Title: Meta-analysis Reveals Genetic Correlates of Osteoporosis Pathogenesis

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Abstract

Objectives: Osteoporosis is a growing health care burden. By identifying osteoporosis-promoting genetic variations, we can spotlight targets for new pharmacologic therapies that will improve patient outcomes. In this meta-analysis, we analyzed mesenchymal stem cell biomarkers in patients with osteoporosis.

Methods: We employed our Search Tag Analyze Resource (STARGEO) platform to conduct a meta-analysis to define osteoporosis pathogenesis. We compared 15 osteoporotic and 14 healthy control mesenchymal stem cell (MSC) samples. We then analyzed the genetic signature in Ingenuity Pathway Analysis.

Results: The top canonical pathways identified which were statistically significant included the SPINK1 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, CTNNB1, CREB1, ERBB2.

Discussion: Although there has been extensive research looking at the genetic basis for inflammatory arthritis, very little literature exists currently that has identified genetic pathways contributing to osteoporosis. Our study has identified several important genes involved in osteoporosis pathogenesis including ESR1, CTNNB1, 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 CTNNB1 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 meta-analysis identifies several genes and pathways that can be targeted to develop new anabolic drugs for osteoporosis treatment.

Introduction

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 65 and older in the United States had osteoporosis and 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 costs 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, gender, 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 to osteoporosis.(12) Previous studies have shown that incidence varies with race and ethnicity.(12) For example, African American women tend to have higher BMD than Caucasian women and this translates to 50% reduced 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 Caucasians.

To date, current evidence defines osteoporosis as a polygenic disease influenced by genetic and environmental factors.(14) Recent studies have 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 are needed to more accurately confirm genetic variations involved in the pathogenesis of osteoporosis.(17) In one genome-wide metanalysis study, Mullin et al. found that genetic polymorphisms of the Wnt ligand secretion (WLS) and the coiled-coil domain containing 170 (CCDC170) gene were significantly associated with low BMD when adjusted for age and gender.(18) However, a review conducted by Al-Bargouthi et al. found 518 independent loci associated with low BMD, but that 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 meta-analysis platform Search Tag Analyze Resource

(STARGEO) (see methods) to mine Gene Expression Omnibus for mesenchymal stem cell (MSC) samples from osteoporosis patients. STARGEO allows to study disease using a larger dataset and allowed for a robust study of osteoporosis.

Methods

The National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) is an open database of millions of biological samples from functional genomics experiments. The curated STARGEO platform enables efficient metanalysis of disease and tissue genomic signatures through tagging of pre-annotated biological samples across various GEO experiments (see Fig. 1). More information on STARGEO and its functionality can be found in our previous paper.(19) We employed the STARGEO platform to conduct a genome-wide metanalysis 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 metanalysis. Samples datasets were taken from series GSE35956, GSE35958, and GSE35959. Approximately 21,000 genes were extracted for each of the metanalyses conducted in STARGEO (see Table 1 for top up- and down-regulated genes). We used standard meta-analysis random and fixed effects models to generate both meta *p*-values and effect sizes across studies as previously described.(19) Briefly, our platform uses inverse variance weighting for pooling of data across studies , and calculates 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 this data, we analyzed gene signature outputs from our meta-analyses using the Ingenuity Pathway Analysis (IPA) tool.(20) Analysis was restricted to genes showing statistical significance ($p < 0.05$) in both and fixed and random effects models and had an absolute experimental log ratio greater than 1.0 between experimental and control samples. A total of 3,787 genes were analyzed in IPA (supplemental table S1). 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 section. P values less than 1E-50 were reported as 0.00 by IPA analysis and are referred to as such in this manuscript.

IPA is based on the QIAGEN knowledge base and highlights relationship between genes, disease processes, phenotype, drug activity, and more which applied to data sourced from genetic studies. The data inputs involve several modalities, including single nucleotide polymorphisms (SNP) and micro-RNA microarrays, RNA-sequencing, proteomic and metabolomic studies, chemical lists, and more. Specifically, we leverage IPA to enable analysis of large-scale data outputs generated in STARGEO in order to dissect complex biological networks that characterize genomic, metabolomics, and proteomic data that would be more difficult otherwise. All data was 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 (IRB) approval was necessary.

