*Full Paper***A Functional RNAi Screen for Runx2-Regulated Genes Associated With Ectopic Bone Formation in Human Spinal Ligaments**

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Abstract. Ossification of the posterior longitudinal ligament of the spine (OPLL) is characterized by ectopic ossification in the spinal ligaments, which enlarges with time and compresses the spinal cord, resulting in serious neurological symptoms. We previously reported that Runx2 expression was enhanced in spinal ligament cells from OPLL patients (OPLL cells). To clarify genes regulated by Runx2, Runx2 expression was first enhanced by culturing primary OPLL cells in osteogenic medium (OS induction) and then inhibited by siRNAs targeted to Runx2. DNA microarray demonstrated that in addition to chondrogenic factors such as connective tissue growth factor and cartilage oligomeric matrix protein, angiopoietin-1 was also significantly increased by OS induction and decreased by siRNAs for Runx2 in OPLL cells, suggesting that these genes are regulated by Runx2. However, these changes were not observed in non-OPLL control cells (from cervical spondylotic myelopathy patients). Furthermore, Runx2 was not decreased by siRNAs for angiopoietin-1. OS induction and RNAi inhibition of angiopoietin-1 expression was also observed in osteoblasts. Both siRNAs for Runx2 and angiopoietin-1 completely inhibited aggrecan-1 expression. These results suggest that angiopoietin-1 is downstream of Runx2 in both OPLL primary cells and osteoblasts. Angiopoietin-1 may play an important role in ectopic ossification.

Keywords: Runx2/Cbfa1, angiopoietin-1, microarray, osteochondrogenesis, ossification of the posterior longitudinal ligament (OPLL)

Introduction

Spinal ligaments sometimes undergo hypertrophy, chondrification and ossification. Ossification of the posterior longitudinal ligament of the spine (OPLL) is an ectopic bone formation in spinal ligaments that is thought to be a part of diffuse idiopathic skeletal hyperostosis (DISH) (1, 2). DISH occurs more commonly in Europeans and North Americans: it occurs at a frequency of 17.6% in males and 11.7% in females over the age of 60 years in Western countries (3). On the other

hand, OPLL is commonly observed in Asia: it is the leading cause of myelopathy in Japan where it is reported to occur at a frequency between 1.8%–4.1% (4). The ossified lesion often enlarges with time and compresses the spinal cord and nerve root, resulting in serious neurological deficiencies (5–9).

Local factors [mechanical stress (10–14)], systemic factors [parathyroid hormone, insulin-like growth factor-I (IGF-I)], and genetic factors have been suggested to play a role in the pathology of OPLL. Recent molecular genetic studies have identified several candidate genes that are differentially expressed in OPLL cells compared to normal ligament cells (15, 16): reports suggest an increase in collagen, type VI, alpha 1 (Col6a1) (17, 18); collagen type XI alpha 2 (Col11a2) (19, 20); nucleotide

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pyrophosphatase (NPPS) (21–24); leptin receptor (22); transforming growth factor-beta 1 (TGF- β 1) (25–27); promyelotic leukemia zinc finger (PLZF) (28, 29); Tumor necrosis factor-alpha-stimulated gene 6 (TSG-6) (29); connective tissue growth factor (CTGF) (12); prostaglandin I₂ (PGI₂) (13) and endothelin-1 (30), amongst others. However, thus far, genetic factors have not been statistically linked to DISH or OPLL, most likely owing to the complexity of these diseases.

Runx2, previously known as core binding factor α 1 (Cbfa1), is a member of the runt family of transcription factors that play a key role in osteoblast differentiation (31). Runx2 binds to the osteoblast-specific *cis*-acting element 2 (OSE2) (32) that is found in the promoter regions of all major osteoblast-specific genes, including alkaline phosphatase (33), type I collagen (34), osteopontin (35), and osteocalcin (32), and controls their expression. In addition, Runx2 can bind to an OSE2 element in its own promoter, suggesting the existence of an autoregulatory feedback mechanism of transcriptional regulation (36).

To determine the relationship between Runx2 and ectopic bone formation, we used DNA microarray to examine gene expression profiles following knock down of Runx2 using RNA interference and confirmed array data by real-time PCR of primary OPLL cells, primary non-OPLL cells, and osteoblasts.

