Functional Relevance of the BMD-Associated Polymorphism rs312009: Novel Involvement of RUNX2 in LRP5 Transcriptional Regulation

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ABSTRACT

LRP5 is an osteoporosis susceptibility gene. Association analyses reveal that individual single-nucleotide polymorphisms (SNPs) determine variation in bone mineral density (BMD) among individuals as well as fracture risk. In a previous study, we identified a lumbar spine BMD-associated SNP, rs312009, located in the LRP5 5' region. A RUNX2 binding site was identified in this region by gel-shift experiments. Here we test the functionality of this SNP and examine whether RUNX2 is indeed a regulator of LRP5 expression. Gene reporter assays were used to test rs312009 functionality. Bioinformatic predictive tools and gel-shift and gene reporter assays were used to identify and characterize additional RUNX2 binding elements in the 3.3-kb region upstream of LRP5. Allelic differences in the transcriptional activity of rs312009 were observed in two osteoblastic cell lines, the T allele being a better transcriber than the C allele. RUNX2 cotransfection in HeLa cells revealed that the LRP5 5' region responded to RUNX2 in a dose-dependent manner and that the previously identified RUNX2 binding site participated in this response. Also, RUNX2 inhibition by RNAi led to nearly 60% reduction of endogenous LRP5 mRNA in U-2 OS cells. Four other RUNX2 binding sites were identified in the 5' region of LRP5. Luciferase experiments revealed the involvement of each of them in the RUNX2 response. The allelic differences observed point to the involvement of rs312009 as a functional SNP in the observed association. To our knowledge, this is the first time that the direct action of RUNX2 on LRP5 has been described. This adds evidence to previously described links between two important bone-regulating systems: the RUNX2 transcription-factor cascade and the Wnt signaling pathway. © 2011 American Society for Bone and Mineral Research.

KEY WORDS: LRP5; RUNX2; SNP; LUCIFERASE; OSTEOPOROSIS

Introduction

The LRP5 gene encodes the low-density lipoprotein receptor-related protein 5 (LRP5), a transmembrane protein that is involved in Wnt signaling. The Wnt pathway has been shown to be related to bone mass and metabolism. Its role as an essential regulator of bone mass was discovered by linkage studies in two rare human diseases, osteoporosis-pseudoglioma syndrome (OPPG) and high bone mass (HBM). In osteoblasts, LRP5 can transduce canonical signals to promote the renewal of stem cells, stimulation of preosteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis by increasing the levels of β-catenin and altering gene expression through LEF/TCF transcription factors. RUNX2 (also known as CBFA1 or AML3) is a transcription factor that is essential for the differentiation of osteoblasts, the cells responsible for bone formation and skeletal development. As demonstrated by several genetic studies in mice and humans, RUNX2 has well-defined roles in mediating osteoblast differentiation and maturation, in controlling proliferation, and in supporting normal osteogenesis. In addition, RUNX2 deficiency and mutations affecting its function cause a severe bone phenotype called cleidocranial dysplasia (CCD). At the molecular level, RUNX2 activates or represses gene expression following a specific interaction with the osteoblast-specific element (OSE) that is present in the promoter or enhancer regions of its targets by organizing protein complexes that can activate or repress mammalian gene expression depending on the cellular and...
promoter/enhancer context.(6–8) RUNX2 factors interact with other transcription factors and recruit numerous chromatin-modifying proteins that regulate gene expression.(9) Among the cofactors that interact with Runx proteins are coactivators such as p300 and CREB-binding protein (CEBP) and co-repressors such as mSin3A, transducin-like enhancer of split proteins (TLEs), and several histone deacetylases (HDacs).

Several RUNX2 responsive elements have been identified in important bone-related genes, such as osteocalcin,(10–16) osteoprotegerin,(17) osteinx(18) and bone sialoprotein,(19–21) among others. More interestingly, functional RUNX2 binding sites have been described in the promoters of genes of other Wnt signaling pathway elements, such as SOST(22) and AXIN2 genes.(23) Sclerostin is a secreted protein that can act as an inhibitor of canonical Wnt signaling by interacting with Lrp5/6 corepressors, whereas Axin2 is a negative regulator of this pathway by another mechanism based on the promotion of β-catenin degradation (reviewed by Liu and colleagues(24)).

Other links between RUNX2 and canonical Wnt signaling pathways have been reported previously. Khaler and colleagues(25) described Lef1, a final effector of Wnt signaling, as a RUNX2 transcriptional regulator. Gaur and colleagues(26) and Reinhold and Naski(27) also reported cooperation between LEF/TCF and RUNX2 factors in RUNX2’s own promoter and the FGF18 promoter, respectively. Recently, McCarthy and Centrella(28) presented novel evidence for bidirectional cross-talk between the Wnt pathway and RUNX2 in osteoblasts. However, regulatory involvement of RUNX2 in LRP5 transcription has not been reported to date.

