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Searching for the Molecular Pathways Regulating Bone Mineral Density in the Proteome and RNA Interference Era

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Abstract

Osteoporosis is a polygenic disorder associated with low bone mineral density and deterioration of bone microarchitecture with increased chance of bone fractures. Although bone matrix mineralization and osteoporosis are closely related, the mineralization of bone matrix is almost a forgotten aspect in osteoporosis research. The complex processes of bone matrix mineralization and bone remodeling are tightly regulated by several transcription factors and signal transduction pathways. However, signal transduction pathways occurring at a protein level that depends not only on mRNA transcriptional regulation but also on a multitude of translational and posttranslational controls. Furthermore, proteomics allow a discerning view of complex molecular pathways, provides an efficient method to determine protein candidates, and elucidates signal transduction pathways that regulate bone mineral density and accelerates the discovery of osteoporosis causative genes. RNA interference is a powerful tool for rapid analysis of gene functions. Therefore, strategies to combine proteomics with RNA interference and transgenic RNAi would greatly improve the efficiency of gene discovery and divulge the molecular pathways involved in osteoporosis pathophysiology. In this review, current methods employed to identify genes involved in osteoporosis, which include linkage analysis, candidate gene association studies, genome wide association studies, transcriptome microarray, and proteomics are evaluated, and a new strategy is proposed.

Abbreviations

Dlx5: Distal-less Homeobox 5; ATF4: Activating Transcription Factor 4; SATB2: Special AT-Rich Sequence-Binding Protein 2; Twist1: a basic helix-lop-helix transcription factor; MITF: Microphthalmia-Associated Transcription Factor

Introduction

Completion of the human genome project more than a decade ago holds great promise for scientific research to excavate the genetic foundation of complex biological processes such as bone matrix mineralization. However, bone matrix mineralization is nearly a forgotten dimension in osteoporosis research [1]. Osteoporosis is a polygenic disorder determined by multiple genes and environmental risk factors, each with modest effects on bone mass and susceptibility to fracture. It is a bone metabolic disorder associated with low Bone Mineral Density (BMD) and deterioration of bone microarchitecture [2,3] with increased chances of bone fracture. BMD changes with age, having a rapid increase during the childhood to reach a peak level by the mid or late twenties in life and declining thereafter in women and elderly men, which is due to unbalanced bone remodeling. Bone matrix mineralization is an important determinant of the stiffness and hardness of the bone material [4,5]. Moreover, it has become evident in recent years that bone mineral and matrix tissue properties play a pivotal role in the overall biomechanical competence of bone [6,7]. The process of bone matrix mineralization is tightly regulated both temporally and spatially [8]. Some factors, such as mineral-binding-extracellular matrix proteins and proteoglycans, mineralization-inhibiting proteins and matrix-vesicles [9,10], are known but still very little is known about the molecular control of bone matrix mineralization. An enhanced understanding of the regulatory mechanisms underlying bone matrix mineralization may improve our understanding of the molecular basis of osteoporosis pathophysiology.

Osteoporosis causes more than 1.5 million fractures annually, with estimated expenditures of $14 billion each year. Moreover, due to the rapid growth of the United States aging population, burden of fractures and and the related costs are expected to double by 2025 [11]. Bone strength is the ultimate measurement of resistance to bone fractures, and it is mainly determined by bone mineral density, bone volume, and bone microarchitecture [12]. However, bone mineral density is a complex phenotype because it is the outcome of the balance between bone resorption and formation during bone remodeling. Bone matrix mineralization is an important determinant of the physical properties of bone. To meet these needs, osteoblasts, cells that make bone, undergo proliferation and maturation required for the proper mineralization of the Extracellular Matrix (ECM). Bone remodeling involves a regulated bone resorption and formation by osteoclasts and osteoblasts [13], which are coordinated by RANK-RANKL-OPG pathway. Unbalanced bone remodeling is the primary factor in determining bone strength and weakness and leads to bone metabolic disorders such as osteopetrosis and osteoporosis [14]. Surprisingly little is yet known about the molecular control of osteoblast matrix mineralization and bone remodeling. Understanding the molecular mechanisms regulating these processes may provide the means to manipulate them for the therapeutic benefit of individuals with osteoporosis where osteoblast matrix

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mineralization is impaired [15].