Materials and Methods

This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine and was conducted according to principles of the Declaration of Helsinki. Informed consent was obtained from each patient.

Clinical diagnosis

The diagnosis of OPLL or non-OPLL (other cervical diseases not related to ectopic ossification, i.e., cervical spondylotic myelopathy) was confirmed using X-rays, computerized tomography, and magnetic resonance imaging of the cervical spine, preoperatively, by spine surgeons. The clinical diagnoses and the spinal ligament tissues used in this study are shown in Table 1.

Spinal ligament primary cell culture

Paraspinal ligaments were harvested aseptically from OPLL patients during surgery to decompress the spinal cord for myelopathy. As a control, spinal ligaments were taken from non-OPLL patients during surgery for cervical spondylotic myelopathy. For cell culture, the ligaments were rinsed with phosphate-buffered saline (PBS), after which the surrounding tissue was carefully

Table 1. Demographics of patients from which tissue samples were taken

No.	Diagnosis	Sex	Age
1	OPLL	male	73
2	OPLL	female	56
3	OPLL	male	48
4	OPLL	male	71
5	OPLL	male	64
6	OPLL	male	55
7	Non-OPLL (CSM)	male	45
8	Non-OPLL (CSM)	male	70
9	Non-OPLL (CSM)	female	75
10	Non-OPLL (CSM)	male	79
11	Non-OPLL (CSM)	male	50
12	Non-OPLL (CSM)	male	59

CSM: cervical spondylotic myelopathy.

removed under a dissecting microscope. The ligaments were extirpated carefully from the non-ossified site to avoid any possible contamination with osteogenic cells. The collected ligaments were minced into approximately 0.5 mm³ pieces, washed twice with PBS, then plated on 100-mm culture dishes and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Nissui Pharmaceutical, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA), 1% L-glutamine, and 1% penicillin/streptomycin. The explants were incubated in a humidified atmosphere containing 95% air and 5% CO₂ at 37°C. The fibroblast-like cells that migrated from the explants were harvested with 0.02% ethylenediamine tetraacetic acid (EDTA)/0.05% trypsin and replated in culture dishes for passage. Fibroblast-like cells were used for experiments after 5 passages. Collected tissue from each patient is generally very small and the cell population migrated from explants is also small. Therefore, it is difficult to use primary cultured cells for any experiment. However, it was confirmed that there was no significant difference in morphology and gene expression induced by osteogenic medium between 2nd and 5th passage cells.

Osteoblast cell culture

Osteoblast cells (NHOst: Clonetics™ Normal Human Osteoblast Cell System) were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA) and cultured using One Normal Human Osteoblast Cell Medium BulletKit® (Clonetics OGM™ BulletKit) according to the manufacturer's instructions.

Table 2. Real-time PCR primer sequences

Gene ID	Primer sequence
Runx2 [NM_001024630] [NM_001015051] [NM_004348]	forward: 5'-AGCCCTCGGAGAGGTACCA-3' reverse: 5'-CGGAGCTCAGCAGAATAATTTTC-3'
ALP (alkaline phosphatase) [NM_000478]	forward: 5'-ACGAGCTGAACAGGAACAACGT-3' reverse: 5'-CACCAGCAAGAAGAAGCCTTTG-3'
ANGPT1 (angiopoietin-1) [NM_001146]	forward: 5'-CAGATCTGCAGCAGATAGGGTAGA-3' reverse: 5'-TCTCTGTGTGACCGTTCAGCAT-3'
VEGF-A [NM_003376]	forward: 5'-AACACAGACTCGCGTTGCAA-3' reverse: 5'-CGGCTTGTCACATCTGCAAGT-3'
COL2A1 (collagen type IIa1) [NM_001844]	forward: 5'-GGAAGAGTGGAGACTACTGGATTGAC-3' reverse: 5'-TCCATGTTGCAGAAAACCTTCA-3'
ACAN (aggrecan-1) [NM_013227]	forward: 5'-GGAAACGTGAATCAGAATCAACTG-3' reverse: 5'-TGACGTGTCCCTCTGTCTCCTT-3'

Chemicals

Dexamethasone was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, USA). All chemicals used in this study were of analytical grade.

