Gene expression profiles for in vitro human stem cell differentiation into osteoblast and osteoclast: a systematic review

Shahrul Hisham Zainal Ariffin [Corresp., Equal first author, 1], **Ker Wei Lim** [Equal first author, 1], **Rohaya Megat Abdul Wahab** [2], **Zaidah Zainal Ariffin** [3], **Rus Dina Rus Din** [4], **Muhammad Ashraf Shahidan** [1], **Anis Nabilah Johari** [1], **Intan Zarina Zainol Abidin** [Corresp. 5]

[1] Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia

[2] Centre of Family Dental Health, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Wilayah Persekutuan Kuala Lumpur, Malaysia

[3] School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia

[4] Forensic Science Programme, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia

[5] Centre for Research and Graduate Studies, University of Cyberjaya, Cyberjaya, Selangor, Malaysia

Corresponding Authors: Shahrul Hisham Zainal Ariffin, Intan Zarina Zainol Abidin
Email address: shahroy8@gmail.com, izzarina7@gmail.com

Background: The potential of stem cells in regenerative medicine has shown promising results in today's scientific research. However, the vast variety of choices of techniques and the lack of a standard approach in analyzing human osteoblast and osteoclast differentiation may lower the efficiency of understanding stem cells as a tool in medical applications. Therefore, this review aims to systematically evaluate the findings based on stem cell differentiation in obtaining a standard analysis method in the gene expression profile approach. **Methods:** A systematic search of the study was conducted, through retrieving of articles in electronic databases, PubMed, and Web of Science (WOS); following article selection and data extraction. The review was performed following PRISMA guideline statements, focused on the gene expression and interaction between cells approaches. **Results:** Six articles were able to be systematically defined, selected, and reviewed based on the inclusion and exclusion criteria. Only original articles of i*n vitro* human stem cell studies on the differentiation of osteoblast and osteoclast cells that involved gene expression profile approach were included in the review; showed that qPCR was the most used technique for gene expression to detect the differentiated human osteoblast and osteoclast cells. A total of 21 genes were found to be involved to identify occurring osteoblast and osteoclast differentiation. **Conclusion:** Qualitative information of genes expression provided by qPCR could become a standard technique to analyze the differentiation of human osteoblast and osteoclast stem cells rather than relative gene expression. Runx2 and CTSK genes can be applied for detecting osteoblast and osteoclast cells, respectively. This review provides better exposure to future researchers on the vast

variety of gene expression approaches in analyzing the differentiation of human osteoblast and osteoclast cells.

Gene Expression Profiles For In Vitro Human Stem Cell Differentiation into Osteoblast and Osteoclast: A Systematic Review

Shahrul Hisham Zainal Ariffin[1*], Ker Wei Lim[1], Rohaya Megat Abdul Wahab[2], Zaidah Zainal Ariffin[3], Rus Dina Rus Din[4], Muhammad Ashraf Shahidan[1], Anis Nabilah Johari[1], Intan Zarina Zainol Abidin[5**]

[1]Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
[2]Centre of Family Dental Health, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz 50300 Kuala Lumpur, Wilayah Persekutuan Kuala Lumpur, Malaysia.
[3]School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia.
[4]Forensic Science Programme, Faculty of Health Sciences, Basement 1, Universiti Kebangsaan Malaysia 43600 Bangi, Selangor, Malaysia.
[5]Centre for Research and Graduate Studies, University of Cyberjaya, 63000 Cyberjaya, Selangor, Malaysia.

[*]First Correspondence to:
Shahrul Hisham Zainal Ariffin
Department of Biological Science and Biotechnology, Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
Telephone number: +60 19-261 8170
Email: hisham@ukm.edu.my or shahroy8@gmail.com

[**]Second Correspondence to:
Intan Zarina Zainol Abidin
[4]Centre for Research and Graduate Studies,
University of Cyberjaya, 63000 Cyberjaya, Selangor, Malaysia.
Telephone number: +60 17-324 8717
Email: izzarina7@gmail.com

Abstract

Background: The potential of stem cells in regenerative medicine has shown promising results in today's scientific research. However, the vast variety of choices of techniques and the lack of a standard approach to analyzing human osteoblast and osteoclast differentiation may lower the efficiency of understanding stem cells as a tool in medical applications. Therefore, this review aims to systematically evaluate the findings based on stem cell

differentiation in obtaining a standard analysis method in the gene expression profile approach.

