Bases Genètiques de l'Osteoporosi: Estudi del gen *LRP5*.

Lídia Agueda Calpena

BASES GENÈTIQUES DE L'OSTEOPOROSI: ESTUDI DEL GEN *LRP5*

Memòria presentada per
Lídia Àgueda Calpena
Per optar al grau de
Doctora per la Universitat de Barcelona

Tesi dirigida per la Dra. Susana Balcells Comas i pel Dr. Daniel Grinberg Vaisman
Al departament de Genètica de la Facultat de Biologia
de la Universitat de Barcelona
Programa de Genètica 2004-2006

Dra. Susana Balcells Comas

Dr. Daniel Grinberg Vaisman

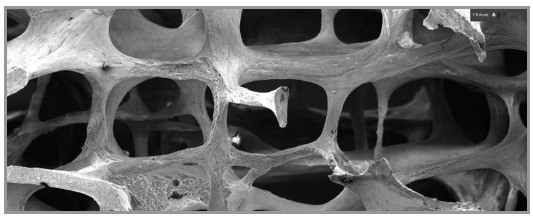
Lídia Agueda Calpena
2010

CONTINGUTS

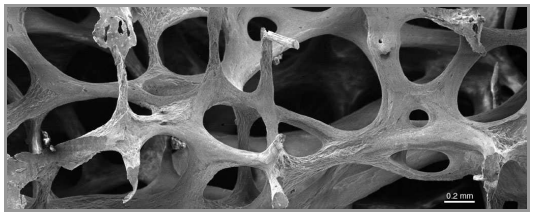
LLISTAT D'ABREVIATURES:

BARCOS	cohort de dones potsmenopàusiques de l'àrea de Barcelona
BMPs	proteïnes morfogèniques de l'os
CBFA1	*core binding factor α 1*
CCD	displàsia cleido-cranial
CD/CV	*common disease / common variant hypothesis*; malaltia comuna / variant comuna
CD/RV	*common disease / rare variant hypothesis*; malaltia comuna / variant minoritària
CNVs	*copy number variants*, variants de nombre de còpia
COL1A1	cadena α 1 del col·lagen tipus I
CHIP	immunoprecipitació de cromatina
DMO/BMD	densitat mineral ossia, *bone mineral denisty*
DXA	Absorciometria dual de rajos X
EMSA	*Electrophoretic mobility shift assay*, assaig de canvi en la mobilitat electroforètica o estudi de retenció en gel
ESR1 ESR2	receptors d'estrògens 1 i 2
FGF	factor de creixement fibroblàstic
FN	*femoral neck,* coll del fèmur
GEFOS	Genetic Factors for Osteoporosis, projecte europeu
GENOMOS	Genetic Markers for Osteoporosis, projecte europeu
GWA	*genome wide association*, associació a nivell de tot el genoma
HapMap	Mapa d'Haplotips del Genoma Humà, projecte internacional
HBM	*high bone mass*, fenotip d'alta massa òssia
HeLa	línia cel·lular d'adenocarcinoma humà
Kb	quilobase
LCT	lactasa
LD	desequilibri de lligament
LRP5	proteïna 5 relacionada amb el receptor de lipoproteines de baixa densitat
LRP6	proteïna 6 relacionada amb el receptor de lipoproteines de baixa densitat
LS	*lumbar spine*, columna
MAF	freqüència de l'al·lel minoritari
M-CSF	factor estimulador de colònies de macròfags
MSC	cèl·lules mare mesenquimals
MTHFR	metilentetrahidrofolat reductasa
OPG	osteoprotegerina
OPPG	síndrome de l'osteoporosi-pseudoglioma
OR	*odds ratio*, oportunitat relativa, raons d'oportunitat
OSE	element específic d'osteoblast
P	nivell de significació
pGL3	vector amb el gen reporter Luciferasa (de Promega)
PTH	hormona paratiroidea
QTL	*quantitative trait locus*, locus de tret quantitatiu
RANK	membre 11A de la superfamília de receptors del TNF
RANKL	lligand del receptor RANK
RFLP	polimorfisme de fragments de restricció de longitud polimòrfica

RUNX2	*runt related transcription factor 2*, factor de transcripció 2 relacionat amb el domini runt (Cbfa1)
SAOS-2	línia cel·lular d'osteosarcoma humà
SNP	*single nucleotide polymorphism*, polimorfisme de canvi puntual de nucleòtid
TCF/LEF	*lymophoid enhancer binding factor/* factor de transcripció específic de cèlules T
TNFs	factors de necrosi tumoral
U-2 OS	línia cel·lular d'osteosarcoma humà
VDR	receptor de la vitamina D_3



RESULTATS



CAPÍTOL 1 : A haplotype-based analysis of the LRP5 gene in relation to osteoporosis phenotypes in Spanish postmenopausal women.

REFERÈNCIA:

Agueda L, Bustamante M, Jurado S, Garcia-Giralt N, Ciria M, Saló G, Carreras R, Nogués X, Mellibovsky L, Díez-Pérez A, Grinberg D, Balcells S.
Journal of Bone and Mineral Research. 2008 Dec; 23(12):1954-63.

RESUM:

LRP5 codifica per la proteïna 5 relacionada amb el receptor de les lipoproteïnes de baixa densitat, una proteïna transmembrana que participa en la via de senyalització de Wnt. *LRP5* és un regulador clau per al creixement i diferenciació dels osteoblasts, i que per tant, afecta a la massa òssia en els vertebrats. Tantmateix, és important saber si la variabilitat comuna en *LRP5* està associada a la variació normal dels valors de densitat mineral òssia (DMO) o als fenotips osteoporòtics. En el present treball, varem utilitzar una aproximació basada en haplotips per a la cerca de variants comunes d'*LRP5* associades amb l'osteoporosi, en una cohort de 964 dones post-menopàusiques espanyoles. Es van seleccionar vint-i-quatre SNPs que cobrien la regió genòmica d'*LRP5*, incloent les variants *missense* p.V667M i p.A1330V. Els SNPs es varen genotipar i se'n va testar l'associació amb la DMO lumbar, la DMO femoral i la presència de fractura osteoporòtica, a nivell d'SNPs individuals i d'haplotips, mitjançant models de regressió. Varem trobar associació amb la DMO lumbar per al SNP 1, rs312009, localitzat a la regió 5' flanquejant del gen (p = 0,011, model recessiu). L'SNP 6, rs2508836, a l'intró 1, també es va trobar associat amb DMO lumbar (p = 0,025, model additiu) i amb DMO femoral (p = 0,031, model recessiu). Dos SNPs es varen mostrar associats amb fractura: l'SNP 11, rs729635, a l'intró 1, I l'SNP 15, rs643892, a l'intró 5 (p = 0,007 model additiu i p = 0,019 model recessiu, respectivament). L'anàlisi per haplotips no va proporcionar associacions addicionals, excepte per a l'haplotip "GC" del bloc localitzat al extrem 3' del gen. Aquest haplotip abarca l'intró 22 i la regió 3' no traduïda i es va trobar associat amb DMO femoral (p = 0,029, una còpia de l'haplotip *versus* cap). Les anàlisis *in silico* varen mostrar que l'SNP1 (rs312009) es troba en un lloc putatiu d'unió de Runx2. Els assaigs de canvi de mobilitat electroforètica varen confirmar la unió de Runx2 en aquesta posició.

INFORMACIÓ SOBRE LA PUBLICACIÓ I INDEX D'IMPACTE:

La revista Journal of Bone and Mineral Research (JBMR) proporciona un fòrum per a articles de la màxima qualitat de l'àmbit de la biologia i la fisiologia òssies, de les hormones que regulen el metabolisme ossi i mineral, i de la patofisiologia i el tractament dels defectes del metabolisme ossi i mineral. El JBMR és la revista oficial de la Societat Americana de Recerca Òssia i Mineral (American Society for Bone and Mineral Research , ASBMR) i la publica mensualment Wiley-Blackwell en nom d'aquesta societat. És la primera en el *ranking* de revistes d'aquest camp, amb un factor d'impacte de **6,443**. El seu editor en cap és Thomas L Clemens (Baltimore, Maryland, USA).

APORTACIÓ PERSONAL A L'ARTICLE:

- Extracció dels DNAs d' aproximadament la meitat de les mostres de la cohort BARCOS. Aquestes mostres han estat usades en d'altres estudis d'associació realitzats pel nostre grup de recerca.
- Selecció dels SNPs a genotipar
- Disseny dels plexes d'SNPlex, en col·laboració amb altres membres del grup i amb el suport del CEGEN
- Reconstrucció dels haplotips, anàlisi estadística i interpretació dels resultats
- Càlculs de poder estadístic i d'estratificació poblacional
- Estudis funcionals per al SNP rs312009
- Elaboració del primer esborrany de l'article i participació en l'elaboració del manuscrit final.

