human BMSCs [16, 18, 20], was decreased (Fold change: 3.81) in osteogenic differentiated ASCs as compared with undifferentiated cells according to our microarray results. To validate the differences in these results and explore the possible function of *H19* in ASCs, we measured dynamic *H19* expression during osteogenic processes of human ASCs and examined the effect of *H19* knockdown on osteogenesis of human ASCs. Consistent with our microarray results, expression of *H19* was decreased significantly at days 7, 14 and 21 when compared with day 0 (Fig. 6A). Additionally, qRT-PCR results confirmed significant inhibition of *H19* expression following siRNA transfection, and significant enhancement of *H19* expression following transfection with pEX-3-H19 plasmid (Fig. 6B, C). Furthermore, knockdown of *H19* resulted in significantly increased expression of *RUNX2* and *ALPL*, but not *COL1A1*, during osteogenic induction of human ASCs (Fig. 6D), whereas a significant decrease in expression of *RUNX2* and *ALPL* was observed after *H19* overexpression (Fig. 6E).

Discussion

An understanding of the molecular mechanisms associated with osteogenesis is fundamental to maintaining osteogenic capacity and realizing the therapeutic potential of human ASCs. Although the functional roles of transcriptional factors, microRNAs and epigenetic factors in osteogenesis have been extensively studied [30-34], few studies focused on the role and the underlying mechanisms of lncRNAs in this biological process [35]. Moreover, there is no report regarding genome-wide expression and function of lncRNAs in osteogenesis of ASCs. In the current study, we revealed the global expression profile of lncRNAs during osteogenic differentiation of human ASCs by microarray and predicted the potential function of the differentially expressed lncRNAs using bioinformatics analysis. In addition, our experimental evidence indicated that lncRNAs may play an important role in osteogenesis of human ASCs.

We selected 10 lncRNAs for qRT-PCR validation of the microarray data. It was noticed that there was variations between lncRNA expression detected by qRT-PCR versus microarray. One explanation may be the inherent pitfalls in the differences between the two methods, including the different normalization procedures and priming methods used for reverse transcription [36]. However, expression of most of the lncRNAs selected for qRT-PCR validation showed similar trends as those observed in the microarray results. Moreover, statistical analysis indicated that the differences in lncRNA expression were significant between differentiated and undifferentiated human ASCs and the fold changes measured by qRT-PCR significantly correlated with those from microarray. Therefore, the qRT-PCR results confirmed the reliability of the microarray data.

Expression of some lncRNAs is altered during osteogenic differentiation of other kinds of MSCs [18, 21, 22]. Wang et al. [18] showed that 687 lncRNAs were significantly upregulated and 519 lncRNAs were significantly downregulated during osteogenesis of human BMSCs. Compared with those findings, we observed much more lncRNAs differentially expressed during osteogenesis of human ASCs. This might be explained by the different cell types used between the two studies, given that lncRNA expression is cell specific [14]. Another explanation could be the differences in microarray platforms utilized in the two studies. That used in this study included 30,586 lncRNAs and detected 17,246 lncRNAs expressed in human ASCs. Moreover, some previous studies investigated the function of particular lncRNA in osteogenesis. It was reported that suppression of lncRNA-ANCR could promote osteogenic differentiation of BMSCs [17]. However, we observed that expression of lncRNA-ANCR decreased slightly and without significance (Fold change: 1.14, $P = 0.080$) during osteogenesis of human ASCs according to our microarray results. Whether lncRNA-ANCR also plays an inhibitory role during osteogenic differentiation of ASCs requires further research. Recently, the Zhou group [23] reported that lncRNA-MIR31HG was induced by nuclear factor (NF)- κ B activation in human ASCs and that lncRNA-MIR31HG, in turn, contributed to NF- κ B activation, which further inhibited bone formation under inflammatory conditions. More importantly, in our current study, we found that lncRNA-MIR31HG was significantly downregulated (Fold change: 2.31, $P = 0.000066$) in osteogenic differentiated human ASCs when compared with undifferentiated cells. Therefore, these results suggested that during normal osteogenic differentiation of ASCs, downregulation of lncRNA-MIR31HG may be required, whereas in an inflammatory environment, MIR31HG may be pathologically upregulated to suppress bone formation. However, more specific experiments are required to test this hypothesis.

