Expression profiling of lncRNAs in C3H10T1/2 mesenchymal stem cells undergoing early osteoblast differentiation

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Abstract. Protein-coding genes and small non-coding microRNAs involved in the guidance of differentiation in mesenchymal stem cells (MSCs) into osteoblasts have been extensively investigated in previous studies. However, long non-coding RNAs (lncRNAs), which account for a large proportion of the genomic sequences in numerous species, have not yet been reported. In the present study, the lncRNA expression profile was analyzed using the Arraystar lncRNA array in C3H10T1/2 MSCs undergoing early osteoblast differentiation and 116 differentially expressed lncRNAs were identified between BMP-2 treated and untreated groups. Among these lncRNAs, 59 were upregulated and 57 were downregulated in BMP-2 treated groups. In addition, 24 cooperatively differentially expressed lncRNAs and nearby mRNA pairs were found. For example, mouselincRNA0231 and its nearby gene, EGFR, were downregulated, while lncRNA NR_027652 and its nearby gene, DLK1, were upregulated. These observations may be part of the regulatory mechanisms of lncRNAs in the control of osteoblast differentiaton. In conclusion, results of the present study indicate that lncRNA expression profiles are significantly altered in C3H10T1/2 undergoing early osteoblast differentiation and these results may provide insight into the mechanisms responsible for osteoblast differentiation.

Introduction

Osteoblast differentiation from mesenchymal stem cells (MSC) is a highly regulated process guided by complex signaling cascades. BMP-2 functions as an effective osteoblast-inducing signal and has been investigated extensively (1). In the past two decades, a number of transcription factors and small non-coding microRNAs involved in BMP-2-induced osteoblast differentiation have been identified (2-5). However, the precise molecular mechanisms of osteoblast differentiation remain largely unknown.

Long non-coding RNAs (long ncRNAs or lncRNAs) are generally considered to represent non-protein coding transcripts of >200 nucleotides (6). An increasing number of studies have reported that lncRNAs participate in diverse biological processes through distinct mechanisms in mammalian biology (7,8). Aberrant lncRNA expression and mutations have been linked to a diverse number of human diseases, including cancer, cardiovascular dieases and Alzheimer's disease (9-11). Specifically, findings of previous studies demonstrated that lncRNAs are extremely important for the control of cell or tissue differentiation (12-15). Although an increasing number of functional lncRNAs have been characterized thus far, the functions of the majority of lncRNAs remain unknown (16).

It is not known whether MSC commitment and differentiation into osteoblasts relies on the modulation of lncRNA expression. To address this question, in the present study, lncRNA expression profiling was performed in MSCs undergoing differentiation into osteoblasts at day 1 and 4. Differentially expressed lncRNAs were then selected for bioinformatic analyses. Results of this study are likely to provide an important foundation for future studies on the lncRNA modulation of osteoblastic differentiation.

Materials and methods

Cell culture and osteoblast differentiation. C3H10T1/2 cells were obtained from the Chinese Academy of Science Cell Bank (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; both Hyclone Laboratories, Inc., Logan, UT, USA) at 37˚C in a humidified atmosphere of 5% CO_2 in air. To induce osteoblast differentiation, the medium was replaced with low-serum medium, consisting of DMEM supplemented with 5% FBS and 200 ng/ml rhBMP-2 (R&D Systems, Minneapolis, MN, USA) and the medium was changed every 2-3 days.

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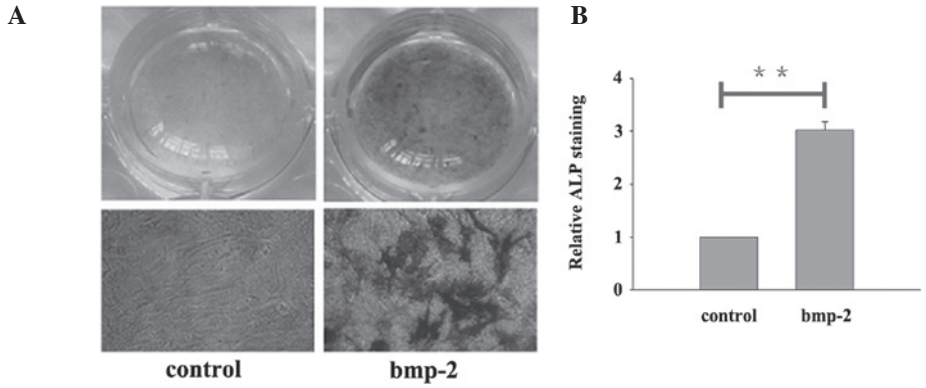