Several allelic variants in the LRP5 gene have been associated with osteoporotic phenotypes such as bone mineral density (BMD) and fracture in different association-analysis approaches, such as single missense single-nucleotide polymorphism (SNP) analysis, gene-wide analysis, and even genome-wide analysis (GWA; reviewed by Li and colleagues(29)). Therefore LRP5 is a confirmed osteoporosis susceptibility gene. In this sense, we have previously reported the association of an LRP5 promoter SNP, rs312009, with lumbar spine BMD.(29) A RUNX2 binding element was identified at the polymorphic site by gel-shift experiments, but there were no differences in the binding capacities of the two possible alleles of rs312009. In this study, we analyzed the putative functional involvement of this polymorphism in the transcriptional activity of the LRP5 5′ region. We also characterized other putative RUNX2 binding elements located in this region and studied their involvement in LRP5 transcriptional regulation.

Materials and Methods

Cell culture

The human osteosarcoma cell lines Saos-2 and U-2 OS and the human adenocarcinoma cell line HeLa were obtained from the American Type Culture Collection (ATCC no. HTB-85, ATCC no. HTB-96, and ATCC no. CCL-2, respectively; ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with l-glutamine (292 mg/L), 10% heat-inactivated fetal calf serum (FCS), and 1% antibiotics (penicillin and streptomycin; Invitrogen, Carlsbad, CA, USA). Immortalized human neonatal calvaria cells (IHNC) were a generous gift of Dr Eric Hay and are described in Ref. 30.

In silico prediction and alignment tools

The presence of putative RUNX2 binding sites was assessed using two predictive tools: MatInspector from Genomatix (Munich, Germany),(31) with the core similarity threshold set at 0.75, and TFSearch (Tokyo, Japan)(32) with the threshold for acceptance set at 85. The DNA sequence conservation of putative RUNX2 binding sites across available eutherian mammals was inspected using the comparative genomic tool BlastZ-net available in the Ensembl database (Wellcome Trust Genome Campus, Hinxton, UK).(33)

Western blot

Nuclear cell extracts from Saos-2, U-2 OS, and HeLa cells were prepared as described previously.(29) Twenty and thirty micrograms of protein were resolved by SDS-PAGE (12.5% polyacrylamide), transferred onto nitrocellulose membranes, and analyzed by standard immunostaining using a horseradish peroxidase–conjugated secondary antibody and chemiluminescence detection. The following primary antibodies were used: RUNX2 M-70 at 1:800 dilution (BD Biosciences, San Jose, CA, USA) for the loading control. Horseradish peroxidase–conjugated secondary antibodies were used (GARPO and SAMPO, respectively; Sigma, St Louis, MO, USA). Immunoreactive bands were detected by incubating the membrane for 2 minutes in the following solution: 10 mL of 100 mM Tris-HCl (pH 9.0), 50 μL of 45 mM p-coumaric acid, 50 μL of luminol, and 10 μL of 30% H2O2.

Real-time quantitative PCR

IHNC, Saos-2 and U-2 OS cells were cultured in 60-mm dishes to confluence. RNA extractions were performed using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA quantity and quality were assessed by spectrophotometry (Nanodrop ND-1000 Spectrophotometer; Nanodrop Technologies Inc., Wilmington, DE, USA). Three micrograms of total RNA were reverse transcribed using M-MLV RT (Invitrogen) with oligo-dT priming according to the manufacturer’s instructions. Five microliters of a 1:20 dilution of the resulting cDNA was used for subsequent amplification in a LightCycler 480 (Roche, Basel, Switzerland) with SYBR Green fluorescent dye (ABsolute Blue QPCR SYBR Green Mix; Thermo Scientific, Waltham, MA, USA) under standard conditions. GAPDH mRNA was used for normalization, and the results were expressed relative to RUNX2 expression in the IHNC cell line. For polymerase chain reaction (PCR) amplification, the following primers were used: GADPH forward: 5′-CGA GAT CCC TCC AAA ATC AA-3’, and reverse: 5′-TGT GGT CAT GAG TCC TTC CA-3’; RUNX2 forward: 5′-TCT GGC CTT CCA CTC TCA GT-3’; and reverse: 5′-GAC TGG CGG GGT GTA AGT AA-3’; LRPS forward: 5′-GAA CAT CAA GCG AGC CAA G-3’, and reverse: 5′-TGG CTC AGA GAG TCC AAA ACA-3’. 

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RUNX2 RNA interference

Two siRNAs against RUNX2 (IDs: s2455 and s2456; Ambion, Austin, TX, USA) were used. Silencer FAM-labeled Negative Control siRNA (Ambion) was used as a reference. U-2 OS and Saos-2 cells were seeded in 6-well plates at \(1 \times 10^5\) cells per well. After 24 hours, the cells were transfected with 75 nM of siRNA and 10 mL of X-tremeGENE (Roche) in DMEM with 5% FBS without antibiotic. Twenty-four hours after transfection, RNA was extracted. Retrotranscription and real-time quantitative PCR conditions were as described earlier.

