

Polymorphisms of the WNT10B Gene, Bone Mineral Density, and Fractures in Postmenopausal Women

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Abstract Wnt ligands are important regulators of skeletal homeostasis. Wnt10B tends to stimulate the differentiation of common mesenchymal precursors toward the osteoblastic lineage, while inhibiting adipocytic differentiation. Hence, we decided to explore the association of WNT10B allelic variants with bone mineral density and osteoporotic fractures. A set of tag SNPs capturing most common variations of the WNT10B gene was genotyped in 1438 Caucasian postmenopausal women, including 146 with vertebral fractures and 432 with hip fractures. We found no association between single SNPs and spine or hip bone mineral density (BMD). In the multilocus analysis, some haplotypes showed a slight association with spine BMD ($P = 0.03$), but it was not significant after multiple-test correction. There was no association between genotype and vertebral or hip fractures. Transcripts of WNT10B and other Wnt ligands were detected in human bone samples by real-time PCR. However, there was no relationship between genotype and RNA abundance. Thus, WNT10B is expressed in the bone microenvironment and may be an important regulator of osteoblastogenesis, but we have not

found evidence for a robust association of common WNT10B gene allelic variants with either BMD or fractures in postmenopausal women.

Keywords Wnt · Gene expression · Association study · Polymorphisms

Bone mass has a strong genetic component [1, 2]. At the tissue level, the maintenance of bone mass depends on the proper balance between bone formation and bone resorption, which are modulated by a number of endocrine and paracrine factors. Following the seminal studies revealing marked skeletal abnormalities in patients with mutations of the lipoprotein receptor-related protein (LRP) 5 gene [3], the Wnt pathway has emerged as an important regulator of bone homeostasis [4, 5]. It includes different ligands, receptors, coreceptors, and inhibitors. Apart from possible variants due to alternative splicing and posttranslational modifications, at least 19 Wnt ligands have been described. They bind a membrane receptor complex formed by a frizzled protein and LRP. Ten different frizzled genes exist, whereas LRP5 and LRP6 are the most extensively studied LRP forms [6]. Binding of Wnt ligands initiates a cascade of events which mediate their cellular effects. The best-known intracellular mechanisms constitute the so-called canonical pathway, which leads to the accumulation of β -catenin [7]. Several Wnts are produced in the bone tissue and exert an influence on bone remodeling, particularly on cells of the osteoblastic lineage. However, the true physiological role of the various Wnt ligands and their relative importance have not been elucidated.

A number of Wnt ligands have been shown to modulate osteoblast differentiation and activity in vitro. FABP4-

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WNT10B mice, which have increased expression of WNT10B in the marrow, show an increased bone mass. On the other hand, WNT10B knockout mice have decreased trabecular bone and osteocalcin levels [8]. Those studies strongly suggest that Wnt10B is an important endogenous regulator of bone formation. The mechanism of action appears to include a shift of cell fate of the mesenchymal precursors toward the osteoblastic phenotype, while the differentiation toward the adipocytic lineage is inhibited [8, 9]. Little is known about the mechanisms regulating Wnt10B synthesis, but it has been demonstrated that loading results in increased expression of several molecules in the Wnt pathway, including WNT10B, thus suggesting its involvement in mechanotransduction [10, 11]. Those data prompted us to study the role of WNT10B as an osteoporosis candidate gene, by analyzing its expression in human bone and exploring the relationship of WNT10B polymorphisms with bone mineral density (BMD) and osteoporotic fractures.

Materials and Methods

Subjects

We studied a group of 1,006 postmenopausal women over 50 years of age (mean, 66 ± 8 years), with an average duration of the menopause of 16 years. They were volunteers recruited by voice and written announcements or women sent to our outpatient clinic to be studied for possible osteoporosis. All participants were living in Cantabria, a region in northern Spain with a population of about 530,000. Subjects taking bisphosphonates, corticosteroids, antiepileptics, estrogens, or other drugs known to modify bone mass, and those with non-Spanish ancestors, were excluded. The study was approved by the Institutional Committee on Ethics in Clinical Research.

We also studied 432 postmenopausal women admitted to hospital with hip fractures (mean age, 79 ± 7 years). Women with fractures due to high-impact trauma (such as traffic accidents and falls from a height) or diseases causing secondary osteoporosis (cancer, rheumatoid arthritis, malabsorption, severe systemic diseases, etc.) were not included.

