ESTUDIS D'ASSOCIACIÓ I FUNCIONALS EN GENS CANDIDATS PER A L'OSTEOPOROSI

Memòria presentada per

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1. ESTUDIS D'ASSOCIACIÓ

1.1. ESTUDIS D'ASSOCIACIÓ EN GENS CANDIDATS CLÀSSICS

1.1.1. La cohort BARCOS

El principal objectiu d'aquesta tesi és aprofundir en el coneixement de quines variants polimòrfiques afecten la determinació de la DMO en població postmenopàusica espanyola. Per aquest motiu vam analitzar una sèrie de polimorfismes situats en els gens candidats clàssics per a l'osteoporosi. Aquests polimorfismes són: Cdx2, *Fok*I, *Bsm*I, *Apa*I i *Taq*I del gen *VDR*; *Pvu*II, *Xba*I i el microsatèl·lit $(TA)_n$ del gen *ESR1*; -800 G/A, -509 C/T, Leu10Pro, Arg25Pro i Thr263Ile del gen *TGFB1* i el polimorfisme Sp1 situat a l'intró 1 del gen *COL1A1.* A més dels polimorfismes anteriors es van analitzar els polimorfismes -1997 G/T i -1663 indelT del promotor del gen *COL1A1*, els quals s'havien trobat associats prèviament a la DMO en una mostra de 256 dones postmenopàusiques espanyoles (Garcia-Giralt i col., 2002).

Els genotips es van obtenir per SNaPshot, per RFLP o per seqüenciació, i el seu efecte es va analitzar en una mostra de 719 dones postmenopàusiques espanyoles (cohort BARCOS). També es va analitzar l'efecte dels respectius haplotips. Els resultats de l'associació entre la DMO i els polimorfismes dels gens clàssics en la cohort BARCOS es van plasmar en l'article que es mostra a continuació.

El polimorfisme -1997 G/T del gen *COL1A1* i el polimorfisme Leu10Pro del gen *TGFB1* es van trobar associats a la DMO lumbar. També es van observar dues interaccions significatives entre els polimorfismes -1997 G/T i Sp1 (o entre -1997 G/T i -1663 indelT) del gen del *COL1A1*; i entre els polimorfismes -1663 indelT (*COL1A1*) i *Apa*I (*VDR*). Finalment l'haplotip GDs del gen *COL1A1* i l'haplotip LPX del gen *ESR1* es van trobar associats a la DMO femoral.

En resum, després d'ampliar la cohort, els polimorfismes del promotor del gen *COL1A1* semblen seguir participant en la determinació de la DMO en població postmenopàusica espanyola.

Referència del treball publicat:

■**Bustamante** M, Nogues X, Enjuanes A, Elosua R, Garcia-Giralt N, Perez-Edo L, Caceres E, Carreras R, Mellibovsky L, Balcells S, Diez-Perez A, Grinberg D. 2007. *COL1A1, ESR1, VDR and TGFB1 polymorphisms and haplotypes in relation to BMD in Spanish postmenopausal women.* **Osteoporos Int.** 18(2):235-43.

Fe d'erratas: El polimorfisme Thr263Pro (rs1800472) hauria de constar com a Thr263Ile.

Aportació personal a l'article:

Obtenció de part dels DNAs a partir de mostres de sang.

Genotipatge de deu de les setze variants polimòrfiques analitzades.

Càlculs estadístics i estima dels haplotips.

Elaboració del primer esborrany de l'article i participació en la maduració del manuscrit final.

1. ESTUDIS D'ASSOCIACIÓ

1.1. ESTUDIS D'ASSOCIACIÓ EN GENS CANDIDATS CLÀSSICS

1.1.2. El projecte GENOMOS

Un dels objectius del consorci GENOMOS és valorar l'efecte sobre la DMO i sobre el risc de patir fractures que tenen diversos polimorfismes situats en els gens clàssics per a l'osteoporosi: *VDR*, *COL1A1*, *ESR1* i *TGFB1*.

L'estudi d'associació es va plantejar com una metaanàlisi prospectiva amb unes 20.000 mostres procedents de diversos centres europeus, entre les quals hi ha les mostres de la cohort BARCOS. Tant les dades dels fenotips com les dels genotips es van estandarditzar i es va valorar la presència d'heterogeneïtat entre cohorts, la qual es va excloure en la majoria dels casos.

Els resultats publicats en els quatre articles que es presenten a continuació van mostrar que només el polimorfisme Sp1 (*COL1A1*) estava associat a la DMO. El genotip "ss" implicava de mitjana una disminució de 21 mg/cm^2 en la DMO lumbar i una disminució de 25 mg/cm^2 en la DMO femoral. D'altra banda es va trobar que els polimorfismes Sp1 (*COL1A1*), *Xba*I (*ESR1*) i Cdx2 (*VDR*) estaven associats al risc de patir fractures vertebrals osteoporòtiques en la totalitat i/o en subgrups de la mostra. Els riscs respectius associats a cada un d'aquests polimorfismes van ser de 1,33 (95% CI: 1-1,77), 0,65 (0,49-0,87) i 0,87 (0,78-0,97).

Referències dels treballs publicats o sotmesos:

■Ralston SH, Uitterlinden AG, Brandi ML, Balcells S, Langdahl BL, Lips P, Lorenc R, Obermayer-Pietsch B, Scollen S, **Bustamante M**, Husted LB, Carey AH, Diez-Perez A, Dunning AM, Falchetti A, Karczmarewicz E, Kruk M, van Leeuwen JP, van Meurs JB, Mangion J, McGuigan FE, Mellibovsky L, del Monte F, Pols HA, Reeve J, Reid DM, Renner W, Rivadeneira F, van Schoor NM, Sherlock RE, Ioannidis JP. 2006. *Large-scale evidence for the effect of the COLIA1 Sp1 polymorphism on osteoporosis outcomes: the GENOMOS study*. **PLoS Med**. 3(3):e90.

■Uitterlinden AG, Ralston SH, Brandi ML, Carey AH, Grinberg D, Langdahl BL, Lips P, Lorenc R, Obermayer-Pietsch B, Reeve J, Reid DM, Amedei A, Bassiti A, **Bustamante M**, Husted LB, Diez-Perez A, Dobnig H, Dunning AM, Enjuanes A, Fahrleitner-Pammer A, Fang Y, Karczmarewicz E, Kruk M, van Leeuwen JP, Mavilia C, van Meurs JB, Mangion J, McGuigan FE, Pols HA, Renner W, Rivadeneira F, van Schoor NM, Scollen S, Sherlock RE, Ioannidis JP. 2006. *The association between common vitamin D receptor gene variations and osteoporosis: a participant-level meta-analysis*. **Ann Intern Med**. 145(4):255-64.

Resultats 1.1.2. Estudis d'associació amb gens candidats clàssics: GENOMOS

■Ioannidis JP, Ralston SH, Bennett ST, Brandi ML, Grinberg D, Karassa FB, Langdahl B, van Meurs JB, Mosekilde L, Scollen S, Albagha OM, **Bustamante M**, Carey AH, Dunning AM, Enjuanes A, van Leeuwen JP, Mavilia C, Masi L, McGuigan FE, Nogues X, Pols HA, Reid DM, Schuit SC, Sherlock RE, Uitterlinden AG. 2004. *Differential genetic effects of ESR1 gene polymorphisms on osteoporosis outcomes*. **JAMA**. 292(17):2105-14.