Results

IPA analysis of MSCs in osteoporotic patients identified several canonical pathways (Fig. 2) including: SPINK1 pancreatic cancer pathway, calcium signaling, pancreatic adenocarcinoma, axonal guidance signaling, glutamate receptor signaling, and LPS/IL-1 mediated inhibition of RXR (retinoid X receptor) function. Additionally, IPA identified estrogen receptor 1 or ER1, catenin beta 1 or CTNNB1, CAMP Responsive Element Binding Protein 1 or CREB1, and Erb-B2 Receptor Tyrosine Kinase 2 or ERBB2. IPA also demonstrated that osteoporotic MSCs had elevated levels of alkaline phosphatase (p-value range 0.15 – 0.00324), ALT (p-value range 0.0205 – 0.017), AST (p-value range 0.317 – 0.0531), hematocrit (p-value 0.0562), and BUN levels (p-value 0.0562) compared to the control samples. Results are summarized in Table 2.

Comparative metanalysis of healthy and osteoporotic MSC samples illustrated significant up- and downregulation of thousands of genes, with 3,787 genes being included in our IPA analysis (see Table S1 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 or MAB21L2 and insulin growth factor 2 (IGF-2).(21,22) We also noted upregulation of the G-protein coupled receptor P2YR10 and transcription factor regulatory factor X4 or 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 protein kinase inhibitor PKIB, PRKC apoptosis regulator protein or PAWR, receptor for trypsin and trypsin-like enzymes F2RL1, zinc

finger protein ZIC1, ankyrin repeats-containing cofactor ANKRD12, and insulin like growth factor 2 receptor (IGF2R).

As CTNNB1 was identified as a top upstream regulator and plays a prominent role in bone mass regulation, we next investigated its downstream signaling.(23–25) Using IPA, we identified several transcription regulators activated by CTNNB1 including CCEN1, SOX2, SOX4, IRF8, TP63, TCF7, and LHX6 (Fig. 3).

Discussion

Although there has been extensive research probing the genetic basis for inflammatory arthritis, few studies exist have investigated and identified genetic pathways contributing to osteoporosis. Our study has identified several important upstream regulators involved in osteoporosis pathogenesis including ESR1, CTNNB1, 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 Pvull) which may play a prominent role in osteoporosis.(27) One metanalysis of 1,838 hip fracture cases and 14,972 healthy controls found that the Pvull allele is significantly associated with increased hip fracture susceptibility in both males and females.(28) A recent study showed that Pvull 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 bone mineral density. Ultimately, our study and others show that ESR1 is a susceptibility gene for fractures and have motivated further studies into the impact of these common genetic variants on osteoporosis. These results suggest ESR1 polymorphisms could be used as a clinical marker in routine osteoporosis fracture risk assessment.

Several genomic studies have identified SNPs in the Wnt/Beta-catenin signaling pathway, which includes the CTNNB1 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 osteo-anabolic target. For example, administration of Romosozumab, a humanized monoclonal antibody to sclerostin, over a one 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 CTNNB1, part of the Wnt signaling pathway, and has been shown regulate osteoblastic differentiation and osteoclastogenesis.(32) We believe targeting CTNNB1 could be used as a novel therapeutic agent for osteoporosis. Pharmacologic inhibitors of CTNNB1 (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 via PlexinB1, resulting in suppression of IGF-1 mediated osteoblast differentiation. Zhang et al. found that SEMA4D serum 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 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. 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 two genes important in osteoporosis pathogenesis, MAB21L2 and IGF-2, 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 impacting MAB21L2 pathway transcriptional activity.(36) It has been found that MAB21L2 expression is significantly higher in osteoporotic human MSCs when compared to a middle age 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 MSC's show downregulation of IGF2R, as well as stark upregulation of IGF-2, which is known to potentiates BMP-9 induced bone formation. Of note, several studies have shown endogenous IGF-2 expression is reduced in osteoporotic MSCs. The upregulation of IGF-2 may likely be due to a cellular response to the downregulation of IGF2R. *In vitro* studies have shown that IGF-2 promotes endochondral ossification and expansion of hypertrophic chondrocyte zone in culture.(37) Together these results suggest that a novel therapy that potentiates IGF-2 and BMP-9 signaling could emerge as an efficacious treatment of osteoporosis and bony defects.