Skeletal Studies

BMD was measured at the spine (L2–L4), femoral neck, and total hip regions by DXA (Hologic QDR4500). Lateral X-rays of the thoracic and lumbar spine were obtained in 646 women; 146 had vertebral fractures, defined as a loss of at least 25% of vertebral body height. X-rays were not obtained in some control women with a normal BMD and

absence of clinical risk factors for osteoporosis or back pain.

Genotyping

DNA was isolated from the peripheral blood or buccal swabs using standard commercial methods and quantified using the Qubit procedure (Invitrogen). We explored the Hapmap database searching SNPs in a 12-kb region including the WNT10B gene and neighboring 5' and 3' regions, with a minimum allelic frequency (MAF) of 10% in the Caucasian population. Then tag SNPs capturing most gene variation were selected using Haploview software, with the “aggressive tagging” option and an r^2 threshold of 0.8 [12]. In addition, we did an in silico search of potentially functional SNPs using the Pupa web tool [13], which explores a number of databases related to SNP function. Those SNPs selected as potentially functional were also included. The SNP set was genotyped using a Sequenom platform, at the Centro Nacional de Genotipado in Santiago de Compostela. In 699 women, randomly chosen, the rs1051886 polymorphism, which was related to bone mass in a recent study [14], was later genotyped using a Taqman assay, with the specific primers and probes designed by the manufacturer (Applied Biosystems). Random samples (about 5%) were genotyped twice to check the consistency of results, which were concordant in more than 99% of cases.

Expression of Wnt Ligands in Bone

Bone samples were obtained from the femoral heads of 14 patients subjected to hip replacement because of severe osteoarthritis (11 women and 3 men; mean age, 73 ± 8 years). Most were taking various analgesics and nonsteroidal antiinflammatory drugs. RNA was isolated with Trizol (Invitrogen, Carlsbad, CA, USA), and further purified using a column adsorption procedure (Qiagen, Hilden, Germany). Contaminant DNA was removed by treating the samples with DNase (Ambion, Austin, TX, USA). Gene expression was analyzed by reverse transcription (RT) real-time polymerase chain reaction (PCR). Aliquots of RNA (250 ng) were reverse-transcribed with the Superscript III kit (Invitrogen). After RT, a real-time PCR was done in an ABI7300 apparatus (Applied Biosystems, Foster City, CA, USA), using specific primers and a FAM-labeled probe for WNT10B (Taqman gene expression assays; Applied Biosystems). The expression of other Wnt ligands was studied also by real-time quantitative PCR using a miniarray with specific primers and SYBR Green to monitor the amount of PCR products (Superarray, Frederick, MD, USA). The results were then normalized to the average expression of housekeeping

genes (GAPDH, ACTB, β 2-microglobulin, and RPL13A). The specific gene expression in bone, relative to a universal reference pool of RNA obtained from 10 human cell lines of various origins (liver, breast, testes, uterus, fat, brain, skin, plasma cells, and lymphoid; Stratagene, La Jolla, CA, USA), was calculated using the formula:

$$2^{\Delta\text{Ct}2-\Delta\text{Ct}1}$$

where $\Delta\text{Ct}1$ is the difference between the gene-of-interest threshold cycle and the housekeeping threshold cycle in the bone sample, and $\Delta\text{Ct}2$ is the difference in the reference RNA [15].

Data Analysis

The genotyping results were first managed with the SNPator Web tool software, which allowed generating data file formats suitable for analysis by several other programs. We used HAPLOVIEW software to compute linkage disequilibrium (LD) measures and construct haplotypic blocks with the method of Gabriel [12]. The single-locus association between the polymorphisms and BMD was assessed assuming a linear trend between alleles and BMD. Age and weight were included as covariates in logistic regression models. The haplotypic analysis was done using sliding windows defined by three consecutive SNPs. PLINK software was used in those analysis [16]. The association with osteoporotic fractures was studied similarly with the Cochran-Armitage trend test [17]. For the hip fracture analysis, the genotype frequency distribution in women with hip fractures was compared with that of a group of women without clinical evidence of vertebral or other nontraumatic fractures after menopause ($n = 707$), selected from the control group. Gene expression was compared across genotypes using a nonparametric Kruskal–Wallis test computed with SPSS software (SPSS Inc., Chicago, IL, USA). QUANTO software was used for power analysis, assuming codominant models [18]. Nominal P -values are shown, unless otherwise stated.