VIST-I-PLAU DELS DIRECTORS DE TESI:

Dra. Susana Balcells Comas Dr. Daniel Grinberg Vaisman

CAPÍTOL 2: Large-scale analysis of association between LRP5 and LRP6 variants and osteoporosis.

REFERÈNCIA:

van Meurs JB, Trikalinos TA, Ralston SH, Balcells S, Brandi ML, Brixen K, Kiel DP, Langdahl BL, Lips P, Ljunggren O, Lorenc R, Obermayer-Pietsch B, Ohlsson C, Pettersson U, Reid DM, Rousseau F, Scollen S, Van Hul W, **Agueda L**, Akesson K, Benevolenskaya LI, Ferrari SL, Hallmans G, Hofman A, Husted LB, Kruk M, Kaptoge S, Karasik D, Karlsson MK, Lorentzon M, Masi L, McGuigan FE, Mellström D, Mosekilde L, Nogues X, Pols HA, Reeve J, Renner W, Rivadeneira F, van Schoor NM, Weber K, Ioannidis JP, Uitterlinden AG; GENOMOS Study.
Journal of the American Medical Association. 2008 Mar 19;299(11):1277-90.

RESUM:

CONTEXT: Mutacions en el gen de la proteïna 5 relacionada amb el receptor de les lipoproteïnes de baixa densitat (LRP5) causen síndromes minoritaries caracteritzades per l'alteració de la densitat mineral òssia (DMO). Algunes variants genètiques comunes en LRP5 podrien afectar al risc de patir osteoporosi en la població general.

OBJECTIU: Generació d'evidències a gran escala de l'associació de dues variants comunes d'LRP5 (Val667Met, Ala1330Val) i una variant d'LRP6 (Ile1062Val) amb DMO i risc de fractura.

DISSENY: Estudi prospectiu, multicèntric i col·laboratiu de dades individuals de cada un dels 37.534 individus procedents de 18 grups europeus i nord-americans participants. La recopilació de dades es va dur a terme entre setembre del 2004 i gener del 2007; l'anàlisi de les dades es va realitzar entre febrer i maig del 2007. La DMO es va determinar per absorciometria dual de raigs X. Les fractures es varen identificar via qüestionari, registres mèdics o documentació radiogràfica; per algunes de les cohorts es disposava de dades sobre les fractures incidents, certificades per mitjà de mètodes de vigilància rutinaris, incloent examen radiològic per les fractures vertebrals.

PRINCIPALS FENOTIPS ESTUDIATS: Densitat mineral òssia lumbar i del coll del fèmur; prevalença de totes les fractures i de fractures únicament vertebrals.

RESULTATS: L'al·lel Met667 d'*LRP5* va resultar associat a DMO lumbar reduïda (n = 25.052 [nombre de participants amb dades disponibles]; per cada còpia de l'al·lel Met667, reducció de la DMO de 20-mg/cm^2; P = 3,3 x 10^{-8}), i també ho va fer l'al·lel Val 1330 (reducció de la DMO de 14-mg/cm^2 per còpia de Val1330, n = 24.812; P = 2,6 x 10^{-9}). Per la DMO femoral es varen observar efectes similars, amb

un descens de 11 mg/cm^2 (P = 3,8 x 10^{-5}) i 8 mg/cm^2 (P = 5,0 x 10^{-6}) per al·lel Met667 i Val1330, respectivament (n = 25 193). Els dos al·lels van resultar associats amb fractura vertebral (odds ratio [OR], 1,26; interval de confiança del 95% [CI], 1,08-1,47 per Met667 [2001 fractures d'entre 20.488 individus] i OR, 1,12; 95% CI, 1,01-1,24 per Val1330 [1988 fractures d'entre 20.096 individus]). El risc per a qualsevol tipus de fractura també es va trobar incrementat per Met667 (OR, 1,14; 95% CI, 1,05-1,24 per al·lel [7876 fractures d'entre 31.435 individus]) i Val1330 (OR, 1,06; 95% CI, 1,01-1,12 per al·lel [7802 fractures d'entre 31.199 individus]). Els efectes foren similars al realitzar ajusts per edat, pes, talla, estat menopàusic i l'ús de teràpies hormonals. Els riscs de fractura es mostraren parcialment atenuats per l'ajust per DMO. L'anàlisi d'haplotips va indicar que les variants Met667 i Val1330 afecten independentment a la DMO. El polimorfisme Ile1062Val d'*LRP6* no es va trobar associat amb cap dels fenotips osteoporòtics. Totes les associacions esmentades anteriorment, excepte per Val1330 i tots els tipus de fractura, continuaven essent significatives després dels ajusts corresponents per múltiples tests.

CONCLUSIONS: Les variants comunes d'*LRP5* es troben associades de manera consistent amb la DMO i el risc de fractura en diferents poblacions blanques. La magnitud de l'efecte d'aquestes associacions és modesta. *LRP5* podria ser el primer gen que supera els nivells de significació *genome-wide* (un nivell de significació conservatiu [aquí P < 10^{-7}, sense ajustar] que té en consideració les possibles comparacions en el genoma humà) per a un fenotip relacionat amb l'osteoporosi.

INFORMACIÓ SOBRE LA PUBLICACIÓ I INDEX D'IMPACTE:

La revista *Journal of the American Medical Association* (JAMA) és una de les publicacions mèdiques més importants del món, publicada ininterrompudament des del 1883 amb 48 edicions l'any. El seu principal objectiu és la promoció de la ciència i la medicina per a la millora de la salut pública. Abarca un rang ampli de temes mèdics. El seu índex d'impacte és de **31,718**. El seu editor en cap és la doctora Catherine DeAngelis (MD, MPH).

APORTACIÓ PERSONAL A L'ARTICLE:

- Extracció dels DNAs d' aproximadament la meitat de les mostres de la cohort BARCOS. Aquestes mostres han estat usades en d'altres estudis d'associació realitzats pel nostre grup de recerca.
- Genotipat dels polimorfismes Val667Met i Ala1330Val d'LRP5 i Ile1062Val d'LRP6 en la cohort BARCOS i en les mostres control del projecte GENOMOS.
- Control de qualitat dels genotips generats en la cohort BARCOS i de les dades associades a cada membre de la cohort.

VIST-I-PLAU DELS DIRECTORS DE TESI:

Dra. Susana Balcells Comas Dr. Daniel Grinberg Vaisman

CAPÍTOL 3: Functional relevance of the BMD-associated polymorphism rs312009. Novel implication of Runx2 in LRP5 transcriptional regulation

REFERÈNCIA:

L. Agueda[1], R. Velázquez-Cruz[1#], R. Urreizti[1], P. Sarrion[1], S. Jurado[2], G. Yoskovitz[2], R. Güerri[2], N. Garcia-Giralt[2], X. Nogués[2], L. Mellibovsky[2], A. Díez-Pérez[2], P. J. Marie[3], S. Balcells[1]*, D. Grinberg[1]*

[1]Dpt Genetics, Faculty of Biology, University of Barcelona, IBUB, CIBER de Enfermedades Raras (CIBERER), Barcelona, SPAIN

[2]Internal Medicine, URFOA, IMIM, Hospital del Mar, Autonomous University of Barcelona, Barcelona, SPAIN

[3]Inserm U606 and University Paris Diderot, Paris, FRANCE

Sotmés a Journal of Bone and Mineral Research.

RESUM:

LRP5 és un dels gens establerts de susceptibilitat a l'osteoporosi. Diversos estudis d'associació han conduit a la identificació de polimorfismes individuals que determinen la variació entre individus en els valors de densitat mineral òssia, així com en el risc de fractura. En un treball anterior varem identificar un polimorfisme associat a la densitat mineral òssia lumbar, anomenat rs312009, i localitzat a la regió 5' d'*LRP5*. Per mitjà d'assaigs de retardament en gel, es va identificar un lloc d'unió de Runx2 en la seva posició. L'objectiu del present treball ha estat testar la funcionalitat d'aquest polimorfisme i analitzar si Runx2 podia ésser un regulador de l'expressió d'*LRP5*.

En una regió de 3,3kb que flanqueja *LRP5* per 5' s'han cercat elements d'unió a Runx2 per mitjà d'eines predictives bioinformàtiques. S'han usat assaigs de retardament en gel i de gen reporter per testar la funcionalitat d'aquests llocs d'unió. Per mitjà de mutagènesi dirigida, així com de cotransfeccions amb Runx2, s'ha volgut determinar la participació de Runx2 en la regulació d'aquesta regió d'*LRP5*. Les diferents construccions s'han transfectat en les línies osteoblàstiques U-2 OS i Saos-2 i en HeLa.

