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Metaanalysis Reveals Genetic Correlates of Osteoporosis Pathogenesis

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ABSTRACT. ***Objective***. Osteoporosis is a growing healthcare burden. By identifying osteoporosis-promoting genetic variations, we can spotlight targets for new pharmacologic therapies that will improve patient outcomes. In this metaanalysis, we analyzed mesenchymal stem cell (MSC) biomarkers in patients with osteoporosis.

Methods. We employed our Search Tag Analyze Resource for the Gene Expression Omnibus (STARGEO) platform to conduct a metaanalysis to define osteoporosis pathogenesis. We compared 15 osteoporotic and 14 healthy control MSC samples. We then analyzed the genetic signature in Ingenuity Pathway Analysis.

Results. The top canonical pathways identified that were statistically significant included the serine peptidase inhibitor kazal type 1 pancreatic cancer pathway, calcium signaling, pancreatic adenocarcinoma signaling, axonal guidance signaling, and glutamate receptor signaling. Upstream regulators involved in this disease process included *ESR1*, dexamethasone, *CTNNβ1*, *CREB1*, and *ERBB2*.

Conclusion. Although there has been extensive research looking at the genetic basis for inflammatory arthritis, very little literature currently exists that has identified genetic pathways contributing to osteoporosis. Our study has identified several important genes involved in osteoporosis pathogenesis including *ESR1*, *CTNNβ1*, *CREB1*, and *ERBB2*. *ESR1* has been shown to have numerous polymorphisms, which may play a prominent role in osteoporosis. The Wnt pathway, which includes the *CTNNβ1* gene identified in our study, plays a prominent role in bone mass regulation. Wnt pathway polymorphisms can increase susceptibility to osteoporosis. Our analysis also suggests a potential mechanism for *ERBB2* in osteoporosis through Semaphorin 4D (*SEMA4D*). Our metaanalysis identifies several genes and pathways that can be targeted to develop new anabolic drugs for osteoporosis treatment.

Key Indexing Terms: genetic studies, metaanalysis, osteoporosis, stem cells

As the world's population longevity continues to increase, new medical challenges are becoming more apparent.[1] One such challenge is the increase in osteoporosis, a skeletal disorder characterized by reduced bone strength and bone mineral density (BMD), conferring an increased risk of fracture or worse to those affected.[2] In 2010, it was estimated that up to 10.2 million adults aged ≥ 65 years in the United States had osteoporosis, and this number is expected to rise annually.[3] Unsurprisingly, this increase in prevalence follows the current increase in the annual incidence of osteoporotic fractures, especially debilitating hip fractures.[4] Hip fractures are particularly concerning, as these events have a yearly mortality rate of 20%.[5] In addition, there has been greater social and economic burden due to increases in prevalence and incidence rates of osteoporosis.[6] For example, the annual cost of caring for osteoporotic fractures exceeds that of caring for myocardial infarction, cerebrovascular accidents, and breast cancer.[7] From 2000 to 2011, annual facility-associated hospital costs related to osteoporotic fractures was $5.1 billion, compared to the next highest disease, myocardial infarction at $4.3 billion.[8]

Osteoporosis is influenced by a variety of risk factors including family history, sex, alcohol and substance abuse, diet, and exercise, and estrogen levels in women.[9] Clinical factors associated with osteoporosis include peak bone mass, of which age is a major determinant.[10] Additionally, other associated secondary medical factors include gastrointestinal diseases, hematologic disorders, and hypogonadal states.[11] Genetic factors also appear to influence BMD, thus predisposing those with higher BMD

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to osteoporosis.[12] Previous studies have shown that incidence varies with race and ethnicity[12]; for example, Black women tend to have higher BMD than White women and this translates to a 50% reduction in osteoporotic-related fracture rates.[13] Similarly, it has also been reported that despite having comparatively reduced hip fracture rates, Asians generally have a lower BMD than White individuals.