Figure 1. ALP staining for C3H10T1/2 mesenchymal stem cell osteoblast differentiation after 7 days of treatment with and without BMP-2. **P<0.05 compared to the control. (A and B) A significant increase in ALP acitivity in BMP-2-induced C3H10T1/2 cells was observed, indicating that BMP-2 stimulates C3H10T1/2 cells into early osteoblast differentiation.

Alkaline phosphatase (ALP) staining. Levels of osteoblast differentiation in C3H10T1/2 cells were determined using ALP staining. For ALP staining, cells were washed with PBS twice, fixed with 70% ethanol for 20 min, rinsed three times with deionized water and then incubated with the BCIP/NBT liquid substrate system (Sigma-Aldrich, St. Louis, MO, USA), an ALP substrate solution, for 30 min. Images of the stained cells were then captured. For quantitative analysis, the ALP stain was extracted with 10% cetylpyridinium chloride for 15 min and quantified by measuring its absorbance at 540 nm. Relative ALP staining was then calculated as a fold change of the control.

RNA isolation. Following incubation with 200 ng/ml BMP-2 for 1 or 4 days, total RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was also extracted from BMP-2 untreated cells. Total RNA from each sample was quantified using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA integrity was assessed using standard denaturing agarose gel electrophoresis.

Microarray detection and analysis. For microarray analysis, the Agilent Array platform (Agilent Technologies, Santa Clara, CA, USA) was employed. Each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method (Quick Amp Labeling kit, One-Color, Agilent Technologies, p/n 5190-0442). The labeled cRNAs were hybridized onto the Mouse lncRNA Array v2.0 (8x60K; Arraystar Inc., Rockville, MD, USA) which was designed for the global profiling of 31,423 mouse lncRNAs and 25,376 protein-coding transcripts. The slides were washed and the arrays were scanned using the Agilent G2505C microarray scanner. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX version 11.5.1 software package (Agilent Technologies). Differentially expressed lncRNAs and mRNAs were identified through fold change filtering.

Category analysis of differentially expressed lncRNAs. According to lncRNA genomic locations relative to protein coding genes, lncRNAs are categorized as: i) sense, ii) antisense, iii) bidirectional, iv) intronic and v) intergenic (17). The differentially expressed lncRNAs identified in this study were categorized based on these groupings.

Bioinformatic analysis of lncRNA relative to nearby protein-coding genes. One important function of lncRNAs is to regulate the expression of nearby protein-coding genes. Therefore, protein-coding genes were searched for differentially expressed lncRNAs using the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). Genes transcribed within 300 kb were considered to represent nearby coding genes and predicted lncRNAs nearby these coding genes were integrated with differentially expressed mRNA in the microarray (fold change ≥2.0 at day 4) (18). The regulatory network between lncRNAs and nearby coding genes was visually presented using the Cytoscape program (http://www.cytoscape.org/).

Statistical analysis. Experiments were repeated three times with the exception of microarray experiments. Two group comparisons were performed using the Student's t test. P<0.05 was considered to indicate a statistically significant difference.

Results

BMP-2 increased osteoblast-specific marker ALP activity. Osteoblast differentiation from MSCs is regulated by various cytokines and growth factors. BMP-2, a transforming growth factor β superfamily member, is well known as one of the most powerful osteoblast promoting factors (19). ALP activity is an early osteoblast differentiation marker. In the present study, ALP activity in C3H10T1/2 cells with and without BMP-2 treatment was analyzed for 7 days to investigate BMP-2-induced C3H10T1/2 cell osteoblast differentiation. A significant increase in ALP acitivity in BMP-2-induced C3H10T1/2 cells was observed (Fig. 1), indicating that BMP-2 stimulates C3H10T1/2 cells into early osteoblast differentiation.