S'ha observat diferències al·lèliques en la capacitat transcripcional del rs312009 en les dues línies osteoblàstiques, on l'al·lel T presentava major capacitat que el C. La transfecció de Runx2 en les cèl·lules HeLa ha demostrat que la regió reguladora estudiada, era capaç de respondre a Runx2 d'una manera dosi-depenent i que el lloc de Runx2 prèviament identificat participava en aquesta resposta. S'han identificat quatre llocs

addicionals d'unió de Runx2 en la regió 5' estudiada. En els experiments de luciferasa, on cada un dels llocs de Runx2 han estat mutats individualment, s'ha posst de manifest la participació de tots ells en la resposta a Runx2.

En conclusió, les diferències al·lèliques observades semblen indicar que rs312009 és un polimorfisme funcional implicat en l'esmentada associació. Que ens consti, aquesta és la primera vegada que es descriu la acció directa de Runx2 sobre el gen *LRP5*. Es doncs una nova evidència de les connexions ja descrites entre la cascada del factor de transcripció Runx2 i la via de senyalització de Wnt, dos importants sistemes reguladors ossis.

INFORMACIÓ SOBRE LA PUBLICACIÓ I INDEX D'IMPACTE:

Veure Capítol 1 de Resultats (pàgina 66).

APORTACIÓ PERSONAL A L'ARTICLE:

- Participació en el disseny dels experiments
- Cultius cel·lulars i tècniques de biologia molecular emprades: Assaigs de retardament en gel, Western Blot, PCR quantitativa, obtenció del vectors emprats (excepte pCMV5-Runx2) i assaig de gen reporter.
- Prediccions de llocs d'unió de factors de transcripció.
- Anàlisi estadístic dels resultats.
- Elaboració del primer esborrany de l'article i participació en l'elaboració del manuscrit final.

VIST-I-PLAU DELS DIRECTORS DE TESI:

Dra. Susana Balcells Comas Dr. Daniel Grinberg Vaisman

FUNCTIONAL RELEVANCE OF THE BMD-ASSOCIATED POLYMORPHISM RS312009: NOVEL INVOLVEMENT OF RUNX2 IN *LRP5* TRANSCRIPTIONAL REGULATION

L. Agueda[1], R. Velázquez-Cruz[1#], R. Urreizti[1], P. Sarrion[1], S. Jurado[2], G. Yoskovitz[2], R. Güerri[2], N. Garcia-Giralt[2], X. Nogués[2], L. Mellibovsky[2], A. Díez-Pérez[2], P. J. Marie[3], S. Balcells[1]*, D. Grinberg[1]*

[1]Dpt Genetics, Faculty of Biology, University of Barcelona, IBUB, CIBER de Enfermedades Raras (CIBERER), Barcelona, SPAIN

[2]Internal Medicine, URFOA, IMIM, Hospital del Mar, Autonomous University of Barcelona, Barcelona, SPAIN

[3]Inserm U606 and University Paris Diderot, Paris, FRANCE

[#]present address: Instituto Nacional de Medicina Genomica, Mexico City, MEXICO

*Equal contributors

ABSTRACT: *LRP5* is an osteoporosis susceptibility gene. Association analyses reveal individual SNPs that 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, 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 the 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. 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.

KEYWORDS: 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 (1-2) . 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) (3). In osteoblasts, LRP5 can transduce canonical signals to promote the renewal of stem cells, stimulation of pre-osteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis by increasing the levels of β-catenin and altering gene expression through the LEF/TCF transcription factors (4).

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 (5). As demonstrated by several genetic studies in mouse and human, 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, named 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 co-factors that interact with Runx proteins are co-activators such as p300 and

CREB binding protein (CEBP); and co-repressors such as mSin3A, transducin-like enhancer of split proteins (TLEs), and several histone desacetylases (Hdacs).

Several Runx2 responsive elements have been identified in important bone-related genes, such as osteocalcin (10-16), osteoprotegerin (17), osterix (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). Sost is a secreted protein that can act as an inhibitor of canonical Wnt signaling by interacting with Lrp5/6 co-repressors whereas Axin2 is a negative regulator of this pathway by another mechanism based on the promotion of β-catenin degradation (reviewed by Liu et al. (24)).

Other links between Runx2 and canonical Wnt signaling pathways have previously been reported. Khaler et al. (12) described Lef1, a final effector of Wnt signaling, as a Runx2 transcriptional regulator. Gaur and collaborators (25) and Reinhold (26) also reported cooperation between LEF/TCF and Runx2 factors in RUNX2's own promoter and the *FGF18* promoter respectively. Recently, McCarthy and Centrella (27) 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 or fracture in different association analysis approaches, such as single missense SNP analysis, gene-wide analysis and even GWA (reviewed by Li et al. (28)). Therefore *LRP5* is a confirmed osteoporosis susceptibility gene. In this sense, we have previously reported the association of a *LRP5* promoter SNP, rs312009, with lumbar spine bone mineral density (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 the present 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# HTB-85, ATCC# HTB-96 and ATCC# CCL-2^{TM}, respectively). 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; Invitrogene).

Immortalized human neonatal calvaria cells (IHNC) were obtained as described previously (30) and were the generous gift of Dr. Eric Haÿ.

In silico *prediction and alignment tools*

The presence of putative Runx2 binding sites was assessed using two predictive tools: MatInspector from GENOMATIX (31) with the core similarity threshold set at 0.75, and TFSEARCH (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 (33).

Western blot

Nuclear cell extracts from Saos-2, U-2 OS and HeLa 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 an HRP-conjugated secondary antibody and chemiluminescence detection. The following primary antibodies were used: Runx2 M-70 at 1:800 dilution (Santa Cruz Biotechnologies, SC-10758x), and nucleoporine NP62 at 1:4000 dilution (BD Biosciences) for the loading control. Horseradish peroxidase-conjugated secondary antibodies were used (GARPO and SAMPO, respectively; SIGMA). Immunoreactive bands were detected by incubating the membrane for 2 min 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% H_2O_2.

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.). Three µg of total RNA were reverse transcribed using M-MLV RT (Invitrogen) with oligo-dT priming according to the manufacturer's instructions. Five µl of a 1/20 dilution of the resulting cDNA were used for subsequent amplification in a LightCycler 480 (Roche) with SYBR Green fluorescent dye (ABsolute Blue QPCR SYBR Green Mix, Thermo Scientific) under standard conditions. *GAPDH* mRNA was used for normalization, and the results were expressed relative to *RUNX2* expression in the IHNC cell line. For PCR amplification the following primers were used: *GAPDH* forward: 5'-CGAGATCCCTCCAAAATCAA-3'; *GAPDH* reverse: 5'-TGTGGTCATGAGTCCTTCCA-3'; *RUNX2* forward: 5'-TCTGGCCTTCCACTCTCAGT-3' and *RUNX2* reverse: 5'-GACTGGCGGGGTGTAAGTAA-3'.

Electrophoretic mobility shift assays (EMSA)

Saos-2 human osteosarcoma cells (ATCC number HBT-85TM) were grown in Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% FCS (Gibco). Nuclear extracts were prepared according to Schreiber et al. (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 °C until use. Single-stranded DNA oligonucleotides were automatically synthesized (Sigma Aldrich) (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 [γ-32P] ATP (GE Healthcare or Perkin Elmer), using the OptiKinase (USB) standard protocol. The unincorporated nucleotides were removed using a quick-spin G-25 Sephadex column (Roche). Binding reactions typically contained 10 µg of nuclear extract, 0.5 µg of double-stranded poly (dI-dC) (Amersham Pharmacia Biotech), 0.5 µg of double-stranded poly (dA-dT) (Roche), 6 µg of acetylated BSA (Promega) and 100,000 cpm of radiolabelled probe. The reaction mixtures were incubated for 30 min at room temperature in a buffer containing 20 mM HEPES pH 7.9, 80 mM KCl, 1 mM EDTA, 1 mM DTT and 10%

glycerol in a total volume of 20 µl. 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 °C in 1× TBE buffer at 20 mA for approximately 3 h. Gels were vacuum-dried and exposed to Storage Phosphor screens (Kodak) at RT for 3—12 h. In competition assays, the binding reactions were performed in the presence of an excess of unlabelled competitor oligonucleotide, as indicated in each case.