■Bente L. Langdahl, André G. Uitterlinden, Stuart H. Ralston, Maria Luisa Brandi, Serena Scollen, Daniel Grinberg, Paul Lips, Roman Lorenc, Barbara Obermayer-Pietsch, Jonathan Reeve, David M. Reid, Antonietta Amidei, Pascal P. Arp, Amelia Bassiti, **Mariona Bustamante**, Lise Bjerre Husted, Alison H. Carey, Adolfo Diez-Perez, Harald Dobnig, Alison M. Dunning, Anna Enjuanes, A. Fahrleitner-Pammer, Yue Fang, Stephen Kaptoge, Elzbieta Karczmarewicz, Marcin Kruk, Johannes P.T.M. van Leeuwen, Carmelo Mavilia, Joyce B.J. van Meurs, Jon Mangion, Fiona E. A. McGuigan, Leif Mosekilde, Huibert A. P. Pols, Wilfried Renner, Fernando Rivadeneira, Natasja M. van Schoor, Rachael E. Sherlock, John P.A. Ioannidis. *Large-scale analysis of association between polymorphisms in the Transforming Growth Factor Beta 1 gene and osteoporosis: The GENOMOS Study.*
Sotmès a la revista: **Journal of Bone and Mineral Research**

Aportació personal als articles:
Obtenció de part dels DNAs de la cohort BARCOS a partir de mostres de sang.
Genotipatge de vuit dels catorze polimorfismes en les mostres de la cohort BARCOS.

1. ESTUDIS D'ASSOCIACIÓ

1.2. ESTUDIS D'ASSOCIACIÓ EN ALTRES GENS CANDIDATS

1.2.1. *RUNX2*

Runx2, un factor de transcripció amb el domini *runt*, és essencial per a la diferenciació dels osteoblasts, pel remodelatge ossi i per la recuperació dels ossos fracturats. El *knock out* de *Runx2* en ratolí comporta una ossificació deficient, mentre que la sobreexpressió produeix un fenotip osteoporòtic. Polimorfismes situats en els promotors del gen *RUNX2* podrien modificar la taxa transcripcional i conseqüentment els nivells de diferenciació osteoblàstica. Aquest fet converteix *RUNX2* en un bon gen candidat pels estudis d'associació relacionats amb l'osteoporosi.

En el present treball es va analitzar el polimorfisme -330 G/T situat al promotor 1 del gen i el polimorfisme -1025 T/C situat al promotor 2 en una cohort de 821 dones postmenopàusiques d'origen espanyol. Aquests dos polimorfismes, els quals disten més de 90 kb i no es troben en desequilibri de lligament, es van escollir partint de la informació publicada prèviament sobre la seva funcionalitat i/o associació a fenotips ossis.

Mentre que el polimorfime -330 G/T (promotor 1) no es va trobar associat a la DMO ni femoral ni lumbar, el polimorfisme -1025 T/C (promotor 2) es va trobar associat a la DMO femoral. Els individus portadors del genotip TC presentaven una mitjana de DMO femoral ajustada major que els individus homozigots pel genotip TT. Aquest resultat concorda amb els resultats d'associació i funcionals descrits per altres grups.

Semblaria ser, doncs, que els polimorfismes del promotor 2 i no els del promotor 1 participen en la determinació de la DMO femoral en humans.

Referència del treball publicat:

■M. Bustamante, X. Nogués, L. Águeda, S. Jurado, A.Wesselius, E. Caceres, R. Carreras, M. Ciria, L. Mellibovsky, S. Balcells, A. Díez-Pérez, D. Grinberg. 2007. *Promoter 2 -1025 T/C polymorphism at RUNX2 gene is associated with FN BMD in a Spanish postmenopausal cohort.* **Calcif Tissue Int.** 81(4):327-32.

Aportació personal a l'article:

Obtenció de part dels DNAs a partir de mostres de sang.

Genotipatge dels dos polimorfismes analitzats.

Càlculs estadístics i estima del LD.

Elaboració del primer esborrany de l'article i participació en la maduració del manuscrit final.

1. ESTUDIS D'ASSOCIACIÓ

1.2. ESTUDIS D'ASSOCIACIÓ EN ALTRES GENS CANDIDATS

1.2.2. *IL6R*

Tant l'osteoporosi com l'obesitat són malalties complexes amb un fort component genètic. Estudis de lligament independents amb la DMO o amb l'IMC han identificat el *locus* 1q21-23 com a possible responsable d'ambdós fenotips. En aquest *locus* es troba situat el gen *IL6R*, el qual codifica per un dels receptors de la interleucina IL6. L'elevació dels nivells sèrics de IL6 i de sIL6R (una forma soluble del receptor) ha estat associada a patologies com l'osteoporosi i l'obesitat. Per tot això, el gen *IL6R* és un bon candidat per a l'osteoporosi.

En aquest treball es van analitzar dos polimorfismes situats en el promotor del gen *IL6R* (-1435 C/T i -208 G/A), així com també un polimorfisme no sinònim situat a l'exó 9 (Asp358Ala). L'efecte d'aquests polimorfismes i dels seus haplotips sobre la DMO lumbar i femoral i sobre l'IMC es va estudiar en una mostra de 559 dones postmenopàusiques d'origen espanyol.

Els dos polimorfismes del promotor es trobaven en fort desequilibri de lligament entre ells però no amb el polimorfisme Asp358Ala. Els polimorfismes del promotor i els seus haplotips C-A i T-G es van trobar associats a la DMO femoral, mentre que el polimorfisme Asp358Ala es va trobar associat a la DMO lumbar. A més, el polimorfisme -208 G/A així com també els haplotips C-G i C-A es van trobar associats a l'IMC i a l'obesitat.

En conclusió, les dades obtingudes suggereixen que polimorfismes del gen *IL6R* participen en certa mesura en la determinació de la massa òssia i de l'IMC en població espanyola.

Referència del treball publicat:

■**Bustamante M**, Nogués X, Mellibovsky L, Agueda L, Jurado S, Cáceres E, Blanch J, Carreras R, Díez-Pérez A, Grinberg D, Balcells S. 2007. *Polymorphisms in the IL6R gene are associated with BMD and BMI in Spanish postmenopausal women.* **Eur J Endocrinol.** 157(5):677-84.

Aportació personal a l'article:

Obtenció de part dels DNAs a partir de mostres de sang.

Genotipatge dels tres polimorfismes analitzats.

Càlculs estadístics i estima dels haplotips.

Elaboració del primer esborrany de l'article i participació en la maduració del manuscrit final.

Effects of BMP2 and CIZ/NMP4 in the *in vitro* regulation of the human *COL1A1* promoter

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Abstract

Collagen is the most abundant protein of bone matrix. Two polymorphisms in the promoter of human *COL1A1* gene (-1997 G/T and -1663 indelT) have been found associated with osteoporotic phenotypes. In addition, allelic differences in binding to nuclear factors and in transcriptional capacity were observed for these two SNPs. The architectural transcription factor CIZ/NMP4 was identified as one of the proteins bound to a *COL1A1* promoter site encompassing the -1663 indelT polymorphism. *CIZ/NMP4* knock-out mice were reported to present increased bone mass due to increased bone formation. In particular, the BMP2-induced osteoblastic differentiation was found to be enhanced, suggesting that CIZ is a suppressor of the BMP2 effects. *In vitro* experiments to test the role of CIZ/NMP4 on *COL1A1* expression yielded controversial results. In the present study we have analyzed the effects of BMP2 and CIZ/NMP4 on a 2.5 kb human *COL1A1* promoter by means of transfection of several reporter constructs in Saos-2 cells.

BMP2 stimulation of *COL1A1* promoter activity was maximal when the -2178 bp to -1634 bp region, containing the two polymorphisms, was present. However, the differences in the percentage of stimulation between the four haplotypes were small. Two putative response elements for BMP2 action (Dlx5 and Smad1 binding sites) present in this region were also tested and neither of them seemed to mediate the BMP2 effect. Several human CIZ/NMP4 cDNAs were observed in different tissues and the two most abundant (21H and 21H-I1) were cloned and stably transfected in Saos-2 cells. While both isoforms slightly increased *COL1A1* promoter activity in the absence of BMP2, no effect was observed in the BMP2-treated cells.