Electrophoretic mobility shift assays (EMSAs)

Saos-2 human osteosarcoma cells (ATCC no. HBT-8STM) were grown in DMEM (Gibco, Billings, MT, USA) supplemented with 10% FCS (Gibco). Nuclear extracts were prepared according to Schreiber and colleagues(34) using a modified buffer C (10% glycerol and 1.5 mM of MgCl\(_2\)). Protein concentrations were determined by the Bradford method, and nuclear extracts were stored at \(-80^\circ\)C until use. Single-stranded DNA oligonucleotides (Sigma-Aldrich, St Louis, MO, USA) were synthesized automatically (both forward and reverse strands). The sequences are listed in Table 1. Double-stranded probes were obtained by annealing complementary oligonucleotides and end labeling with \([\gamma^{32}P]\)ATP (GE Healthcare, Piscataway, NJ, USA) or PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) using the OptiKinase (USB, Santa Clara, CA, USA) standard protocol. The unincorporated nucleotides were removed using a quick-spin G-25 Sephadex column (Roche). Binding reactions typically contained \(10\mu\)g of nuclear extract, \(0.5\mu\)g of double-stranded poly(dI-dC) (Amersham Pharmacia Biotech, Waukesha, WI, USA), \(0.5\mu\)g of double-stranded poly(dA-dT) (Roche), \(6\mu\)g of acetylated BSA (Promega, San Luis Obispo, CA, USA), and 100,000 cpm of radiolabeled probe. The reaction mixtures were incubated for 30 minutes at room temperature in a buffer containing 20 mM HEPES (pH 7.9), 80 mM KCl, 1 mM EDTA, 1 mM MgCl\(_2\), and 10% glycerol in a total volume of 20 \(\mu\)L. Protein-DNA complexes were resolved from the free probes in non-denaturing 7% polyacrylamide (29:1) gels containing 2.5% glycerol. Electrophoresis was performed at 4 \(^\circ\)C in 1 \(\times\) TBE buffer at 20 mA for approximately 3 hours. Gels were vacuum dried and exposed to storage phosphor screens (Kodak, Rochester, NY, USA) at room temperature for 3 to 12 hours. In competition assays, the binding reactions were performed in the presence of an excess of unlabeled competitor oligonucleotide, as indicated in each case. For supershift experiments, 1 \(\mu\)g of the RUNX2 polyclonal antibody (Calbiochem, Merck KGaA, Darmstadt, Germany) or 0.5 \(\mu\)L of SP1 polyclonal antibody (H0000667-A01; Abnova, Taipei City, Taiwan) was preincubated with nuclear proteins in the binding buffer on ice for 15 minutes before adding the probe.

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Reporter constructs and gene-reporter assays

To generate several constructs with the LRP5 5' region linked to a reporter gene, an initial PCR amplicon spanning Chr11 positions 68,078,243 to 68,080,193 bp (according to the February 2009, GRCh37/hg19 UCSC genome assembly) corresponding to positions \(-1879\) to \(+1\) from \(A(\pm 1)G\) in the LRP5 promoter region was obtained from Saos-2 DNA using the following primers: F8: 5'-TGG ATG TCC CAG CAG AGA ACA GC-3'; and R7: 5'-GGT GCC TCC ATG TTG TCC C-3'. This fragment was cloned in pUC18/ Smal (Amerham Pharmacia Biotech) by blunt-end ligation and subcloned by Adel and Ncol digestion in the pGL3 basic vector/ Smal (Promega). This clone was named MP and contained positions \(-49/-1826\) of LRP5. Subsequent digestion of MP with BpiI and Ncol produced a 729-bp fragment that was recloned in the pGL3 basic vector/Smal to obtain the BP construct (\(-49/-778\)). A second PCR fragment was obtained using the following primers: forward: 5'-TGG ATT CCT CGG CCT CAG C-3'; and reverse: 5'-TCA TGC GTC CCC ACT TGC T-3' (Chr11: 68,076,318 to 68,078,510, 2192 bp) again from Saos-2 DNA. This fragment was digested with Adel and Avrl and subcloned to an Nhel- and Adel-digested MP to give the LP-C construct (\(-49/-3274\)). The LP-T construct was obtained by site-directed mutagenesis using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA, USA). Site-directed mutagenesis also was used to incorporate 2-bp substitutions in the RUNX2 binding site core sequences to obtain the MUT1-C, MUT1-T, MUT2, MUT3, MUT4, and MUT5 constructs. All constructs were verified by automatic sequencing.