Mesenchymal stem cells differentiate through specific signal transduction pathways into osteoblasts, chondrocytes and adipocytes [16-18]. Also, bone marrow-derived mesodermal progenitor cells differentiate into osteoblasts, chondrocytes, adipocytes, myocytes, and endothelial cells [19]. Osteoblasts are the bone forming cells that are responsible for synthesis, deposition, and mineralization of the extracellular matrix of bone. Bone forms through endochondral ossification that involves a cartilage anlagen and intramembranous ossification that forms directly from mesenchymal cells condensations. The process of bone formation is highly regulated and involves the differentiation of mesenchymal stem cells into osteoblasts under the control of Core binding factor α-1 (Cbfa1) and Osterix (Osx) transcription factors.

Osteoblast commitment and differentiation are regulated by several transcription factors and complex signal transduction pathways that elicit a cascade of gene expression. Osteoblast differentiation is essential for bone formation and defects in this process result in weak bone with an increased chance for fractures [20]. Osteoblast matrix mineralization is an important determinant of the stiffness and hardness of bone tissue [4]. Transcriptional control of osteoblast growth and differentiation is also tightly regulated both temporally and spatially [8]. Understanding the molecular events leading to bone matrix mineralization is clinically relevant to bone metabolic disorders, tissue engineering and gene therapy. While gene therapy applications in bone regeneration are in early stages, pioneer studies by our group have established that genetically modified muscle and fat grafts are capable of repairing defects in bone and cartilage [21-26].

Understanding the molecular mechanisms that control bone matrix mineralization is essential for gaining knowledge about the pathogenesis of osteoporosis and may provide the means to develop anabolic therapies for bone metabolic disorders. In this review, current methods to identify genes influencing osteoporosis are evaluated, their advantages and limitations are summarized in Table 1, and a new strategy is proposed.

Methods for Identifying Osteoporosis Genes

In the proteome era, diverse methods have been currently employed to identify genes involved in complex genetic disorders, which include linkage analysis, candidate gene association studies, genome wide association studies, transcriptome microarray, and proteomics.

A. Linkage analysis: Genetic linkage analysis is a powerful tool to spot the chromosomal location of a locus responsible for a monogenic disorder by identifying other markers that co-segregate through families with the risk of the disorder. Successively, molecular methods are employed to identify the particular gene linked to the genetic disorder.

Most linkage analysis studies have focused on BMD as the phenotype of osteoporosis. It is true that the use of linkage analysis for monogenic disorder mapping was very successful, which was due to the development of high-throughput genotyping technology [27]. Two positional candidate genes have been identified through linkage analysis: Low density lipoprotein receptor-related protein 5 (LRP5) and bone morphogenetic protein 2 (BMP2) [28,29]. Linkage analysis [30-32] has been successful in identifying numerous quantitative trait loci involved in BMD regulation, but these findings have not been replicated between linkage studies and most of the genetic variables resulting from linkage signals remain to be identified. However, until now, identification of genes influencing osteoporosis by linkage analysis proved difficult with few successes [33], and positional cloning of genes underlying the associated trait loci appears intricate. Furthermore, meta-analysis of 11,842 subjects from nine linkage studies found no loci were associated with BMD [34]. Therefore, linkage analysis has ultimately failed to identify the causative genes of complex genetic disorders such as osteoporosis [3], which illustrates the need for new strategies [35]. Several genes that regulate vulnerability to osteoporosis have been discovered through studies of rare bone disorders. Although extensive progress over the past eighteen years has been accomplished in identifying the genes and loci that may regulate BMD, most of the genetic variables that regulate susceptibility to osteoporosis remain to be discovered [3]. Furthermore, the correlation between genomic DNA sequences and protein levels are insignificant, which is due to transcriptional regulation, alternate splicing and posttranslational modifications [36]. Therefore, it could be inferred that linkage analysis is an inadequate method to study polygenic disorders efficiently and cost effectively, primarily because most contributions from individual genetic variants are negligible alone and it is the combined effects which result in bone changes [27]. Linkage analysis lacks the power to identify such small effects. The conclusive failure of linkage studies to identify causative genetic factors in polygenic disorders has led scientists to focus on candidate gene association studies.

Table 1: Evaluation of methods for identifying genes associated with osteoporosis.