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 67,080,193 bp (according to Feb 2009, GRCh37/hg19 UCSC genome assembly) corresponding to positions -1879 to +11, from A(+1)TG in the *LRP5* promoter region, was obtained from Saos-2 DNA using the following primers F8: 5'-GAGTCCCAGCAGAGAACAGC-3', R7:5'-GCTGCCTCCATGTTGTCC-3'. This fragment was cloned in pUC18/SmaI (Amersham Pharmacia Biotech) by blunt-end ligation and subcloned by *Ade*I and *Nco*I digestion in the pGL3 basic vector/*Sma*I (Promega). This clone was named **MP** and contained positions -49/-1826 of *LRP5*. Subsequent digestion of **MP** with *Blp*I and *Nco*I produced a 729-bp fragment that was recloned in the pGL3 basic vector/*Sma*I to obtain the **BP** construct (-49/-778). A second PCR fragment was obtained using the following primers F: 5'-GTGATTCTCCCGCCTCAGC-3' and R: 5'-TCATGCGTCCCCACTTGCT-3' (Chr11: 68,076,318-68,078,510; 2192 bp) again from Saos-2 DNA. This fragment was digested with *Ale*I and *Avr*II and subcloned to an *Nhe*I- and *Ale*I-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). Site-directed mutagenesis was also 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, $5x10^4$ 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 µg of reporter construct or 0.875 µg of reporter construct plus 0.875 µg of effector and 50 ng of normalizing beta-galactosidase expression vector (Upstate

Biotechnology) were co-transfected using 3 μl of Transfast transfection reagent (Promega). The effector was pCMV-Runx2, a mouse Runx2 isoform II (MASNS) cDNA cloned in the pCMV5 expression vector (pCMV-Runx2) (kindly provided by Dr. Karsenty, Columbia University, NY, USA). This form has 97.6% homology at the amino acid level with the orthologous isoform in humans. Empty pCMV5 vector (pCMV-EV) was also cotransfected in order to provide the same total amount of DNA in cotransfection experiments.

Twenty-four/forty-eight hours after transfection, luciferase and beta-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 Figure 2C). The results were expressed as relative luciferase units (RLU) 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 ± standard error of the mean (x ± s.e.m.). Statistical significance was determined by unpaired Student *t* tests (for comparisons between different constructs) and by paired Student *t* tests (for comparison of the same construct in different cotransfection conditions). Significance is denoted as follows: * (or §) for $p<0.05$; ** (or §§) for $p<0.01$; *** (or §§§) for $p<0.001$ and **** (or §§§§) for $p<0.0001$.

RESULTS

Binding of Runx2 to the putative Runx2-responsive element at -2.9 kb of LRP5

The presence of a Runx2 binding site at -2.9 kb from the transcription start site of *LRP5* has previously been reported (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 Figure 1A and B. Equivalent patterns of shifted bands appeared for the BS1T (Figure 1A) and BS1C (Figure 1B) probes, and the same level of competition was observed with specific (BS1T or BS1C) and non-specific cold probes (GRE, SP1). Cross-competition with the other allele did not show any allelic differences either. Specific competition with the osteoblast-specific element from the osteocalcin gene promoter (OSE) revealed the participation of Runx2 in the retained protein complex (Figure 1A and B). 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, MUT1C was not able to compete the binding at BS1C at any concentration, and MUT1T showed some degree of competition for the BS1T binding, when added at high molar excess (x500).

Levels of Runx2 mRNA and Runx2 protein in human osteoblast-like cells and in non-osteoblastic HeLa

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 (Figure 2A). Using IHNC as a reference, the levels of RUNX2 mRNA in U-2 OS were approximately 60% of those in Saos-2. This difference was even greater at the protein level (Figure 2B). The non-osteoblastic 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 LRP5 5' region in three cellular contexts

The performance of the *LRP5* 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 *LRP5* (BP (up to -778), MP (up to -1826) and LP-C (up to -3274, bearing the C allele of rs312009)) were transfected and luciferase activity was measured (Figure 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, showing more than three-fold higher transcriptional activity than pGL3-EV ($p<0.0001$). In U-2 OS and HeLa this activity was double that of the empty vector ($p<0.0001$). In all cellular contexts, MP doubled the transcriptional activity of the LP-C construct ($p<0.0001$ in Saos-2, $p=0.0130$ in U-2 OS, and $p=0.0002$ in HeLa). BP also led to increased luciferase activity relative to LP-C in all cell lines ($p<0.0001$ in Saos-2 and HeLa, and $p=0.0002$ in U-2 OS) and to MP in the two osteosarcoma cell lines ($p=0.0003$ in Saos-2, and $p=0.0493$ in U-2 OS), 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 the 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* 5' region were obtained: i) LP-C, with the C allele in rs312009; ii) LP-T, with the T allele in rs312009; iii) MUT1-C, with mutated Runx2 BS1 core sequence and the C allele in rs312009; and iv) MUT1-T, with mutated Runx2 BS1 core sequence and the T allele in rs312009. A schematic representation of the constructs is shown in Figure 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% CI) were: 40.7 (±26.5), $p=0.0029$ in Saos-2, and 57.8 (±57.6), $p=0.049$ in U-2 OS. 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, 38.27 (±34.7), p=0.031; in U-2 OS, 74.1 (±41.1), p=0.0011; and in HeLa, 333.3 (±209.0), p=0.0022]. On the contrary, MUT1-T activity was significantly lower than LP-T only in the U-2 OS cell line [-63.9 (±63.2), p=0.0476].

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. Luciferase activity was tested at 24 and 48 h after transfection. Figure 4A shows a dose-dependent stimulatory effect caused by increasing amounts of Runx2 vector. This effect was higher at 24 h than at 48 h. Cotransfection of the maximal pCMV-Runx2 concentration tested (0.875 μg) caused a more than five-fold increase at 24 h (p=0.0013). 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 (Figure 4B). LP-T, but not the mutant constructs, was stimulated by Runx2 (p=0.0298), 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 (Figure 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.

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'-T**GPyGG**TPy-3', where GPyGG is the core) and were also consistent with the most frequent sequence 5'-TGTGGTT-3'. 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 5 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 non-specific competitors, were not able to erase the shifted bands. Specific competition with OSE 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 (Figure 6). 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. 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 cells types: around a two-fold increase in Saos-2 and U-2 OS, and a four-fold increase in HeLa. The differences between means were 108.4±32.6 in Saos-2 ($p<0.0001$), 88.5±54.0 in U-2 OS ($p=0.0026$), and 380.3±131.5 in HeLa ($p<0.0001$). A similar effect, although somewhat lower, was observed for site 1 (MUT1-C), as previously shown (see Figure 3). MUT2 and MUT4 led to a small but significant decrease in luciferase activity only in U-2 OS (differences between means: -37.0±34.7, $p=0.038$, and -40.1±33.8, $p=0.0223$, respectively), while MUT3 led to a small but significant increase in Saos-2 (difference between means: 30.2±21.2, $p=0.0058$).

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 (Figure 7). A general reduction in the Runx2 stimulatory effect was observed for all mutants compared to 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, while MUT2 and MUT5 were slightly stimulated (p=0.028 and p=0.018, respectively). Interestingly, a significant reduction in the activity of MUT4 was observed after Runx2 cotransfection (p=0.018).

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 widely used 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 *LRP5* and lumbar spine bone mineral density (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 *LRP5* rs312009 polymorphism in the above-mentioned association. We hypothesized that Runx2 binding at the SNP site (BS1) might 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 co-transfection and site-directed mutagenesis, we showed the implication of Runx2 in the regulation of the *LRP5* promoter.

We demonstrated for the first time that *LRP5* 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 (25). On the other hand, the inhibitor of the Wnt signaling pathway SOST is a target of Runx2. It harbors a Runx2 binding site acting as transcriptional activator in its promoter (22). Furthermore, Axin 3 is under Runx2 regulation (23). 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, both in Saos-2 and in 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 due 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 h than at 48 h, 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 (36), in both MUT1-C and MUT1-T.

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 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 et al. (37), 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 individually mutated, altered the Runx2-stimulatory effect observed after LP-C Runx2 cotransfection. Runx2 acts as a

repressor or activator depending on the presence of different co-factors or genomic contexts (38). Further study of the genomic proximity of each Runx2-binding site and the presence of possible co-factor interactions may allow dissecting 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, since the luciferase activity was inversely proportional to the amount of 5' sequence included in the construct. These repressors need to be further characterized. To our knowledge, only one previous study has addressed the dissection of the human *LRP5* promoter (39). In general, our results in the U-2 OS cell line are consistent with those of Li *et al.*

One of the limitations of this work is that transcription gene reporter assays are known to be dependent on the cellular systems employed. Other cells that were not studied here might be relevant. The work by Yadav et al. (40) 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 (41) and it would be useful to discern whether they interact with the BSs described here. It would also be important to assess Runx2 activity in these cells.

In conclusion, the current 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.