Results

SNPs in the WNT10B gene region showed strong LD and were grouped in a single haplotypic block (Fig. 1). The allelic frequencies were similar to those reported in other Caucasian populations (Table 1) and there was no evidence for departure from Hardy–Weinberg equilibrium.

Association with Bone Mineral Density

Allelic variations of the SNPs analyzed were not associated with BMD at either the lumbar spine or the hip (Fig. 2). In

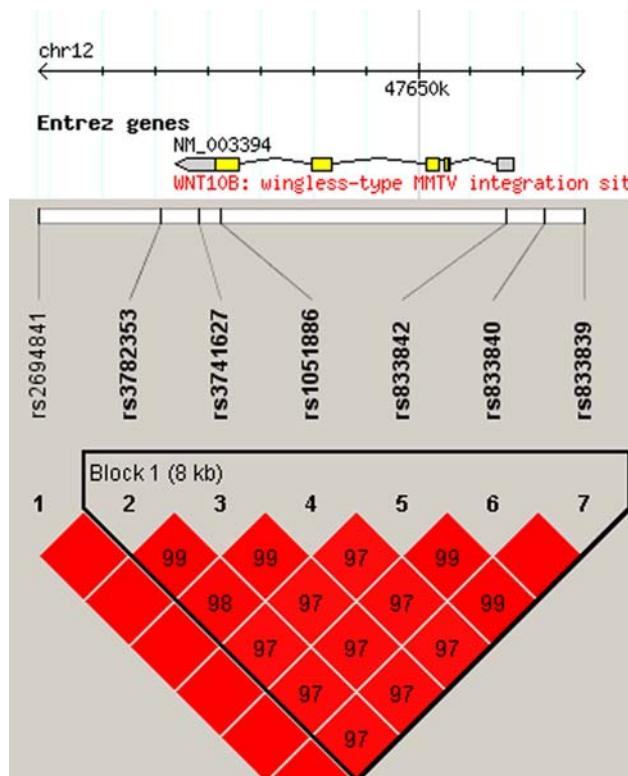


Fig. 1 Linkage disequilibrium and resulting haplotypic block structure of the WNT10B region. The numbers represent the D' distances

Table 1 Polymorphisms included in the present study

| SNP | Position | Location | Alleles | MAF |
|-----------|----------|---------------|---------|------|
| rs2694841 | 47642801 | 3' downstream | A/G | 0.03 |
| rs3782353 | 47645147 | 3' downstream | A/G | 0.45 |
| rs3741627 | 47645854 | 3'-UTR | G/T | 0.45 |
| rs1051886 | 47646256 | Exon 5 | T/C | 0.45 |
| rs833842 | 47651690 | 5'-UTR | G/T | 0.38 |
| rs833840 | 47652417 | 5' upstream | C/G | 0.39 |
| rs833839 | 47653157 | 5' upstream | A/T | 0.45 |

MAF minor allelic frequency

the multilocus analysis, several haplotypes were associated with spine BMD, with P -values between 0.03 and 0.05 (Fig. 2), but they were no longer significant after multiple-test adjustment. The strongest association was found for the GTG haplotype (loci rs3782353, rs3741627, and rs833842). Spine BMD was 2.4% higher in women bearing at least one copy of the GTG haplotype as in women with no copies. The inclusion of all six SNPs in the haplotypic block established by the Gabriel method did not result in a stronger association. The polymorphisms were not associated with hip BMD, hip BMC, or average femoral neck diameter.