For the luciferase activity assays, 5 \(\times\) 10^5 Saos-2, U-2 OS, or HeLa cells were plated in 24-well plates and cultured in DMEM with 10% FCS. On the following day, 1.75 \(\mu\)g of reporter construct or 50 ng of normalizing \(\beta\)-galactosidase expression vector (Upstate Biotechnology, Billerica, MA, USA) were cotransfected using 3 \(\mu\)L of Transfast transfection reagent (Promega). The effector was pCMV-Runx2, a mouse Runx2 isofrom II (MASNS) CDNA cloned in the pCMV5 expression vector (pCMV-Runx2; kindly provided by Dr Karsenty, Columbia University, New York, NY, USA). This form has 97.6% homology at the amino acid level with the orthologous isoform in humans. Empty pCMV5 vector (pCMV-EV) also was cotransfected in order to provide the same total amount of DNA in cotransfection experiments.

Table 1. Sequences of the Probes Used in the EMSA Experiments (Only the Forward Strand is Shown)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS1C</td>
<td>5'-CCTTGTCTCCGTGGCCCGGA-3'</td>
</tr>
<tr>
<td>MUT1C</td>
<td>5'-CCTTGTCTCCGTAAACCGGA-3'</td>
</tr>
<tr>
<td>BS1T</td>
<td>5'-CCTTGTCTCCTGTGGCGTGGA-3'</td>
</tr>
<tr>
<td>MUT1T</td>
<td>5'-CCTTGTCTCCTGAACCTGA-3'</td>
</tr>
<tr>
<td>BS2</td>
<td>5'-TGGGGGCTGTGGCTTCGAT-3'</td>
</tr>
<tr>
<td>MUT2</td>
<td>5'-TGGGGGGCTGTTCGATGCT-3'</td>
</tr>
<tr>
<td>BS3</td>
<td>5'-TGTGAAACCCAGACAGACAC-3'</td>
</tr>
<tr>
<td>MUT3</td>
<td>5'-TGTGAACTCAGACAGACAC-3'</td>
</tr>
<tr>
<td>BS4</td>
<td>5'-GAAGAAGGTTGGTGGTGACA-3'</td>
</tr>
<tr>
<td>MUT4</td>
<td>5'-GAAGAAGGGTGATGGTGACA-3'</td>
</tr>
<tr>
<td>BS5</td>
<td>5'-GGCGCTGATGTTGGCTTCCA-3'</td>
</tr>
<tr>
<td>MUT5</td>
<td>5'-GGCGCTGATGTTGGCTTCCA-3'</td>
</tr>
<tr>
<td>OSE</td>
<td>5'-CAGCGGCTCCCAACACCATACCAC-3'</td>
</tr>
<tr>
<td>GRE</td>
<td>5'-TAATATGAGAAGAGTTCTGTTCTAATGACCA-3'</td>
</tr>
<tr>
<td>SP1</td>
<td>5'-ATTGCGATCGGCGGGGCGGCGGAC-3'</td>
</tr>
</tbody>
</table>
Twenty-four/forty-eight hours after transfection, luciferase and β-galactosidase activities were measured in a plate luminometer (either SAFAS-Xenius XL, SAFAS, Monaco, or a GloMax-Multi Detection System, Promega) using the corresponding standard commercial kits (Luciferase Assay System, Promega, and Chemiluminescent Beta-Gal Reporter Gene Assay, Roche). The luciferase activity was normalized to the β-galactosidase activity to correct for transfection efficiency. The luciferase activity of the empty pGL3 vector (a measure of background signal) was subtracted from normalized experimental values (except in Fig. 2C). The results were expressed as relative luciferase units (RLUs) in terms of the long promoter construct with the more frequent allele of rs312009 (LP-C). Transfections were performed in quadruplicate in at least three independent experiments.

Statistical analyses

The results of the quantitative PCR and luciferase assays are expressed as means ± SEM. Statistical significance was determined by unpaired Student’s t tests (for comparisons between different constructs) and by paired Student’s t tests (for comparison of the same construct in different cotransfection conditions). Significance is denoted as follows: *(or *) for p < .05; **(or **) for p < .01; ****(or *****) for p < .001; and *****(or ******) for p < .0001.

Results

Binding of RUNX2 to the putative RUNX2-responsive element at −2.9 kb of LRPS

The presence of a RUNX2 binding site at −2.9 kb from the transcription start site of LRPS has been reported previously.(29) In contrast to that indicated by predictive tools (Table 2), the presence of this binding site (BS1) was not conditioned by the presence of the T allele of the rs312009 polymorphism, as shown in gel-shift experiments in Fig. 1A, B. Equivalent patterns of shifted bands and competition effects were observed for the BS1T and BS1C probes. In both cases, the same level of competition was observed with the specific probes (BS1T or BS1C) without any cross-competition differences, whereas nonspecific cold probes (GRE or SP1) displayed nearly no competition at all. Specific competition with the osteoblast-specific element (OSE) from the osteocalcin gene promoter revealed the participation of RUNX2 in the retained protein complex. In addition, when the core sequence of the predicted RUNX2 binding site was mutated (MUT1-C and MUT1-T) and used as a cold oligonucleotide competitor (Fig. 1C), MUT1C was not able to compete with the binding at BS1C at any concentration, and MUT1T showed some degree of competition for the BS1T binding when added at high molar excess (×500).