To date, current evidence defines osteoporosis as a polygenic disease influenced by genetic and environmental factors.[14] A previous study has shown that peak bone mass variations can be genetic in up to 60–70% of cases.[15] Thus far, genome-wide investigations have identified over 500 loci associated with bone phenotypes, but only a few specific genes have been mapped and explored.[16] While other studies have shown similar gene association results, more research with greater sample sizes is needed to more accurately confirm the genetic variations involved in the pathogenesis of osteoporosis.[17] In 1 genome-wide meta-analysis, Mullin, *et al* found that genetic polymorphisms of the Wnt ligand secretion and the coiled-coil domain containing 170 (*CCDC170*) gene were significantly associated with low BMD when adjusted for age and sex.[18] However, a review conducted by Al-Bargouthi and Farber found 518 independent loci associated with low BMD, but that these only accounted for 20% of observed phenotypic variation.[16] Thus, further studies are needed to continue examining genetic and cell-signaling pathways associated with the pathogenesis of osteoporosis. By identifying osteoporosis-promoting genetic variations, we can spotlight targets for new pharmacologic therapies that will reduce disease-associated adverse effects and improve patient outcomes. We used the novel metaanalysis platform Search Tag Analyze Resource for the Gene Expression Omnibus (STARGEO; see Methods) to mine the Gene Expression Omnibus (GEO) for mesenchymal stem cell (MSC) samples from patients with osteoporosis. STARGEO enabled us to study disease using a larger dataset and allowed for a robust study of osteoporosis.

METHODS

The National Center for Biotechnology Information GEO is an open database of millions of biological samples from functional genomics experiments. The curated STARGEO platform enables efficient metaanalysis of disease and tissue genomic signatures through tagging of preannotated biological samples across various GEO experiments (Figure 1). More information on STARGEO and its functionality can be found in our previous paper.[19] We employed the STARGEO platform to conduct a genomewide metaanalysis in order to define genetic contributions to osteoporosis pathogenesis based on publicly available data. We tagged 15 osteoporotic and 14 healthy control MSC samples for the metaanalysis. Dataset samples were taken from series GSE35956, GSE35958, and GSE35959. Approximately 21,000 genes were extracted for each of the metaanalyses conducted in STARGEO (Table 1 shows top up- and downregulated genes). We used standard metaanalysis random and fixed effects models to generate both metaanalysis *P* values and effect sizes across studies as previously described.[19] Briefly, our platform used inverse variance weighting for pooling of data across studies, and calculated weights for estimates of random effects with continuous data using the DerSimonian-Laird estimate.[19] All STARGEO data and information on analyses can be found at http://stargeo.org.

To evaluate these data, we analyzed gene signature outputs from our metaanalyses using the Ingenuity Pathway Analysis (IPA) tool.[20] Analysis was restricted to genes showing statistical significance ($P < 0.05$) in both fixed and random effects models and those that had an absolute experimental log ratio greater than 1.0 between experimental and control samples. A total of 3787 genes were analyzed in IPA (Supplementary Table 1, available from the authors on request). Top up- and downregulated genes were further assessed in IPA to elucidate biological processes, mechanisms of disease, and potential biomarkers and therapeutic targets that will be highlighted in our Results and Discussion sections. *P* values $< 1 \times 10^{-50}$ were reported as 0.00 by IPA analysis and are referred to as such in this manuscript.

Table 1. Summary of the most up- and downregulated genes from the metaanalysis of mesenchymal stem cells of patients with osteoporosis. Experimental log ratios indicating magnitude of change from control samples are shown.