Methods: A systematic search of the study was conducted, by retrieving articles in electronic databases, PubMed, and Web of Science (WOS); following article selection and data extraction. The review was performed following PRISMA guideline statements, focused on the gene expression and interaction between cells approaches.

Results: Six articles were able to be systematically defined, selected, and reviewed based on the inclusion and exclusion criteria. Only original articles of i*n vitro* human stem cell studies on the differentiation of osteoblast and osteoclast cells that involved gene expression profile approach were included in the review; showed that qPCR was the most used technique for gene expression to detect the differentiated human osteoblast and osteoclast cells. A total of 21 genes were found to be involved to identify occurring osteoblast and osteoclast differentiation.

Conclusion: Qualitative information of genes expression provided by qPCR could become a standard technique to analyze the differentiation of human osteoblast and osteoclast stem cells rather than relative gene expression. Runx2 and CTSK genes can be applied for detecting osteoblast and osteoclast cells, respectively. This review provides better exposure to future researchers on the vast variety of gene expression approaches in analyzing the differentiation of human osteoblast and osteoclast cells.

Keywords: Molecular analysis, Stem cell, Differentiation, Osteoblast, Osteoclast, Human.

Introduction

Bone, one of the hardest tissues in the body serves three important functions; provide mechanical support, act as a shield to internal organs and carry out metabolic processes such as providing storage for minerals and hematopoiesis (Ansari, Ito & Hofmann 2021; Konukoğlu 2019). As bone tissues are of paramount importance to the human body, the bones must be replenished continuously to retain their strength and structural integrity. This process is known as bone remodelling, which involves two main mechanisms, i.e., bone matrix formation and resorption. Osteoblasts are responsible for bone formation while osteoclasts are involved in bone resorption (Konukoğlu 2019; Phan, Xu & Zheng 2004). Faulty regulation of these two mechanisms will disrupt the bone remodelling cycle making them potential targets for pharmacological interventions in disease states such as osteoporosis (Kenkre & Bassett 2018).

Stem cells have currently shown a positive outlook in tissue regeneration and have been considered to be applied in the medical fields, such as repairing defective tissues and organs including bone tissues. Several types of isolated mesenchymal stem cells (MSC) isolated from various organ stem cells are suggested as a source of osteoblast progenitors, such as dental pulp tissues (Koh et al. 2021; Shahrul et al. 2016) and peripheral blood (Shahrul et al. 2010; Shahrul Hisham et al. 2019). Osteoblast and osteoclast were the main cells that exist in the organic phase of bone tissue (Iaquinta et al. 2019).

Osteoblast cells are derived from the differentiation of MSC that has been induced by regulatory factors such as bone morphogenic proteins (BMP). Osteoblasts produce bone matrix proteins wherein type 1 collagen is the most abundant extracellular protein of bone and is responsible for tissue mineralization. Therefore, human osteoblast differentiation was observed through the expression of various kinds of bone-related extracellular matrix proteins, such as type 1 collagen (*Col1)*, osteocalcin (*OCN*), osteopontin (*OPN*), and bone sialoprotein (BSP). In addition, an increase in alkaline phosphatase (*ALP*) enzymatic profile activity is believed to be the major contributor to its characteristics (Intan et al. 2010; Katagiri & Takahashi 2002).

Osteoclasts are formed via the fusion of monocyte lineage cells, activating bone-resorption osteoclast cells. Myriads of factors such as cytokines, signaling molecules, and transcription factors aid osteoclast differentiation especially macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL), which were produced by osteoblasts, are crucial to activate the differentiation of osteoclast cells. The survival of osteoclasts is maintained through the binding of RANKL to the nuclear factor-κB receptor which induces the formation of multinuclear osteoclasts (Kang et al. 2014).