H19, one of the most well-known imprinted genes, encodes a 2.3-kb polyadenylated RNA that does not produce any known protein [37]. Several studies reported that *H19* was upregulated during osteogenesis in BMSCs [16, 18, 20]. In addition, *H19* promotes osteogenic differentiation of BMSCs via the transforming growth factor β -1/Smad3/histone deacetylase pathway, with microRNA (miR) -675 derived from *H19* partially responsible for this pro-osteogenic effect [16]. However, another study showed that instead of promoting osteogenesis, miR-675-5p negatively regulates osteogenesis of BMSCs. The authors argued that this might be due to the negative-feedback loop associated with *H19* and miR-675-5p. Meanwhile, they revealed another mechanism for *H19* in the enhancement of osteogenic differentiation of BMSCs that concerned *H19*-mediated activation of Wnt signaling by acting as a microRNA sponge for miR-141 and miR-22, both of which negatively regulated β -catenin [20]. Here, we found that *H19* expression decreased significantly during osteogenic differentiation of human ASCs. To the best of our knowledge, only one study showed reduced expression of *H19* in ASCs as compared with BMSCs [38]; however, no report exists regarding *H19* expression during osteogenesis of ASCs. This discrepancy might be explained by evidence showing that *H19* expression is regulated in a tissue- and cell-specific manner during embryogenesis [39]. However, the specific role of *H19* in ASC osteogenesis has not been studied and it might be different from that of BMSCs, given its different expression patterns observed between the two cell types. In the present study, we showed that at early stage of osteogenesis of human ASCs, knockdown of *H19* led to upregulation of osteogenesis-

related genes, including *RUNX2* and *ALPL*, while overexpression of *H19* played a contrasting role. These findings suggested a negative regulatory role for *H19* in early osteogenesis of human ASCs. It was reported that *H19* also served as a competing endogenous RNA for the let-7 family of microRNAs and its deletion resulted in overexpression of let-7 [40]. Moreover, let-7 has been showed to enhance osteogenesis and suppress adipogenesis by negatively regulating high mobility group AT hook 2 (*HMGA2*) in ASCs [41]. Therefore, we attempted to determine whether *H19* could act as a molecular sponge for let-7 to regulate the function of *HMGA2* in ASCs. However, manipulation of *H19 in vitro* has no significant effects on expression of let-7 or *HMGA2* during early stage of osteogenic differentiation of human ASCs (data not shown), which may imply that let-7 and *HMGA2* are not targets of *H19* during ASC osteogenesis. Therefore, further investigation to determine the direct targets and detailed mechanism of *H19* during osteogenesis of human ASCs is required.

There were some limitations in the present study. First, we only chose cells after a 14-day osteogenic induction as osteogenic differentiated cells. This might result in losing information about the dynamic pattern of lncRNA expression over the course of the entire osteogenic process. However, this level of testing would be costly and increase the difficulty in comparing the gene expression profiles of different time points using microarray analysis. On the other hand, comparison of differentiated and undifferentiated stem cells has been widely used to investigate the differentiation process. Moreover, we monitored the expression of *H19* at different time points during osteogenic induction and will measure the dynamic expression patterns of other candidate lncRNAs in future studies. Another limitation was that we did not identify the direct target of *H19*. Nonetheless, we have provided evidence of the potential role of *H19* in early osteogenesis of human ASCs. Further studies will be conducted to investigate the detailed mechanism. Finally, although the study identifies *H19* as a negative regulator of human ASC osteogenic differentiation, further studies are still required to determine what impact it has on matrix mineralization by ASC-derived osteoblasts.

In summary, to our knowledge, this is the first study revealing the comprehensive expression profiles of lncRNAs during osteogenic differentiation of human ASCs. The potential functions and mechanisms of the dysregulated lncRNAs were also investigated by bioinformatics analysis and lncRNA transfection experiments. Although the specific role and mechanism of these candidate lncRNAs in osteogenesis of human ASCs require further studies, our findings extended the information known about the osteogenic process of ASCs and suggested directions for further investigations on the regulation of osteoblastic differentiation of ASCs as well as treatments for osteoporosis and other bone diseases.

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Disclosure Statement

The authors declare that they have no competing interests.