Osteogenic induction, RNA preparation, and cDNA synthesis

Cells were cultured in osteogenic medium (OSM: DMEM + 10% FCS + 10^{-7} M dexamethasone) to induce osteogenesis or DMEM + 10% FCS (control) for 48 h (37, 38). Total RNA was extracted from cells with an RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The purity and integrity of the total RNA was verified using an RNA 6000 Nano assay with Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Total RNA was treated with RNase-free DNase I (Life Technologies Inc., Gaithersburg, MD, USA) and first strand cDNA was synthesized from 1 μ g of total RNA using standard random hexamer priming techniques. The cDNAs were stored at -20°C until required.

Real-time PCR analysis

Real-time PCR was carried out with Power SYBR Green PCR Master Mix on an ABI Prism[®] 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The conditions for PCR were: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, and 60°C for 1 min. All samples were analyzed for glycerol 3-phosphate dehydrogenase (G3PDH) expression in parallel in the same run. Real-time PCR data were represented as Ct values, where Ct is defined as the cycle number at which the amount of amplified product exceeds the threshold level. To compare the different RNA samples in an experiment, we used the

comparative Ct method and compared the RNA expression in samples to that of the control in each experiment. The primers were constructed so that the dynamic range of both the targets and the G3PDH reference were similar over a wide range of dilutions (1:1 – 10,000). Reactions were performed in quadruplet for each cell preparation. Six independent experiments were performed using cell preparations from six OPLL and six non-OPLL patients (see Table 1). The results were expressed as the mean $\pm$ S.E.M. ($n = 6$) and graphs show the relative expression levels compared with the control (non-OPLL patient). Primers were designed to the sequences given in GenBank using Primer Express (version 1.5) software (Applied Biosystems), as recommended by the manufacturer, to ensure suitability for use with the ABI Prism 7000 sequence detection system. The sequences of primers used in this study are shown in Table 2. Primers for Runx2-real time PCR are matched in 3 isoforms of Runx2 sequences (NM_001024630, NM_001015051, and NM_004348).

RNAi

Specific inhibition of gene expression was carried out with Stealth[™] RNA (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. OPLL cells, non-OPLL cells, and osteoblasts were transfected with 100 nM Stealth[™] RNA (siRNA) or Stealth[™] RNAi Negative Control Duplexes (NC) using Lipofectamine[™] 2000 (5 μ l) (Invitrogen). The cells (90% – 95% confluent) were incubated in Lipofectamine at 37°C in a CO_2 incubator for 24 h before gene knockdown. The following Stealth[™] RNA primers sequence (siRNA) were used: Runx2 (HSS101402) (5' to 3'); UAGUG GCAGAAUGGAUGAAUCUGUU, angiopoietin-1 (HSS100477) (5' to 3'); ACACAAGGAAGAGUUG

GACACCUUA. The former RNAi sequence is matched in 3 isoforms (transcript variants 1-3) of Runx2 sequences (NM_001024630, NM_001015051, and NM_004348).

Oligonucleotide DNA microarray analysis

A 500-ng sample of total RNA was reverse transcribed into cDNA and used as a template to generate cRNA labeled with cyanine 3-CTP (Cy3) or cyanine 5-CTP (Cy5) (Perkin-Elmer/NEN Life Science) using an In Situ Hybridization Kit Plus (Agilent Technologies). Labeled cRNAs were hybridized with an In Situ Human 1A ver.2 Oligonucleotide DNA microarray kit (Agilent Technologies). Arrays were hybridized with cRNA from the following: array 1: non-OPLL cells vs OS induced non-OPLL cells; array 2: OS induced OPLL cells vs OPLL cells; array 3: OS induced OPLL cells vs OS induced OPLL treated with siRNA for 24 h. Microarray analysis was repeated with different samples: the first analysis compared non-OPLL sample No. 8 with OPLL sample No. 4 and the second analysis compared non-OPLL sample No. 9 and OPLL sample No. 5 (see Table 1). Microarrays were scanned in the Cy3 and Cy5 channels with an Affymetrix® 428™ Array Scanner. Raw array data were normalized according to the manufacturer's guidelines using BioDiscovery Microarray analysis Software (ImaGene ver.6.0, GeneSight ver.4.1). The average values were used for subsequent analysis. The ratio of normalized intensity ≥ 2.0 or ≤ 0.5 ($P < 0.01$) was used to generate a list of genes with significant changes in expression during osteoblastic differentiation.