Osteoblast and osteoclast differentiation can be observed using various gene expression profile approaches. Gene expression profiling is the study of the pattern of gene expression at the gene transcription level. The expression of genes that contain biological information about the organisms resulted in the synthesis of ribonucleic acid (RNA) followed by the synthesis of protein molecules (Brown 2012). Hence, the analysis of gene expression can be directly correlated to the end products of the genes. These analyses enable researchers to understand the process, development, behavior of the cells, and interaction among cells.

In recent studies, many known methodologies have proven to be able to analyze the differentiation of human osteoblastic and osteoclastic cells from stem cells by using gene expression approaches. However, there is currently no establishment of standard analysis methods given the vast variety of molecular techniques used to analyze the differentiation of human osteoblast and osteoclast stem. Hence, this systematic review aims to collect and evaluate the findings of each study. This systematic review is able to obtain the best gene expression marker and analyzing technique during human osteoblast and osteoclast differentiation.

Materials and Methods

This systematic review was performed following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statements (Page et al. 2021). The PICOS question was aligned as follows: “Amongst different methodologies and techniques and genes available, which are the most suitable techniques and genes to be suggested as standard analysis in detecting the gene expression of human osteoblast and osteoclast cells from the differentiation of stem cells?” Two independent observers (Lim, K.W. and Anis, N.J.) performed the searches and the evaluation of the articles to determine

the eligibility of the articles while another observer (Shahrul, H.Z.A.) was included to sort out any discrepancies from the stated methods.

Data Search

The studies included in this systematic review were retrieved from PubMed and Web of Science (WOS) databases. Independent keywords together with their combinations were applied to the search engines of these databases. A detailed customized search strategy was established for each electronic database as shown in *Table 1*. All data gathered such as the title, abstract, authors' names and affiliations, journal name, and year of publication were then exported to a *Microsoft Excel* spreadsheet. The two observers then screened the title and abstract, followed by eligibility assessment independently. During this phase, disagreements between observers were discussed and resolved by consensus. If no agreement could be reached, a third observer (Shahrul, Z.A.) would have been taken consideration.

Selection Criteria

Inclusion and exclusion criteria were aligned and followed. Only original articles that were published in the English language from the year 2016 to 2022 were included while review articles and duplicated articles from other databases were excluded from this review. For this systematic review, *in vitro* studies involving the potential of only human stem cells to differentiate into osteoblasts and osteoclasts were included whereas studies using any cell lines or primary cultures from animals were excluded. *In vivo* studies were also not considered in this review as well. Studies that had combinations of both animal and human cultures were included but only considered the section on human studies.

Data Extraction and Screening Process

Data screening was performed, and final inclusion and exclusion criteria were decided as stated. An author independently reviewed the full texts of each study retrieved from the databases that were considered eligible for inclusion. The data extraction process was conducted following the PRISMA guidelines (Page et al. 2021). The data extracted included the following: study characteristics (first author, year of publication, language study design), organism and cell lines, and the methods on gene expression profiles. The duplication of articles from other publications was removed. The following screening was done to sort out any review articles and articles published other than in the English language. Next, studies performed without utilizing human stem cells and did not match the parameters of osteoblastic and osteoclastic differentiation were also removed. All the remaining articles were then thoroughly screened for their eligibility.

Risk of Bias Assessment

The quality of methodology of the included studies was evaluated by an author (Lim, K.W.) following the "Modified CONSORT checklist of items for reporting *in vitro* studies" with

slight modifications to fit the study. The main domains were as listed as follows: 1) Structured summary in abstract, 2) Specific objectives or hypothesis, 3) Study population, 4) Further description of interventions, 5) Primary and secondary outcome, 6) Results, 7) Limitation, 8) Sources of funding, 9) Availability of protocol. Disagreements between reviewers were resolved after discussion. Each criterion was marked as follows: present (+), absent (-), unclear (?), not stated (/), or not applicable (NA).

Results

Data Extraction Results

Searches with keywords related to stem cell osteogenic differentiation (*Table 1*) made in electronic databases PubMed and Web of Science (WOS) produced a total of 52 articles altogether which comprise 17 results from PubMed and 35 results from Web of Science. Every potential article was assessed independently based on the inclusion and exclusion criteria. After removing five duplicates between the two databases, 47 articles remained. A review article was also excluded. A single article in Japanese and a single article in German were further excluded followed by 12 articles that are not relevant to human stem cells and 26 articles which did not match the parameter of human study and were also excluded. After a thorough screening, a total of six articles were eligible for qualitative synthesis for this current systematic review. *Fig.1* shows the flowchart of the articles selection process.