Differentially expressed profiles of the lncRNAs in BMP-2-induced C3H10T1/2 cell osteoblast differentiation. To investigate the expression profiles of lncRNAs during MSC osteoblast differentiation, total RNA was extracted from

Table I. Upregulated expression of 59 lncRNAs in BMP-2 treated C3H10T1/2 cells for 1 and 4 days.

lncRNA symbol/name	lncRNA symbol/name	lncRNA symbol/name
uc009odu.1	uc009gzn.1	uc009bxq.1
uc008drt.1	AK144695	BC023483
uc007djg.1	uc007cyp.1	NR_027652
CB272499	AV570737	AK201409
AK043290	AK032849	AK032137
AK030568	mKIAA0031	NR_033578
AK148154	uc.77	AK148935
AA656495	uc007rco.1	ENSMUST00000122262
ENSMUST00000122215	ENSMUST00000121177	ENSMUST00000170943
ENSMUST00000120905	ENSMUST00000120431	ENSMUST00000120163
ENSMUST00000120127	ENSMUST00000120017	ENSMUST00000118988
ENSMUST00000118701	ENSMUST00000118061	ENSMUST00000117850
ENSMUST00000074110	ENSMUST00000053965	ENSMUST00000172364
ENSMUST00000171670	ENSMUST00000119457	ENSMUST00000080271
ENSMUST00000165702	ENSMUST00000135189	ENSMUST00000163579
ENSMUST00000160955	ENSMUST00000153890	ENSMUST00000151866
ENSMUST00000170806	ENSMUST00000170714	ENSMUST00000143202
ENSMUST00000121036	ENSMUST00000119775	ENSMUST00000081067
ENSMUST00000126138	MM9LINCRNAEXON11209-	MM9LINCNAEXON10436+
MM9LINCRNAEXON11971-	MM9LINCRNAEXON11966-	

lncRNAs, long non-coding RNAs.

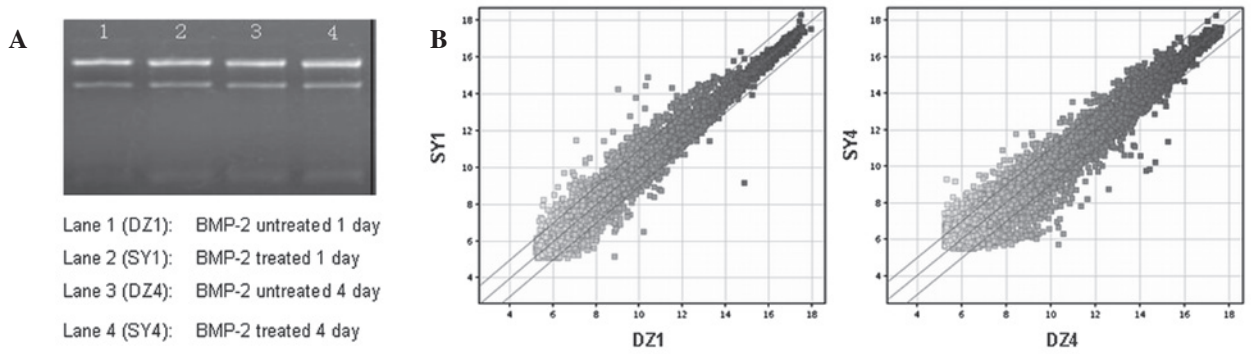

Figure 2. (A) Gel electrophoresis was used to determine the quality of total RNA. (B) Scatter plot analysis of the microarray data between BMP-2-treated and untreated cells.