Statistical analysis

All data were expressed as the mean $\pm$ S.E.M. Data were analyzed by ANOVA and significant interactions were further examined with Dunnett's post-hoc test for multiple comparisons. $P < 0.05$ was considered significant.

Results

OS induction and siRNA inhibition of Runx2 expression in OPLL cells

Runx2 expression in OPLL cells was higher than in non-OPLL, as reported previously (10) (Fig. 1). After OS induction, Runx2 expression was further enhanced in OPLL cells. In addition, expression of the osteogenic marker alkaline phosphatase (ALP) was elevated. siRNA of Runx2 in OS-induced OPLL cells decreased expression to levels equivalent with unstimulated OPLL cells. Moreover, siRNA of Runx2 resulted in significant downregulation of ALP. Negative control siRNA was without effect.

Fig. 1. siRNA knockdown of Runx2 on Runx2 (A) and ALP (B) expression in OPLL cells after OS induction. OPLL cells were treated with Runx2 (siRunx) or negative control siRNA (NC) for 24 h and then cultured in normal or osteogenic medium for 48 h. mRNA levels were analyzed by real-time PCR. Data are reported as the mean $\pm$ S.E.M. * $P < 0.05$ ($n = 6$), ** $P < 0.01$ ($n = 6$).

Oligonucleotide DNA microarray analysis of gene expression in OPLL and non-OPLL cells after OS induction with or without siRNA targeted to Runx2

Microarray analysis was performed to identify genes that are regulated by Runx2 in OPLL cells (Fig. 2). The temporal pattern of gene expression during early-stage osteoblastic differentiation (OS induction for 48 h) was examined with an oligonucleotide DNA microarray that represents 22,575 human genes. The expression of 123 genes was up-regulated more than 2-fold and 124 genes were down-regulated less than 0.5-fold in OPLL cells by OS induction. We focused on the genes that were OPLL specific, that is, unchanged in non-OPLL cells (black in array 1, Fig. 2) and significantly influenced by siRNA-mediated knockdown of Runx2 (green or red in Array 3, Fig. 2). Consequently, 24 genes that were up-regulated more than 2-fold and 23 genes that were down-regulated less than 0.5-fold in OS-induced OPLL cells successfully met the criteria (Table 3). These genes included chondrogenic factors such as connective tissue growth factor (CTGF), cartilage oligomeric matrix protein (COMP), growth arrest-specific 6 (GAS-6) and the angiogenic factor angiopoietin-1 (Table 3). In addition, several chondrogenic and angiogenic genes were not specific to OPLLs but seemed to be regulated by Runx2 (Table 4).

Effects of OS induction on the osteoblastic differentiation of OPLL cells and non-OPLL cells

Microarray data were confirmed by real-time PCR. After OS induction, expression of osteogenic marker genes (Runx2 and ALP), chondrogenic marker genes

Fig. 2. Hierarchical clustering: oligonucleotide DNA microarray analysis of OPLL cells and non-OPLL cells after OS induction with or without siRNA-mediated knockdown of Runx2. Total RNA was extracted from non-OPLL or OPLL cells cultured in normal medium or osteogenic medium, with or without siRNAs to Runx2 (siRunx). cRNAs were labeled with Cy3 or Cy5, hybridized to microarrays, and spot intensity was analyzed. Microarrays were repeated with different RNA samples and the average expression levels were used for subsequent analysis. Red: up-regulated genes with an intensity ratio ≥ 2.0 ($P < 0.01$); green: down-regulated genes with an intensity ratio ≤ 0.5 ($P < 0.01$); and black: unchanged genes with an intensity ratio between 0.5 and 2.0.