Study Design

A total of six gene expression profile approach articles were published between 2016 and 2022. The six articles were screened and eligible for this review. In this systematic observation, four studies focused on the differentiation of osteoblast, and another two focused on the combination of both osteoblast and osteoclast differentiation. However, no studies focused specifically on the differentiation of osteoclast. All six studies were based on i*n vitro* studies. The cultured cells used in the *in vitro* studies include hematopoietic stem cells (HSC), CD34+ peripheral blood stem cells (Srikanth et al. 2016), and human mesenchymal stem cells (hMSC) (Bradamante et al. 2018; Höner et al. 2018; Xie et al. 2021; Xu et al. 2018). A single study includes more than one cultured cell which is hMSC and human blood peripheral mononuclear cells (hBPMC) (Höner et al. 2018).

Risk of Bias Assessment

The results from the risk of bias assessment are tabulated (*Table 2*). The included studies presented an abstract with a brief rationale and clear objective or hypotheses and introduction. Study populations and results were also presented in the included studies. 3 studies did not state the limitation of the studies while 4 studies did not state the funding.

Gene Expression Profile of Osteoblasts and Osteoclasts

The methods used for analyzing the differentiation of osteoblast and osteoclast focus on the expression of genes using quantitative polymerase chain reaction (qPCR) (Hashimoto et al. 2018; Höner et al. 2018; Srikanth et al. 2016; Xie et al. 2021; Xu et al. 2018) microarray analysis, and Next Generation Sequencing (NGS) (Bradamante et al. 2018). Certain gene expression profiles irrespective of the variants of cells and methodologies were detected through differentiation of osteoblast and osteoclast cells. Some genes were expressed during both osteoblastic and osteoclastic differentiation and some genes were specifically expressed either during osteoblasts or osteoclasts differentiation.

The genes that were expressed during the differentiation of both osteoblast and osteoclast was nuclear factor κB ligand (*RANKL*). Meanwhile, runt-related transcription factor 2 *(Runx2*), osterix, osteonectin (*SPARC*), miR-142-5p, collagen type 1 *(COL1a*), bone sialoprotein (*BSP*), osteopontin (*OPN*), miR-139-5p, alkaline phosphatase (*ALP*), osteoprotegerin (*OPG*), osteocalcin (*OCN*), Chordin Like 1 (*CHRDL1*), secreted phosphoprotein 1 (*SPP1*), lamin A/C (*LMNA*), *CYP19A1*, *CYP24A1*, *HSD11B1*, *AKR1B1*, *MIRLET7A12*, miR-940, and *Four and a half LIM domains 2 (FHL2* gene) were only expressed specifically during osteoblast differentiation. Cathepsin K (*CTSK*), *Notch1*, *Hes1,* and *Hey1* were expressed specifically during osteoclast differentiation. *Table 3* shows the articles involved in gene expression, techniques, and the type of stem cells applied which indicate the presence of osteoblast and osteoclast differentiation whilst *Table 4* presents the gene markers used by various studies.

Upregulation of markers or the increase in transcription of genes involved *Runx2*, *Osterix, RANKL, SPARC, OCN, CHRDL1, SPP1, CYP19A1, CYA24A1, HSD11B1, AKR1B1, MIRLET7A2, LMNA, BSP, COLA1, Col1, OPN, OPG,* hsa-miR-940*, ALP*, and *FHL2* gene showed osteoblasts differentiation. The *OPG* gene was highly expressed in osteoblastic differentiation and halted osteoclastic differentiation. *MIR31* was the only marker that was downregulated to promote osteoblasts differentiation.

The *CTSK* gene was upregulated showing osteoclast differentiation. Genes of the Notch signaling mechanism; *Notch1, Hey1,* and *Hes1* showed downstream regulation resulting in suppressed osteoblast differentiation. Genes such as *COL11A1, COL12A1, ITGA11, and THBS1* were downregulated showing the occurrence of osteoclast differentiation.