BMP-2 treated and untreated cells at day 1 and 4. $OD_{260/280}$ ratios were close to 2.0 and $OD_{260/230}$ ratios were >2.0 for all the samples. The quality of total RNA was checked by gel electrophoresis (Fig. 2A), confirming that the RNA was of good quality. Scatter plot analysis of the microarray data is shown in Fig. 2B. Microarray expression analysis of lncRNAs was then performed.Firstly, lncRNAs upregulated or downregulated by >1.5-fold in BMP-2 treated or untreated C3H10T1/2 cells for 1 day were screened, revealing 886 upregulated and 825 downregulated lncRNAs in the BMP-2 treated group. Secondly, 595 upregulated and 548 downregulated lncRNAs by >2-fold were identified at day 4. Continuously upregulated or downregulated lncRNAs following prolonged BMP-2 treatment may be more important for the control of osteoblast differentiation. We also identified 59 upregulated (Table I) and 57 downregulated lncRNAs (Table II) following 1 and 4 days of BMP-2 treatment in accordance with the described thresholds.

Category analysis of differentially expressed lncRNAs. Based on a previous study by Ponting *et al* (17), of the 116 differentially expressed lncRNAs identified in this study, 60.3% were categorized as intergenic. In addition, 20.7% were catagorized as sense. Details of the categories are presented in Table III.

Bioinformatic analysis of lncRNAs relative to osteoblast differentiation. Unlike proteins or microRNAs, lncRNA function cannot be inferred from sequence or structure. Studies have hypothesized a number of regulatory paradigms to explain

Table II. Downregulated expression of 57 lncRNAs in BMP-2 treated C3H10T1/2 cells for 1 and 4 days.

lncRNA symbol/name	lncRNA symbol/name	lncRNA symbol/name
uc009rnr.1	uc009qfj.1	BC099572
AK044623	mD53	uc007cpz.1
BC038927	AK142678	C130026I21Rik
BC038927	AK137923	mouselincRNA0231
mouselincRNA0106	mouselincRNA0243	mouselincRNA1280
AK043049	BM942986	AK156713
AK143058	AK137040	AK136357
AK134233	AK089560	AK054249
AK046177	AK044187	AK043294
AK039250	AK032775	AK031656
AK031010	AK030271	AK029300
AK020801	AK014853	AK013539
AI551087	ENSMUST00000125230	ENSMUST00000122459
ENSMUST00000116393	ENSMUST00000170072	ENSMUST00000165050
ENSMUST00000162276	ENSMUST00000160831	ENSMUST00000160674
ENSMUST00000156260	ENSMUST00000154798	ENSMUST00000148528
ENSMUST00000147939	ENSMUST00000137032	ENSMUST00000133270
ENSMUST00000129933	MM9LINCRNAEXON10823+	MM9LINCRNAEXON11750+
MM9LINCRNAEXON11996+	MM9LINCRNAEXON11765+	MM9LINCRNAEXON11642-

lncRNAs, long non-coding RNAs.

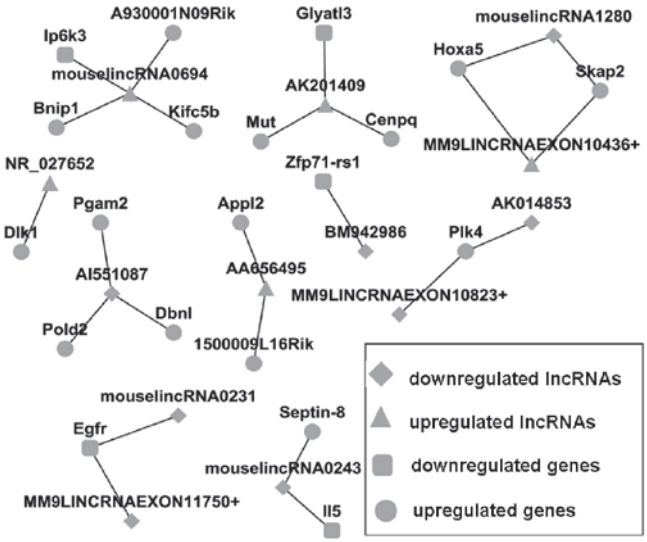

Figure 3. Regulatory network between differentially expressed lncRNAs and nearby protein-coding genes pairs. lncRNAs, long non-coding RNAs.

the mechanism by which lncRNAs function. In the present study, the genomic context of lncRNAs was highlighted. Differentially expressed nearby mRNAs were combined and 24 differentially expressed lncRNAs and nearby coding genes pairs were identified for 13 differentially expressed lncRNAs and 20 differentially expressed mRNAs. Using the Cytoscape program, a regulatory network was constructed between differentially expressed lncRNA and nearby coding genes (Fig. 3).