(aggrecan-1 and collagen-2a1), and angiopoietin-1 were significantly up-regulated in OPLL cells compared to non-OPLL cells (Fig. 3).

Effects of siRNA-mediated gene silencing of Runx2 on gene expression in OPLL cells and osteoblasts

OS induction induced a significant increase in Runx2 expression both in OPLL cells and osteoblasts (Fig. 4A). siRNA targeted to Runx2 inhibited the increase in Runx2 expression after OS induction to levels comparable with the control (before OS induction). In the case of angiopoietin-1, OS induction induced a significant increase in angiopoietin-1 expression both in OPLL cells and osteoblasts, and siRNA for Runx2 attenuated this effect (Fig. 4B). Negative control siRNA was without effect.

Effects of siRNA-mediated gene silencing of angiopoietin-1 on Runx2 expression in OPLL cells

OS-induced angiopoietin-1 expression was significantly down-regulated by siRNA for angiopoietin-1 in OPLL cells (Fig. 5A). However, Runx2 expression was not influenced by angiopoietin-1 siRNA, suggesting that angiopoietin-1 functions downstream of Runx2 in OPLL cells (Fig. 5B). Negative control siRNA was without effect.

Effects of siRNAs for Runx2 and angiopoietin-1 on the expression of aggrecan-1 and VEGF-A in OPLL cells

We used siRNA to Runx2 and angiopoietin-1 to investigate their role in osteochondrogenesis and angiogenesis in OPLL cells. As shown in Fig. 6A, OS induction significantly increased aggrecan-1 expression in OPLL cells but not in non-OPLL cells; siRNA for