Method	Advantages	Limitations
Linkage analysis	Suitable for identifying gene responsible for monogenic disorders	lacks the sensitivity to identify genes underlying polygenic disorders.
Candidate genes association studies	Several candidate genes in a signaling pathway may be studies simultaneously.	Inconsistent, spurious, and insignificant replication of the association study results.
Genome wide association studies	Suitable to investigate genetic architecture of polygenic disorders arising from nucleotide polymorphisms. Offers the possibility to identify novel susceptibility genes and pathways.	It does not identify individual causal genes, nor does it provide functional information required for discovering a therapy, and the occurrence of false negatives is highly significant.
Gene microarray	Offers insights into the global patterns of gene expression, and provides a panoramic analysis of gene expression alterations.	Signal transduction occurs at protein level, and the correlation between mRNA and protein abundance in the cell is extremely poor.
Proteomics	An efficient method to determine protein candidates, and elucidates signal transduction pathways that regulate bone mineral density and accelerates the discovery of osteoporosis causative genes.	Still under development and show certain limitations, which include the capabilities to identify challenging protein groups such as low-abundance, hydrophobic and basic proteins.

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B. Candidate genes association studies (CGAS): The candidate gene methodology is based on conducting genetic association studies to test whether one of the candidate gene alleles is more observed in individuals affected with the genetic disorder than the general population. The candidate genes are regularly selected for study based on previous knowledge about the impact of their functions on the disorder under investigation. Candidate genes are divided into three groups: functional, expressional and positional candidate genes.

A large candidate gene association study has found that there are neither associations nor linkage between Vitamin D receptor (VDR) polymorphisms and bone mass in their twin populations [37,38]. Like the situation of VDR studies, subsequent studies have also yielded contradictory results for collagen IαI (COLIAI), estrogen receptors, calcitonin, interlukin-1, interlukin-6, HS-glycoprotein, transforming growth factor beta 1 (TGF-β1), and apoliprotein E [39]. However, recent association studies have revealed that VDR, COLIAI, TGF-β1 among others gene's polymorphisms are linked to bone mineral density [31,40]. Also, meta-analyses reveal inconclusive results regarding VDR association with bone mineral density [41]. In large-scale genome wide meta-analysis from a study which involved 19,195 individuals, 9 of 150 candidate genes were associated with BMD regulation, but, nearly all the candidate genes had an inconsistent association with BMD [42]. A major advantage of this approach is that several candidate genes in a signaling pathway may be studied simultaneously [43,44]. The primary limitation of this approach is that many critical biological candidate genes regulating bone metabolism may still go undiscovered [45]. Also, the inconsistent, spurious, and insignificant replication of the candidate gene association studies results [42,45,46] illustrate the limitations of the method and the need for alternative approaches. One such alternative approach is genome wide association studies.

C. Genome wide association studies (GWAS): So far, linkage analysis and candidate gene studies have not substantially contributed to elucidating the genetic factors contributing to osteoporosis pathology. GWAS examine genetic variations (markers) through the whole genome of many individuals to identify genetic markers associated with a specific disorder. This methodology is predominantly useful in identifying genetic variations that contribute to polygenic disorders.

Advances in Single Nucleotide Polymorphisms (SNPs) genotyping technologies have made it possible to perform GWAS by analyzing several hundred of thousands of SNPs distributed closely across the whole genome, instead of focusing on CGAS [47]. GWAS is a powerful tool to investigate the genetic architecture of polygenic disorders arising from nucleotide polymorphisms and have been successful in identifying osteoporosis predisposition genes [29,48,49]. GWASs have recently identified nearly 100 independent associations for osteoporosis susceptibility traits [33]. GWAS has a major advantage over the CGAS by offering the possibility to identify novel susceptibility genes and pathways. However, the disadvantages of GWAS include; the current marker sets cannot exclude the possibility that rare polymorphisms within the gene of interest may influence osteoporosis [47], it does not identify individual causal genes, nor does it provide the functional information required to discover new therapies, the occurrence of false negatives is highly significant [42], and positional cloning of genes underlying the associated trait loci appears very challenging.

D. Transcriptome analysis: Unlike the genome, which is approximately fixed for a given cell, the transcriptome includes all mRNA transcripts of genes that are being actively expressed in a cell at any given time that may vary according to internal and external stimuli. There are several techniques for transcriptome analysis, which include gene microarray, Serial Analysis of Gene Expression (SAGE), RNA sequencing (RNA Seq), mRNA subtractive hybridization, mRNA differential display, PCR array, Chip assay, polyribosome analysis and polyribosome analysis..