Discussion

Stem Cells Analysis

The cell type that had been used was mostly human mesenchymal stem cell (hMSC) (Bradamante et al. 2018; Hashimoto et al. 2018; Höner et al. 2018; Srikanth et al. 2016; Xu et al. 2018). On the other hand, hMSC isolated from bone marrow, i.e., bone marrow-derived mesenchymal stem cells (hBMSC) (Bradamante et al. 2018; Xie et al. 2021) were the most used cell source, followed by hMSC cell line from the human umbilical cord (Hashimoto et al. 2018), primary culture of hMSC from femoral head (Höner et al. 2018) and primary culture of hMSC from blood peripheral monocytes (Xu et al. 2018).

The properties of hBMSC such as ease of isolation from bone marrow without causing an immunological problem and the ability to reach confluence in a short period make them the best model for *in vitro* osteogenic differentiation studies (Bhat et al. 2021; Ouryazdanpanah et al. 2018). Studies by Ansari et al. (2021) have shown rapid osteogenic differentiation under biochemical and/or mechanical stimuli will significantly increase the gene expression specific to osteoblast differentiation (Ansari, Ito & Hofmann 2021). The other type of adult stem cell variant obtained from the observation is hematopoietic stem cells (HSC) (Srikanth et al. 2016).

Hematopoietic stem cells on the other hand are the most thoroughly characterized tissue-specific stem cells that possess potential in regenerative medicine (Zakrzewski et al. 2019). Monocytes derived from HSC which comprise 10-20% of peripheral blood have been used during *in vitro* studies as osteoclast precursor cells. It is also shown that HSC and monocytes can be isolated and purified based on the expression of their specific surface markers such as CD34 and CD14. However, unlike MSC, the cell isolation procedures that involved HSC are time-consuming and might lead to a low number of cells obtained, resulting in a larger volume of peripheral blood needed (Ansari, Ito & Hofmann 2021).

Gene Expression Profile

Genes will be upregulated and downregulated during cell-specific differentiation. These gene expressions can be detected by quantitative polymerase chain reaction (qPCR) and DNA microarray analysis. qPCR can detect a single expressed gene on the other hand DNA microarray detects multiple gene expressions at once, enabling efficient evaluation of cell-specific differentiation (Nguyen, Nag & Wu 2010). However, most of the studies used qPCR as compared to DNA microarray analysis to detect differentiation of osteoblasts and osteoclasts because it provides quantitative information which only shows relative gene expression. In addition, DNA microarray analysis requires a large amount of RNA to produce a result or outcome hence PCR amplification may be needed to amplify the amount of RNA, which may result in losing the RNA samples' integrity. Therefore, qPCR is the most chosen technique to be used. Only a single study uses next-generation sequencing (NGS) which generates more complete and accurate data than other conventional profiling techniques. These techniques allow researchers to detect multiple genes and reveal new transcripts (Bisgin et al. 2018) and are not suitable for rapid detection.

Osteoblastic and Osteoclastic Differentiation

RANKL stimulates osteoclast formation and activity which induces the expression of *RANKL* by osteoblastic stromal cells (Konukoğlu 2019; Mohammad et al. 2020). *RANKL* together with its receptor, *RANK* is essential for bone remodelling. *RANKL* is highly expressed in osteoblasts while it also serves its importance in osteoclastogenesis in which dysregulation of *RANKL* signaling may impair bone resorption (Ono et al. 2020). Osteoblasts regulate bone resorption through *RANKL* expression (Konukoğlu 2019). *RANKL,* part of the *RANKL/RANK/OPG* signaling pathway secreted by osteoblast embedded to its receptor (*RANK)* on osteoclasts and

increases osteoclastic differentiation resulting in bone resorption and bone loss (*Fig.2*) (Roumeliotis et al. 2020). Osteoprotegerin (*OPG)* on the other hand, could bind to *RANKL* inhibiting osteoclastogenesis (Mohammad et al. 2020).