Levels of RUNX2 mRNA and RUNX2 protein in human osteoblast-like cells and in nonosteoblastic HeLa cells

In order to choose the appropriate cell lines for transfection studies, two different osteoblastic cell lines, Saos-2 and U-2 OS, were assessed for RUNX2 expression at the RNA and protein levels. Initially, RUNX2 transcript levels were assessed in the two osteoblastic cell lines by real-time PCR (Fig. 2A). Using IHNC as a reference, the levels of RUNX2 mRNA in U-2 OS cells were approximately 60% of those in Saos-2 cells. This difference was even greater at the protein level (Fig. 2B). The nonosteoblastic cell line HeLa showed no detectable expression of RUNX2 protein, as expected, given the osteoblast-specific expression pattern of RUNX2.

Promoter activity of different fragments of the LRPS 5′ region in three cellular contexts

The performance of the LRPS promoter region was assessed in the two osteoblastic lines as well as in HeLa cells. Three different reporter constructs bearing increasing amounts of the 5′ region of LRPS [BP (up to −778), MP (up to −1826), and LP-C (up to −3274, bearing the C allele of rs312009)] were transfected, and

Table 2. The Five Predicted RUNX2 Binding Sites Found in the 3-kb LRPS 5′ Region

<table>
<thead>
<tr>
<th>RUNX2 binding sites</th>
<th>Chromosomal Position (Feb. 2009, GRCh37/hg19)</th>
<th>Matrix similarity according to Genomatix prediction</th>
<th>Score according to TFSearch prediction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS 1</td>
<td>Chr11: 68,077,228–68,077,242 (−2.9 kb)</td>
<td>MS:0.846 (only in presence of T allele of rs312009)</td>
<td>&lt;.85 (not predicted)</td>
<td>TCCCTGGGCYGGAGb</td>
</tr>
<tr>
<td>BS 2</td>
<td>Chr11: 68,077,403–68,077,417 (−2.4 kb)</td>
<td>MS:0.954</td>
<td>100</td>
<td>GGGTGTTCTTGCAc</td>
</tr>
<tr>
<td>BS 3</td>
<td>Chr11: 68,078,103–68,078,117 (−1.8 kb)</td>
<td>MS:0.859</td>
<td>&lt;.85 (not predicted)</td>
<td>TGAGAACCCACAG</td>
</tr>
<tr>
<td>BS 4</td>
<td>Chr11: 68,078,138–68,078,152 (−1.7 kb)</td>
<td>MS:0.949</td>
<td>100</td>
<td>AAGKGTGGTTGGAGCd</td>
</tr>
<tr>
<td>BS 5</td>
<td>Chr11: 68,078,794–68,078,808 (−1.3 kb)</td>
<td>MS:0.900</td>
<td>100</td>
<td>TGATGTGGTTCCTCC</td>
</tr>
</tbody>
</table>

*aFor all RUNX2 sites, the optimized matrix similarity was 0.84 and the core similarity was 1.

bY stands for C/T rs312009, validated frequent SNP (CEU HapMap frequency C 0.776 and T 0.224).

cY stands for A/G rs4988327, validated rare SNP. Only the A allele (CEU HapMap frequency 0.941) was considered further.

dK stands for T/G rs4988329, described in dbSNP but without frequency validation (only T allele considered).
luciferase activity was measured (Fig. 2C). pGL3-EV (empty vector) was used for normalization. All constructs showed significantly higher luciferase gene activity than pGL3-EV in all cell lines. The LP-C promoter activity was highest in Saos-2 cells, showing more than threefold higher transcriptional activity than pGL3-EV ($p < .0001$). In U-2 OS and HeLa cells, this activity was double that of the empty vector ($p < .0001$). In all cellular contexts, MP doubled the transcriptional activity of the LP-C construct ($p < .0001$ in Saos-2 cells; $p = .0130$ in U-2 OS cells; and $p = .0002$ in HeLa cells). BP also led to increased luciferase activity relative to LP-C in all cell lines ($p < .0001$ in Saos-2 and HeLa cells, and $p = .0002$ in U-2 OS cells) and to MP in the two osteosarcoma cell lines ($p = .0003$ in Saos-2 cells; and $p = .0493$ in U-2 OS cells) but not in HeLa cells. These data point to the presence of one or several repressor elements between positions −778 and −1826 and between positions −1826 and −3270. In general, the LRP5 promoter activity was higher in Saos-2 cells than in other cell lines for all three constructs.

**Functional analysis of the rs312009 polymorphism**

Given the genomic localization of SNP rs312009 in the LRP5 promoter region, we hypothesized that it could have functional implications and that these might be mediated by the binding of RUNX2. To test these hypotheses, several reporter-gene assays were performed.