Cbfa1 and Osx have been identified as master regulators of osteoblast differentiation and absence of either one, leads to complete lack of bone matrix mineralization [50-52]. Several other transcription factors are involved in osteoblast regulation including Hedgehog, Dlx5, Twist1, ATF4, SATB2 and Shn3 [53-58]. Also, other factors [59,60], and signaling pathways [61] are essential for osteoblast differentiation. These regulators interact with each other as networks to trigger diverse signals and orchestrate the transcription of genes crucial to define osteoblastic lineage and differentiation [62-64].

Gene microarray has offered insight into the global patterns of gene expression during osteoblast matrix mineralization *in vitro*, as well as osteoblast and osteoclast regulation, which is essential to comprehend the pathogenesis of bone metabolic disorders [65]. Furthermore, it provides a panoramic analysis of gene alterations underlying the complex process of bone formation [66]. Therefore, several groups have used microarray to unravel the molecular pathways that regulate osteoblast differentiation and bone formation using a number of cellular models [19,67-83]. However, it has been reported that the correlation between mRNA and protein abundance in the cell is extremely poor [12,84,85], which is due to transcriptional and translational controls, posttranslational modifications and protein decay. Furthermore, several alterations may occur in proteins without reflecting the changes at the mRNA levels [86].

E. Proteomics: The proteome represents the whole set of proteins that are expressed in a given cell under certain conditions. While, proteomics is defined as the qualitative and quantitative analysis of proteomes under diverse conditions to unravel molecular pathways of polygenic disorders.

Since genes influence disease through the proteins they encode, proteomics is a powerful tool to discover candidate genes that underlie a genetic disorder [36]. Thus, the expression levels of all proteins provide the most relevant data set characterizing a biological system [87]. Applying proteomics to investigate bone disorders offers the prospects that proteomics technologies will overcome the limitations of the current approaches [88]. While calls for proteomic profiling of human disorders have been made a decade ago [36,89], bone proteomics is still in early stages.

Proteomic profiling of diseased tissues by protein microarray is an emerging technology, and successful application of antibody microarray to analyze protein expression of the squamous cell carcinomas of the oral cavity has been reported [90]. Mass spectrometric profiling of proteins present in the extracellular matrix of rat bone revealed the presence of 108 and 25 proteins in the metaphysis and diaphysis, respectively. Twenty-one of them were bone specific and appeared in both samples including: osteopontin, bone sialoprotein, osteocalcin, osteoregulin, and type I collagen [91].

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Two dimensional gel electrophoresis and mass spectrometric analysis revealed 52 proteins responsible for the differentiation of mesenchymal stem cells into osteoblasts [92]. These proteins were separated into several groups including metabolism, transcription, protein folding, calcium-binding proteins, protein degradation and signal transduction. Proteomic analysis of osteonecrotic femoral head bone tissues revealed 141 upregulated and 56 downregulated proteins compared to the controls [93].

We have successfully used proteomic differential display and mass spectrometric analysis and identified several differentially expressed proteins (Table 2) in mineralizing osteoblast [94]. One of these proteins, transketolase, was among the proteins responsible for the differentiation of mesenchymal stem cells into osteoblasts [92]. Additionally, vimentin, calreticulin and lamin a/c have been noted for biological functions in osteoblast differentiation [95-97], which further confirm their roles in osteogenesis.

New Strategy

Proteomics is a promising approach to increase understanding about the molecular pathways which underlie the complex process of bone matrix mineralization, and offers the prospective to overcome the limitations of the genome and transcriptome based approaches. Moreover, proteomics provides an efficient way to elucidate the signal transduction pathways regulating bone mineral density. The WNT pathway is a key regulator of skeletogenesis and osteoblast differentiation. Meanwhile, Receptor Activator of NF-Kappab (RANK) pathway regulates osteoclasts and bone homeostasis. Both WNT and RANK ligand signalling pathways pathways are linked to osteoporosis [98-100].

RNA interference (RNAi) is a powerful tool for rapid analysis of gene functions [101]. RNAi is a process of sequence specific posttranscriptional gene silencing that initiates by a duplex of small interfering RNA (siRNA), which is homologous to the target gene sequence [102]. One of the siRNA strands binds a nuclease complex to form an RNA-Induced Silencing Complex (RISC). The latter recognizes the gene transcript by base pairing and cleaves it [103] which makes the cleaved transcript unavailable for translation to protein. Strategies to emerge proteomics with RNAi and transgenic RNAi would greatly improve the efficiency of gene discovery and elucidate gene functions and signal transduction pathways involved in osteoporosis pathophysiology.