References

- 1 Lane NE: Epidemiology, etiology, and diagnosis of osteoporosis. *Am J Obstet Gynecol* 2006;194:S3-11.
- 2 Ito H: Clinical considerations of regenerative medicine in osteoporosis. *Curr Osteoporos Rep* 2014;12:230-234.
- 3 Antebi B, Pelled G, Gazit D: Stem cell therapy for osteoporosis. *Curr Osteoporos Rep* 2014;12:41-47.
- 4 You L, Pan L, Chen L, Gu W, Chen J: MiR-27a is Essential for the Shift from Osteogenic Differentiation to Adipogenic Differentiation of Mesenchymal Stem Cells in Postmenopausal Osteoporosis. *Cell Physiol Biochem* 2016;39:253-265.
- 5 Mueller SM, Glowacki J: Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. *J Cell Biochem* 2001;82:583-590.
- 6 Stenderup K, Justesen J, Clausen C, Kassem M: Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone* 2003;33:919-926.
- 7 Rodriguez JP, Montecinos L, Rios S, Reyes P, Martinez J: Mesenchymal stem cells from osteoporotic patients produce a type I collagen-deficient extracellular matrix favoring adipogenic differentiation. *J Cell Biochem* 2000;79:557-565.
- 8 Tapp H, Hanley EN, Jr, Patt JC, Gruber HE: Adipose-derived stem cells: characterization and current application in orthopaedic tissue repair. *Exp Biol Med (Maywood)* 2009;234:1-9.
- 9 Chen HT, Lee MJ, Chen CH, Chuang SC, Chang LF, Ho ML, Hung SH, Fu YC, Wang YH, Wang GJ, Kang L, Chang JK: Proliferation and differentiation potential of human adipose-derived mesenchymal stem cells isolated from elderly patients with osteoporotic fractures. *J Cell Mol Med* 2012;16:582-593.
- 10 Zhu M, Kohan E, Bradley J, Hedrick M, Benhaim P, Zuk P: The effect of age on osteogenic, adipogenic and proliferative potential of female adipose-derived stem cells. *J Tissue Eng Regen Med* 2009;3:290-301.
- 11 Mirsaidi A, Genelin K, Vetsch JR, Stanger S, Theiss F, Lindtner RA, von Rechenberg B, Blauth M, Muller R, Kuhn GA, Hofmann Boss S, Ebner HL, Richards PJ: Therapeutic potential of adipose-derived stromal cells in age-related osteoporosis. *Biomaterials* 2014;35:7326-7335.
- 12 Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassady JP, Cabili MN, Jaenisch R, Mikkelsen TS, Jacks T, Hacohen N, Bernstein BE, Kellis M, Regev A, Rinn JL, Lander ES: Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* 2009;458:223-227.
- 13 Ponting CP, Oliver PL, Reik W: Evolution and functions of long noncoding RNAs. *Cell* 2009;136:629-641.
- 14 Li YP, Wang Y: Large noncoding RNAs are promising regulators in embryonic stem cells. *J Genet Genomics* 2015;42:99-105.
- 15 Cui Y, Lu S, Tan H, Li J, Zhu M, Xu Y: Silencing of Long Non-Coding RNA NONHSAT009968 Ameliorates the Staphylococcal Protein A-Inhibited Osteogenic Differentiation in Human Bone Mesenchymal Stem Cells. *Cell Physiol Biochem* 2016;39:1347-1359.
- 16 Huang Y, Zheng Y, Jia L, Li W: Long Noncoding RNA H19 Promotes Osteoblast Differentiation Via TGF-beta1/Smad3/HDAC Signaling Pathway by Deriving miR-675. *Stem Cells* DOI:10.1002/stem.2225.
- 17 Zhu L, Xu PC: Downregulated LncRNA-ANCR promotes osteoblast differentiation by targeting EZH2 and regulating Runx2 expression. *Biochem Biophys Res Commun* 2013;432:612-617.
- 18 Wang L, Wang Y, Li Z, Li Z, Yu B: Differential expression of long noncoding ribonucleic acids during osteogenic differentiation of human bone marrow mesenchymal stem cells. *Int Orthop* 2015;39:1013-1019.
- 19 Zhuang W, Ge X, Yang S, Huang M, Zhuang W, Chen P, Zhang X, Fu J, Qu J, Li B: Upregulation of lncRNA MEG3 Promotes Osteogenic Differentiation of Mesenchymal Stem Cells From Multiple Myeloma Patients By Targeting BMP4 Transcription. *Stem Cells* 2015;33:1985-1997.
- 20 Liang WC, Fu WM, Wang YB, Sun YX, Xu LL, Wong CW, Chan KM, Li G, Waye MM, Zhang JF: H19 activates Wnt signaling and promotes osteoblast differentiation by functioning as a competing endogenous RNA. *Sci Rep* 2016;6:20121.
- 21 Zuo C, Wang Z, Lu H, Dai Z, Liu X, Cui L: Expression profiling of lncRNAs in C3H10T1/2 mesenchymal stem cells undergoing early osteoblast differentiation. *Mol Med Rep* 2013;8:463-467.
- 22 Dong R, Du J, Wang L, Wang J, Ding G, Wang S, Fan Z: Comparison of long noncoding RNA and mRNA expression profiles in mesenchymal stem cells derived from human periodontal ligament and bone marrow. *Biomed Res Int* 2014;2014:317853.