Table III. Category analysis of differentially expressed lncRNAs.

	lncRNAs		
Category	Upregulated	Downregulated	%
Sense	14	10	20.7
Antisense	7	12	16.4
Bidirectional	2	1	2.6
Intronic	0	0	0.0
Intergenic	36	24	60.3

lncRNAs, long non-coding RNAs.

Discussion

Specific lncRNAs, including linc-MD1, TINCR and ANCR, have been previously reported to be involved in the control of cell or tissue differentiation (12-15). In the present study, the Arraystar microarray analysis was used to identify differentially expressed lncRNAs associated with BMP-2 stimulated osteoblast differentiation. To the best of our knowledge, this is the first study to demonstrate genome-wide differentially expressed lncRNA profiling in MSCs undergoing early osteoblast differentiation.

Although lncRNAs may have an important impact on a diverse range of human diseases, the current understanding of the molecular mechanisms by which lncRNAs function remain largely unknown. Previous studies have demonstrated

that lncRNAs may function by controlling the transcriptional regulation of neighboring coding genes (7,17,20). For example, the ncRNA, Evf2, forms a complex with the transcription factor, Dlx2, to induce the expression of adjacent protein-coding genes (21). Identifying differentially expressed nearby coding mRNA may enhance understanding of the function and potential regulatory mechanisms for lncRNAs. In the current study, 24 differentially expressed lncRNAs and nearby coding genes pairs were identified. Among the regulatory network between differentially expressed lncRNAs and nearby protein-coding genes pairs, specifc nodes have been previously reported to be involved in osteoblast differentiation or bone metabolism. For example, the protein-coding gene, EGFR, a nearby coding gene for differentially expressed lncRNA, mouselincRNA0231, was downregulated by 2.2- and 2.8-fold following BMP-2 treatment for 1 and 4 days, respectively. A previous study reported that EGFR signaling suppresses osteoblast differentiation by inhibiting the expression of master osteoblastic transcription factors, Runx2 and Osterix (22). We hypothesized that mouselincRNA0231 may negatively regulate osteoblast differentiation by affecting EGFR signaling. DLK1 is a novel regulator of bone mass and inhibits bone formation and stimulates bone resorption (23,24) and was upregulated in the BMP-2 treatment group. This upregulation may have reduced the action of BMP-2. DLK1 is a nearby coding gene for differentially expressed lncRNA NR_027652. Therefore, a synergistic effect on osteoblast differentiation may exist between DLK1 and NR_027652. IL-5, is a nearby coding gene for mouselincRNA0243 and is a T cell-derived factor. Macias *et al* previously reported that overexpression of IL-5 in a transgenic mouse line mediated bone formation through the mobilization of marrow-derived osteogenic progenitors (25). These pairings between lncRNA and nearby coding protein may represent one of the regulatory mechanisms by which lncRNAs control osteoblast differentiaton through the regulation of neighboring osteoblast-related gene expression. However, further studies must be performed to prove this hypothesis.

In conclusion, 116 continuously differentially expressed lncRNAs were identified in this study during BMP-2-stimulated osteoblast differentiation for 1 and 4 days in C3H10T1/2 mensenchymal stem cells. In addition, potential regulatory mechanisms by which lncRNAs control osteoblast differentiation were identified by bioinformatic analysis. Although more studies are required to demonstrate the precise role and mechanisms of lncRNAs in osteoblast differentiation, lncRNAs appear to be potent candidates for osteoblast differentiation or therapeutic agents for osteogenic disorders in the future.

Acknowledgements

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