Table 2: Main biological functions and chromosomal locations of osteoporosis candidate genes identified through the proteomics of mineralizing osteoblasts [94].

Candidate genes	Main Biological functions	Chromosomal locations
High density lipoprotein	RNA binding protein	*2q37.3*
Nucleobindin 1	Calcium homeostasis	*19q13.33*
Phosphoglycerate kinase 1	Tooth germ development	*Xq21.1*
Protein disulfide isomerase A3	Calcium binding	*15q15.3*
Transketolase	Pentose phosphate pathway	*3p21.1*
Prolyl 4-hydroxylase α1	Collagen biosynthesis	*3p21.31*
Prolyl 4-hydroxylase α2	collagen stabilization	*5q31.1*
Pyruvate kinase muscle	Glucose metabolism	*15q23*
Vimentin	Osteoblast differentiation	*10p13*
Calreticulin	Calcium signaling	*19p13.2*
Lamin A/C	Bone formation	*1q22*
Lysyl-tRNA synthestase	MITF transcription activities	*16q23.1*
Coronin 1B	Actin binding protein	*11q13.2*
Phosphoenolpyruvate CK2	Glyceroneogenesis	*14q11.2*

Conclusions

Genetic factors play important roles in the development of osteoporosis, but the genes and mutations conferring osteoporotic risk remain largely unknown. Linkage analysis, first developed to map gene alterations causing monogenic bone disorders like osteogenesis imperfecta, appear to lack the sensitivity to define genes underlying polygenic bone disorders. Until now, few causative genes have been discovered and most of the genetic variables leading to osteoporosis remain to be identified, which is mainly because linkage analysis lacks the sensitivity to map genes responsible for polygenic disorders. The conclusive failure of linkage analysis to identify causative genetic factors in polygenic disorders has led scientists to focus on candidate gene association studies.

The advantage of candidate gene association studies is that several candidate genes in a signalling pathway may be studied simultaneously. However, several critical candidate genes regulating bone metabolism may remain undiscovered, and the inconsistent, spurious, and insignificant replications of the candidate gene association studies results illustrate the limitations of the method. Genome wide association studies are powerful tool to investigate the genetic architecture of polygenic disorders arising from nucleotide polymorphisms. The limitations of this method, which include the inability to identify genes that are not polymorphic, but play a functional role in osteoporosis pathogenesis, makes it most unlikely that this approach would identify the majority of the genes involved in osteoporosis.

Transcriptome analysis provides more information about mRNA level and explores the relationship between certain genes and the biological pathways that regulate bone matrix mineralization. The downsides of transcriptome analysis are that signal transduction pathways occur at a protein level and the abundance of mRNA is not a real indicator of a gene's role in cellular functions, and the correlation between mRNA and protein abundance is extremely poor.

The dynamic properties of the bone tissue proteome provide incentives to analyze gene expression in bone disorders at a protein, rather than mRNA, level. The application of proteomics in bone research holds a great promise to increase our understanding of protein expression, dynamics, decay, posttranslational modifications, and signal transduction pathways that regulate bone matrix mineralization. Identification of novel proteins that may be associated with bone matrix mineralization presents vital new information toward deciphering the precise mechanisms regulating this process. Moreover, proteomic profiling provides an efficient method to determine protein candidates and elucidate the signal transduction pathways regulating bone mineral density. Therefore, proteomic profiling of osteoblasts, chondrocytes, and osteoclasts would greatly enhance our knowledge about the molecular pathways regulating

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bone growth and remodelling. The discovery of genes involved in the risk of osteoporosis has promising scientific, therapeutic, and public health benefits.

Proteomic alterations in bone tissue due to a genetic disorder may occur in several ways that are not predictable from either genome or transcriptome analysis, and it is obvious that a deeper view of these alterations will impact medicine in the field of bone metabolic disorders. Identifying proteins and pathways that are crucial to osteoporosis pathophysiology is clinically relevant to provide a potential therapy.

Therefore, the utilization of proteomics and RNAi in osteoporosis research is a solution which will accelerate discovery, streamline the process of developing therapeutic strategies to reduce the incidence of fractures amongst the aging population and thus, reduce health care costs overall, in hope to improve the quality of elderly individual's life.

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