Four reporter constructs containing 3270 bp of the LRP5 S' region were obtained: (1) LP-C, with the C allele in rs312009, (2) LP-T, with the T allele in rs312009, (3) MUT1-C, with mutated RUNX2 BS1 core sequence and the C allele in rs312009, and (4) MUT1-T, with mutated RUNX2 BS1 core sequence and the T allele in rs312009. A schematic representation of the constructs is shown in Fig. 3 together with their relative luciferase activities after transfection in the above-mentioned cells.

In both osteoblastic cell lines, the promoter bearing the T allele (LP-T) showed significantly higher transcriptional activity than LP-C. The differences between means (95% confidence interval [CI]) were 40.7 (± 26.5), $p = .0029$, in Saos-2 cells, and 57.8 (± 57.6), $p = .049$, in U-2 OS cells. The difference between LP-C and LP-T did not reach statistical significance in HeLa cells.

Mutation of the RUNX2 core sequence was tested for its effect on the transcriptional activity of the LP-C and LP-T constructs. The transcriptional capacity of MUT1-C was significantly higher than LP-C in the three cell lines [differences between means (95% CI) in Saos-2 cells, 38.27 (± 34.7), $p = .0031$; in U-2 OS cells, 74.1 (± 41.1), $p = .0011$; and in HeLa cells, 333.3 (± 209.0),
p = .0022]. On the contrary, MUT1-T activity was significantly lower than LP-T activity only in the U-2 OS cell line (C63.9, p = .0476).

Effect of RUNX2 cotransfection on transcriptional activity of the LRP5 5’ region

To assess the response of the LRP5 5’ region to exogenous RUNX2, HeLa cells (known to lack endogenous RUNX2 expression) were cotransfected with increasing amounts of pCMV5-RUNX2 expression vector together with the LP-C construct, which contains the major allele of the polymorphism. Luciferase activity was tested at 24 and 48 hours after transfection. Figure 4A shows a dose-dependent stimulatory effect caused by increasing amounts of RUNX2 vector. This effect was higher at 24 hours than at 48 hours. Cotransfection of the maximal pCMV-Runx2 concentration tested (0.875 μg) caused a more than fivefold increase at 24 hours (p = .0013). This experimental condition was used in subsequent cotransfection experiments.

The stimulatory effect of RUNX2 expression observed on the LP-C construct was then tested on the LP-T construct, as well as on MUT1-C and MUT1-T (Fig. 4B). LP-T, but not the mutant constructs, was stimulated by RUNX2 (p = .0298), although to a lesser extent. The differences between means were 244.2 (95% CI 416.9–71.6) for LP-C and 102.2 (95% CI 192.9–11.4) for LP-T. As already seen in the simple transfection experiments (Fig. 3), the transcriptional activity of MUT1-C and MUT1-T was higher than that of LP-C and LP-T in the absence of RUNX2.

Taken together, these results indicate that the LPR5 5’ region is able to respond to RUNX2 and that BS1 participates in this response.

Effect of RUNX2 inhibition on the expression of the endogenous LRP5 gene

To analyze the effect of RUNX2 on endogenous LRP5 gene expression, RUNX2 was inhibited by RNAi, both in U-2 OS and Saos-2 cells. These cell lines were transfected with siRNA s2456 against RUNX2 (Fig. 5), achieving 85% to 90% inhibition (p < .005). In U-2 OS cells, the reduction in RUNX2 mRNA levels led to nearly 60% reduction in LRP5 mRNA levels (p = .001). In contrast, in Saos-2 cells, LRP5 mRNA levels were not modified. Similar results were observed with a second siRNA against RUNX2, (siRNA s2455; not shown).
Search for additional RUNX2 binding sites in the LRP5 5′ region

After identifying the first RUNX2 binding site (BS1), we looked for other putative RUNX2 binding sites in the 3.3-kb LRP5 upstream region. The Genomatix and TFSearch prediction tools identified four additional putative RUNX2 binding sites, as summarized in Table 2. BS2, BS4, and BS5 were perfect matches to the RUNX2 consensus binding site (5′-TGPyGGTPy-3′, where GPyGG is the core) and also were consistent with the most frequent sequence 5′-TGTTGGT3′. On the other hand, BS1 and BS3 differed by one base, next to the core. Evolutionary conservation was assessed for the five predicted sites (data not shown), and all showed a certain degree of conservation in closely related primates. Interestingly, none was conserved in mouse or rat.

In vitro assessment of RUNX2 binding to the LRP5 upstream region

In vitro binding of the RUNX2 transcription factor at the other four predicted binding sites (BS2, BS3, BS4, and BS5) was assessed by electromobility gel-shift assays. Figure 6A shows the presence of specific binding at all the sites tested. While wild-type competitors had a clear competitive effect, this was not the case for the mutated probes. GRE and SP1, used as nonspecific competitors, were not able to erase the shifted bands. Specific competition with OSE and supershift experiments (Fig. 6B) confirmed the involvement of RUNX2 in the protein complexes retained in the gel shift.