In conclusion, BMP2 stimulated human *COL1A1* promoter in Saos-2 cells, independently of the putative Dlx5 and Smad1 binding sites. The two promoter polymorphisms did not play a major role on this effect, either. CIZ/NMP4 did not inhibit the *COL1A1* stimulation produced by BMP2.

Keywords: *COL1A1*, BMP2, CIZ/NMP4, bone, Saos-2, transfection, Smad

Introduction

COL1A1 is one of the most abundantly expressed genes in osteoblasts. Two polymorphisms in its promoter (-1997 G/T and -1663 indelT) were found to be associated with bone mineral density (BMD) (Bustamante et al. 2007; Garcia-Giralt et al. 2002) and to display allelic differences in nuclear factor binding and transcriptional capacity (Garcia-Giralt et al. 2005).

The DNA region bearing the -1663 indelT polymorphism, a tract of 7 or 8 Ts, is homologous to the rat *site B*, which was shown to bind the CIZ/NMP4 protein (Alvarez et al. 1998). Similarly, a human probe containing -1663 indelT was also shown to bind this protein (Garcia-Giralt et al. 2005). CIZ/NMP4, also called ZNF384, is an architectural transcription factor that binds poly-dT sequences through its Krüppel zinc fingers and bends the DNA (Alvarez et al. 1998). Several CIZ/NMP4 isoforms, which mainly differ in the presence or absence of two inserts (I1 and I2) and in the presence or absence of the 4th and 5th zinc finger (4+5 ZNF), have been described (Nakamoto et al. 2000; Thunyakitpisal et al. 2001; Torrungruang et al. 2002). While the 28H isoform does not bind DNA, the 11H, the 21H and the 13H do so. However, only the 21H and the 13H isoforms were shown to be active in transcription regulation (Thunyakitpisal et al. 2001).

The different CIZ/NMP4 isoforms are expressed in several tissues (Nakamoto et al. 2000; Thunyakitpisal et al. 2001). In bone, CIZ/NMP4 stimulates the transcription of metalloproteinase 13 (*MMP-13)* (Fan et al. 2006; Nakamoto et al. 2000; Shah et al. 2004) and has a controversial role on *COL1A1* expression *in vitro* (Furuya et al. 2000; Shen et al. 2002; Thunyakitpisal et al. 2001). *In vivo*, *CIZ/NMP4* knock-out mice showed increased bone mass and increased bone formation, without alteration in the rate of osteoclastogenesis (Morinobu et al. 2005). More precisely, the action of CIZ/NMP4 was shown to be the inhibition of the osteoblastic differentiation induced by BMP2. *In vitro*, it has also been observed an inhibition of the expression of *COL1A1* and *RUNX2* by CIZ/NMP4 after BMP2 treatment (Shen et al. 2002).

BMP2 is a member of the transcription growth factor β family that regulates several embryologic processes such as bone development, and also adult bone formation (Chen et al. 2004). The BMP2 signal transduction pathway includes the combination of two membrane receptors (type I and type II), which transmit the signal through several R-Smad proteins (1, 5 and 8) that interact with a Co-Smad (Smad4) (Miyazono et al. 2005). The Smad complex translocates to the nucleus and acts as a transcription factor that interacts with Smad binding elements (SBEs) or GCs rich sequences (Alvarez Martinez et al. 2002; Liberatore et al. 2002; Miyazono et al. 2005). Additionally, the overexpression of Smad1 or Smad5 stimulates the expression of Dlx5, a transcription factor that may be an upstream regulator of Runx2 and Osterix, two proteins essential for osteoblast differentiation (Ryoo et al. 2006). It was shown that CIZ/NMP4 was able to block Smad-mediated transcriptional activation (Shen et al. 2002).

The aims of the present study were to assess the effects of BMP2 and CIZ/NMP4 on 2.5 kb of the human *COL1A1* promoter in Saos-2 cells.

Materials and methods

RNA extraction and RT PCR

RNA from MG-63, Saos-2 and primary human osteoblasts (OB) was obtained with the RNeasy Mini Kit (Quiagen). The RNA was converted to cDNA with oligo-dT (Gene Link) and M-MLV RT retrotransciptase (Promega).

PCR to evaluate the CIZ/NMP4 isoforms

The presence of different CIZ/NMP4 isoforms in a cDNA tissue panel (Clontech), and in cDNA from MG-63 and Saos-2 osteosarcoma cell lines and from primary human osteoblasts was evaluated by PCR amplification. Four pairs of primers were designed: one pair for the total variability present in the isoforms, one pair for the insert 1 (I1), one pair for the insert 2 (I2) and one for the 4th and 5th zinc finger (4+5 ZNF). These primers were: TOTAL_F: 5´-GCCGTCTACAGGACTGATGACT-3´ and TOTAL_R: 5´-CAGATTGGAGAGATTGTGTAAG-3´; I1_F: 5´-GCCGTCTACAGGACTGATGACT-3´ and I1_R: 5´-AGGTCTTGCCGTCTTTCTGAT-3´; I2_F: 5´-ATGTCCTCTCCCCTGAGGAT-3´ and I2_R: 5´-GCGGAAGGATTTCTCACAGA-3´; 4/5_F: 5´-ACAAGTGCCCACATTGCTCC-3´ and 4/5_R: 5´-GCACATTTGTATGGTCTATCACC-3´. The CIZ/NMP4 PCR fragments were generated in a 25-µl reaction volume, containing 2.5 µl of cDNA, 1x indicated buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer and 1.25 U Taq DNA polymerase (Promega). The PCR reactions involved an initial denaturation step of 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 40 s, annealing at 60ºC for 30 s, and polymerization at 72ºC for 40 s, with a final elongation step at 72ºC for 5 min.

The PCR fragments were resolved in a 2.5% agarose gel and purified using the GFX^{TM} DNA PCR and Gel Band Purification kit (Amersham). Fragments were sequenced with BigDye v3.1 (Applied Biosystems) in an ABI PRISM 3700 DNA Analyser (Applied Biosystems) using the previously mentioned oligonucleotides.

Constructs

21H and 21H-I1 CIZ/NMP4 constructs

The human 21H and 21H-I1 CIZ/NMP4 isoforms were PCR amplified from MG-63 cDNA using primers containing an *Eco*RI (forward) or an *Xba*I (reverse) restriction site at the 5' end, and cloned in the pcDNA3 vector (Innogenetics). These primers were: F: 5'-CGGAATTCGTAGA**ATG**GAAGAATCTCACTTC-3' and R: 5'-GCTCTAGAGCTGGCAGCACGGATCT**CTA**AG-3', the restriction sequences are underlined and the translation start site and the STOP codon are in bold. The PCR consisted in an initial denaturation step of 94ºC for 5 min, followed by 10 cycles of denaturation at 94ºC for 30 s, annealing at 59ºC for 40 s, and polymerization at 72ºC for 1 min, and by 30 cycles of denaturation at 94ºC for 30 s, annealing at 64ºC for 40 s, and polymerization at 72ºC for 1 min with a final elongation step at 72ºC for 5 min. Fifty to one-hundred ng of cDNA, 1x indicated buffer, 5% of DMSO, 1mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer and 1.25 U Taq DNA

polymerase (Promega) were added in a final volume of 50 µl. The PCR fragments and the vector were digested with *Eco*RI and *Xba*I (Takara) and ligated using the DNA Ligation Kit (Takara).

