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New insights into the gene function of osteoporosis

Li You[1], Lin Chen[1], Ling Pan[1], Jin-Yu Chen[1]

[1]Department of Endocrinology and Metabolism, Shanghai First People's Hospital, Shanghai Jiao Tong University School of Medicine, 100 Haining Road, Shanghai 200080, China

TABLE OF CONTENTS

1. ABSTRACT

Osteoporosis is a common disease characterized by low bone mass, microarchitectural deterioration of bone tissue and an increased risk of fracture. Population-based and case-control studies have identified polymorphisms in several candidate genes that have been associated with bone mass or osteoporotic fracture, including the vitamin D receptor (VDR), estrogen receptor (ER), oestrogen α receptor (ESR), transforming growth factor (TGF)-β, and type I collagen. The Wnt signaling and receptor activator of nuclear factor κB (RANK)/RANK ligand (RANK-L)/osteoprotegerin (OPG) pathways have been shown to play critical roles in determining bone mass and strength. An important aim of future work will be to further clarify the mechanisms involved in the interaction between candidate genes and environmental variables leading to osteoporosis via signaling pathways in individual patients. Hence preventative therapy, particularly gene therapy, could be targeted in patients at greatest risk of osteoporosis.

2. INTRODUCTION

Osteoporosis has a strong genetic component that is characterized by multiple pathogenetic mechanism-mediated convergence to cause reduction in bone mineral density (BMD), microarchitectural deterioration of skeletal structure and an increased risk of fracture. Indeed, osteoporosis has recently become a major clinical problem. The clinical importance of osteoporosis lies in its association with bone fractures. It has been estimated that osteoporosis results in > 1.3 million osteoporotic fractures per year in the US, and > 40% of postmenopausal women will have a fracture (1, 2).

Two forms of osteoporosis have been widely proposed as follows: osteoporosis related to estrogen deficiency at the menopause; and osteoporosis related to calcium deficiency and aging of the skeleton, particularly in the elderly (3). These factors, coupled with an increased risk of falls, contribute to the high incidence of osteoporotic

fractures, particularly of the hip and wrist. Skeletal fragility can result from the following: (a) failure to produce a skeleton of optimal mass and strength during growth; (b) excessive bone resorption, resulting in decreased bone mass and microarchitectural deterioration of the skeleton; and (c) an inadequate formation response to increased resorption during bone remodeling. Because the resorption and reversal phases of bone remodeling are short and the period required for osteoblastic replacement of the bone is long, any increase in the rate of bone remodeling would result in a loss of bone mass (4). Hence, the disequilibrium of the remodeling with inadequate formation and excessive bone resorption plays an essential role in the pathogenesis of osteoporosis.

Within the last decade the identification of many regulatory mechanisms associated with osteoporosis has resulted from preclinical genetic studies; however, these studies presented conflicting data, partly because of sample size and differences in the genetic background of control and disease subjects. Because osteoporosis is such a complex disorder, specific gene polymorphisms may be clinically important and contribute to a further understanding of the mechanisms involved in the disease.

3. CANDIDATE GENES

Twin and family studies have reported that the differences in skeletal traits between individuals of the same age are largely attributable to differences in genetic factors and not differences in environmental exposures (5-8). In keeping with this finding, population-based studies have demonstrated that a family history of fracture is a significant risk factor for fracture by mechanisms that are partly independent of bone density (9, 10). Therefore, it is clear that genetic factors are extremely important in the pathogenesis of osteoporosis, and it is likely that the effect is mediated by a combination of several candidate genes rather than a single gene. Candidate gene association studies in osteoporosis have logically tested the main regulators of bone metabolism, and several classic candidate genes, such as vitamin D receptor (VDR), estrogen receptor (ER), oestrogen α receptor (ESR), transforming growth factor (TGF)-β, and type I collagen, have been most widely studied (11). These factors act through the imbalance in bone remodeling by inhibiting osteoblast activation and/or increasing osteoclast function, leading to osteoporosis.