ACKNOWLEDGMENTS

The authors are grateful to M. Cozar and M. Bustamante for technical help and R. Rycroft for revising the English. LA was the recipient of a fellowship from the Catalan Government and an ECTS Travel Grant. RVC was supported in part by a grant from the Foundation Carolina of Spain and from the Consejo Nacional de Ciencia y Tecnología (CONACYT: SALUD-2008-C01-87331). This study was supported by grants from the Carlos III Health Institute (ISCIII), Spanish Ministry of Science and Innovation (PI030708), the Spanish Ministry of Education and Science (SAF2007-64654) and the Catalan Government (2005SGR 00848 and

2009SGR971). The authors are also grateful for the support from the Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER) and the Red Temática de Investigación Cooperativa en Envejecimiento y Fragilidad (RETICEF), which are initiatives of the ISCIII.

BIBLIOGRAPHY

1. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Juppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML 2001 LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell **107**(4)**:**513-23.

2. Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, Manning SP, Swain PM, Zhao SC, Eustace B, Lappe MM, Spitzer L, Zweier S, Braunschweiger K, Benchekroun Y, Hu X, Adair R, Chee L, FitzGerald MG, Tulig C, Caruso A, Tzellas N, Bawa A, Franklin B, McGuire S, Nogues X, Gong G, Allen KM, Anisowicz A, Morales AJ, Lomedico PT, Recker SM, Van Eerdewegh P, Recker RR, Johnson ML 2002 A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. Am J Hum Genet **70**(1)**:**11-9.

3. Ralston SH, de Crombrugghe B 2006 Genetic regulation of bone mass and susceptibility to osteoporosis. Genes Dev **20**(18)**:**2492-506.

4. Krishnan V, Bryant HU, Macdougald OA 2006 Regulation of bone mass by Wnt signaling. J Clin Invest **116**(5)**:**1202-9.

5. Karsenty G 2008 Transcriptional control of skeletogenesis. Annu Rev Genomics Hum Genet **9:**183-96.

6. Ducy P 2000 Cbfa1: a molecular switch in osteoblast biology. Dev Dyn **219**(4)**:**461-71.

7. Lian JB, Stein GS, Javed A, van Wijnen AJ, Stein JL, Montecino M, Hassan MQ, Gaur T, Lengner CJ, Young DW 2006 Networks and hubs for the transcriptional control of osteoblastogenesis. Rev Endocr Metab Disord **7**(1-2)**:**1-16.

8. Schroeder TM, Kahler RA, Li X, Westendorf JJ 2004 Histone deacetylase 3 interacts with runx2 to repress the osteocalcin promoter and regulate osteoblast differentiation. J Biol Chem **279**(40)**:**41998-2007.

9. Schroeder TM, Jensen ED, Westendorf JJ 2005 Runx2: a master organizer of gene transcription in developing and maturing osteoblasts. Birth Defects Res C Embryo Today **75**(3)**:**213-25.

10. Ducy P, Karsenty G 1995 Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. Mol Cell Biol **15**(4)**:**1858-69.

11. Javed A, Gutierrez S, Montecino M, van Wijnen AJ, Stein JL, Stein GS, Lian JB 1999 Multiple Cbfa/AML sites in the rat osteocalcin promoter are required for basal and vitamin D-responsive transcription and contribute to chromatin organization. Mol Cell Biol **19**(11)**:**7491-500.

12. Kahler RA, Westendorf JJ 2003 Lymphoid enhancer factor-1 and beta-catenin inhibit Runx2-dependent transcriptional activation of the osteocalcin promoter. J Biol Chem **278**(14)**:**11937-44.

13. Lee JS, Thomas DM, Gutierrez G, Carty SA, Yanagawa S, Hinds PW 2006 HES1 cooperates with pRb to activate RUNX2-dependent transcription. J Bone Miner Res **21**(6)**:**921-33.

14. Makita K, Ishitani K, Ohta H, Horiguchi F, Nozawa S 2005 Long-term effects on bone mineral density and bone metabolism of 6 months' treatment with gonadotropin-releasing hormone analogues in Japanese women: comparison of buserelin acetate with leuprolide acetate. J Bone Miner Metab **23**(5)**:**389-94.

15. Pregizer S, Baniwal SK, Yan X, Borok Z, Frenkel B 2008 Progressive recruitment of Runx2 to genomic targets despite decreasing expression during osteoblast differentiation. J Cell Biochem **105**(4)**:**965-70.

16. Xiao G, Jiang D, Ge C, Zhao Z, Lai Y, Boules H, Phimphilai M, Yang X, Karsenty G, Franceschi RT 2005 Cooperative interactions between activating transcription factor 4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression. J Biol Chem **280**(35)**:**30689-96.

17. Thirunavukkarasu K, Miles RR, Halladay DL, Yang X, Galvin RJ, Chandrasekhar S, Martin TJ, Onyia JE 2001 Stimulation of osteoprotegerin (OPG)

gene expression by transforming growth factor-beta (TGF-beta). Mapping of the OPG promoter region that mediates TGF-beta effects. J Biol Chem **276**(39)**:**36241-50.

18. Nishio Y, Dong Y, Paris M, O'Keefe RJ, Schwarz EM, Drissi H 2006 Runx2-mediated regulation of the zinc finger Osterix/Sp7 gene. Gene **372:**62-70.

19. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G 1997 Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell **89**(5)**:**747-54.

20. Javed A, Barnes GL, Jasanya BO, Stein JL, Gerstenfeld L, Lian JB, Stein GS 2001 runt homology domain transcription factors (Runx, Cbfa, and AML) mediate repression of the bone sialoprotein promoter: evidence for promoter context-dependent activity of Cbfa proteins. Mol Cell Biol **21**(8)**:**2891-905.

21. Roca H, Phimphilai M, Gopalakrishnan R, Xiao G, Franceschi RT 2005 Cooperative interactions between RUNX2 and homeodomain protein-binding sites are critical for the osteoblast-specific expression of the bone sialoprotein gene. J Biol Chem **280**(35)**:**30845-55.

22. Sevetson B, Taylor S, Pan Y 2004 Cbfa1/RUNX2 directs specific expression of the sclerosteosis gene (SOST). J Biol Chem **279**(14)**:**13849-58.

23. Li Q, Lin S, Wang X, Lian G, Lu Z, Guo H, Ruan K, Wang Y, Ye Z, Han J, Lin SC 2009 Axin determines cell fate by controlling the p53 activation threshold after DNA damage. Nat Cell Biol **11**(9)**:**1128-34.

24. Liu B, Yu HM, Hsu W 2007 Craniosynostosis caused by Axin2 deficiency is mediated through distinct functions of beta-catenin in proliferation and differentiation. Dev Biol **301**(1)**:**298-308.

25. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB 2005 Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem **280**(39)**:**33132-40.

26. Reinhold MI, Naski MC 2007 Direct interactions of Runx2 and canonical Wnt signaling induce FGF18. J Biol Chem **282**(6)**:**3653-63.

27. McCarthy TL, Centrella M 2010 Novel links among Wnt and TGF-beta signaling and Runx2. Mol Endocrinol **24**(3)**:**587-97.

28. Li WF, Hou SX, Yu B, Li MM, Ferec C, Chen JM 2010 Genetics of osteoporosis: accelerating pace in gene identification and validation. Hum Genet **127**(3)**:**249-85.

29. Agueda L, Bustamante M, Jurado S, Garcia-Giralt N, Ciria M, Salo G, Carreras R, Nogues X, Mellibovsky L, Diez-Perez A, Grinberg D, Balcells S 2008 A haplotype-based analysis of the LRP5 gene in relation to osteoporosis phenotypes in Spanish postmenopausal women. J Bone Miner Res **23**(12)**:**1954-63.

30. Hay E, Lemonnier J, Modrowski D, Lomri A, Lasmoles F, Marie PJ 2000 N-and E-cadherin mediate early human calvaria osteoblast differentiation promoted by bone morphogenetic protein-2. J Cell Physiol **183**(1)**:**117-28.

31. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T 2005 MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics **21**(13)**:**2933-42.

32. Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA, Podkolodny NL, Kolchanov NA 1998 Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res **26**(1)**:**362-7.

33. Schwartz S, Kent WJ, Smit A, Zhang Z, Baertsch R, Hardison RC, Haussler D, Miller W 2003 Human-mouse alignments with BLASTZ. Genome Res **13**(1)**:**103-7.

34. Schreiber E, Matthias P, Muller MM, Schaffner W 1989 Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res **17**(15)**:**6419.

35. Marie PJ 2008 Transcription factors controlling osteoblastogenesis. Arch Biochem Biophys **473**(2)**:**98-105.

36. Ehret GB, Reichenbach P, Schindler U, Horvath CM, Fritz S, Nabholz M, Bucher P 2001 DNA binding specificity of different STAT proteins. Comparison of in vitro specificity with natural target sites. J Biol Chem **276**(9)**:**6675-88.