Involvement of the identified RUNX2 binding sites in the transcriptional activity of the LRP5 5′ region

The functional involvement of the five RUNX2 binding sites was tested by gene-reporter assays (Fig. 7). The wild-type construct (LP-C) was compared with five other constructs, each containing the five RUNX2 sites, one of which was mutated (MUT1-C, MUT2, MUT3, MUT4, and MUT5). Transfections were carried out in the two osteoblastic cell lines and in HeLa cells. The mutation of any of the sites resulted in significant changes in transcription activity in at least one of the cell lines. Mutation of BS5 (MUT5) had the greatest effect in the three cell types: around a twofold increase in Saos-2 and U-2 OS cells and a four-fold increase in HeLa cells. The differences between means were 108.4 ± 32.6 in Saos-2 cells (p < .0001), 88.5 ± 54.0 in U-2 OS cells (p = .0026), and 380.3 ± 131.5 in HeLa cells (p < .0001). A similar effect, although somewhat lower, was observed for site 1 (MUT1-C), as shown previously (Fig. 3). MUT2 and MUT4 led to a small but significant decrease in luciferase activity only in U-2 OS cells (differences between means: −37.0 ± 34.7, p = .038, and −40.1 ± 33.8, p = .0223, respectively), whereas MUT3 led to a small but significant increase in Saos-2 cells (difference between means: 30.2 ± 21.2, p = .0058).

The RUNX2 expression vector was cotransfected with each of the mutants, and the transcriptional activity was plotted as the fold change relative to that produced by cotransfection with an empty vector (Fig. 8). A general reduction in the RUNX2 stimulatory effect was observed for all mutants compared with that of the LP-C wild type, suggesting a functional role for all sites. In particular, MUT1-C and MUT3 were not significantly stimulated by RUNX2 cotransfection, whereas MUT2 and MUT5 were slightly stimulated (p = .028 and p = .018, respectively). Interestingly, a significant reduction in the activity of MUT4 was observed after RUNX2 cotransfection (p = .018).

Discussion

Osteoporosis is a complex disease in which bone quality is impaired and bones are prone to fracture. As in other multifactorial diseases, a complex interplay between genetic and environmental factors determines the phenotype. Genetic association analyses have been used widely to study its genetic component, and LRP5 has been demonstrated to be one of the most relevant osteoporosis genes at the genome-wide level. A
previous study by our group reported a positive association between the SNP rs312009 located in the 5’ region of LRPS and lumbar spine BMD, and we identified a RUNX2 binding site at this SNP position.\(^{(29)}\) RUNX2 is a master regulator in bone biology and targets many important bone genes. Here we assessed the possible functional role of the LRPS rs312009 polymorphism in the above-mentioned association. We hypothesized that RUNX2 binding at the SNP site (BS1) may be allele-dependent, leading to differential allele-specific transcriptional capacity. Using gene-reporter assays, we showed allele-specific differences in transcriptional activity between the C and T alleles of this SNP. In addition, with cotransfection, site-directed mutagenesis, and RNA interference, we illustrated the implications of RUNX2 in regulation of the LRPS promoter.

We demonstrated for the first time that LRPS is modulated by RUNX2. RUNX2 and the Wnt signaling pathway, together with osterix, are key regulators of osteoblast differentiation and function.\(^{(7,35)}\) Some interconnections between these pathways have already been described. For example, the family of transcription factors LEF/TCF that are downstream effectors of the Wnt β-catenin signaling pathway have been shown to interact with RUNX2 on some promoters\(^{(12,26)}\) and also to act on
the RUNX2 promoter itself. On the other hand, the inhibitor of the Wnt signaling pathway sclerostin is a target of RUNX2. It harbors a RUNX2 binding site, acting as a transcriptional activator in its promoter. Furthermore, AXIN3 is under RUNX2 regulation. Here we propose another level of regulation in which RUNX2 acts on the LRP5 promoter. Interactions between these two master regulatory networks may be crucial for osteoblast maturation and mature function.

Reporter-gene experiments showed allelic differences, the T allele being a better transcriber, in both Saos-2 and U-2 OS cells. This result is consistent with the fact that the T allele contains a RUNX2 binding site that is more similar to the consensus than that of the C allele. These differences could not be detected by the EMSA experiments presumably owing to the limited sensitivity of the technique.

Importantly, cotransfection of pCMV-Runx2 in HeLa cells stimulated both the LP-C and LP-T promoters, revealing for the first time the effect of this transcription factor on LRP5 expression. The effect was stronger at 24 hours than at 48 hours, which is consistent with a direct effect of RUNX2 on the LRP5 promoter. The selective mutation of BS1 (in both allele contexts) abolished this response, highlighting the relevance of this binding site for RUNX2 stimulation. However, the mutation of BS1 resulted in increased expression in HeLa cells in a RUNX2-independent manner. These changes may be explained either by the destruction of a repressor binding site or by the artificial creation of an activator binding site. Indeed, an in silico search of transcription binding sites in the mutated sequence revealed the possibility of a STATx recognition element, most probably STAT6, in both MUT1-C and MUT1-T.