Top Upregulated Genes	Log Ratio	Top Downregulated Genes	Log Ratio
MAB21L2	3.460	*HSP90B1*	–3.933
IGF2	3.302	*ALG5*	–3.850
P2YR10	3.238	*PKIβ*	–3.845
RFX4	3.165	*PAWR*	–3.689
PTPRD-AS1	3.141	*WFDC21P*	–3.608
CKM	2.976	*F2RL1*	–3.571
TMEM59L	2.934	*ZIC1*	–3.517
ZNF503	2.908	*SIAE*	–3.499
LINC01234	2.865	*ANKRD12*	–3.466
CSMD2	2.823	*THRAP3*	–3.355

IPA is based on the QIAGEN knowledge base and highlights relationship between genes, disease processes, phenotypes, drug activity, and others that applied to data sourced from genetic studies. The data inputs involve several modalities, including single-nucleotide polymorphisms (SNPs) and micro-RNA microarrays, RNA sequencing, proteomic and metabolomic studies, and chemical lists, among others. Specifically, we leveraged IPA to enable analysis of large-scale data outputs generated in STARGEO in order to dissect complex biological networks that characterize genomic, metabolomic, and proteomic data that would be more difficult otherwise. All data were sourced from GEO. All presented data is publicly available and thus access to identifiable private patient information as well as human subject interaction/intervention was not required. As such, no institutional review board approval was necessary.

RESULTS

IPA analysis of MSCs in osteoporotic patients identified several canonical pathways (Figure 2) including: serine peptidase inhibitor kazal type 1 pancreatic cancer pathway, calcium signaling, pancreatic adenocarcinoma signaling, axonal guidance signaling, glutamate receptor signaling, and lipopolysaccharide/interleukin-1–mediated inhibition of retinoid X receptor function. Additionally, IPA identified estrogen receptor 1 (*ESR1*), catenin β-1 (β-catenin; *CTNNβ1*), cyclic AMP (cAMP) responsive element-binding protein 1 (*CREB1*), and erb-B2 receptor tyrosine kinase 2 (*ERBB2*). IPA also demonstrated that osteoporotic MSCs had elevated levels of alkaline phosphatase (ALT; *P* range = 0.15–0.00324), alanine aminotransferase (*P* range = 0.0205–0.017), aspartate aminotransferase (*P* range = 0.317–0.0531), hematocrit (*P* = 0.0562), and blood urea nitrogen levels (*P* = 0.0562) compared to the control samples. Results are summarized in Table 2.

Comparative metaanalysis of healthy and osteoporotic MSC samples illustrated significant up- and downregulation