37. Twells RC, Metzker ML, Brown SD, Cox R, Garey C, Hammond H, Hey PJ, Levy E, Nakagawa Y, Philips MS, Todd JA, Hess JF 2001 The sequence and gene characterization of a 400-kb candidate region for IDDM4 on chromosome 11q13. Genomics **72**(3)**:**231-42.

38. Otto F, Lubbert M, Stock M 2003 Upstream and downstream targets of RUNX proteins. J Cell Biochem **89**(1)**:**9-18.

39. Li J, Yang Y, Jiang B, Zhang X, Zou Y, Gong Y 2010 Sp1 and KLF15 regulate basal transcription of the human LRP5 gene. BMC Genet **11:**12.

40. Yadav VK, Ryu JH, Suda N, Tanaka KF, Gingrich JA, Schutz G, Glorieux FH, Chiang CY, Zajac JD, Insogna KL, Mann JJ, Hen R, Ducy P, Karsenty G 2008 Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum. Cell **135**(5)**:**825-37.

41. Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, Yamamoto H, Yamashita N, Yano T, Ikeda T, Itohara S, Inazawa J, Abe T, Hagiwara A, Yamagishi H, Ooe A, Kaneda A, Sugimura T, Ushijima T, Bae SC, Ito Y 2002 Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell **109**(1)**:**113-24.

TABLES

Table 1. Sequences of the probes used in the EMSA experiments (only the forward strand is shown).

Name	Sequence (5'→3')
BS1C	5'-CCTTTGTTCCT**GTGG**CCGGA-3'
MUT1C	5'-CCTTTGTTCCTGTAACCGGA-3'
BS1T	5'-CCTTTGTTCCT**GTGG**CTGGA-3'
MUT1T	5'-CCTTTGTTCCTGTAACTGGA-3'
BS2	5'-TGGGGGGT**GTGG**TTTGCAAT-3'
MUT2	5'-TGGGGGGTGTAATTTGCAAT-3'
BS3	5'-TTGAGAAC**CCAC**AGAGACAC-3'
MUT3	5'-TTGAGAACTTACAGAGACAC-3'
BS4	5'-GAAGAAGT**GTGG**TTGGAGCA-3'
MUT4	5'-GAAGAAGTGTAATTGGAGCA-3'
BS5	5'-GGCCTGAT**GTGG**TTCCTCCC-3'
MUT5	5'-GGCCTGATGTAATTCCTCCC-3'
OSE	5'-CGCAGCTCCCAA**CCAC**ATACC-3'
GRE	5'-TAATGAGAGAAGATTCTGTTCTAATGACCA-3'
SP1	5'-ATTCGATCGGGGCGGGGCGAGC-3'

Table 2. The five predicted Runx2 binding sites found in the 3-kb LRP5 5' region.

Runx2 binding sites	**Chromosomal Position** (Feb 2009, GRCh37/hg19)	**Matrix similarity according to GENOMATIX prediction ***	**Score according to TFSEARCH prediction**	**Sequence**
BS 1	Chr11: 68,077,228 - 67,077,242 (-2.9 kb)	MS:0.846 (only in presence of T-allele of rs312009)	<85 (Not predicted)	TTCCT**GTGG**CYGGAG[(a)]
BS 2	Chr11: 68,077,403 - 67,077,417 (-2.4 kb)	MS:0.954	100	GGGT**GTGG**TTTGCRA[(b)]
BS 3	Chr11: 67,078,103 - 67,078,117 (-1.8 kb)	MS:0.859	<85 (Not predicted)	TGAGAAC**CCAC**AGAG
BS 4	Chr11: 67,078,138 - 67,078,152 (-1.7 kb)	MS:0.949	100	AAGK**GTGG**TTGGAGC[(c)]
BS 5	Chr11: 67,078,794 - 67,078,808 (-1.3 kb)	MS:0.900	100	TGAT**GTGG**TTCCTCC

* For all Runx2 sites the Optimized Matrix Similarity was 0.84 and the Core Similarity was 1.
[(a)] Y stands for C/T rs312009, validated frequent SNP (CEU HapMap frequence C 0.776 and T 0.224).
[(b)] R stands for A/G rs4988327, validated rare SNP. Only the A allele (CEU HapMap frequence 0.941) was considered further.
[(c)] K stands for T/G rs4988329, described in dbSNP but without frequency validation (only T allele considered).

FIGURE LEGENDS

Figure 1. The DNA sequence containing the predicted Runx2 binding site 1 (BS1) binds nuclear proteins present in osteoblast nuclear extracts, including Runx2. DNA binding was analyzed by gel shift experiments. **A)** Labeled double-stranded oligonucleotide containing the T allele of rs312009 (BS1T*) was incubated with 10 µg of Saos-2 nuclear extract. Competition experiments were performed with cold T-allele (BS1T) or C-allele (BS1C) oligonucleotides; with the nonspecific competitors GRE and Sp1; and with OSE, an oligonucleotide containing the Runx2 consensus sequence of the osteocalcin promoter. **B)** An equivalent experiment was performed with the double-stranded oligonucleotide containing the C allele (BS1C*). **C)** Oligonucleotides, BS1C* and BS1T*, competed by the corresponding cold probes, either wild-type or mutated (MUT1C, MUT1T).

Figure 2. Characterization of Runx2 mRNA and protein levels and assessment of LRP5 promoter constructs in different cell lines. **A)** Real-Time PCR quantification of *RUNX2* isoform II mRNA levels in immortalized human neonatal calvaria cells (IHNC), Saos-2 and U-2 OS. *GAPDH* was used as the internal normalization gene; results were expressed relative to *RUNX2* in IHNC. **B)** Western blot analysis of Runx2 protein levels in the different cell lines employed. Twenty and 30 µg of each nuclear extract were subjected to SDS-PAGE. Antibody Runx2 (M-70, SCBT) was used as the primary antibody, and Nucleoporine NP62 was used for the loading control. **C)** The long promoter construct (LP-C) and two deletion constructs (MP and BP) were assayed for transcriptional activity in the indicated cell lines. Luciferase values were normalized to β-galactosidase activity. The results are represented relative to the pGL3 empty vector (pGL3-EV) for each cellular type. The bars represent the average luciferase activity of at least three independent experiments with four replicates each and the error bars represent the standard error of the mean. The significance, according to the unpaired Student *t* test, is represented by asterisks for comparisons with the LP-C construct within each cell line and by "§" for the comparison of BP with MP, within a given cell line.

Figure 3. Functional effect of rs312009 on the transcriptional activity of the *LRP5* 5' region. The LP construct in the two possible allelic forms of rs312009 (LP-C and LP-

T) was assayed for luciferase activity in three cell lines (Saos-2, U-2 OS and HeLa). Additional constructs were obtained by selective mutation of the Runx2 binding site core sequence (MUT1-C and MUT1-T). The Runx2-binding site core sequence is depicted in bold, the rs312009 position is in bold and italic, and the mutated base pairs are underlined. The results are expressed relative to LP-C, after β-galactosidase normalization and subtraction of pGL3-EV values. The bars represent the average luciferase activity of at least three independent experiments with four replicates each; the error bars represent the standard error of the mean. The significance, according to the unpaired Student *t* test, is represented by asterisks for comparisons with LP-C, except for MUT-1T values, which are compared with those of LP-T, in the corresponding cell line.

Figure 4. Effect of Runx2 cotransfection on the transcriptional activity of the *LRP5* 5' region. A) Transfection was performed in HeLa cells and luciferase activity was measured at 24 h (black bars) and 48 h (dashed bars). Increasing amounts of the Runx2 expression vector were cotransfected with the LP-C construct as indicated in the table below. The asterisks indicate the statistical significance (according to the paired Student *t* test) of the difference between each transfection and that of LP-C with only pCMV-EV, except for the one specified by a connector. B) 0.875 μg of the Runx2 expression vector (dashed bars) or 0.875 μg of the empty vector (black bars) was cotransfected with the following long promoter constructs: LP-C, LP-T, MUT1-C or MUT1-T, and luciferase activity was measured after 24 h. The asterisks indicate the statistical significance of the difference between the cotransfection with the Runx2 expression vector and that of the empty vector for each long promoter construct, using the paired Student *t* test. The "§" symbols indicate the significance of the difference between MUT1-C and LP-C or MUT1-T and LP-T, cotransfected with the empty vector, according to the unpaired Student *t* test. The bars represent the average luciferase activity of at least three independent experiments with four replicates each; the error bars represent the standard error of the mean.

Figure 5. 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, 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.

