

Potential of 20 unexplored genes to be candidates for osteogenic regulators as novel therapeutic strategies in bone diseases

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Abstract

Background: Bone homeostasis is maintained by a balance between bone formation by osteoblasts and bone resorption by osteoclasts. As a therapeutic agent for osteoporosis, a strategy of suppressing bone resorption by inhibiting the function of osteoclasts is used; however, long-term use causes problems, such as decreased formation of osteoblasts and osteonecrosis of the jaw. Therefore, various growth factors and hormones have been used to promote the proliferation of osteoblasts, but the current situation is that long-term use cannot be applied owing to issues such as carcinogenic risk. To establish an appropriate osteogenesis-promoting strategy, it is essential to ensure the fate of progenitor cells in the mature osteoblasts.

Purpose: To investigate potential osteogenic regulators based on published single-cell transcriptome data as an alternative approach to bone diseases treatment.

Methods: a systematic review using articles through several databases with descriptions related to various genes were identified based on single-cell transcriptome data as candidates for osteogenic regulators.

Results: 20 unexplored genes were identified based on single-cell transcriptome data as candidates for osteogenic regulators.

Conclusions: Increasing osteoblast activity through the promotion of osteoblast differentiation shows promise as a novel approach for treating bone-formation-related diseases.

Keywords: Bone Homeostasis; Osteoblast; Bone Diseases; Gene Expression.

1. Introduction

Bone homeostasis is maintained by a balance between bone formation by osteoblasts and bone resorption by osteoclasts. Osteoporosis is one of the diseases caused by an imbalance in the activities of osteoblasts and osteoclasts [1].

Strategies for suppressing bone mass and density loss are used as therapeutic agents for osteoporosis, such as administration of the antiresorptive agent bisphosphonate, which mechanism of action reduces resorption by inhibiting

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the function of osteoclasts, directly promoting osteoblast proliferation, and increasing osteoblast differentiation. However, long-term use causes adverse events, such as osteonecrosis of the jaw (ONJ), atypical fractures, oesophageal irritation, and cancer when administered orally [2].

Therefore, we aimed to identify an alternative therapeutic agent for osteoporosis that promotes osteogenesis. We will utilize this information to develop a more effective and safer treatment option for patients with osteoporosis. Our approach involves the discovery of genes associated with osteoblast differentiation from stem cells. To establish an appropriate osteogenesis-promoting strategy, it is essential to ensure the fate of progenitor cells in the mature osteoblasts.

According to the published paper by Yoshioka, Venus-positive (Venus+) cells expressing the fluorescent protein Venus under the control of the 2.3-kb Col1a1 promoter were isolated from new-born mouse calvariae and subjected to single-cell RNA sequencing. Following clustering and pseudo time ordering analyses, osteoblasts were categorized into four groups. Of these, three clusters (clusters 1–3) exhibited similarities in the expression of osteoblast markers, whereas cluster 4 was distinctly different. Cells of cluster 4 expressed Cd34 and Cxcl12 with relatively low levels of osteoblast markers, suggesting that this cell type differs from active bone-forming osteoblasts and retains or reacquires progenitor properties [3].

2. Methods

Topic of the article was determined. The papers that included are articles on bone remodelling, bone diseases, and mouse cell lines; internationally accredited journals; and articles that meet the criteria after title and abstract screening. The author utilized online international journal databases as sources in obtaining the included articles.

Browsing on online journal databases to find relevant articles were performed using keywords of “Bone Remodelling”, “Bone Formation Diseases”, “Osteoblast Dysfunction in Bone Diseases”, “Therapeutic Approaches in Bone Diseases”, “Gene Expression in Mouse Cells”, “Osteoblast Differentiation”, and “Novel Osteogenic Regulators”.

The authors conducted the screening process by reviewing the titles and abstracts of relevant journals. Then candidate articles were selected based on predetermined inclusion and exclusion criteria. Finally, selected articles were reviewed systematically in this study.

3. Results

3.1. Article Search Results

The following diagram illustrates the process of selecting articles for discussion in the systematic review, using predetermined inclusion and exclusion criteria.

The figure 1 illustrates the process of selecting articles for a systematic review. Following the initial step of database search, a total of 71 articles were identified. However, only 10 articles met the author's predetermined criteria and were included in this study.