Osteoblastic Differentiation

The gene markers that are most frequently used within the articles to detect osteoblastic differentiation are *Runx2* (Bradamante et al. 2018; Höner et al. 2018; Srikanth et al. 2016; Xie et al. 2021; Xu et al. 2018) and *Col1a* (Bradamante et al. 2018; Höner et al. 2018; Xie et al. 2021; Xu et al. 2018). A Runt-related transcription factor was the master transcription factor that communicates with the target genes via promoters which are facilitated by its Runt domain. Positive and negative regulation of *Runx2* was crucial in the phenomena of bone formation (Narayanan et al. 2019). Runx2 protein is first detected in pre-osteoblasts, and the expression is upregulated in immature osteoblasts but downregulated in mature osteoblasts (*Fig.3*). *Runx2* was the first transcription factor required for the determination of the osteoblast lineage during the differentiation of multipotent mesenchymal stem cells into immature osteoblasts (Komori 2009). *Runx2* is one of the members of the Runx family that have the common evolution-conserved domain which serves to communicate with the promoters of its target genes. *Runx2* is attributed to the structure of the protein encoded by its mRNA sequence. The domains or sites of *Runx2* facilitate bone remodelling as each distinctive function is based on its interaction with proteins and DNA sequences (Narayanan et al. 2019). *Runx2* gene encodes multiple transcripts which are derived from two promoters (P1 & P2) and alternative splicing. P1 (distal) and P2 (proximal) are the two promoters that initiate expression of the major *Runx2* isoforms, type II and type I respectively. The double promoter maintains the conservative of its structure in both human and murine *Runx2* genes. *Runx2-I* is expressed by osteoblast at consistent levels throughout osteoblast differentiation while *Runx2-II* expression is increased during osteoblast differentiation under the induction BMP (Schroeder, Jensen & Westendorf 2005).

Type 1 collagen is a bone matrix protein that facilitates morphological changes and transformation of pre-osteoblasts into mature osteoblasts which also serves as an early marker for osteoblasts (*Fig.3*) (Narayanan et al. 2019). Collagen is a triple helical structure in which the procollagen forms the first helical structure during collagen synthesis. Protease removed the amino and carboxyl ends of the molecule, forming tropocollagen followed by cross-links. PYD and DYP cross-links are cross-linking collagen polypeptides that maintain and stabilize the collagen, providing mechanical support for type 1 collagen. These cross-linkages affect the differentiation of osteoblasts. DYP is shown to be a more specific and sensitive marker as it is found specifically in bones and dentin (Konukoğlu 2019).

Some osteoblastic markers such as *RANKL* and osteonectin (*SPARC)* (Roumeliotis et al. 2020) projected high expression only at the later stages of osteoblast differentiation. *SPARC* regulates extracellular matrix (ECM) assembly and the formation of matrix metalloproteinases (MMPs) and collagen and is needed for fibronectin-induced integrin-linked kinase activation as ECM development needs an organized fibronectin matrix (Purnachandra Nagaraju et al. 2014).

Four and a half LIM domains 2 protein encoded by the FHL2 gene interacts with integrins and transcription factors, to control osteoblast differentiation. Overexpression of the FHL2 gene rapidly differentiates stem cells into osteoblast and increases the expression of osteoblast markers whilst knocking out the FHL2 gene showed downregulation of osteoblast markers (Lai et al. 2006; Xie et al. 2021).

The other markers that also showed upregulation during osteoblastic differentiation were *Osterix*, which is an osteoblast-specific transcription factor required for osteoblast differentiation and bone formation (*Fig.3*). *Osterix* is considered a major effector in skeletal formation, *Osterix* interacts with the nuclear factor of activated T (NFAT) cells which then form a complex that improves osteoblastic bone formation via the activation of *Col1* α1 promoter (Han et al. 2016).

Other than that, osteocalcin (*OCN*) which is commonly used as a serum marker for osteoblastic bone formation is synthesized by the induction of vitamin D. The *OCN* serum gives an important indication of high osteoblastic activity as it is secreted by mature osteoblasts (*Fig.3*). Most of this synthesized calcium-binding peptide enters the bone matrix, but a small amount is released into the bloodstream. During synthesis, vitamin dependent-dependent carboxylation happens in specific glutamate residues of molecules, allowing them to bind to calcium after posttranslational modifications. *OCN* is rather unstable in the analysis as it provides minimal and limited information on bone metabolism given a high degree of biological variation (Konukoğlu 2019).