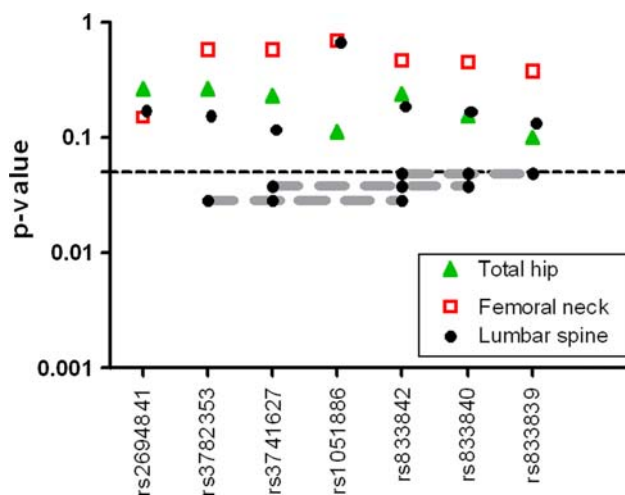


Fig. 2 Association of WNT10B polymorphisms with BMD at the spine or the hip. Nominal *P*-values of the single-locus and haplotype analysis (Armitage–Cochran trend test)

Association with Vertebral and Hip Fractures

There was no significant association with vertebral fractures in the single-locus (Table 2) or the haplotypic analysis (not shown). Likewise, no association with hip fractures was detected in either analysis. Since women with hip fractures tended to be older than those in the control group, the analysis was repeated including only women <80 years of age (the median in the hip fracture group) and selecting age-matched controls or adjusting by age as a model covariate. No association between genotype and hip fractures was detected in those analyses.

Wnt Ligand Expression in Bone Tissue

The expression of several Wnt ligands in bone samples is shown in Fig. 3. WNT3A, WNT4, WNT7A, and WNT10A were preferentially expressed in bone, in comparison with the reference RNA (nominal *P*-values <0.0015 and <0.05 after multiple-test correction). WNT10B expression in

Table 2 Lack of association of WNT10B polymorphisms with osteoporotic fractures (nominal *P*-values)

| SNP | Vertebral fractures | Hip fractures |
|-----------|---------------------|---------------|
| rs2694841 | 0.31 | 0.85 |
| rs3782353 | 0.18 | 0.09 |
| rs3741627 | 0.18 | 0.11 |
| rs1051886 | 0.60 | 0.70 |
| rs833842 | 0.27 | 0.28 |
| rs833840 | 0.24 | 0.21 |
| rs833839 | 0.25 | 0.31 |

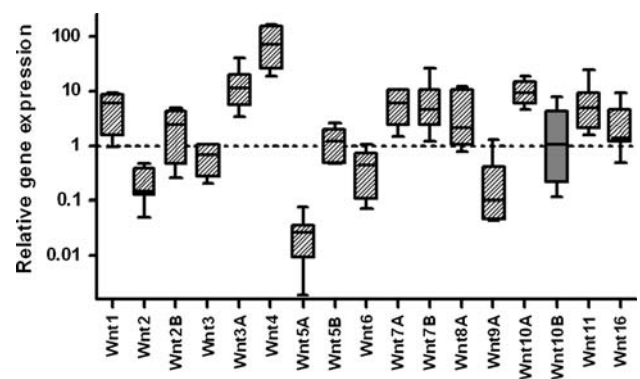


Fig. 3 Relative expression of Wnt ligands in human bone tissue (in comparison with universal reference RNA). The box-whisker plots represent the median, 25th and 75th percentiles, and minimum and maximum values

bone was similar to that in reference RNA. No significant correlations existed between WNT10B expression in bone samples and the alleles present at the seven polymorphic loci studied (not shown).

Discussion

The Wnt pathway has emerged as an important regulator of skeletal development and is also involved in bone homeostasis in adult organisms. The role of WNT10B in human skeletal development has been revealed by the identification of an homozygous missense WNT10B mutation as a cause of the Split-Han/Foot malformation syndrome [19]. In general, Wnt pathway activation has an anabolic effect on adult bone, which is the consequence, at least in part, of a positive influence on the differentiation of osteoblast precursors. However, differences among the various Wnt molecules exist. Whereas some Wnt ligands stimulate differentiation toward the osteoblastic lineage, others, such as Wnt4 and Wnt5A, tend to increase the expression of adipogenesis-related genes [20]. Wnt10B has been shown to be one of the osteoblast-differentiating ligands, whereas it inhibits the differentiation of common precursors toward the adipocytic lineage, in part by suppressing the expression of C/EBP α and PPAR γ [21]. Studies with transgenic mice have shown that increased WNT10B expression is associated with a higher bone mass, an effect primarily due to increased osteoblastogenesis [22]. Although the mechanisms controlling WNT10B expression are largely unknown, gene expression is increased by mechanical loading [10, 23].