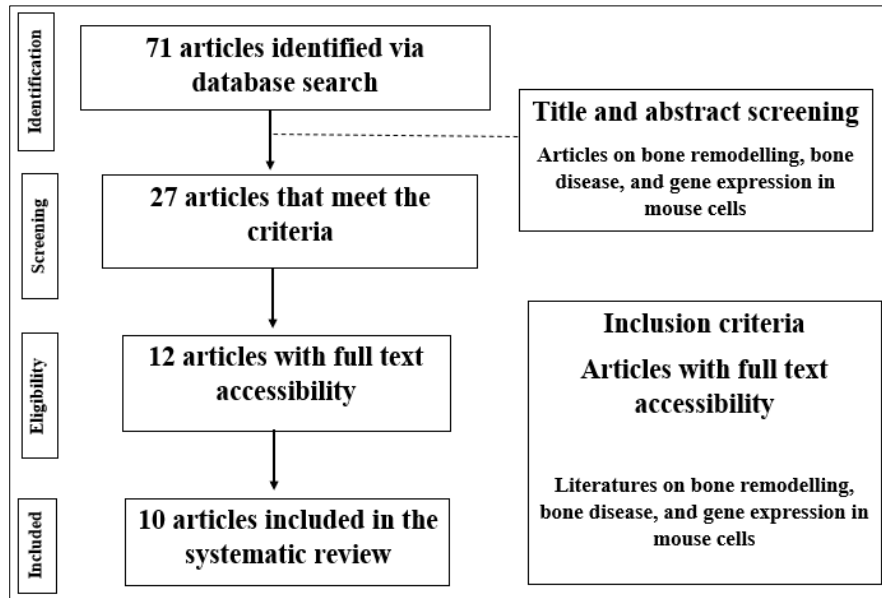


Figure 1 The Flow chart of article selection based on the PRISMA protocol

3.2. Candidate Osteogenic Regulators Selection

Among 252 genes whose expression fluctuated significantly between clusters 4 and 1, 190 genes have been reported in academic papers on osteoblast differentiation according to the PubMed database. Therefore, we focused on unknown 62 genes which were likely to be involved in the regulation of differentiation. In this study, we selected 10 upregulated or downregulated genes based on the fold change between two clusters and explored potential targets for osteogenesis regulators.

Table 1 A schematic figure of genes in differentiation from cluster 4 to cluster 1

| Category | Symbol | Full Name | Cluster 1 vs. Cluster 4 | |
|----------|----------------|---|-------------------------|----------|
| | | | log fold-change | p-value |
| Up | <i>Cgrefl</i> | cell growth regulator with EF hand domain 1 | 2.569 | 1.13E-17 |
| Up | <i>Golph3</i> | golgi phosphoprotein 3 | 1.439 | 3.02E-11 |
| Up | <i>Mamdc2</i> | MAM domain containing 2 | 1.324 | 2.56E-13 |
| Up | <i>Sppl2a</i> | signal peptide peptidase like 2A | 1.231 | 3.45E-11 |
| Up | <i>Myo10</i> | myosin X | 1.202 | 1.50E-11 |
| Up | <i>Gprc5c</i> | G protein-coupled receptor, family C, group 5, member C | 1.200 | 2.31E-14 |
| Up | <i>Cd1d1</i> | CD1d1 antigen | 1.181 | 2.11E-11 |
| Up | <i>Chac1</i> | ChaC, cation transport regulator 1 | 1.123 | 9.65E-09 |
| Up | <i>Mlip</i> | muscular LMNA-interacting protein | 1.089 | 4.12E-12 |
| Up | <i>Cpq</i> | carboxypeptidase Q | 1.046 | 1.34E-10 |
| Down | <i>Nfib</i> | nuclear factor I/B | -1.740 | 1.03E-12 |
| Down | <i>Itih5</i> | inter-alpha (globulin) inhibitor H5 | -1.940 | 1.72E-23 |
| Down | <i>Nrep</i> | neuronal regeneration related protein | -1.990 | 1.22E-13 |
| Down | <i>Fbln2</i> | fibulin 2 | -2.133 | 3.00E-15 |
| Down | <i>Agtr2</i> | angiotensin II receptor, type 2 | -2.138 | 4.27E-15 |
| Down | <i>Gfpt2</i> | glutamine fructose-6-phosphate transaminase 2 | -2.182 | 4.29E-10 |
| Down | <i>Capn6</i> | calpain 6 | -2.360 | 1.48E-14 |
| Down | <i>Col6a6</i> | collagen, type VI, alpha 6 | -2.509 | 6.57E-13 |
| Down | <i>Coll4a1</i> | collagen, type XIV, alpha 1 | -2.565 | 2.84E-20 |
| Down | <i>Nid1</i> | nidogen 1 | -2.910 | 1.65E-20 |