- 23 Jin C, Jia L, Huang Y, Zheng Y, Du N, Liu Y, Zhou Y: Inhibition of lncRNA MIR31HG Promotes Osteogenic Differentiation of Human Adipose-Derived Stem Cells. *Stem Cells* DOI:10.1002/stem.2439.
- 24 Gamez B, Rodriguez-Carballo E, Graupera M, Rosa JL, Ventura F: Class I PI-3-Kinase Signaling Is Critical for Bone Formation Through Regulation of SMAD1 Activity in Osteoblasts. *J Bone Miner Res* 2016;31:1617-1630.
- 25 Kim JH, Yoon SM, Song SU, Park SG, Kim WS, Park IG, Lee J, Sung JH: Hypoxia Suppresses Spontaneous Mineralization and Osteogenic Differentiation of Mesenchymal Stem Cells via IGFBP3 Up-Regulation. *Int J Mol Sci* 2016;17:
- 26 Xi JC, Zang HY, Guo LX, Xue HB, Liu XD, Bai YB, Ma YZ: The PI3K/AKT cell signaling pathway is involved in regulation of osteoporosis. *J Recept Signal Transduct Res* 2015;35:640-645.
- 27 Faghihi MA, Wahlestedt C: Regulatory roles of natural antisense transcripts. *Nat Rev Mol Cell Biol* 2009;10:637-643.
- 28 Orom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang Q, Guigo R, Shiekhattar R: Long noncoding RNAs with enhancer-like function in human cells. *Cell* 2010;143:46-58.
- 29 Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, Rinn JL: Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev* 2011;25:1915-1927.
- 30 Yang L, Ge D, Cao X, Ge Y, Chen H, Wang W, Zhang H: MiR-214 Attenuates Osteogenic Differentiation of Mesenchymal Stem Cells via Targeting FGFR1. *Cell Physiol Biochem* 2016;38:809-820.
- 31 Huang J, Meng Y, Liu Y, Chen Y, Yang H, Chen D, Shi J, Guo Y: MicroRNA-320a Regulates the Osteogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells by Targeting HOXA10. *Cell Physiol Biochem* 2016;38:40-48.
- 32 Zhang H, Li L, Dong Q, Wang Y, Feng Q, Ou X, Zhou P, He T, Luo J: Activation of PKA/CREB Signaling is Involved in BMP9-Induced Osteogenic Differentiation of Mesenchymal Stem Cells. *Cell Physiol Biochem* 2015;37:548-562.
- 33 Xiao WL, Zhang DZ, Fan CH, Yu BJ: Intermittent Stretching and Osteogenic Differentiation of Bone Marrow Derived Mesenchymal Stem Cells via the p38MAPK-Osterix Signaling Pathway. *Cell Physiol Biochem* 2015;36:1015-1025.
- 34 Zhou N, Hu N, Liao JY, Lin LB, Zhao C, Si WK, Yang Z, Yi SX, Fan TX, Bao W, Liang X, Wei X, Chen H, Chen C, Chen Q, Lin X, Huang W: HIF-1alpha as a Regulator of BMP2-Induced Chondrogenic Differentiation, Osteogenic Differentiation, and Endochondral Ossification in Stem Cells. *Cell Physiol Biochem* 2015;36:44-60.
- 35 Tye CE, Gordon JA, Martin-Buley LA, Stein JL, Lian JB, Stein GS: Could lncRNAs be the missing links in control of mesenchymal stem cell differentiation? *J Cell Physiol* 2015;230:526-534.
- 36 Morey JS, Ryan JC, Van Dolah FM: Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. *Biol Proced Online* 2006;8:175-193.
- 37 Brannan CI, Dees EC, Ingram RS, Tilghman SM: The product of the H19 gene may function as an RNA. *Mol Cell Biol* 1990;10:28-36.
- 38 Ravid O, Shoshani O, Sela M, Weinstock A, Sadan TW, Gur E, Zipori D, Shani N: Relative genomic stability of adipose tissue derived mesenchymal stem cells: analysis of ploidy, H19 long non-coding RNA and p53 activity. *Stem Cell Res Ther* 2014;5:139.
- 39 Gabory A, Jammes H, Dandolo L: The H19 locus: role of an imprinted non-coding RNA in growth and development. *Bioessays* 2010;32:473-480.
- 40 Kallen AN, Zhou XB, Xu J, Qiao C, Ma J, Yan L, Lu L, Liu C, Yi JS, Zhang H, Min W, Bennett AM, Gregory RI, Ding Y, Huang Y: The imprinted H19 lncRNA antagonizes let-7 microRNAs. *Mol Cell* 2013;52:101-112.
- 41 Wei J, Li H, Wang S, Li T, Fan J, Liang X, Li J, Han Q, Zhu L, Fan L, Zhao RC: let-7 enhances osteogenesis and bone formation while repressing adipogenesis of human stromal/mesenchymal stem cells by regulating HMGA2. *Stem Cells Dev* 2014;23:1452-1463.