These data pointed toward WNT10B as an appealing candidate gene for osteoporosis. However, in the present study we found no consistent evidence for an association

between several polymorphisms of the gene and either BMD or osteoporotic fractures in Caucasian postmenopausal women. Thus, our results do not corroborate a recent report in men with African ancestry. In that study, Zmuda et al. sequenced the WNT10B gene and found several polymorphisms that had not been previously reported. Then they genotyped a set of eight SNPs in two groups, each one including about 1000 men with a mean age of 62 years. They found two SNPs (rs3741627 and rs1051886) to be associated with hip BMD in both groups, although the statistical significance was moderate ($P < 0.05$). They also studied the bone structure at the distal radius by QCT and found that those SNPs were associated with BMC and bone size but not with trabecular volumetric BMD [14].

SNPs selected in the present study included tag SNPs capturing most common allelic variants of those represented in the Hapmap Caucasian database, as well as some SNPs predicted to have functional consequences on gene transcription or translation. The tagging approach may result in the selection of different SNPs depending on the database used for reference. Thus, our SNP set included several SNPs different from those selected by Zmuda et al. but also the two SNPs found to be slightly associated with hip BMD in males in the study by Zmuda et al. However, in women we have not been able to demonstrate a consistent association between SNPs and BMD strong enough to resist multiple-test adjustment. The failure to replicate the results of Zmuda et al. suggests that the influence of WNT10B variants may depend on the gender or race of the individuals. In fact, the allelic frequencies found in this study were similar to those found in other Caucasian populations and to the frequencies reported by Zmuda et al. in a small Caucasian group but quite different from the results in individuals of African ancestry. On the other hand, there appears to be ethnicity-dependent differences in the linkage disequilibrium pattern in the Wnt10b region. Thus, in our study, as well as in the CEU (Caucasian) Hapmap population, the SNPs were grouped in a single haplotypic block. On the contrary, in populations of African origin (such as the Yoruba Hapmap population and the individuals studied by Zmuda et al.), two separate blocks can be recognized.

WNT10B expression was readily detected in human trabecular bone samples, in similar amounts to a reference mixture of RNA from 10 different cell lines. However, we did not find genotype-related differences in gene expression. Similarly, we did not find an association between genotype and gross measures of skeletal size (such as height or average neck diameter of the femoral neck estimated from DXA output). Although BMD measurements by DXA are obviously influenced by both cortical and trabecular bone components, we do not have data on

specific measurements of the cortical bone structure by qCT. Since, according to Zmuda et al. [14], the WNT10B polymorphisms may preferentially influence the cortical bone, it could also be argued that gender and racial factors influence the power to detect the genetic effects, which could be more readily apparent in black men with thick cortices than in Caucasian postmenopausal women with thinner bones. Nevertheless, this remains speculative at the moment, and although we cannot exclude the possibility that WNT10B polymorphisms exert some influence on the cortical bone in Caucasian women, such influence is probably small, if any, as we found no association with hip fractures.

Our study has some limitations. We excluded patients with diseases that could impact bone homeostasis, but we did not exclude some subclinical disorders such as vitamin D insufficiency that might interact with the genetic factors. The age distributions of patients with hip fractures and controls were somewhat different. This might have had some influence toward the null effect, as some individuals included in the control group could fracture in the future. Nevertheless, we tried to limit this influence by performing age-adjusted analyses and subgroup age-restricted analyses (to compare age-matched patient and control groups), which failed to show an association of genotypes with fractures. With an α level of 0.05, the study had 90% power to detect an association of SNPs with bone phenotypes, if the genotypes explained at least 1% of BMD variance or were associated with odds ratios >1.3 for hip fractures or >1.5 for vertebral fractures. The sample size does not allow the exclusion of a small effect below those thresholds. On the other hand, we cannot exclude an influence of very rare allelic variants. But even if they have marked effects at the individual level, the results of resequencing studies [14] suggest that such variants are unlikely to play an important role at the population level. The limited number of bone samples available to study gene expression and the wide interindividual variations limited the power to analyze the genotype influence on gene expression (which could be roughly estimated to be just above 30% for 1-log differences across opposing homozygotes).