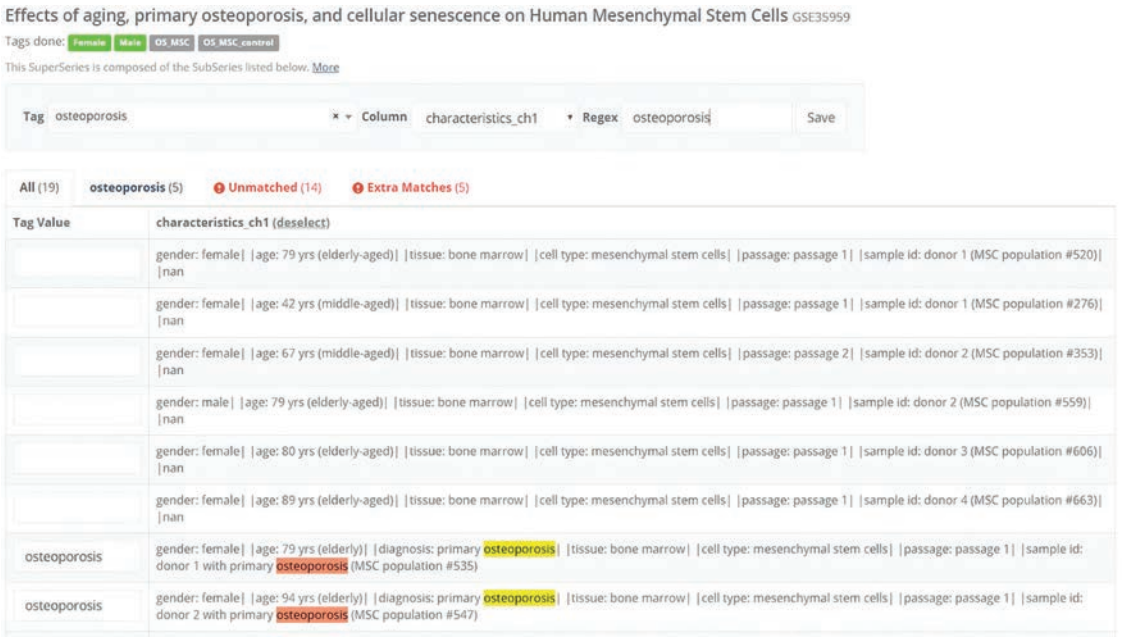

Figure 1. Screen capture of tagging mesenchymal stem cell samples using STARGEO. STARGEO: Search Tag Analyze Resource for the Gene Expression Omnibus.

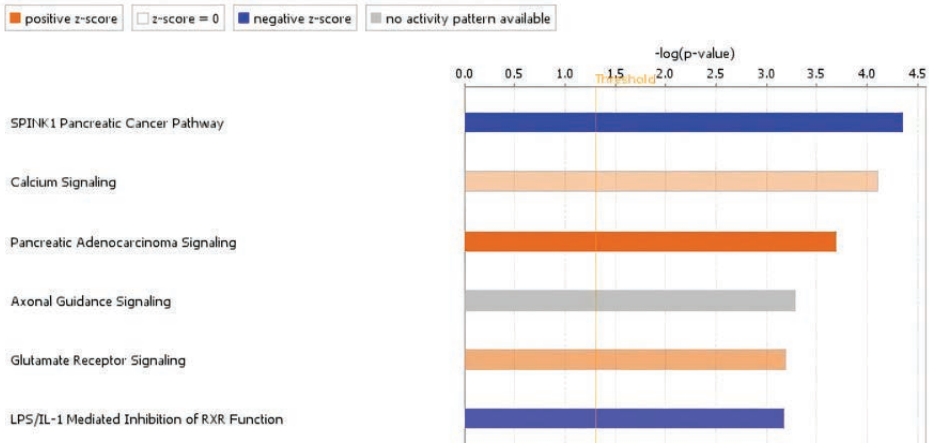

Figure 2. Top canonical pathways identified by Ingenuity Pathway Analysis from the osteoporotic MSC analysis. *Z* score is illustrated in the legend. LPS/IL-1: lipopolysaccharide/interleukin-1; MSC: mesenchymal stem cells; *SPINK1*: serine peptidase inhibitor kazal type 1.

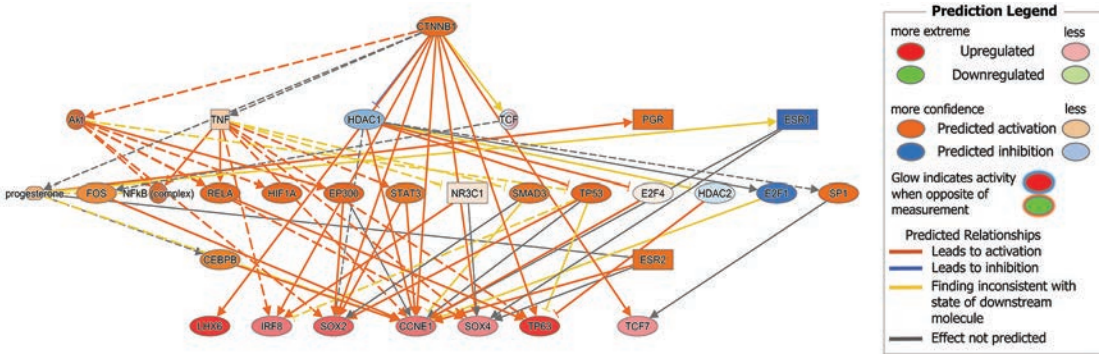

Figure 3. Ingenuity Pathway Analysis of osteoporotic MSCs shows several transcription regulators activated by *CTNNB1*, a top upstream regulator and gene implicated in bone homeostasis. Prediction legend illustrates relationships between genes. MSC: mesenchymal stem cells.