Dysregulation of calcium signaling pathways have also been established as a major player in the pathogenesis of osteoporosis as 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 activities of osteoblasts and osteoclasts. This results in an unsteady rate of bone formation and resorption, and therefore osteoporosis.(38) Serum calcium levels directly regulate parathyroid hormone (PTH) secretion for the parathyroid glands via calcium sensing receptors (CaSR).(39,40) Specifically, low calcium levels stimulate the release of PTH via CaSR inhibition, which when bound to its receptor, stimulates osteoblast secretion of RANKL. RANKL further stimulates osteoclast formation from progenitors through activation of RANK receptors, ultimately resulting in bone resorption and stabilization of serum calcium

concentrations.(41) In addition, activation of phospholipase C (PLC)-coupled receptors results in the production of inositol-1,4,5- trisphosphate (IP3), leading to calcium release from the endoplasmic reticulum (ER) upon bindings its receptor. IP3 has also been shown to induce the release of calcium from bone cells including osteoclasts and osteoblasts to increase resorption.(42) Although the effect of CaSR on MSCs are not well described, a rat study suggests that CaSR activity is implicated in cellular proliferation, survival, and ERK1/2 signaling.(43) Thus, a decrease in CaSR may limit mesenchymal stem cell 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.(40) Consequently, further studies examining the role calcium signaling pathways influence 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 does not contain all the information on the patients that can help limit confounding variables. For example, patients may have been taking medications at the time of collection or have other co-morbidities that may have an effect of gene expressions in their MSCs. Additionally, there may be significant differences between how samples are processed and analyzed between studies that may further complicate the meta-analysis. Lastly, public data on MSCs in osteoporosis is limited and so our sample size was relatively low. Despite our lower sample size, the results of the meta-analysis showed stark differences in gene expression between patient 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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Figure Legends

Table 1: Summary of the most up- and down-regulated genes from the metanalysis of mesenchymal stem cells of osteoporosis patients. Experimental log ratios indicating magnitude of change from control samples are shown.

Table 2: Summary of top canonical pathways identified with Ingenuity Pathway Analysis.

Figure 1: Screen capture of tagging mesenchymal stem cell samples using STARGEO.

Figure 2: Top canonical pathways identified by Ingenuity Pathway Analysis from the osteoporotic MSC analysis. Z-score is illustrated in the legend.

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

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	PKIB	-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

Canonical Pathway	p-value, z-score
SPINK1 pancreatic cancer pathway	p-value 4.35 E-05, z-score -3.273
Calcium signaling	p-value 7.74 E-05, z-score 1.372
Pancreatic adenocarcinoma	p-value 2.02 E-04, z-score 3.695
Axonal guidance signaling	p-value 5.18 E-04, z-score NaN
Glutamate receptor signaling	p-value 6.35 E-04, z-score 2.121
LPS/IL-1 mediated inhibition of RXR (retinoid X receptor) function	p-value 6.52 E-04; z-score -2.353
Estrogen receptor 1 (ER1)	p-value 2.32 E-12, with predicted inhibition
Catenin beta 1 (CTNNB1)	p-value 9.31 E-12, with predicted activation
CAMP Responsive Element Binding Protein 1 (CREB1)	p-value 2.74 E-09, with predicted activation
Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2)	p-value 3.05 E-09