3.1. VDR

VDR was the first candidate gene to be studied in osteoporosis. VDR was chosen because it acts as an important regulator of calcium metabolism and bone cell function by binding to 1, 25-dihydroxyvitamin D3 [1, 25-(OH)2D3], the biologically active metabolite of vitamin D (12). A large number of studies have since been carried out regarding the relationships between the VDR genotype and bone mineral density (BMD). Studies by several investigators have essentially supported the positive effect of VDR on BMD, although others found no significant association between VDR alleles and BMD (13-15). Indeed, there is evidence suggesting that the relationship between VDR alleles and BMD might be modified by high calcium intake, although the studies performed have been small and need to be repeated in larger populations (16, 17). Frequently studied markers of VDR in relation to osteoporosis include BsmI, TaqI, Cdx2, and FokI. Ivanova *et al.* (18) examined the effect of FokI and BsmI polymorphisms of VDR, and found that the stature of BMD is closely related to the VDR genotype at the forearm and lumbar spine. In another study conducted by Casado-Diaz *et al.* (19), the Cdx2 polymorphism was protective on BMD in a cross-sectional study of 229 osteoporotic postmenopausal women. *In vitro* evidence has also suggested that the VDR Cdx-2 polymorphism is functional, although this has not been extensively investigated in clinical studies (20). However, conflicting studies revealed that the association was not found between VDR alleles, such as BsmI, FokI, ApaI, and TaqI, and calcium absorption, fracture, or BMD (21-23). In summary, the studies that have been performed to date have indicated that allelic variation at the VDR gene locus plays some role in the genetic regulation of BMD, although the molecular mechanisms responsible for these remain unclear.

3.2. ER

Estrogens play an important role in regulating bone homeostasis, bone turnover, and maintenance of bone mass. The effects of estrogens on skeletal structure are mediated via binding to two different ERs, which are encoded by the ER-α and -β genes. Both ERs are highly expressed in bone (24). The concept that estrogen deficiency is critical to the pathogenesis of osteoporosis was based initially on the fact that postmenopausal women, whose estrogen levels decline naturally, are at the highest risk for developing the disease (4). ER deficiency continues to play a role in bone loss in women in the 8th and 9th decades of life, as evidenced by the fact that estrogen treatment rapidly reduces bone breakdown in older women (25). Single nucleotide polymorphisms (SNPs) of ER-α are associated with a significant reduction of fracture risk in men and women, as well as BMD (26-29). Other studies have suggested that SNPs of ER-α can affect BMD and rates of bone loss as well as fracture risk.

3.3. ESR

ESR, by interacting with its ligand oestrogen, acts as a strong candidate gene for osteoporosis because of the relationship between oestrogen deficiency and bone loss, as well as the positive effects of oestrogen in regulating skeletal growth and maintenance of bone mass. By interfering with the ESR locus, osteoporosis is observed with reduced BMD and increased risk of fracture (30-33), prompting further research on the relationship between polymorphisms at the ESR locus and bone mass. Sano *et al.* (34) reported a positive association between a TA repeat polymorphism in the ESR promoter and bone mass in some Japanese women and similar results were also observed in an American population (35). Other investigators have reported positive associations between haplotypes defined by PvuII and/or XbaI polymorphisms in intron 1 of the ESR gene and bone mass as well as age in menopausal women (36-38). These polymorphisms are in strong linkage disequilibrium with each other; however, the molecular

mechanism by which these polymorphisms influence bone mass are unclear. Functional studies will be required to address this issue and to define the molecular mechanisms responsible for the association with BMD.

3.4. TGF-β

Several polymorphisms of the TGF-β gene have been identified, and many of these polymorphisms are associated with BMD and/or osteoporotic fractures (39, 40). The best functional candidate is a C/T polymorphism, which causes a proline-leucine substitution at amino acid 10 in the TGF-β coding region. The C allele is associated with increased BMD and a reduced frequency of osteoporotic fractures in two Japanese populations (41). Further, the C allele is associated with circulating levels of TGF-β, suggesting that the C allele may influence protein secretion or stability, but the underlying mechanisms have not been investigated at the molecular level. Loots *et al.* (42) reported that TGF-β controls SOST transcription in mature osteoblasts, suggesting that sclerostin may mediate the inhibitory effect of TGF-β upon osteoblast differentiation. Of note, negative effects have also been reported by Hubacek *et al.* (43), demonstrating that variants in genes for TGF-β are not associated with low BMD in postmenopausal Czech Caucasian females.