Figure 6. Effect of the specific mutation of each of the five Runx2 binding sites on the transcriptional capacity of LP-C. The LP-C construct was assayed for luciferase activity and compared with that of the MUT1-C, MUT2, MUT3, MUT4 and MUT5 constructs, in three different cellular contexts (Saos-2, U-2 OS and HeLa). A schematic representation of the constructs is shown on the left. The mutated nucleotides are underlined. Luciferase values were normalized as in Figure 3. The bars represent the average luciferase activity of at least three independent experiments with four replicates each; the error bars represent the standard error of the mean. The significance according to the unpaired Student *t* test is indicated by asterisks for differences with LP-C, in the same host cell.

Figure 7. Effect of each individual Runx2 binding site mutation on the response to Runx2 cotransfection. Hela cells were cotransfected with 0.875 μg of the different long promoter constructs (LP-C, MUT1-C, MUT2, MUT3, MUT4 and MUT5) and the same amount of pCMV-EV or pCMV-Runx2, and were assayed for luciferase activity at 24 h. The dashed bars represent the fold-induction of luciferase activity after Runx2 cotransfection, and the asterisks indicate the statistical significance according to the paired Student *t* test (for each construct with or without Runx2 cotransfection). The bars represent the average luciferase activity of at least three independent experiments with four replicates each; and the error bars represent the standard error of the mean.

FIGURES

Figura 1.

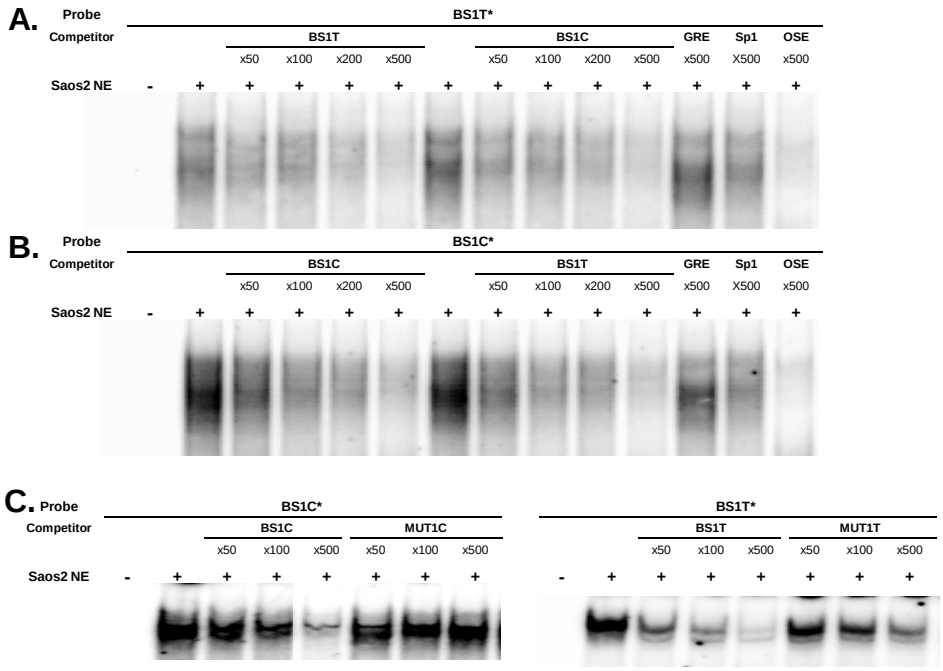


Figura 2.

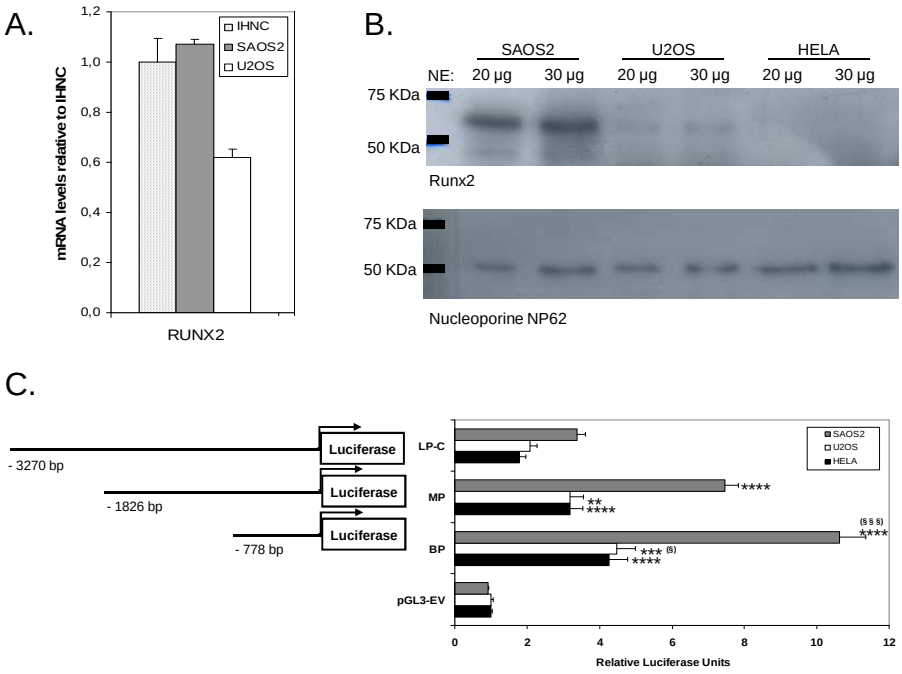


Figura 3.

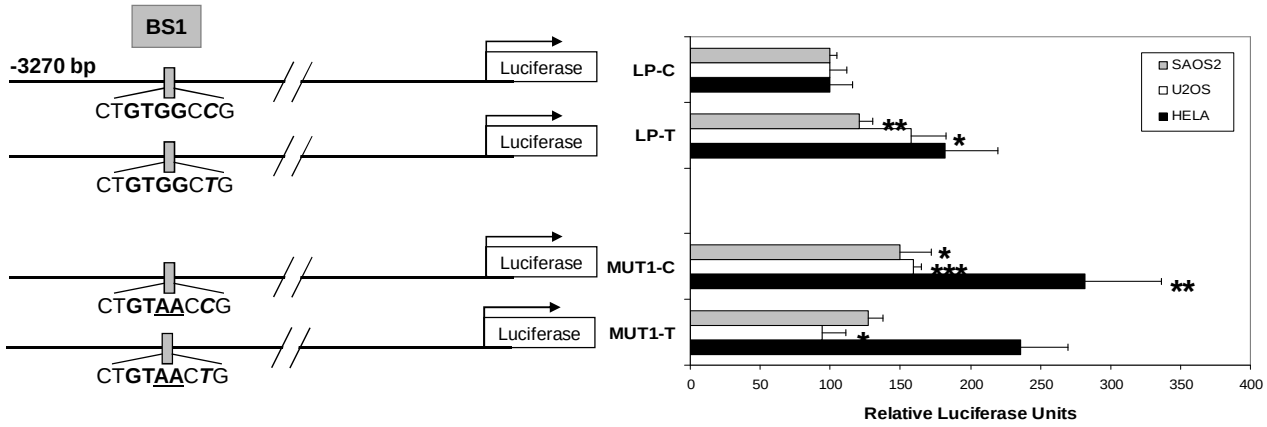
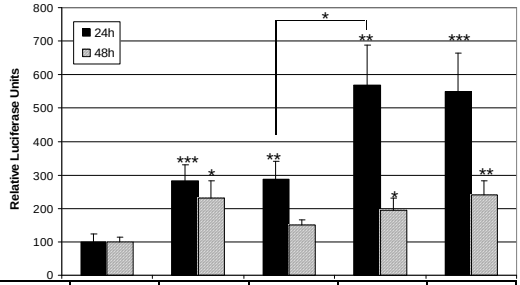


Figura 4.

A.



LP-C	0.875 µg	0.875 µg	0.875 µg	0.875 µg	0.875 µg
pCMV-Runx2	-	0.250 µg	0.500 µg	0.750 µg	0.875 µg
pCMV-EV	0.875 µg	0,625 µg	0,375 µg	0.125 µg	-

B.

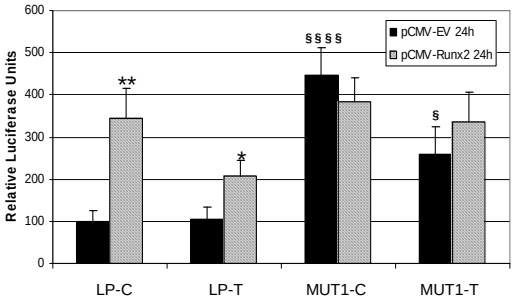


Figura 5.