Two more constructs were obtained by adding an in frame Myc epitope at the N-terminus of the cDNA of each CIZ/NMP4 isoforms. The oligos used to create the epitope were MYC_F: 5'-GATCC**ATG**GAGCAGAAACTCATCTCTGAAGAAGATCTGG-3' and MYC_R: 5'-AATTCCAGATCTTCTTCAGAGATGAGTTTCTGCTC**CAT**G-3', underlined are the protuberant *Eco*RI- and *Bam*HI-compatible ends and in bold the translation start site. They were annealed and ligated to *Bam*HI- and *Eco*RI-digested vectors, containing the CIZ/NMP4 isoforms. These constructs were named pcDNA3-21H-myc and pcDNA3-21H-I1-myc.

COL1A1 promoter constructs

The four main constructs containing the *COL1A1* promoter fused to the Luciferase gene (pGL3-COL1A1) were the ones described in Garcia-Giralt et al. (2005). Briefly, their names and characteristics are: 1) SP (basal promoter; +40 to -220); 2) LP (long promoter; +40 to -2483); 3) IR [long promoter without the polymorphisms; LP∆(-1284 to -2178)]; and 4) IRD [total promoter without a repressor fragment; LP∆(-254 to -1634)] (Figure 1 A). The two constructs that contained the polymorphisms -1997 G/T and -1663 indelT (LP and IRD) were available in the four possible haplotypic combinations (G-7T, T-7T, G-8T, T-7T).

To create stable cells expressing the *COL1A1* promoter fused to the Luciferase gene, the neomycin resistance gene *neo*^{r} (in a 2.7 kb *Bam*HI-*Pci*I fragment from pcDNA3) was inserted downstream of the Luciferase gene in the pGL3-COL1A1 constructs described above, giving rise to the pGL3-COL1A1-Neo plasmids.

Two mutant versions of the LP_G-8T construct were obtained as follows: 1) a putative Dlx5 consensus binding site (according to GenoMatix (Cartharius et al. 2005)) situated in the minus strand from -1776 bp to -1764 bp (ggctatAATTaaa) was mutagenized to ggctatACCTaaa using the QuickChange II XL kit (Stratagene) and the oligonucleotides MUT_D_F: 5'-AACTCTATATTTTCCCTTTTAGGTATAGCCCCTGCAGTCTCCC-3' and its complementary MUT_D_R following the manufacturers protocol (the mutagenizated nucleotides are the ones underlined); 2) the same protocol was used to mutagenize the putative Smad consensus binding site situated in the plus strand from -1699 bp to -1692 bp (caGCCGaCGtg) according to Alvarez Martinez et al. (2002). The oligonucleotides used were MUT_S_F: 5'-GCCAGCCGGCCAGTTTACGTGGCTCCCTCCC-3' and its complementary MUT_S_R.

All constructs were verified by automatic sequencing using the BigDye v.3.1 kit (Applied Biosystems). Furthermore, the mutagenized *COL1A1* promoter fragments were subcloned into new pGL3-Basic vectors (that had not undergone any PCR amplification) to ensure that no further mutations had been introduced into them during the mutagenesis process.

Cell Culture

Cells were grown in DMEM medium (Gibco-BRL) supplemented with 10% Bovine Serum (Gibco-BRL), 1% Ampicilin-Streptovidin (Gibco-BRL) and 1% Piruvate (Gibco-BRL). Medium was changed every two-three days. Cells were cultured at 37ºC in 5% CO_2.

Stable transfection

A Geneticin (Gibco-BRL) mortality curve was performed for Saos-2 cells and a 400 µg/ml concentration was selected for the obtention of stably transfected clones.

Cells bearing the COL1A1 promoter fused to the Luciferase gene

To create stably transfected cells, 6-8 µg of the different pGL3-COL1A1-Neo constructs were linearized with *Pci*I (Roche) and transfected into cells at 80% of confluence using 18 µl of Fugene6 (Roche). At 48h post-tranfection, cells were treated with 400 µg/ml of Geneticin for approximately 15 days. For each construct a minimum of 250 clones were obtained and collected as pools. Five pools of stably transfected cells were created: Saos-2-pGL3, Saos-2-SP, Saos-2-LP_G-8T, Saos-2-IR and Saos-2-IRD_G-8T.

To confirm the presence and integrity of the transfected constructs, genomic DNA was extracted and PCR amplifications using a forward primer from the pGL3 vector and different reverse primers corresponding to the different *COL1A1* promoter regions were performed.

Cells expressing the 21H and 21H-I1 CIZ/NMP4 isoforms

Stably transfected cells expressing CIZ/NMP4 isoforms were created using 6-8 µg of pcDNA3-21H-myc, pcDNA3-21H-I1-myc or the empty pcDNA3 vector, all linearized with *Pci*I enzyme (Roche). The transfection conditions were similar to the ones described above. The cells were collected as pools of more than 250 clones each.

For experiments comparing different stable transfectants, cells were counted using a Neubauer chamber and plated at a density of 100.000-200.000 cells/well in 6 or 12 well plates.

Transient transfections

Saos-2 cells were transiently transfected 24 h after plating (60%-80% confluence) using fresh medium. For transfections, 2-3 µl of Fugene6 (Roche), 600-1800 ng of the different pGL3-COL1A constructs and 50-150 ng of phRG-TK Renilla Luciferase normalization vector (Promega) were used. In experiments comparing the effects of diffent constructs, equal copy numbers of each pGL3-COL1A construct were used. For all transient transfections, the proportion between µg of DNA and µl of Fugene6 was kept constant (2:3).

For cells stably expressing CIZ/NMP4 isoforms, transfections with pGL3-COL1A1-LP construct were performed after plating at a density of 100.000-200.000 cells/well. Transfection conditions were as described above.

BMP2 treatment

BMP2 (R&S) treatment (1, 2 or 5 nM) was performed 6 hours after transfection, in a medium essentially depleted of Bovine Serum (0.1%). After assaying 12, 24 or 48-hour treatments, the 24 h time-point was selected for all the experiments. Control cells were equally treated with BMP2 reconstitution buffer (4mM HCl + 0.1% BSA).

Luciferase activity measurements

For transiently transfected cells, Luciferase activity was measured 24 h after BMP2 (or mock) treatment. For stably transfected cells, Luciferase was measured 48 h after plating. Dual-Glo Luciferase System (Promega) and Single Luciferase System (Promega) were used for transient and stable Luciferase measurements, respectively. The protocols were the ones provided by the manufacturer with minor modifications. Each Luciferase measurement was the mean of two lecture replicas in a FB 12 Luminometer (Berthold). For stable transfections, Luciferase activity was normalized by total protein, measured with Bradford (Biorad) in a NanoDrop ND-1000 Spectrophotometer (Nucliber).

ALP activity measurements

A 1.5 µl volume of cells resuspended in Luciferase lysis buffer (PLB) was mixed with 200 µl of pNPP (Sigma) and incubated for 30 min at 37°C. The reaction was stopped by adding 200 µl of 3N NaOH. The blank reaction was performed using 1.5 µl of PLB buffer. ALP activity was quantified at 405 nm in a NanoDrop ND-1000 Spectrophotometer and normalized by the total protein content measured by the Bradford method (Biorad).