Table 2. Summary of top canonical pathways identified with Ingenuity Pathway Analysis (IPA).

Canonical Pathway	*P*	*Z*
SPINK1 pancreatic cancer pathway	4.35×10^{-5}	−3.273
Calcium signaling	7.74×10^{-5}	1.372
Pancreatic adenocarcinoma	2.02×10^{-4}	3.695
Axonal guidance signaling	5.18×10^{-4}	NA
Glutamate receptor signaling	6.35×10^{-4}	2.121
LPS/IL-1–mediated inhibition of RXR function	6.52×10^{-4}	−2.353
ESR1	2.32×10^{-12}, with predicted inhibition	
CTNNβ1	9.31×10^{-12}, with predicted activation	
CREB1	2.74×10^{-9}, with predicted activation	
ERBB2	3.05×10^{-9}	

CREB1: cAMP responsive element-binding protein 1; *CTNNB1*: Catenin β-1; *ERBB2*: erb-B2 receptor tyrosine kinase 2; *ESR1*: estrogen receptor 1; LPS/IL-1: lipopolysaccharide/interleukin-1; NA: not available; RXR: retinoid X receptor; *SPINK1*: serine peptidase inhibitor kazal type 1.

of thousands of genes, with 3787 genes being included in our IPA analysis (see Supplementary Table 1, available from the authors on request, for *P* values and experimental log ratios). The top gene candidates have various roles in cell signaling, with several not previously described in the context of osteoporosis or bone homeostasis. The most upregulated genes that are implicated in bone morphogenic protein (BMP) signaling include Mab-21-like-2 (*MAB21L2*) and insulin-like growth factor-2 (*IGF2*).[21,22] We also noted upregulation of the G protein–coupled receptor *P2YR10* and transcription factor regulatory factor X4 (*RFX4*). Other notable top upregulated genes include the zinc finger protein *ZNF503* and type 1 membrane glycoprotein *TMEM59L*.

Similarly, top downregulated genes are also implicated in cell signaling and other essential cellular processes. The most downregulated gene is the chaperone protein *HSP90B1*. Other notable top downregulated genes include cAMP-dependent protein kinase inhibitor β (*PKIβ*); PRKC apoptosis Wilms tumor 1 regulator protein (*PAWR*); receptor for trypsin and trypsin-like enzymes, *F2RL1*; zinc finger protein, *ZIC1*; ankyrin repeats–containing cofactor, *ANKRD12*, and *IGF2* receptor (*IGF2R*).

As *CTNNβ1* was identified as a top upstream regulator and plays a prominent role in bone mass regulation, we next investigated its downstream signaling.[23,24,25] Using IPA, we identified several transcription regulators activated by *CTNNβ1* including CCEN1, SOX2, SOX4, IRF8, TP63, TCF7, and LHX6 (Figure 3).

DISCUSSION

Although there has been extensive research probing the genetic basis for inflammatory arthritis, few studies have investigated and identified genetic pathways contributing to osteoporosis. Our study has identified several important upstream regulators involved in osteoporosis pathogenesis including *ESR1*, *CTNNβ1*, *CREB1*, and *ERBB2*.