3.5 Type i collagen

Type I collagen is the major structural protein of bone. The genes encoding type I collagen (COLIA1 and COLIA2) are candidates for the genetic regulation of bone mass. A G/T polymorphism, which affects a Sp1 binding site, has been identified in the first intron of the COLIA1 gene (44). Positive associations between the COLIA1 Sp1 polymorphism and femoral neck geometry, reduced bone mass, or osteoporotic fractures have subsequently been reported (45-48). Functional studies have shown that the polymorphism increases the affinity of Sp1 binding to DNA, and is associated with increased allele-specific transcription (49). Other potentially functional polymorphisms associated with BMD have recently been identified in the COLIA1 promoter (50); however, several studies have also demonstrated there is no significant association between the COLIA1 Sp1 polymorphism and bone mass or fractures (51, 52). Taken together, the mechanism by which the Sp1 polymorphism predisposes to osteoporosis has yet to be fully defined.

4. SIGNALING PATHWAY

The current emphasis on the development of new therapies for osteoporosis is directed at modifying the effects of relative signaling on osteoblast differentiation and bone formation. Local signaling that results in bone formation during remodeling takes place in several ways.

4.1. Wnt signaling pathway

One pathway that has been identified by human genetic studies as a major determinant of bone mass and strength by controlling osteoblast differentiation, is the Wnt signaling pathway, which is crucial for the regulation of cell growth, differentiation, and apoptosis (53-55). Together with one of the Wnt ligand receptors frizzled, LDL receptor–related protein 5 (LRP5) serves as a cell-membrane co-receptor for Wnt proteins consisting of Axin, adenomatous polyposis Coli (APC), and glycogen synthase kinase (GSK) 3, and plays an essential role in transducing intracellular Wnt signals (56-60). In the inactive state of Wnt signaling, Wnt is inhibited by several secreted frizzled-related proteins (sFRPs) and GSK3 phosphorylates β-catenin, which is then degraded via the ubiquitin/proteosome pathway. In the active state of Wnt signaling, the protein complex is disrupted and phosphorylation does not occur (Figure 1). After a series of biochemical events within the cell, Wnt signaling leads to stabilization of β-catenin, the accumulation of which promotes transcriptional events that increase osteoblast number and activity, and ultimately bone formation (61).

The human disease, sclerosteosis, is associated with a homozygous mutation in the SOST gene, which encodes sclerostin and results in moderate increases in bone mass and fewer skeletal complications (62, 63). By inactivating the mutation in the LRP5 gene, impaired bone mass and severe osteoporosis occurs (56). In contrast, a greatly increased bone mass of normal shape and architecture is associated with an activating mutation of the same gene (58). LRP5 can be inhibited by the Wnt antagonists, Dickkopf (Dkk)-1 or Dkk-2. *In vitro* studies have shown that inhibition of Wnt signaling by Dkk-1 is defective in the presence of an activating mutation of LRP5, providing a molecular explanation for the increased activity of the Wnt signaling pathway (64). Confirming the importance of this pathway, over-expression of Dkk-1 in osteoblasts of mutant mice reduced bone formation and leads to significant osteopenia (65). The connection between Wnt signaling and bone formation was apparent, according to a recent study demonstrating that phosphorylation steps and a combination of chromatin modifications result in repression of PPAR-γ, with prevention of adipogenesis and enhanced osteoblast differentiation (66). *In vitro* studies also showed the direct effects of Wnt signaling on osteoblast differentiation and co-operative effects with bone morphogenetic proteins (67-69) ; however, the control mechanisms for this pathway have not been delineated. The very complexity, the number of participants, and the likely involvement of this pathway in cell proliferation control and in neoplastic processes in some tissues, provide major challenges, which will be discussed further.

4.2. Cell proliferation

TDSCs, similar to other MSCs, proliferate faster than terminally differentiated cells *in vitro*. Human and mouse TDSCs proliferate faster than bMSCs isolated from the same source (5). TDSCs isolated form different tendons in the same individual also have different proliferative potential (26).