SDS-PAGE Western Blotting Analysis

To check for the expression of the CIZ/NMP4 isoforms in the stable transfectants, nuclear and cytoplasmatic protein fractions were obtained according to the protocol by Schreiber et al. using a modified buffer C (10% glycerol and 1.5 mM $MgCl_2$) (Schreiber et al. 1989). Fifty µg of protein were subjected to SDS-PAGE (12.5% polyacrylamide) and electrophoretically transferred onto PVDF membranes (Millipore). Each membrane was blocked by an overnight incubation in 5% non-fat milk in PBS, containing 0.1% Tween20 at 4ºC and subsequently incubated for 2 h with Rabbit Anti-Myc-Tag polyclonal antibody (MBL) (1:1000). Membranes were washed 3 times with PBS-0.2% Tween and 3 times with PBS, for 10 min each, and incubated for 2 h at room temperature with goat anti-rabbit antibody conjugated with horseradish peroxidase (Sigma). After 6 washes identical to the previous ones, immunoreactive bands were detected by incubating the membrane for 2 min in the following solution: 10 ml 100mM Tris-HCl pH 9, 50 µl 45mM p-Coumaric Acid, 50 µl 250 mM Luminol and 10 µl 30% H_2O_2.

Statistical analyses

All analyses were performed using the SPSS v.11.5 statistical software package (SPSS, Inc). The Mann-Whitney U test was performed to compare all transcriptional activities, except related linked data. For these data the Wilcoxon signed-rank test was used. A p value of 0.05 was considered significant.

Results

Human COL1A1 promoter constructs transiently or stably transfected in Saos-2

The expression levels of different *COL1A1* constructs transiently transfected in Saos-2 cells were similar to those previously observed in MG-63 cells (Garcia-Giralt et al. 2005) (data not shown). In both cell lines, the short promoter construct (SP) showed a transcriptional activity twice as high as either the long promoter (LP) or the IR constructs. These results were indicative of the presence of a repressor element between positions -220 bp and -1284 bp. The IRD construct, which does not contain the repressor, displayed a transcriptional activity similar to that of the SP construct. The presence of the two polymorphims had no major effects on this system, as demonstrated by the equivalent activity of the LP and IR constructs or the IRD and SP constructs.

No differences to this general pattern were observed when the different *COL1A1* promoter constructs were integrated in the genome in stably transfected cells. Figure 1 depicts the different human *COL1A1* promoter constructs fused to Luciferase (A) and the relative transcriptional activities for each construct stably transfected in Saos-2 cells (B).

BMP2 treatment of Saos-2 cells transiently transfected with human COL1A1 promoter constructs

Saos-2 cells were transiently transfected with LP_G-8T and subsequently treated with different BMP2 concentrations (1 nM, 2 nM and 5 nM) for 24 h in 0.1% Bovine Serum. Both the LP transcriptional activity and ALP activity were increased by the different BMP2 concentrations. Since no significant differences could be observed between the 2 and 5 nM treatments (data not shown), BMP2 was used at 2 nM concentration in all subsequent experiments.

The human *COL1A1* promoter constructs were transiently transfected in Saos-2 cells and treated with BMP2 for 24 h. Figure 2 shows the relative normalized Luciferase activity (mean and SD) for the different constructs in the presence or absence of BMP2 (A) and the percentage of stimulation produced by BMP2 in each construct (B). All constructs, except the empty pGL3 vector, were significantly affected by BMP2. While SP was found to be repressed ($p=0.015$), the remaining three constructs (IR, LP_G-8T and IRD_G-8T) were found to be stimulated ($p=0.015$, $p=0.002$ and $p=0.003$, respectively). However, the percentage stimulation of the IR construct was approximately 20%, whereas the stimulation of either the LP or the IRD constructs was about 60%. These two last constructs contain the -2178 to -1634 bp region, where the polymorphisms are located, suggesting that these play a role in BMP2 stimulation. When the transcriptional activities of the 4 haplotypes were tested in Saos-2 cells, no significant differences were observed, either within the BMP2 treated group or within the non-treated group (Figure 3). The four constructs were significantly stimulated by BMP2 ($p\leq0.003$). The percentages of stimulation were in the range of 50 to 70% (Figure 3). Marginally significant differences were observed between the LP haplotype T-8T and either T-7T ($p=0.052$) or G-8T ($p=0.039$).

Putative binding sites that could direct the BMP2 effect: Dlx5 and Smad1

In silico searches of binding sites for transcription factors that might be downstream effectors of BMP2, within the -2178/-1634 sequence, yielded two positive results: a putative Dlx5 binding site (ggctatAATTaaa, from -1776 bp to -1764 bp) and a putative Smad1 consensus sequence (GCCGaCG, from -1698 bp to -1692 bp). To test whether any of them mediate BMP2 stimulation of *COL1A1* promoter activity, both sites were mutagenized separately and their relative transcription activities measured and compared with the wild type (Figure 4). None of the two putative binding sites seemed to direct the BMP2 stimulation observed on the activity of the *COL1A1* promoter.

Expression pattern of CIZ/NMP4 isoforms in different human tissues and in osteoblast like cells

To know which CIZ/NMP4 isoforms were present in human tissues and in human osteoblast-like cells, RT-PCR amplifications encompassing the regions alternatively spliced (insert 1, insert 2 and zinc fingers 4 and 5) were performed with different sets of primers (Figure 5 A). Up to four CIZ/NMP4 isoforms were observed in pancreas, kidney, skeletal muscle, liver, lungs, placenta, brain, heart, Saos-2 and MG-63 human osteosarcoma cell lines and human primary osteoblasts (Figure 5 B). They could be identified as 21H, 21H-I1, 11H and CIZ6.1, either by sequencing of amplified products (21H and 21H-I1) or by deduction from amplicon sizes (Figure 5 C). In general, the 21H appeared to be the most abundant isoform. The 21H-I1 isoform was also abundant in osteoblast-like cells (Figure 5 C).

Effect of CIZ/NMP4 on 2.5 kb of the human COL1A1 promoter in Saos-2 cells

Saos-2 cells stably transfected with a tagged CIZ/NMP4 gene (isoform 21H or 21H-I1 fused to a Myc epitope) were created as described in Materials and Methods, and expression of the CIZ/NMP4 isoforms was verified by Western-blot (Figure 6 A). Both isoforms could be detected in the enriched nuclear protein extract but were absent from the cytoplasm fraction. These cells were then transiently transfected with LP_G-8T and grown in the presence or absence of 2 nM BMP2 for 24 h. *COL1A1* transcriptional activity was slightly increased in the cells expressing 21H or 21H-I1 CIZ/NMP4 and not treated with BMP2 when compared with the control pcDNA3-transfected cells ($p \leq 0.026$) (Figure 6 B). However, when cells were treated with BMP2 no significant differences in the transcriptional levels were detected.

Discussion

In the present study, we have analyzed the roles of BMP2 and CIZ/NMP4 on a 2.5 kb human *COL1A1* promoter, which contains two polymorphisms, -1997 G/T and -1663 indelT, associated with BMD.

CIZ/NMP4, a nuclear matrix protein, is an architectural transcription factor that remodels chromatin to repress or to activate transcription (Bidwell et al. 2001). The DNA packing may be as

important as the primary sequence to regulate gene expression and might vary between episomal and integrated vectors. To test this possibility, Saos-2 cell lines with different *COL1A1* promoter constructs integrated in the genome were created. The integration in the genome was not site-specific, so one or more copies of the construct per cell could be integrated in euchromatin or heterochromatin. A minimal pool of 250 stable foci was obtained for each *COL1A1* promoter construct. The relative promoter activities of the different constructs were similar in transient versus stable transfections. These results are in agreement with those of other studies that showed the normal packing of DNA of transiently transfected vectors (Reeves et al. 1985),

As previously observed in MG-63 cells (Garcia-Giralt et al. 2005) and other cell types (Buttner et al. 2004; Jimenez et al. 1994), the effects of a repressor element between positions -1284 bp and -254 bp of the *COL1A1* promoter were observed both in transiently and in stably transfected Saos-2 cells. The polymorphisms did not seem to play a major role in promoter activities in this system.