Osteoporosis is a complex disease associated with a variety of risk factors and has a poorly understood etiology. Genetic factors may account for up to 50–85% of the risk in developing osteoporosis among postmenopausal women. Among these genetic factors, estrogen receptor gene polymorphisms (i.e., *ESR1*, *ESR2*) are the most well known.[26] For example, *ESR1* contains numerous polymorphisms (Xbal and PvuII), which may play a prominent role in osteoporosis.[27] One metaanalysis of 1838 hip fracture cases and 14,972 healthy controls found that the PvuII allele is significantly associated with increased hip fracture susceptibility in both males and females.[28] A recent study showed that PvuII and Xba1 *ESR1* gene haplotypes are correlated with decreased femoral neck *t* scores and may be predictive of osteoporosis in female patients with inflammatory bowel disease.[29] Similarly, a European study looking at *ESR1* genotyping in osteoporosis found that a homozygous absence of the Xbal recognition site reduced overall fractures by 19% and vertebral fractures by 35%.[27] These effects were independent of BMD. Ultimately, our study and others show that *ESR1* is a susceptibility gene for fractures and have motivated further studies to examine the effect of these common genetic variants on osteoporosis. These results suggest *ESR1* polymorphisms could be used as clinical markers in routine osteoporosis fracture risk assessment.

Several genomic studies have identified SNPs in the Wnt/β-catenin signaling pathway, which includes the *CTNNβ1* gene identified in our study, a known contributor to BMD and osteoporosis susceptibility.[30] Most notably, inhibition of sclerostin, a Wnt antagonist secreted by osteocytes, has been proven in clinical trials to be a pharmacologically efficacious osteoanabolic target. For example, administration of romosozumab, a humanized monoclonal antibody to sclerostin, over a 1-year period was shown to increase bone formation and density by greatly reducing osteoclast-mediated bone resorption, ultimately decreasing fracture risk in treated patients.[31] This promising agent highlights the Wnt pathway as a suitable candidate for therapeutic intervention to increase bone mass, especially in postmenopausal women. As our study spotlighted *CTNNβ1*, part of the Wnt signaling pathway that has been shown to regulate osteoblastic differentiation and osteoclastogenesis,[32] we believe targeting *CTNNβ1* could be used as a novel therapeutic agent for osteoporosis. Pharmacologic inhibitors of *CTNNβ1* (CWP232291 and PRI-724) are currently in clinical trials for treating cancer, and results from this study may provide attractive alternative uses.[33]

Previous studies have shown that inhibition of semaphorin 4D (SEMA4D) increases bone formation in a mouse model of osteoporosis.[34] SEMA4D is a transmembrane protein found on osteoclasts that activates osteoblast *ERBB2* through PlexinB1, resulting in suppression of IGF-1–mediated osteoblast differentiation. Zhang, *et al* found that serum SEMA4D levels in osteoporotic postmenopausal women were negatively correlated with lumbar spine BMD and bone turnover markers such as serum ALP.[35] These results suggest that SEMA4D and *ERBB2* play an

important role in the pathogenesis of postmenopausal osteoporosis by enhancing bone resorption while negatively influencing bone formation. These findings have been confirmed by other studies that have evaluated circulating serum SEMA4D levels in postmenopausal women with low BMD following denosumab and teriparatide treatments.[36] Anastasilakis, *et al* concluded that circulating SEMA4D levels increased following denosumab treatment and decreased following teriparatide treatment.[37] Taken together, these results suggest SEMA4D and *ERBB2* could potentially mediate the coupling effect that occurs after both antiresorptive and osteoanabolic treatments. Thus, *ERBB2* may be an attractive target for osteoporosis therapy as no studies to date have investigated the effects of the principle *ERBB2* inhibitor, trastuzumab, on bone density. A better understanding of the role of SEMA4D and *ERBB2* in this context will be important for developing future therapeutic strategies.

Our study has also identified 2 genes important in osteoporosis pathogenesis, *MAB21L2* and *IGF2*, as significantly upregulated in osteoporosis MSCs. *MAB21L2*, like sclerostin, is a novel repressor of BMP-induced transcription. In fact, some postulate that the osteoporosis-associated aging process is epigenetically affecting *MAB21L2* pathway transcriptional activity.[36] It has been found that *MAB21L2* expression is significantly higher in osteoporotic human MSCs when compared to a middle-aged control group.[26] There is considerable intracellular crosstalk primarily involving BMP, Wnt, and parathyroid hormone receptor signaling, which are major regulators of the bone regeneration process. Given the positive results from the sclerostin antagonism clinical trials, there may be clinical utility in further investigating *MAB21L2*-mediated repression of BMP signaling for therapeutic purposes.