The proliferation of TDSCs is also affected by age. Aged tendon tissues have fewer TDSCs and aged TDSCs have a longer population doubling time (PDT; 17). Analysis of the cell cycle phase distribution has shown that aged TDSCs contain an arrest in G2⁄M which could result from accumulated genetic and/or epigenetic damage. Cited2 is a transcription factor implicated in the control of

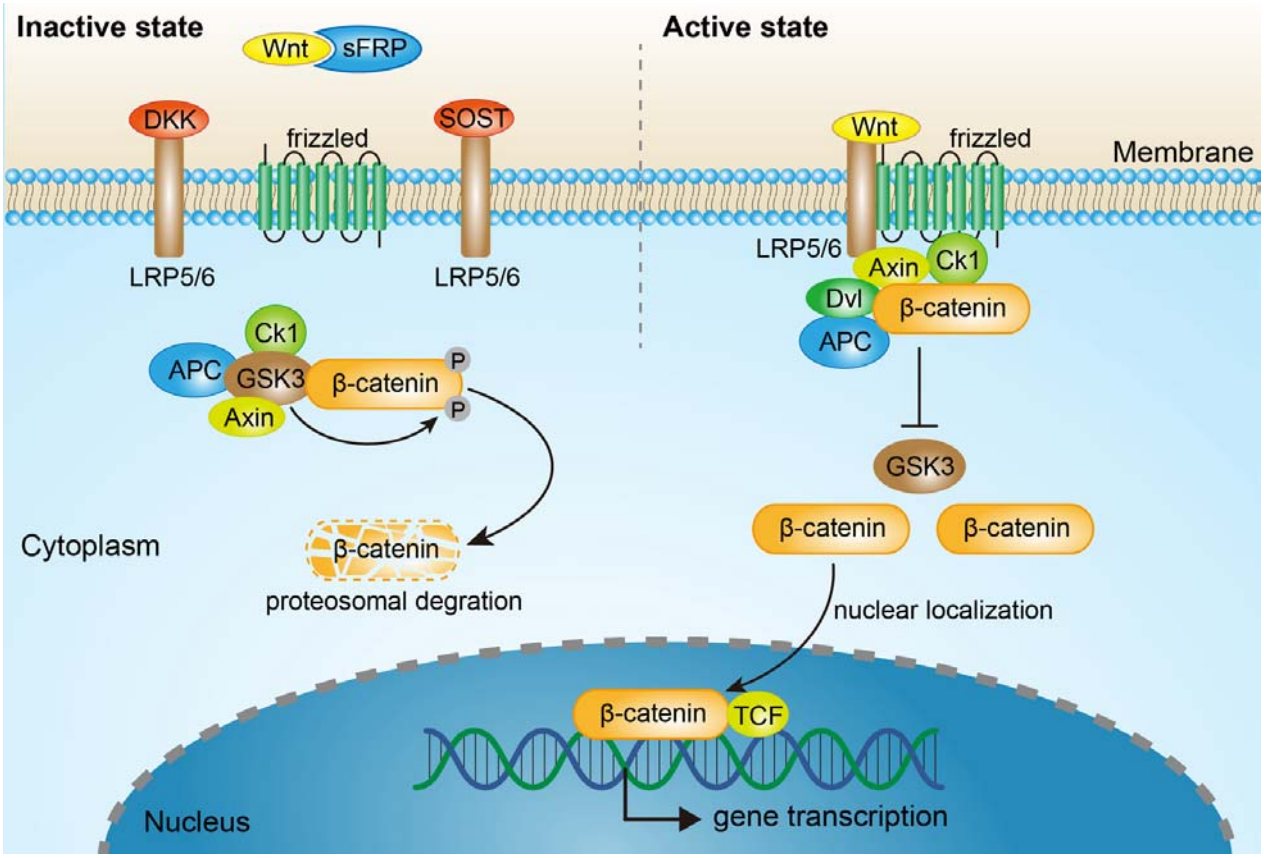

Figure 1. Wnt signaling pathway. A. In the inactive state of the Wnt signaling pathway, Wnt is inhibited by a decoy sFRP. LRP5/6 is bound by sclerostin encoded by SOST or Dkk. GSK-3 results in proteosomal degradation of β-catenin. B. In the inactive state of the Wnt signaling pathway, LRP5/6 is engaged in the receptor complex after Wnt binding, resulting in disruption of the GSK-3 inhibitory complex, stabilization of β-catenin and translocation to the nucleus where LRP5/6 activates transcription through TCF. Ck1, casein kinase 1; Dvl, disheveled; TCF, T-cell factor

growth and senescence in several cell types (27-30). Previous studies have suggested that Cited2 expression in aged TDSCs is reduced, consistent with positive roles for Cited2 in TDSC self-renewal (17). Additionally, differences in apoptotic rates between young and old TDSCs could also contribute to the observed disparities in population size (17).