BMP2, an osteoblastic differentiation factor, was found to up- or downregulate the *COL1A1* promoter, depending on the construct analyzed. BMP2 could induce a 60% stimulation of the transcription through the sequence from -2178 bp to -1634 bp. Several studies have shown that BMP2 stimulates collagen expression (Lecanda et al. 1997; Nissinen et al. 1997; Shen et al. 2002). However, other studies have failed to replicate this (Li et al. 2004). Signalling by BMP2 can be transmitted through the Smad or the MAPK pathways or through molecules such as Dlx5 (Chen et al. 2004; Ryoo et al. 2006). Smad proteins are known to interact with DNA at Smad binding elements (SBE) or at GCs rich sequences. A putative Smad1 binding site was identified in the *COL1A1* promoter fragment stimulated by BMP2. However, in the present work, this sequence did not seem to direct the BMP2 effect in Saos-2 cells, as deduced from a mutagenesis experiment. Dlx5 is a bone inducing transcription factor coexpressed with BMP2. Dlx5 has been proposed as a crucial mediator of BMP2-induced osteoblast differentiation (Ryoo et al. 2006). A putative consensus sequence for Dlx5, located in the same *COL1A1* promoter fragment, was also tested and found not to be implicated in the BMP2 effect in Saos-2 cells, either. It could be that Dlx5 and Smad1 boxes have redundant functions in relation to BMP2 stimulation, so that if only one box is mutagenized no differences in *COL1A1* transcriptional activity are observed. Another possibility is that sequences different from the putative binding sites for Smad1 or Dlx5 may regulate the BMP2 stimulation of the *COL1A1* promoter. Proteins regulated by Dlx5, such as Runx2 or Osterix, or other BMP2 downstream effectors could act on this region.

In relation to haplotype transcription capacities, we did not observe significant differences among the four LP haplotypes in Saos-2 cells either treated or not treated with BMP2. In contrast, Garcia-Giralt et al. (2005) found that LP_G-7T had slightly increased transcriptional capacity as compared with LP_T-8T or LP_T-7T in MG-63 cells. Regarding the percentage of BMP2 stimulation in Saos-2 cells, small differences between LP haplotypes were observed. The LP_T-8T exhibited higher stimulation than LP_T-7T or LP_G-8T, but not LP_G-7T. The differences in transcription between haplotypes were very small, both in MG-63 and in Saos-2, so experimental artefacts cannot be ruled out. A possible explanation for these results is that these polymorphisms

do not have a direct function on the collagen promoter, but are in linkage disequilibrium with others. Alternatively, the functionality of the polymorphisms may not be readily elucidated *in vitro* if their effects depend on, for example, age or menopausal status, conditions that are not replicable in the cell culture system (Rebbeck et al. 2004).

To study the effect of CIZ/NMP4 on human *COL1A1* promoter, we first characterized the CIZ/NMP4 isoforms expressed in different human tissues and cells. As in rats, in human, CIZ/NMP4 was found to be ubiquitously expressed and several isoforms were observed in each tissue analyzed. The main isoforms identified in humans were 11H, CIZ6.2, 21H and 21H-I1. In a study performed with rat osteoblast-like cells, the main CIZ/NMP4 isoforms expressed were 10H, 11H, CIZ6.2, 21H and 22H (Torrungruang et al. 2002). The rat 10H isoform was not detected in human tissues and the 22H isoform, which was detected in less quantity in rat cells, could be, as its size suggests, the same that has been named 21H-I1 in humans. The CIZ/NMP4 21H and 21H-I1 seemed majoritary in osteoblast-like cells, and for this reason we decided to clone them and analyze their effects on *COL1A1* transcription. Saos-2 cells expressing 21H and 21H-I1 tagged isoforms, presented them in the nucleus, the common location for CIZ/NMP4 as described previously (Feister et al. 2000; Torrungruang et al. 2002).

In this study, overexpression of CIZ/NMP4 slightly increased the *COL1A1* transcription in Saos-2 cells untreated with BMP2. The isoforms 21H and 21H-I1 developed the same effect. In agreement with the present results, a CIZ/NMP4 stimulatory effect on collagen expression was described in MC3T3E1 cells (Furuya et al. 2000). In contrast, Thunyakitpisal et al. (2001) showed that rat CIZ/NMP4 13H and 21H isoforms, but not 11H or 28H, inhibited the transcriptional activity of 3.5 kb of the *COL1A1* promoter in UMR-106 cells. The CIZ/NMP4 inhibitiory effect was carried out through the *site B*. Shen et al. (2002) also observed a decrease in the *COL1A1* mRNA level in MC3T3E1 cells expressing CIZ/NMP4, as comparing with the control ones, when they were BMP2-treated. Surprisingly, in absence of BMP2 treatment, the cells expressing CIZ/NMP4 showed a small increse in the *COL1A1* mRNA level at the first days of culture (7 days). These results could point out a dual role for CIZ/NMP4 on *COL1A1* regulation: stimulating its expression at the first days of an undifferentiating culture, and repressing it when cells are manteined in culture longer or under a differentiating treatment.

The present study has some limitations. For instance, it is important to note that the CIZ/NMP4 isoforms contained a Myc tag in their N-terminal domain and, although the tag has only 15 aminoacids, it could modify CIZ/NMP4 structure or function. On the other hand, many of the cited studies undertaken to elucidate the CIZ/NMP4 role on *COL1A1*, were perfomed at mRNA level. In the present study, we have analyzed the CIZ/NMP4 effect on transcription from a 2.5 kb *COL1A1* promoter, but three putative CIZ/NMP4 boxes, that could participate in the regulation of *COL1A1*, are present upstream of the 2.5 kb analyzed. Finally, although previous studies on rat promoter showed that the 21H and 13H isoforms regulated *COL1A1* transcription, here, only the effect of 21H and 21H-I1 isoforms was analyzed, so it cannot be ruled out that isoforms different from the studied ones have an effect on *COL1A1* regulation in humans.

In conclusion, no different *COL1A1* transcriptional patterns were detectable beteween transient or stable transfections. The maximal BMP2 stimulation occurred through the positions from -2178 bp to -1634 of the human *COL1A1* promoter. That stimulation was independent of the polymorphisms and of the putative Dlx5 and Smad1 boxes present in that region. Finally, in Saos-2 cells, CIZ/NMP4 isoforms did not repress a 2.5 kb *COL1A1* promoter after BMP2 treatment, but slightly stimulated it in the absence of treatment.

Acknowledgments

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Figures

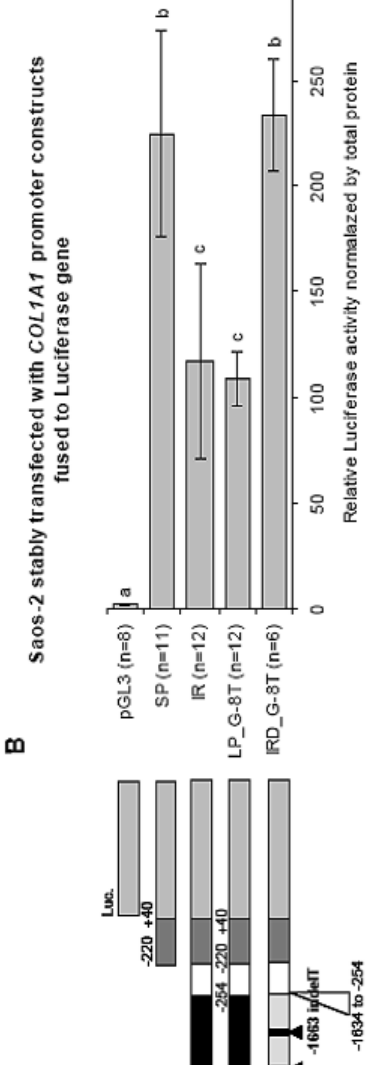

Figure 1. A. The main human *COL1A1* promoter constructs fused to the Luciferase gene used in the present study. **B.** Saos-2 cells were stably transfected with pGL3-COL1A1-Neo promoter constructs or pGL3-Neo empty vector (as control) and Luciferase activity was measured. Mean relative Luciferase activities, normalized by total protein, and standard deviations are shown. Number of replicates (n=6-12) correspond to 2 to 4 independent experiments, with 3 replicates in each. Letters a, b and c denote significantly different values ($p \leq 0.001$), according to the Mann-Whitney U test.