MSCs are bone marrow stromal cells that self-renew and differentiate into osteogenic, chondrogenic, and adipogenic lineages. Osteogenic differentiation of MSCs involves the coordination of several bone morphogenic proteins, of which BMP-9 has been shown to be essential for this process. Specifically, our analyses of osteoporotic MSCs show downregulation of *IGF2R*, as well as stark upregulation of *IGF2*, which is known to potentiate BMP-9–induced bone formation. Of note, several studies have shown that endogenous *IGF2* expression is reduced in osteoporotic MSCs. The upregulation of *IGF2* may be due to a cellular response to the downregulation of *IGF2R*. *In vitro* studies have shown that *IGF2* promotes endochondral ossification and expansion of hypertrophic chondrocyte zone in culture.[38] Together these results suggest that a novel therapy that potentiates *IGF2* and BMP-9 signaling could emerge as an efficacious treatment of osteoporosis and bone defects.

Dysregulation of calcium signaling pathways have also been established as major players in the pathogenesis of osteoporosis, since calcium availability modulates bone formation and resorption rates. Osteoclasts regulate bone resorption, while osteoblasts regulate bone formation. Intercellular calcium signaling occurs between osteoclasts and osteoblasts as a regulatory pathway. Thus, dysregulation of calcium signaling disrupts the stasis between osteoclasts and osteoblasts, leading to disproportionate osteoblast and osteoclast activities. This results in an unsteady rate of bone formation and resorption, and therefore osteoporosis.[39] Serum calcium levels directly regulate parathyroid hormone (PTH) secretion for the parathyroid glands through calcium-sensing receptors (CaSR).[40,41] Specifically, low calcium levels stimulate the release of PTH through CaSR inhibition, which, when bound to its receptor, stimulates osteoblast secretion of receptor activator of nuclear factor-κB ligand (RANKL). RANKL further stimulates osteoclast formation from progenitors through activation of RANK receptors, ultimately resulting in bone resorption and stabilization of serum calcium concentrations.[42] In addition, activation of phospholipase C–coupled receptors results in the production of inositol-1,4,5-trisphosphate (IP3), leading to calcium release from the endoplasmic reticulum upon binding its receptor. IP3 has also been shown to induce the release of calcium from bone cells including osteoclasts and osteoblasts to increase resorption.[43] Although the effect of CaSR on MSCs is not well described, a rat study suggested that CaSR activity is implicated in cellular proliferation, survival, and ERK1/2 signaling.[44] Thus, a decrease in CaSR may limit MSC proliferation and further drive osteoporosis pathogenesis. While calcium signaling pathways are already targets of current drugs in the treatment of bone-related pathologies, including osteoporosis, the physiologic role of osteoclast and osteoblast CaSR expression remains incompletely understood.[41] Consequently, further studies examining the role of calcium signaling pathways in influencing the regulation of bone anabolic and catabolic pathways will be critical in future targeted drug therapy.

There are limitations to this study. Annotations for the MSCs in our analysis do not contain all the information on patients that can help limit confounding variables. For example, patients might have been taking medications at the time of collection, or may have other comorbidities that could have had an effect on gene expressions in their MSCs. Additionally, there may be significant differences in how samples are processed and analyzed between studies that would further complicate the metaanalysis. Last, public data on MSCs in osteoporosis is limited so our sample size was relatively low. Despite the lower sample size, the results of our metaanalysis showed stark differences in gene expression between patients' and controls' MSCs, with experimental log ratios ranging from an impressive –4 to 4. Even with these limitations, our results offer exceptional insights to osteoporosis pathogenesis.

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