4.3. Receptor activator of the nuclear factor KB (RANK)/rank ligand (RANK-L)/osteoprotegerin (opg) pathway

The RANK/RANK-L/OPG signaling pathway has been shown to mediate the production and activity of cells in the osteoclast lineage (70). RANK is a primary mediator of osteoclast differentiation, activation, and survival. By interacting with RANK, RANK-L is responsible for osteoclast-mediated bone resorption in a broad range of conditions. The soluble protein, OPG, the key endogenous regulator released by osteoblasts into the microenvironment, can block the interaction between RANK-L and RANK as a decoy receptor (71).

There is positive evidence that blocking RANK-L with a monoclonal antibody leads to prolonged inhibition of bone resorption in postmenopausal women (72, 73). It has also been shown that the level of RANK-L is increased on the surface of bone marrow cells from women in early postmenopause who are estrogen deficient (74). OPG-deficient transgenic mice develop early onset osteoporosis with a high incidence of fractures (75). Polymorphisms in the OPG gene have been associated with osteoporotic fractures and differences in BMD (76). As OPG levels are not consistently altered, however, it has been difficult to clarify the role of OPG deficiency in the pathogenesis of osteoporosis.

5. GENE THERAPY

Although bone is one of the few organs in the body that can spontaneously heal and restore function without scarring, it has been recognized that bone healing is not always satisfactory, with failure rates of up to 50% when the treatment of fractures associated with osteoporosis is performed (77). Until recently, prevention and treatment of bone loss was dependent entirely on the use of drugs that inhibit bone resorption, a mechanism that does not restore bone structure or bone that has already been lost (77). Osteoinductive cytokines comprise the bulk

of known osteoinductive molecules as a class of relatively small secreted molecules that are capable of promoting the formation of bone; however, a single exposure to an exogenous growth factor may not induce an adequate osteogenic signal in many clinical situations. Delivery of exogenous growth factors need to be sustained because these factors have exceedingly short biological half-lives, usually on the order of minutes or hours rather than the days or weeks needed to stimulate a complete osteogenic response. Moreover, delivery of exogenous growth factors also requires avoidance of local ectopic ossification and other undesirable side effects. Therefore, gene therapy may be one of the tools available to provide more sustained protein release when necessary, and to deliver protein in a more physiologic manner than recombinant proteins (78, 79). Both *in vivo* and *ex vivo* gene therapy strategies have been investigated for bone regeneration (80, 81).

5.1. *In vivo* gene delivery strategy

In vivo gene delivery involves direct delivery of the gene into a specific anatomic site locally or systemically by transducing local cells. The advantages of this strategy are that *in vivo* gene delivery is a relatively simple technique with lower costs, which favor clinical use. The disadvantages of *in vivo* gene delivery are the difficulties in targeting specific cells for transduction or achieving high transduction efficiency. Two commonly used *in vivo* gene delivery strategies involve gene activated matrix (GAM) technologies and the direct injection of a viral vector. GAM, incorporated with plasmid DNA, when surgically implanted directly into segmental gaps of adult rat femurs, promote the formation of new bone (82, 83). Although human clinical trials using GAM technology have not been conducted, these data hold great promise for patients with osteoporosis.