Figure 2. A. Saos-2 transiently transfected with different *COL1A1* constructs and treated (black code) or not (grey code) with 2 nM of BMP2 for 24 h. Relative Luciferase activity (mean and SD) and number of replicates (n=11-12) (each from an independent transfection) are shown. pGL3 did not experiment any change, SP was repressed and IR, LP_G-8T and IRD_G-8T were stimulated ($p \leq 0.015$) according to Wilcoxon test. **B.** Relative BMP2 stimulation experimented by different *COL1A1* constructs. Significant differences between percentages of stimulation are indicated (a to c) according to Mann-Whitney U tests.

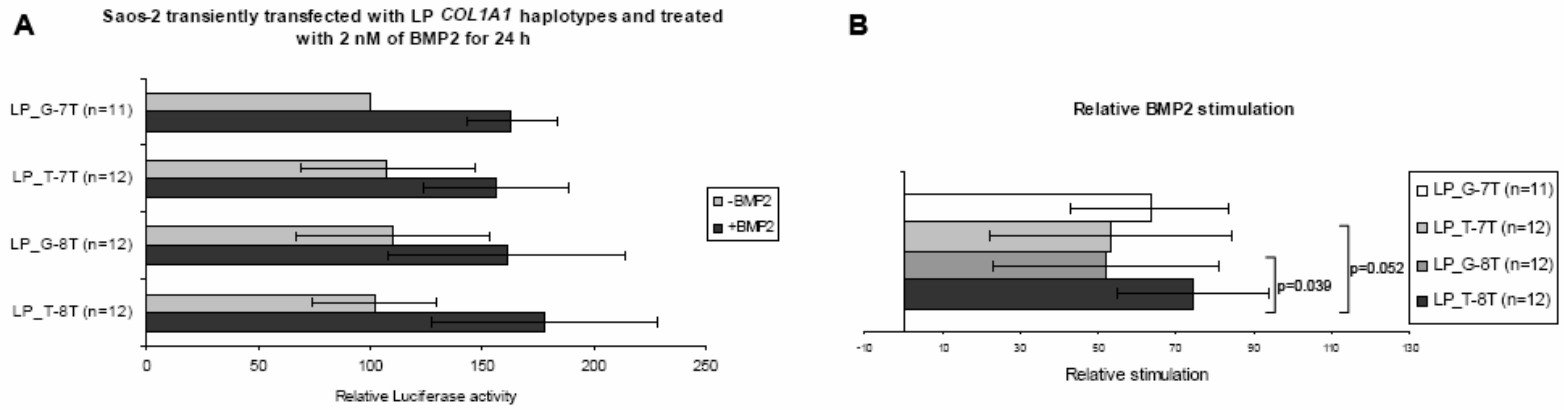

Figure 3. A. Saos-2 transiently transfected with LP *COL1A1* haplotypes and treated (black code) or not (grey code) with 2 nM of BMP2 for 24 h. Relative Luciferase activity (mean and SD) and number of replicates (n=11-12) (each from an independent transfection) are shown. All LP haplotypes were stimulated with BMP2 ($p \leq 0.003$ according to Wilcoxon test). **B.** Relative BMP2 stimulation experimented by different LP *COL1A1* haplotypes. Haplotypes are indicated on the right and number of replicates of each haplotype (n=11-12) are indicated in brachets. Haplotypes exhibited nearly significant differences (as indicated with Mann-Whitney U test p values). LP_T-8T showed higher stimulation than LP_T-7T and LP_G-8T (p=0.052 and p=0.039, respectively).

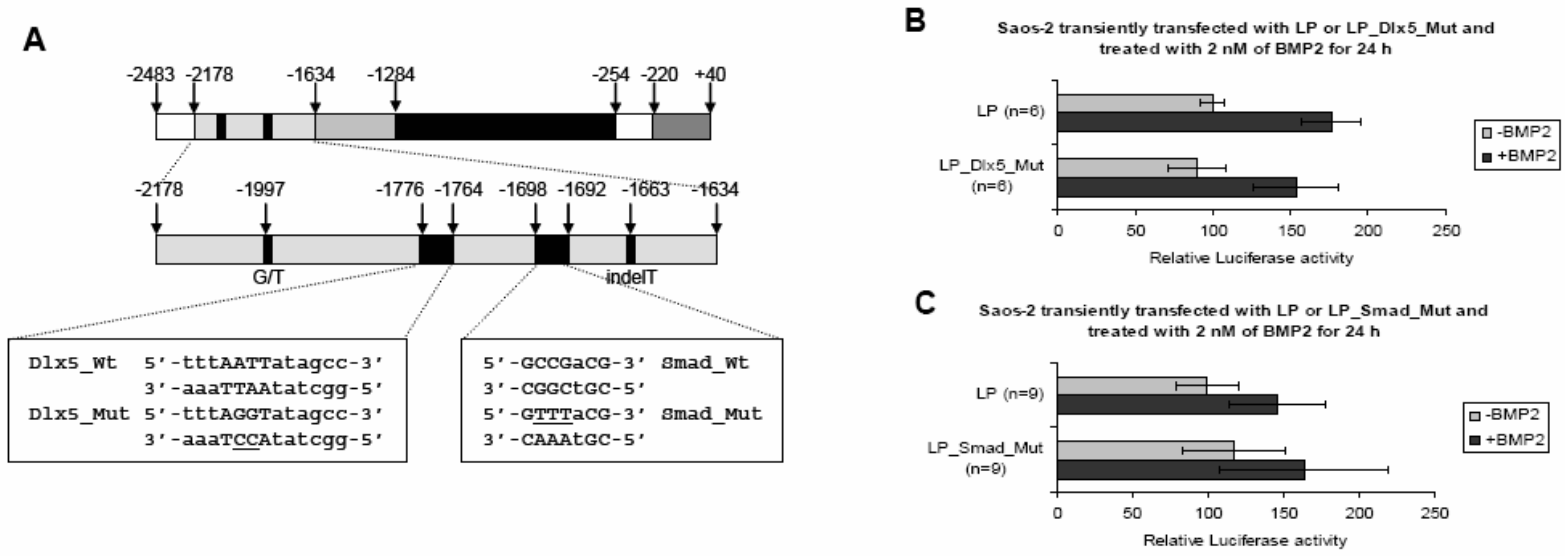

Figure 4. A. Scheme of 2.5 kb of the human *COL1A1* promoter with the putative Dlx5 and Smad1 consensus sequences. **B and C.** Relative Luciferase activity obtained with the mutagenized Dlx5 consensus site or Smad1 consensus site. Saos-2 cells were transiently tranfected with the wild type LP_G-8T or the mutagenized one and treated for 24 h with 2 nM of BMP2 (black code) or non treated (grey code). The mean and SD of the relative Luciferase activity and the number of replicates (n=6-9) (from 2-3 independent transfections with three replicates in each) are indicated. There were no significant differences according tot Mann-Whitney U test, between mutaginized and wild type constructs, either for BMP2 treated or untreated cells.