Furthermore, the inhibition of the endogenous RUNX2 mRNA by siRNAs in U-2 OS cells led to a decrease of the endogenous

![Figure 5](image-url)

**Fig. 5.** Effect of RUNX2 mRNA inhibition on endogenous LRP5 gene expression. Transfection of the siRNA s2456 against RUNX2 was performed in U-2 OS and Saos-2 cells, and LRP5 mRNA levels were quantified by qPCR 24 hours after transfection. GAPDH was used as a reference gene. Each cell line transfected with the Silencer FAM-labeled negative control siRNA was taken as reference value (control). The bars represent the average mRNA levels relative to the control cells of two independent experiments with at least two replications each. The error bars represent SD. The asterisks indicate the statistical significance (according to an unpaired Student’s t test) of the difference between the control and RUNX2-inhibited cells.

![Figure 6](image-url)

**Fig. 6.** (A) DNA oligonucleotides containing BS2, BS3, BS4, or BS5 specifically bind nuclear proteins that are present in osteoblastic extracts. DNA binding was analyzed by gel-shift assays. Labeled double-stranded oligonucleotide probes were incubated with 10 μg of Saos-2 nuclear extract. Competition experiments were performed with the respective cold probes (wt) or mutated cold probes (MUT2, MUT3, MUT4, and MUT5, respectively) at increasing molar excesses. Probes containing GRE or the Sp1-binding site were used as nonspecific competitors and the OSE probe as a RUNX2-specific competitor. (B) An anti-RUNX2 antibody and not an anti-SP1 antibody is able to generate supershifts (arrowheads) for all four binding sites.

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LRP5 mRNA levels, showing a rate-limiting role of RUNX2 on LRP5 regulation. However, in Saos-2 cells, this effect was not observed, probably owing to the high levels of RUNX2 present in this cell line.

To further characterize the effect of RUNX2 on the LRP5 promoter, we looked for additional RUNX2 binding sites within the 3.3-kb region included within the LP construct. Predictive tools allowed the identification of four other RUNX2 binding sites (BS2, BS3, BS4, and BS5), which were confirmed by EMSA and supershift experiments. These are located more than 1 kb upstream of the transcription start site and are present in primates but not in other mammals, in agreement with data reported by Twells and colleagues, which showed the lack of LRP5 promoter conservation between human and mouse. This lack of evolutionary conservation may indicate that human and mouse LRP5 promoters are subjected to different regulatory controls with different regulatory boxes or with the same boxes but organized differently.

A series of reporter gene experiments in which each BS was selectively mutated revealed the participation of each of them in the RUNX2-response. All BS mutations affected transcriptional activity, but again, the effects differed according to the site and the cell type used. Moreover, all BSs, when mutated individually, altered the RUNX2-stimulatory effect observed after LP-C RUNX2 cotransfection. RUNX2 acts as a repressor or activator depending on the presence of different cofactors or genomic contexts. Further study of the genomic proximity of each RUNX2 binding site and the presence of possible cofactor interactions may allow determination of the precise role played by each BS in the different cell types. Furthermore, our study of the 3.3-kb region upstream of the LRP5 gene by means of serial deletions suggests the existence of several repressor elements because the luciferase activity was inversely proportional to the amount of 5' sequence included in the construct. These repressors need to be characterized further. To our knowledge, only one previous study has addressed the dissection of the human LRP5 promoter. In general, our results in the U-2 OS cell line are consistent with those of Li and colleagues.

One of the limitations of this work is that transcription gene-reporter assays are known to depend on the cellular systems employed. Both Saos-2 and U-2 OS cells are osteosarcoma cell lines generally used as osteoblast models. They represent different differentiation states, and because they are transformed, there are important genetic differences between them and with normal osteoblasts. We can only speculate with the participation of other proteins/factors that could be present in different quantities in these cell lines. On the other hand, Hela cell line is not an osteoblastic cell, and it was chosen as a model of cells lacking RUNX2 expression. The complete pattern of transcription factors expressed by this cell type is obviously different form that in osteoblastic cells. Other cells that were not studied here might be relevant. The work by Yadav and colleagues suggests that the effect of LRP5 on bone depends on its expression in the enterochromaffin cells in the duodenum.
Thus experiments on the regulation of the LRP5 promoter in this cell type should be very interesting. Other members of the RUNX family of transcription factors are expressed in gastroepithelial cells, and it would be useful to discern whether they interact with the BSs described here. It also would be important to assess RUNX2 activity in these cells.

In conclusion, this analysis provides functional evidence of the involvement of the BMD-associated SNP rs312009 in LRP5 promoter activity. Our functional analysis also suggests a role for the RUNX2 transcription factor in LRP5 transcriptional regulation in osteoblast-like cells.

Disclosures

All the authors state that they have no conflicts of interest.

Acknowledgments

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