In summary, WNT10B is expressed in human bone tissue, but we have not been able to demonstrate a robust association of several common polymorphisms in the WNT10B region with BMD or fractures in postmenopausal women. These results argue against a major influence of common polymorphisms of the WNT10B gene on bone mass and the risk of osteoporosis in Caucasian postmenopausal women.

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References

1. Ralston SH, de Crombrughe B (2006) Genetic regulation of bone mass and susceptibility to osteoporosis. *Genes Dev* 20:2492–2506
2. Lei SF, Jiang H, Deng FY et al (2007) Searching for genes underlying susceptibility to osteoporotic fracture: current progress and future prospect. *Osteoporos Int* 18:1157–1175
3. Little RD, Carulli JP, Del Mastro RG et al (2002) A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *Am J Hum Genet* 70:11–19
4. Glass DA, Karsenty G (2007) In vivo analysis of Wnt signaling in bone. *Endocrinology* 148:2630–2634
5. Krishnan V, Bryant HU, MacDougald OA (2006) Regulation of bone mass by Wnt signaling. *J Clin Invest* 116:1202–1209
6. Wang HY, Liu T, Malbon CC (2006) Structure-function analysis of Frizzleds. *Cell Signal* 18:934–941
7. Kikuchi A, Yamamoto H, Kishida S (2007) Multiplicity of the interactions of Wnt proteins and their receptors. *Cell Signal* 19:659–671
8. Bennett CN, Longo KA, Wright WS et al (2005) Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc Natl Acad Sci USA* 102:3324–3329
9. Zhou H, Mak W, Zheng Y et al (2008) Osteoblasts directly control lineage commitment of mesenchymal progenitor cells through Wnt signaling. *J Biol Chem* 283:1936–1945
10. Robinson JA, Chatterjee-Kishore M, Yaworsky PJ et al (2006) Wnt/beta-catenin signaling is a normal physiological response to mechanical loading in bone. *J Biol Chem* 281:31720–31728
11. Bonewald LF, Johnson ML (2008) Osteocytes, mechanosensing and Wnt signaling. *Bone* 42:606–615
12. Barrett JC, Fry B, Maller J et al (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21:263–265
13. Conde L, Vaquerizas JM, Dopazo H et al (2006) PupaSuite: finding functional single nucleotide polymorphisms for large-scale genotyping purposes. *Nucleic Acids Res* 34:W621–W625
14. Zmuda JM, Yerges LM, Kammerer CM et al (2009) Association analysis of WNT10B with bone mass and structure among individuals of African ancestry. *J Bone Miner Res* 24:437–447
15. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 25:402–408
16. Purcell S, Neale B, Todd-Brown K et al (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81:559–575
17. Neale BM, Ferreira MAR, Medland SE, Posthuma D (2007) *Statistical genetics: gene mapping through linkage and association*. Taylor and Francis, London
18. Gauderman WJ (2002) Sample size requirements for association studies of gene-gene interaction. *Am J Epidemiol* 155:478–484
19. Ugur SA, Tolun A (2008) Homozygous WNT10b mutation and complex inheritance in split-hand/foot malformation. *Hum Mol Genet* 17:2644–2653
20. Nishizuka M, Koyanagi A, Osada S et al (2008) Wnt4 and Wnt5a promote adipocyte differentiation. *FEBS Lett* 582:3201–3205
21. Kang S, Bennett CN, Gerin I et al (2007) Wnt signaling stimulates osteoblastogenesis of mesenchymal precursors by suppressing CCAAT/enhancer-binding protein alpha and peroxisome proliferator-activated receptor gamma. *J Biol Chem* 282:14515–14524
22. Bennett CN, Ouyang H, Ma YL et al (2007) Wnt10b increases postnatal bone formation by enhancing osteoblast differentiation. *J Bone Miner Res* 22:1924–1932
23. Liu J, Zou L, Wang J et al (2009) Hydrostatic pressure promotes Wnt10b and Wnt4 expression dependent and independent on ERK signaling in early-osteoinduced MSCs. *Biochem Biophys Res Commun* 379:505–509