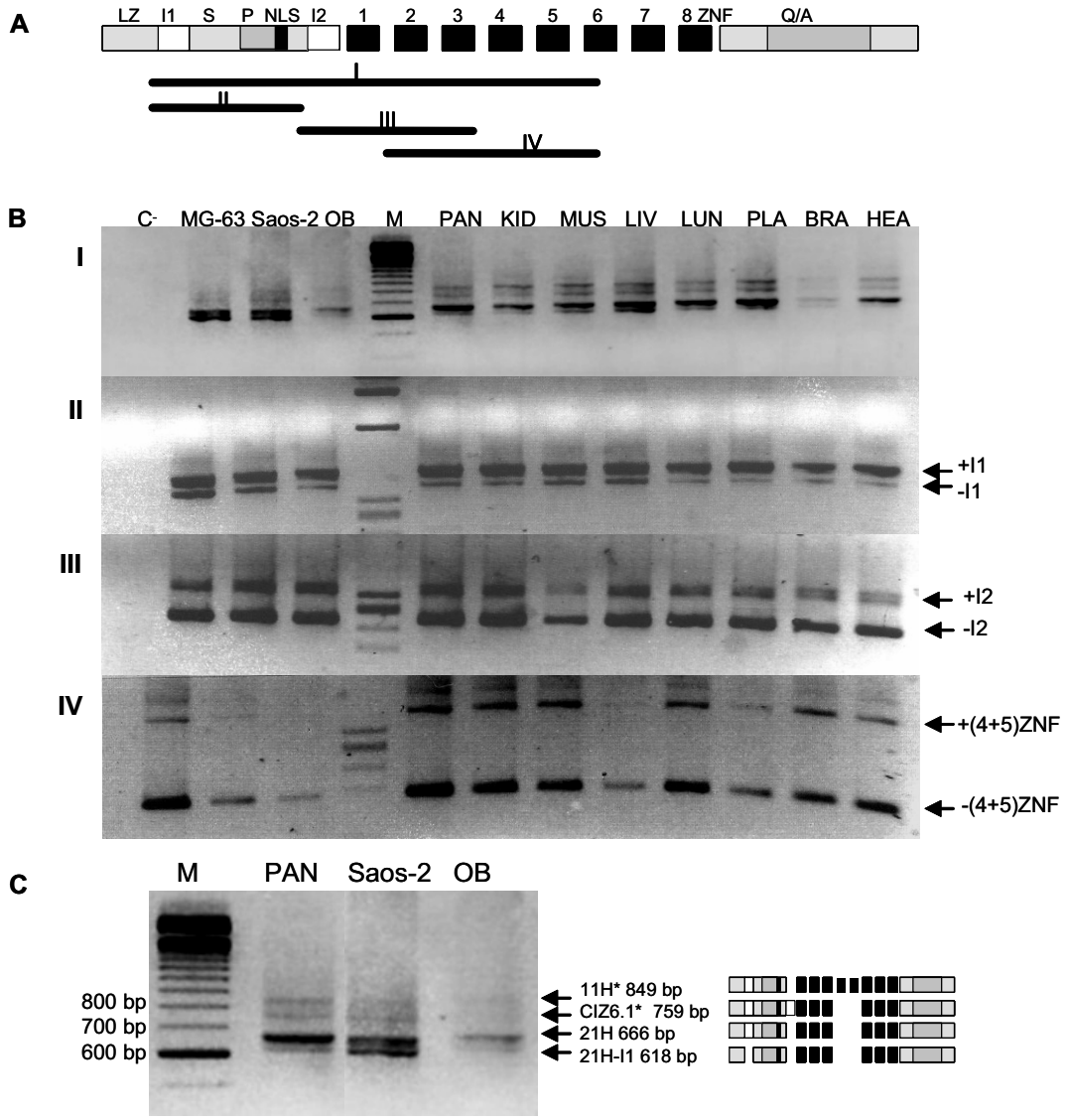

Figure 5. CIZ/NMP4 expression in a human cDNA tissue panel and in human osteoblast-like cells. **A.** Scheme of the CIZ/NMP4 protein that includes all the known variability: insert 1 (I1), insert 2 (I2), and 4th and 5th zinc finger (4+5 ZNF). The four different cDNA fragments amplified by RT PCR are indicated with solid lines and labelled I-IV. **B.** CIZ/NMP4 mRNA expression pattern in different human tissues and osteoblast-like cells (numbers according to the RT PCR amplified fragments represented in **A**). Variants of each amplified PCR fragment are indicated on the right with arrows. **C.** Partial enlarged image of the RT-PCR that includes all the possible variations (fragment I). The isoforms observed and their sizes are indicated on the right. All the PCR fragments were sequenced except the ones indicated with an asterisk (*), which were deduced based on their size.

LZ: leucine zipper, S: serine rich region; P: proline rich region; NLS: nuclear location signal; ZNF: zinc finger; Q/A: glutamine and alanine rich region; OB: primary osteoblasts; PAN: pancreas; KID: kidney; MUS: skeletal muscle; LIV: liver; LUN: lung; PLA: placenta; BRA: brain; HEA: heart; M: molecular weight marker.

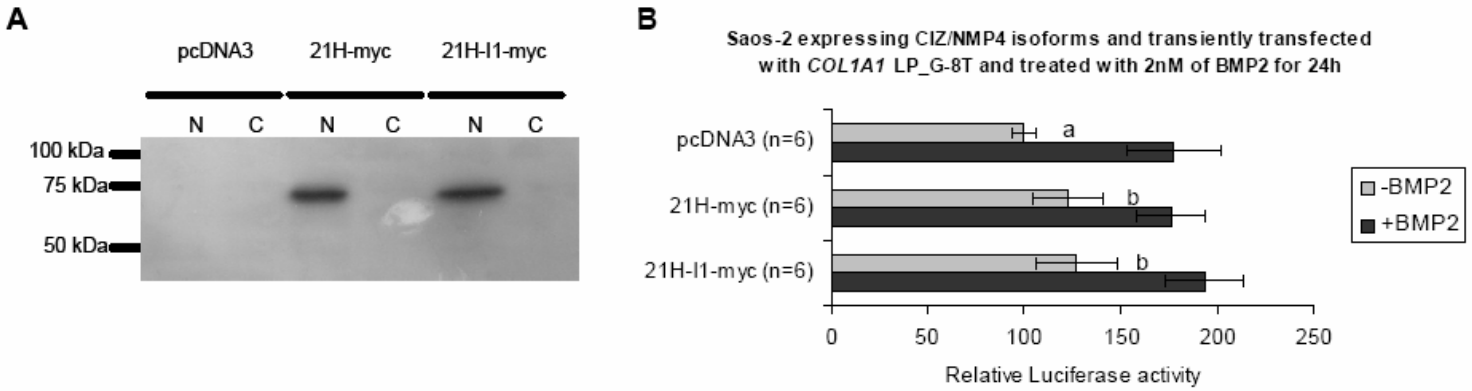

Figure 6. Effect of CIZ/NMP4 isoforms on 2.5 kb of the human *COL1A1* promoter in Saos-2 cells. **A.** Western-blot showing the expression and the nuclear location of two isoforms of CIZ/NMP4 (21H and 21H-I1 containing an N-terminal Myc tag) in stably transfected Saos-2 cells. Empty pcDNA3 stably transfected was used as negative control. **B.** Transient transfection of Saos-2 cells, expressing 21H or 21H-I1 CIZ/NMP4 isoforms or the empty vector, with LP_G8T and treated (black code) or not (grey code) with 2 nM of BMP2 for 24 h. Relative Luciferase activity (mean and SD) and number of replicates (n=6) (from three independent experiments with two replicates in each) are indicated. Expression of these isoforms increased the collagen transcription in non treated cells ($p \leq 0.026$ according to Mann-Whitney U test, indicated with an a-b code), and no significant differences were observed in the BMP2 treated cells.

N: nuclear protein extract; C: cytoplasmatic protein extract.