Table 3. Candidate genes thought to be regulated by Runx2 in OPLL cells

Gene ID	Array 1 (non-OPLL)	Array 2 (OPLL)	Array 3 (OPLL + siRunx)
Angiopoietin 1 (ANGPT1), transcript variant 1, mRNA [NM_001146]	1.80	3.91	0.27
Cartilage oligomeric matrix protein (COMP), mRNA [NM_000095]	1.00	3.53	0.27
Protein phosphatase 1, regulatory (inhibitor) subunit 14A (PPP1R14A), mRNA [NM_033256]	1.66	3.43	0.28
Rho-related BTB domain containing 3 (RHOBTB3), mRNA [NM_014899]	1.72	3.19	0.13
Androgen receptor (AR), transcript variant 1, mRNA [NM_000044]	1.00	2.96	0.32
Carboxypeptidase M (CPM), transcript variant 1, mRNA [NM_001874]	1.61	2.87	0.37
Vitrin (VIT), mRNA [NM_053276]	1.00	2.87	0.43
Connective tissue growth factor (CTGF), mRNA [NM_001901]	1.56	2.72	0.11
Likely ortholog of mouse limb-bud and heart gene (LBH), mRNA [NM_030915]	1.85	2.67	0.28
mRNA for KIAA0938 protein, partial cds. [AB023155]	1.77	2.65	0.39
Activated leukocyte cell adhesion molecule (ALCAM), mRNA [NM_001627]	1.53	2.64	0.34
Chromosome 21 open reading frame 70 (C21orf70), mRNA [NM_058190]	1.68	2.63	0.45
Cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA [NM_001554]	1.99	2.48	0.41
Calponin 2 (CNN2), transcript variant 1, mRNA [NM_004368]	1.78	2.36	0.23
Crystalline, alpha B (CRYAB), mRNA [NM_001885]	1.97	2.28	0.38
Glutathione S-transferase theta 2 (GSTT2), mRNA [NM_000854]	1.35	2.25	0.44
Arg/Abl-interacting protein ArgBP2 (ARGBP2), transcript variant 2, mRNA [NM_021069]	1.21	2.23	0.27
Pig11 (PIG11) mRNA, complete cds. [AF010315]	1.36	2.16	0.40
LIM and cysteine-rich domains 1 (LMCD1), mRNA [NM_014583]	1.45	2.13	0.16
Keratin associated protein 1-5 (KRTAP1- 5), mRNA [NM_031957]	1.69	2.07	0.41
cDNA clone IMAGE:4150464 5', mRNA sequence [BF344333]	1.48	2.06	0.24
Growth arrest-specific 6 (GAS-6), mRNA [NM_000820]	1.05	2.06	0.33
Tropomyosin 2 (beta) (TPM2), transcript variant 2, mRNA [NM_213674]	1.90	2.03	0.41
Hypothetical protein LOC55924 (LOC55924), transcript variant 1, mRNA [NM_019099]	1.02	2.01	0.47
Thrombospondin 2 (THBS2) mRNA, complete cds. [L12350]	1.19	0.49	3.78
Stathmin-like 3 (STMN3), mRNA [NM_015894]	0.92	0.49	2.42
Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II (P4HA2), transcript variant 1, mRNA [NM_004199]	0.93	0.49	2.20
Lipoma HMGIC fusion partner-like 2 (LHFPL2), mRNA [NM_005779]	0.94	0.48	2.06
Angiotensinogen (serine or cysteine) proteinase inhibitor, member 8 (AGT), mRNA [NM_000029]	0.82	0.47	5.48
Chemokine (C-C motif) ligand 2 (CCL2), mRNA [NM_002982]	0.74	0.46	4.27
Sestrin 2 (SES2), mRNA [NM_031459]	0.77	0.45	2.46
Family with sequence similarity 46, member A (FAM46A), mRNA [NM_017633]	0.93	0.45	2.98
Rho GTPase activating protein 22 (ARHGAP22), mRNA [NM_021226]	0.57	0.44	2.38
Procollagen C-endopeptidase enhancer 2 (PCOLCE2), mRNA [NM_013363]	0.62	0.44	3.03
B-cell CLL/lymphoma 3 (BCL3), mRNA [NM_005178]	0.70	0.44	2.50
Eukaryotic translation initiation factor 5 (EIF5), transcript variant 1, mRNA [NM_001969]	0.63	0.44	2.20
Clone IMAGE:4477067, mRNA, partial cds. [BC012029]	0.90	0.44	6.43
Insulin-like growth factor binding protein 1 (IGFBP1), transcript variant 1, mRNA [NM_000596]	0.70	0.42	2.42
Chromosome 9 open reading frame 132 (C9orf132), mRNA [NM_203305]	0.81	0.41	2.42
Potassium channel tetramerisation domain containing 12 (KCTD12), mRNA [NM_138444]	0.82	0.40	2.09
Protease inhibitor 16 (PI16), mRNA [NM_153370]	0.94	0.37	4.88
Plasminogen activator, urokinase (PLAU), mRNA [NM_002658]	0.78	0.36	2.08
Putative lymphocyte G0/G1 switch gene (G0S2), mRNA [NM_015714]	0.87	0.33	16.36
Hypothetical protein FLJ14834 (FLJ14834), mRNA [NM_032849]	0.57	0.32	3.76
Bradykinin receptor B ₂ (BDKRB2), mRNA [NM_000623]	0.56	0.31	8.27
Chromosome 9 open reading frame 150 (C9orf150), mRNA [NM_203403]	0.53	0.29	2.59
Myosin binding protein H (MYBPH), mRNA [NM_004997]	0.68	0.21	3.40