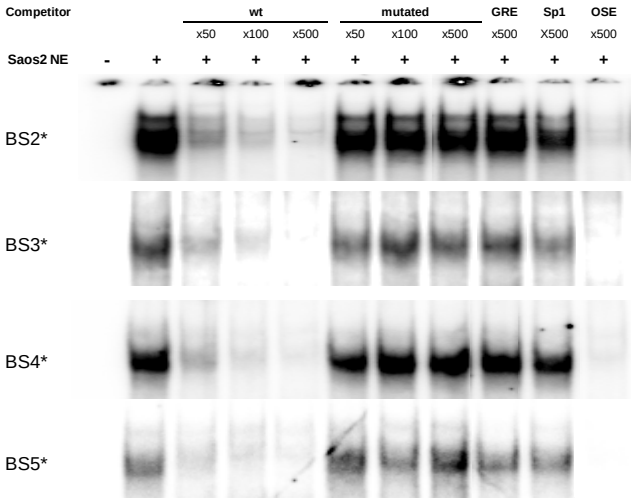


Figura 6.

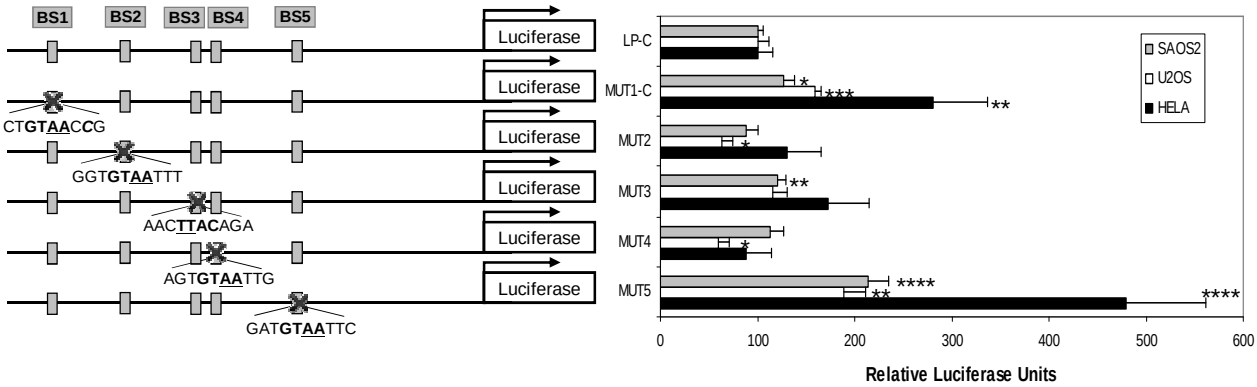
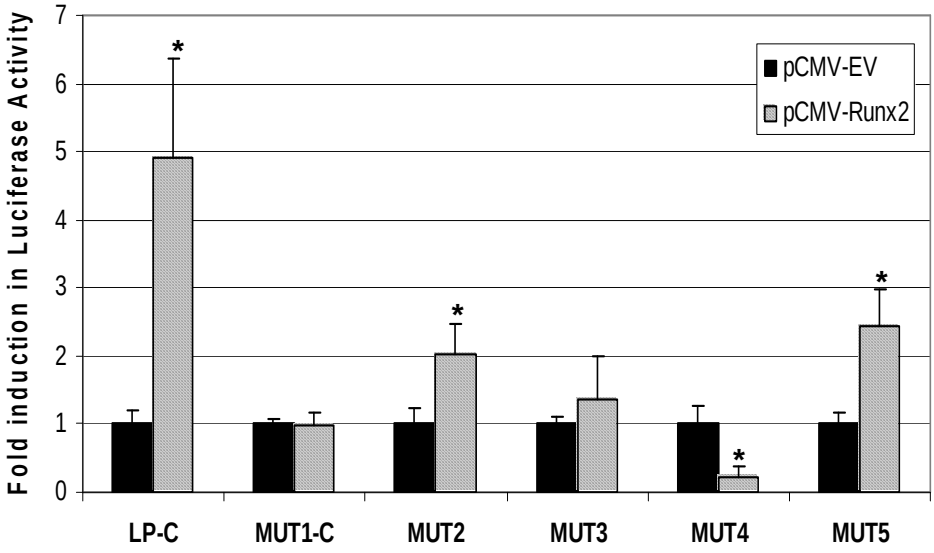


Figura 7.


Fold induction in Luciferase Activity
0
1
2
3
4
5
6
7
pCMV-EV
pCMV-Runx2
*
*
*
*
LP-C
MUT1-C
MUT2
MUT3
MUT4
MUT5

CAPÍTOL 4: Analysis of three functional polymorphisms in relation to osteoporosis phenotypes: replication in a Spanish cohort

REFERÈNCIA:

Lídia Agueda, Roser Urreizti, Mariona Bustamante, Susana Jurado, Natàlia Garcia-Giralt, Adolfo Díez-Pérez, Xavier Nogués, Leonardo Mellibovsky, Daniel Grinberg, Susana Balcells
Calcified Tissue International, 2010 Apr 14. [Epub ahead of print]

RESUM:

L'Osteoporosi és una malaltia complexa en que participen diversos factors genètics. Els estudis d'associació de polimorfismes (SNPs) funcionals constitueixen una eina molt important per a la seva identificació. No obstant, aquesta aproximació sovint es veu afectada per la manca de poder estadístic, l'estratificació poblacional i altres impediments que acaben donant lloc a resultats discordants. La replicació en cohorts independents és essencial.

En aquest treball, es va dur a terme una anàlisi d'associació de tres polimorfismes funcionals prèviament associats amb fenotips ossis: Ala222Val del gen *MTHFR*, Ile1062Val del gen *LRP6* i -13910C>T del gen *LCT*, en una cohort de 944 dones espanyoles postmenopàusiques, totes elles amb dades de densitat mineral òssia (DMO) lumbar, i per la majoria d'elles dades de DMO femoral i fractura. Només es varen trobar diferències significatives entre els genotips del polimorfisme de *MTHFR* i el subgrup de factures vertebrals, amb un OR de 2,27 (1,17-4,38) per al genotip TT contra els genotips CC/CT, p=0,018.

Es presenten dades sobre la freqüència al·lèlica i genotípica del polimorfisme -13910C>T d' *LCT* en una població espanyola, amb una freqüència de l'al·lel T (que confereix persistència de la lactasa) del 38.6%. Aquestes freqüències genotípiques foren consistents amb la clina observada a Europa i amb la prevalença de la no-persistència de la lactasa. El polimorfisme *LCT* -13910C>T es va trobar associat significativament amb alçada i pes, on les portadores de l'al·lel T eren 0,88 cm més altes (95% CI=0,08–1,59 cm, p=0,032, ajustant per edat) que les dones CC, i les homozigotes TT pesaven 1.91 Kg més que les dones CC/CT (95% CI=0,11–3,71 Kg, p=0,038, ajustant per edat).

En conclusió, no es varen observar associacions significatives entre els polimorfismes estudiats i la DMO lumbar i femoral en dones postmenopàusiques espanyoles; només el polimorfisme *MTHFR* Ala222Val va resultar associat amb les fractures vertebrals.

INFORMACIÓ SOBRE LA PUBLICACIÓ I INDEX D'IMPACTE:

La revista *Calcified Tissue International*, fundada l'any 1976, pública articles de recerca originals posant especial èmfasi en temes relacionats amb l'estructura i la funció de l'os i altres sistemes mineralitzats dels organismes vius. Aquesta revista serveix com a fòrum per explorar els aspectes bioquímics biofísics, moleculars i clínics de l'estructura, la funció i el metabolisme ossi i d'altres sistemes mineralitzats. El seu factor d'impacte actual és de **2.737** I el seu editor en cap és el Prof. Stuart Ralston.

APORTACIÓ PERSONAL A L'ARTICLE:

- Extracció dels DNAs d' aproximadament la meitat de les mostres de la cohort BARCOS. Aquestes mostres han estat usades en d'altres estudis d'associació realitzats pel nostre grup de recerca.
- Elecció d'alguns dels SNPs estudiats
- Genotipat dels polimorfismes per diferents tècniques.
- Control de qualitat, anàlisi estadístic i interpretació dels resultats
- Càlculs de poder estadístic i d'estratificació poblacional
- Elaboració del primer esborrany de l'article i participació en l'elaboració del manuscrit final.

NOTA: les dades de genotipat del polimorfisme Ile1062Val del gen *LRP6* són les mateixes que varen ser emprades en el treball presentat en el capítol 2 de Resultats. En aquell cas formaren part d'una meta-anàlisi multi cèntrica (GENOMOS) i ara s'analitzen només a nivell de la cohort BARCOS individualment.

VIST-I-PLAU DELS DIRECTORS DE TESI:

Dra. Susana Balcells Comas Dr. Daniel Grinberg Vaisman