4. Discussion

The structural components of bone are porous mineralized structures consisting of an extracellular matrix (largely mineralized), collagen, and cells. Depending on the type and location of bones, their proportions vary. There are two types of bone, cortical and trabecular, both of which have identical chemical compositions despite their distinct macroscopic and microscopic features. Genes play an important role in controlling cellular differentiation processes that give rise to the skeleton. Mesenchyme and cartilage are first used to set the pattern of the skeleton before being replaced by bone through osteoblast differentiation [4].

Bone provides important functions, such as mechanical support of soft tissues, levers for muscle action, protection of the central nervous system, release of calcium and other ions required for the maintenance of a constant ionic environment in the extracellular fluid, and housing and support for haematopoiesis [1].

To serve these crucial functions, the shape, quality, and size of the skeleton must be maintained. This maintenance involves bone remodelling, in which old bone is continuously replaced by new bone structure by coordinated actions of osteoclasts and osteoblasts organized in bone multicellular units (BMU) in the sequence of activation-resorption-formation events [4].

Bone homeostasis is a complex process in which bone tissue is in a constant state of remodelling to ensure a constant, homeostatically controlled amount of bone. The balance between bone destruction is called resorption by osteoclasts, followed by bone formation by osteoblasts [5].

Bone remodelling is regulated by both systemic and local mechanisms. The systemic mechanism involves parathyroid hormone (PTH) as the key role. When the serum calcium level falls, PTH increases the serum calcium concentration by increasing bone resorption as a deposit of calcium and minerals, increasing calcium reabsorption in renal tubules, and renal calcitriol production. As a result, when PTH is administered infrequently, it induces bone formation; however, when administered excessively, bone resorption is highly induced. One local mechanism is the OPG/RANKL/RANK system. RANK stands for Receptor activator of nuclear factor kappa-B, it is expressed on the surface of osteoclastic precursor cells, while RANKL which is ligand for the RANK receptor is expressed on the preosteoblastic/stromal cells. When RANKL binds to RANK, it induces differentiation, fusion into multinucleated cells, and the activation and survival of osteoclastic cells. Osteoprotegerin (OPG) blocks the effects of RANKL, thereby inhibiting osteoclast differentiation and activation [4].

Osteoclasts are giant multinucleated cells responsible for bone resorption. They are derived from hematopoietic cells of the mononuclear lineage, can grow up to 100 μ m in diameter [4], and share the same differentiation pathway as macrophages and dendritic cells. Thus, depending on the type of activator signal it is exposed to, a promyeloid precursor can differentiate into an osteoclast, a macrophage, or a dendritic cell. When it is exposed to receptor activator of NF- κ B ligand (RANKL; also called tumor necrosis factor-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL) or osteoclast differentiation factor (ODF) macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) will induce a precursor to differentiate into osteoclasts, macrophages, or dendritic cells [6].

Bone resorption is the process of destruction of old bone structures, which starts with osteoclasts attached to the bone matrix with the folded plasma membrane called the ruffled border, releasing lysosomal enzymes such as tartrate-resistant acid phosphatase and cathepsin K. With acids, osteoclasts dissolve crystalline hydroxyapatite, and proteases degrade the organic bone matrix, which is rich in collagen fibers. After degradation, collagen and other matrix components are endocytosed, transported through the cell, and exocytosed via a functional secretory domain. This transcytotic pathway enables the removal of significant amounts of matrix degradation products by osteoclasts, without compromising their strong connection to the underlying bone [6].

Osteoblasts originate from multipotent mesenchymal stem cells, are responsible for bone formation, and produce bone matrix constituents. Osteoblasts are found in clusters along the bone surface, lining the bone matrix layer. Fifteen percent of mature osteoblasts are entrapped in the new bone matrix and further differentiate into osteocytes, whereas the cells remaining on the bone surface develop into flat bone-lining cells [4].