Table 4. Chondrogenic or angiogenic genes related to Runx2

Gene ID	Array 1 (non-OPLL)	Array 2 (OPLL)	Array 3 (OPLL + siRunx)
Vascular endothelial growth factor B (VEGFB) [NM_003377]	0.75	0.76	2.05
Collagen, type VI, alpha 3 (COL6A3), transcript variant 1 [NM_004369]	0.96	0.78	1.84
Collagen, type XVIII, alpha 1 (COL18A1), transcript variant 1 [NM_030582]	1.04	0.65	1.84
Collagen, type IV, alpha 2 (COL4A2) [NM_001846]	1.14	0.92	0.68
Collagen, type XII, alpha 1 (COL12A1) [NM_004370]	0.92	0.97	0.66
Hyaluronan and proteoglycan link protein 1 (HAPLN1) [NM_001884]	0.54	0.66	0.61
Collagen, type VII, alpha 1 (COL7A1) [NM_000094]	1.75	1.81	0.59
Angio-associated, migratory cell protein (AAMP) [NM_001087]	1.10	1.09	0.54
Collagen, type V, alpha 1 (COL5A1) [NM_000093]	1.00	0.66	0.53
Angiopoietin-like 4 (ANGPTL4), transcript variant 2 [NM_016109]	1.65	1.05	0.49
Proteoglycan 1, secretory granule (PRG1) [NM_002727]	1.20	1.27	0.45
Collagen, type XI, alpha 1 (COL11A1), transcript variant B [NM_080629]	2.12	5.50	0.42
Cysteine-rich, angiogenic inducer, 61 (CYR61) [NM_001554]	1.99	2.48	0.41
Angiomotin like 2 (AMOTL2) [NM_016201]	1.40	1.37	0.37
Collagen, type VIII, alpha 2 (COL8A2) [NM_005202]	3.26	2.86	0.35
HSCSP cartilage specific proteoglycan (600 AA) (Homo sapiens), complete [THC2435904]	1.39	1.32	0.31
Cartilage oligomeric matrix protein (COMP) [NM_000095]	1.00	3.53	0.27
Aggrecan 1 [NM_013227]	1.39	1.48	0.18

Runx2 and angiopoietin-1 completely inhibited the expression of aggrecan-1. On the other hand, OS induction reduced the expression of VEGF-A, a typical angiogenic factor, in both OPLL and non-OPLL cells (Fig. 6B). In contrast, siRNA for Runx2 increased the expression of VEGF-A over control levels, but siRNA for angiopoietin-1 restored VEGF-A expression to control levels after OS induction, suggesting VEGF-A is negatively controlled by Runx2 and this may occur via angiopoietin-1, in part at least.

Discussion

Runx2 is a key factor in osteochondrogenesis (31–36). We previously reported that expression of Runx2 in OPLL cells is elevated and reconfirmed this finding in the present study (10). However, the mechanisms of Runx2 signaling and its role in OPLL remain to be clarified. The Runx family of transcription factors was recently shown to be up-regulated by angiogenic cytokines in a murine endothelial cell model where they are thought to regulate the expression of angiopoietin-1 (39), an angiogenic factor that is important in endothelial cell migration and differentiation (40). The current study demonstrated that the expressions of various osteochondrogenic factors, including Runx2 and angiopoietin-1, were induced by osteogenic stimulation and suppressed by siRNA for Runx2 in OPLL cells, suggesting these genes are regulated by Runx2.

Furthermore, siRNA for angiopoietin-1 failed to inhibit Runx2 expression but completely inhibited the expression of aggrecan-1, an osteochondrogenic factor. In addition, siRNA for Runx2 suppressed the expression of angiopoietin-1 significantly, but not completely. Therefore, angiopoietin-1 may function downstream of Runx2 to mediate, at least in part, the osteochondrogenic function of Runx2 in OPLL cells.

Osteogenic medium have been frequently used to induce a transformation into osteoblastic cells (37, 38, 41). However, the detailed mechanism had remained to be clarified. Effects of siRNA on gene expressions evoked by osteogenic stimulation other than osteogenic medium such as mechanical stress and osteogenic cytokines including BMP-2 are now in progress. In addition, it is likely that the same mechanism is involved in the normal osteochondrogenesis. Indeed, siRNA for Runx2 diminished both increases in collagen type I and osteocalcin, which are important factors in Runx2-regulated process of normal ossification, induced by osteogenic medium (our unpublished observation). However, further investigation is required on this point.

Histological and biochemical evidence suggests that ectopic ossification in spinal ligaments occurs and progresses through endochondral ossification processes, whereby cartilage is first formed and then replaced by new bone (12, 42, 43). OPLL cells are thought to have a potential to differentiate into chondrogenic cells. Microarray data presented here support this idea because

Fig. 3. Effects of OS induction on gene expression in OPLL cells and non-OPLL cells. After OS induction, expression of osteogenic marker genes (Runx2 and ALP) (A), chondrogenic marker genes (aggrecan-1 and collagen-2a1) (B), and angiopoietin-1 (C) in non-OPLL and OPLL cells were analyzed by real-time PCR. Data are reported as the mean $\pm$ S.E.M. * P <0.05 (n = 6)

the expression of several chondrogenic factors such as CTGF were induced by culturing OPLL cells in osteogenic medium and their expression was suppressed by siRNA for Runx2. Together, these data indicate that