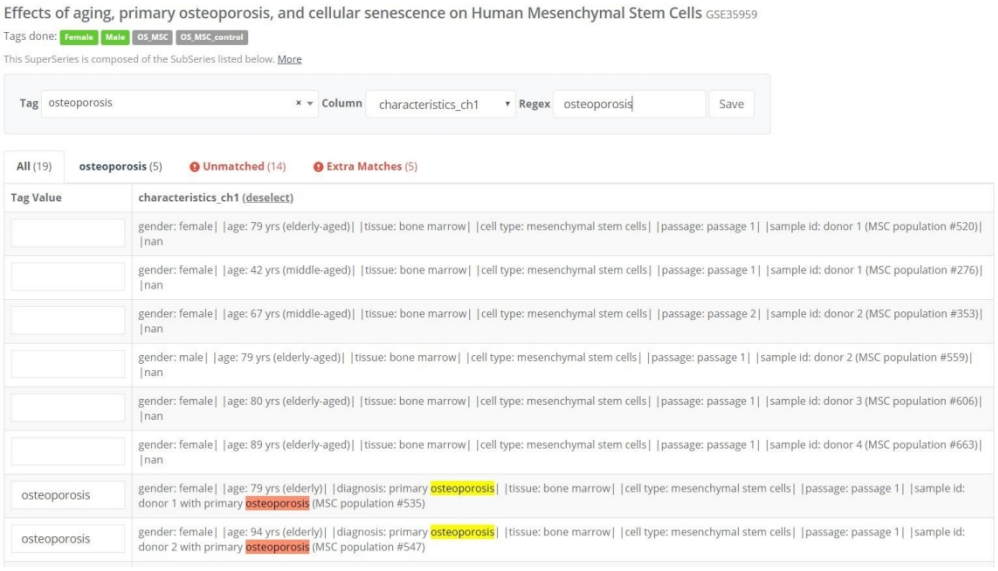

Figure 1: Screen capture of tagging mesenchymal stem cell samples using STARGEO.

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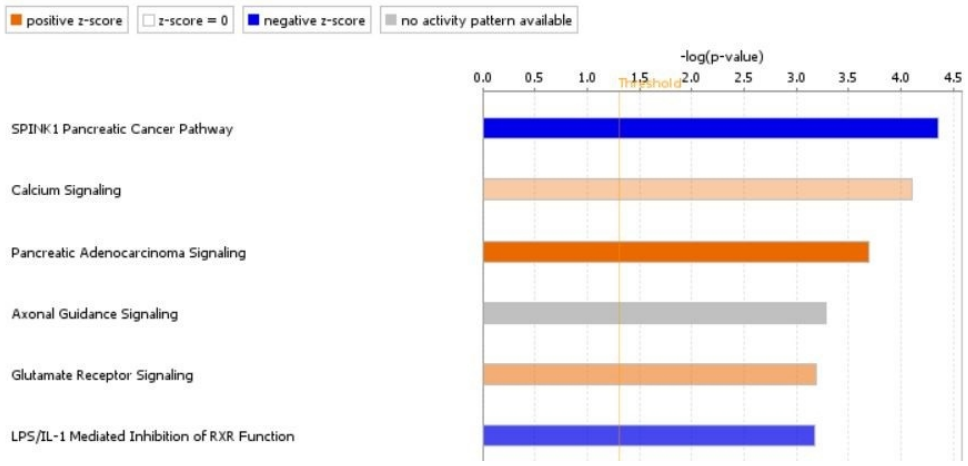

Figure 2: Top canonical pathways identified by Ingenuity Pathway Analysis from the osteoporotic MSC analysis. Z-scores are illustrated in the legend.

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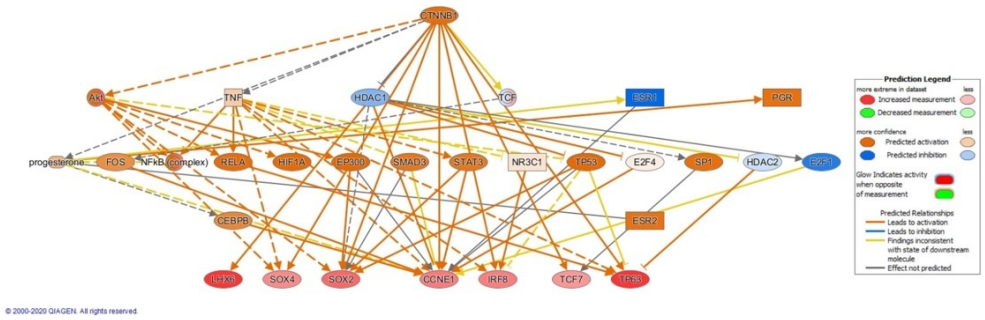

Figure 3: Ingenuity Pathway Analysis of osteoporotic mesenchymal stem cells shows several transcription regulators activated by CTNNB1, a top upstream regulator and gene implicated in bone homeostasis. Prediction legend illustrates relationships between genes.

191x64mm (150 x 150 DPI)