Adenovirus vector is a powerful tool for gene therapy and invades cells by binding to the coxsackievirus and adenovirus receptor (CAR), which has been induced in regenerating tissues, particularly in immature osteoblasts, suggesting a reasonable option for enhancing fracture healing (84). When using an adenoviral vector, there is concern about the development of an immune response to the viral particles injected directly into the anatomic site and to the infected cells, which can inhibit transgene expression. Ectopic bone formation has been induced by injecting a first-generation adenoviral vector containing the cDNA encoding bone morphogenetic protein (BMP)-2 into the thigh muscle of nude mice and rats; however, bone formation was inhibited in immunocompetent rats, with signs of inflammation observed in these animals (85, 86). Nevertheless, the extent of the immune response against viral vectors was modified by the route of administration (87, 88). Baltzer *et al.* (89) showed that intraosseous administration is able to heal critical-size defects in the femurs of immunocompetent rabbits by the direct, intralesional injection of adenovirus carrying BMP-2 cDNA. The immune response to adenovirus may be further blunted by delivering the virus in conjunction with a collagenous matrix in a modified matrix strategy. Both Franceschi *et al.* (90) and Sonobe *et al.* (91) have successfully used this tactic to form bone intramuscularly and subdermally in immunocompetent rodents.

As an alternative to adenovirus, lentiviruses are a specialized class of retrovirus, which are capable of infecting nondividing cells. Several studies demonstrated that lentiviral vectors expressing BMP-2 from a murine leukemia virus long terminal repeat promoter led to significantly greater BMP-2 production *in vitro* and ectopic bone formation *in vivo* compared that from cytomegalovirus promoter (92-94).

5.2. *Ex vivo* gene delivery strategy

Ex vivo strategies involve the harvesting of a specific population of cells from the patient, followed by genetic modification of these cells under *in vitro* conditions and subsequent implantation into the site of injury. Most investigators are prone to use *ex vivo* approaches, which may be safer in a clinical setting than direct injection of viral particles *in vivo*.

Ex vivo approaches based on different cell-types, gene delivery vectors, and target genes have been extensively explored for bone repair applications. One strategy that has shown particular promise is the implantation of bone marrow stromal cells (BMSCs) genetically engineered to over-express BMP-2 into critical-sized defects (95-98). In an independent study, Tsuchida *et al.* (99) investigated the bone healing capacity of allogeneic BMSCs infected with a BMP-2 adenoviral vector in a rat femoral segmental defect. Two studies based on constitutive retroviral overexpression of BMP-4 demonstrated that intramedullary injection of BMSCs expressing BMP-4 healed critical-sized calvarial defects or increased trabecular bone mineral density at endosteal bone sites with a normal histologic appearance in animal models (98, 100). Collectively, these results suggested that *ex vivo* genetic manipulation of BMSCs may provide an effective strategy for bone formation than the direct implantation of these cells alone. Other investigators also confirmed the success of the *ex vivo* approach using cells transduced, in addition to BMP-2 and BMP-4, other osteogenic genes (BMP-7) (90, 101).

6. CONCLUSIONS

Osteoporosis is a complex disease that is thought to be mediated by an interaction between environmental factors and different genes that individually have modest effects on BMD and other aspects of fracture risk. Genetic factors are extremely important in regulating BMD and other determinants of osteoporotic fracture risk. From a clinical standpoint, advances in our knowledge about the candidate genes of osteoporosis and signaling pathways are critical because they offer the prospect of developing genetic markers for assessment of the fracture risk and identifying molecular targets for the design of new therapies that can be used to prevent or treat osteoporosis. The applications of gene therapy provide a good likelihood of early clinical success.

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Abbreviations: VDR: vitamin D receptor; ER: estrogen receptor; ESR: oestrogen α receptor; TGF: transforming growth factor; BMD: bone mineral density; SNPs: single nucleotide polymorphisms; APC: adenomatous polyposis Coli; GSK: glycogen synthase kinase; sFRPs: secreted frizzled-related proteins; Dkk: Dickkopf; LDL receptor–related protein 5 (LRP5); RANK: receptor activator of nuclear factor κB; RANK-L: RANK ligand; OPG: osteoprotegerin; BMP: morphogenetic protein; GAM: gene activated matrix; CAR: coxsackievirus and adenovirus receptor; BMSCs: bone marrow stromal cells; Ck1: casein kinase 1; Dvl: disheveled; TCF: T-cell factor

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Send correspondence to: Li You, Department of Endocrinology and Metabolism, Shanghai First People's Hospital, Shanghai Jiao Tong University School of Medicine, 100 Haining Road, Shanghai 200080, China, Tel: 86-21-63240090, Fax: 86-21-63240090, E-mail: youlisky2002@yeah.net