Fig. 4. Effects of siRNA targeted to Runx2 on Runx2 (A) and angiopoietin-1 (B) expression in OPLL cells and osteoblasts. After OS induction in the presence or absence of siRNA targeted to Runx2 (siRunx), expression of Runx2 and angiopoietin-1 in OPLL cells and osteoblasts were analyzed by real-time PCR. Data are reported as the mean $\pm$ S.E.M. * P <0.05 (n = 6).

development of OPLL may result from a metaplasia of spinal ligament cells through a mechanism of endochondral ossification in which Runx2 plays an important role.

Invasion of blood vessels into the cartilaginous template plays an indispensable role in physiological endochondral ossification of bone tissue because migration of osteoblasts and osteoclasts occurs through the newly formed blood vessels (12, 44 – 47). Angiogenesis may also play a role in ectopic ossification (48). Clinical reports indicate that patients with OPLL have a bleeding tendency in the perioperative period (49, 50) and significantly greater blood loss after surgery, when compared to non-OPLL patients, despite the fact that bleeding time and levels of coagulation factors (prothrombin level and activated partial thromboplastin time) are not significantly different between OPLL patients and non-OPLL patients (unpublished observations). These observations suggest that microangiopathic dysfunction un-

Fig. 5. Effects of siRNA targeted to angiopoietin-1 on angiopoietin-1 (A) and Runx2 (B) expression in OPLL cells. After OS induction with or without siRNA targeted to angiopoietin-1 (siANG), gene expression in OPLL cells was analyzed by real-time PCR. Data are reported as the mean $\pm$ S.E.M. * P < 0.05 (n = 6). NS: not significant (n = 6).

Fig. 6. Effects of siRNA targeted to Runx2 and angiopoietin-1 on expression of aggreCAN-1 (A) and VEGF-A (B) in non-OPLL and OPLL cells. After OS induction with or without siRNA targeted to Runx2 (Runx) or angiopoietin-1 (ANG), each gene expression was analyzed by real-time PCR. Data are reported as the mean $\pm$ S.E.M. * P < 0.05 (n = 6), ** P < 0.01 (n = 6).

derlies OPLL and is regulated by angiogenic factors. Interestingly, VEGF-A was downregulated by OS induction and up-regulated, beyond control levels, by siRNA for Runx2. Until the matrix of chondrocytes calcifies, penetration of blood vessels is suppressed in parallel with angiogenic factor release, including VEGF, in the early stages of osteochondrogenesis (51, 52). OS induction in this study was relatively short (48 h) and may represent an early stage in the process of endochondral ossification. This notion is supported by the fact that chondrogenic factors other than osteogenic factors were induced by this regime. In middle- or late-

stage endochondral ossification, in which the invasion of blood vessels may occur, the relationship between Runx2, angiopoietin-1, and VEGF (or other angiogenic factors) may differ from that of the early-stage. Further studies are clearly necessary.

Runx2 regulation of angiopoietin-1 in OS-induced OPLL cells seems to be a little controversial. The OS-induced increase in angiopoietin-1 expression was significantly inhibited by siRNA for Runx2. The increase in the expression of aggreCAN-1 induced by OS induction was completely inhibited by both siRNAs for Runx2 and angiopoietin-1. There was a difference between the effects of siRNA for Runx2 and angiopoietin-1 on VEGF-A expression after OS induction. These results suggest that the regulation of aggreCAN-1, and possibly chondrogenesis by Runx2, occurs mainly via angiopoietin-1, but regulation of VEGF-A, and possibly angiogenesis, is only partly regulated via angiopoietin-1. Further studies are required to investigate the signaling pathways of Runx2 and angiopoietin-1 in ectopic ossification.

In summary, osteogenic induction induced a Runx2-dependent increase in several osteochondrogenic factors and angiopoietin-1 in OPLL cells. Angiopoietin-1 may mediate, at least in part, Runx2 signaling in ectopic ossification of human spinal ligaments.

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