In bone remodelling, after bone is destroyed by resorption by osteoclasts, osteoblasts lay down new bone structures until the resorbed bone is completely replaced. There are three phases of bone formation: production and maturation of the osteoid matrix by rapidly depositing collagen, followed by an equal rate of mineralization of the matrix. Importantly, to maintain a balance between matrix production and mineralization, these phases are performed at the

same rate until the final stage, when the rate of collagen synthesis decreases and mineralization continues until the osteoid is entirely mineralized [1].

Since the maintenance of bone structure and strength is highly dependent on bone resorption and formation within the bone remodelling process, imbalance in any part of this process causes defects and disorders in the bone. Bone resorption by osteoclasts is higher than bone formation by osteoblasts, which results in the reduction of bone matrix, including bone mineral density (BMD) and bone marrow cells (BMCs), which increases the risk of bone fracture and joint destruction in several diseases, including osteoporosis and rheumatoid arthritis (RA). However, excessive osteoblastic activity produces an osteosclerotic phenotype and weakens its action primes to osteomalacia and rickets [7].

Osteoporosis is characterized by increased bone resorption; when the bone formation rate is insufficient to compensate for resorption, it results in trabecular bone perforation and bone loss, and an increased risk of bone fracture is associated with sex hormone deficiency at the menopause stage. In addition, age-related bone loss, characterized by decreased recruitment and differentiation of osteoblast progenitor cells, as well as decreased lifespan, number, and bone-forming capacity of mature osteoblasts, contributes to osteoblast dysfunction in age-related bone loss [8].

By understanding the mechanisms of osteoblast dysfunction in osteoporosis, we aimed to develop effective and safe therapeutic strategies to promote bone formation, particularly during the osteoblast differentiation stage.

A few of the current therapeutic strategies against osteoporosis include increasing the number of osteoblasts by promoting the recruitment of osteoblast precursor cells, reducing osteoblast death from apoptosis, and increasing osteoblast activity in bone formation [9]. Several factors and signalling pathways that are considered to promote osteoblastogenesis include Insulin Growth Factor-1 (IGF-1), Fibroblast Growth Factor (FGF), transforming growth factor- β (TGF β), Bone Morphogenetic Proteins (BMPs), NOTCH, and WNT signalling [10].

Careful attention and sufficient precautions are necessary before the administration of therapeutic agents that are associated with adverse effects, such as bisphosphonate, which has a high risk of causing ONJ [11] and activation of NOTCH, which can result in bone cancer [12].

Stochastic fluctuations in the expression levels of genes and/or proteins drive cell behavior such as metabolism, growth, and fate determination [13]. Similarly, fluctuations in gene expression strongly influenced the differentiation of Cluster 4 to Cluster 1. Therefore, our aim to identify an alternative therapeutic agent for osteoporosis that promotes osteogenesis by identification of candidate genes which play roles as osteogenic regulators.

MC3T3-E1 is an osteoblastic cell line derived from C57BL/6 mouse calvaria. These cells possess osteoblast and osteocyte differentiation capabilities and have demonstrated the ability to form calcified bone tissue in *in vitro* studies. They are fibroblastic in shape and are approximately 20–50 μm in diameter. MC3T3-E1 cells have very low ALP activity in the growing state, but enzyme activity increased several hundredfold after the cultures reached a confluent state. These cells can differentiate into osteoblast, deposit hydroxyapatite in bone matrix and further differentiate into osteocytes [14].

KUSA-A1 cells are osteoblastic cell lines derived from bone marrow stromal stem cells that have the ability to differentiate into osteoblasts, chondrocytes, and myotubes under inducing conditions while maintaining an immature stage under non-inductive conditions [15]. Based on their characteristics and capacity, MC3T3-E1 and KUSA-A1 cell lines were selected as models to study osteoblast differentiation *in vitro*.

5. Conclusion

Bone diseases have negative impact on bone structures that can lead to bone loss and bone fracture. Increasing osteoblast activity through the promotion of osteoblast differentiation shows promise as a novel approach for treating bone-formation-related diseases. This approach has potential as to significantly impact medical treatment as an alternative to current therapeutic approaches that can have adverse effects. The authors have selected 20 previously unexplored genes, whose expressions show fluctuations during osteoblast differentiation, as potential candidates for regulating osteogenesis.

Compliance with ethical standards

Disclosure of conflict of interest

There is no conflict of interest declared by authors in this study.

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