Alkaline phosphatase (*ALP*) (Hashimoto et al. 2018; Höner et al. 2018) is an early marker for osteoblastic differentiation produced by osteoblasts and its elevated level is positively correlated with bone formation rate (*Fig.3*). *ALP* increases the inorganic phosphate local rates and aids in mineralization all the while reducing extracellular pyrophosphate, which is an inhibitor of mineral formation (Vimalraj 2020).

Osteopontin (*OPN*) and bone sialoprotein (*BSP*) are co-expressed in osteoblasts and osteoclasts, which promote adhesion of the cells to the bone matrix through RGD (Arg-Glu-Asp) cell adhesion sequence. *OPN* is an acidic molecule in which the central section of the molecule consists of sequences that communicate and interact with seven integrins. *OPN* is a crucial factor in bone remodelling and settling osteoclasts to the bone matrix (Zhao et al. 2018). On the other hand, *BSP* which is also highly negatively charged can isolate the calcium ions while conserving polyglutamate regions with hydroxyapatite crystal nucleation potential. Through the RGD cell adhesion motif sequence, *BSP* allows the attachment and activation of osteoclasts (Huang et al. 2005).

Osteoprotegerin (*OPG*) (Iaquinta et al. 2019) is a decoy receptor for *RANKL*. It is secreted by osteoblast in which the inhibition of osteoclastic differentiation occurs via the binding of *OPG* to *RANKL* and this process will block the interaction between *RANK/RANKL* (Kenkre & Bassett 2018). However, in Kang et al. (2014), *OPG* expression which was rarely reported for osteoclastogenesis was detected in osteoclasts that were involved during alveolar bone

resorption. *OPG* expression is shown to play a role in autoregulation in the later phase of osteoclastogenesis (Kang et al. 2014).

Genes that are responsible for the pathway of *Metabolism of Steroid Hormones and Vitamins A and D* (*CYP19A1, CYA24A1, HSD11B1, AKR1B1*) and *Integrin Cell Surface Interactions* pathways (*CHRDL1, SPP1, CYP19A1, CYA24A1, HSD11B1*) were upregulated, favouring osteoblastogenesis [4]. Overexpression of *CYP24A1* which is an enzyme that regulates the levels of vitamin D content aids in osteoblastogenesis as vitamin D3 induces osteoblastic differentiation.

Several studies have shown the role of microRNAs in bone turnover, such as miR-940, which significantly promoted osteoblastic differentiation during *in vitro* analysis of hMSC (Hashimoto et al. 2018; Konukoğlu 2019). However, the roles of microRNAs are very complicated, and many studies are needed to understand them better. As *Runx2* is the master transcription factor, no bone is formed in the absence of *Runx2*, making *Runx2* the preferred standard marker for osteoblast differentiation.

Osteoclastic Differentiation

The notch signaling pathway is highly conserved, and it regulates the process of cell proliferation and differentiation, determines cell fate, and is involved in cellular processes in adult tissues, including skeletal tissue development and regeneration (Luo et al. 2019). Notch pathway regulates bone marrow mesenchymal progenitors by suppressing osteoblast differentiation and the overexpression of *Notch1* inhibits osteoblastogenesis in stromal cells. Hence, the activation of Notch signaling had a negative effect on osteogenic differentiation. Under the exposure of an osteogenic induction medium, MSC was forced to undergo epigenetic modifications, resulting from the upregulation of miR-139-5p which will inhibit Notch1 mediated signaling activity, triggering osteoclastic differentiation (Xu et al. 2018). However, *Notch1* deletion indirectly promotes osteoclast differentiation through the enhancement of osteoblastic lineage cell-mediated stimulation of osteoclastogenesis (Konukoğlu 2019).

OSCAR, RANK, NFATc, and cathepsin K (*CTSK*) which were not expressed showed no osteoclastic activity as these genes are predominantly expressed by active osteoclasts (Konukoğlu 2019; Srikanth et al. 2016). *CTSK* expression is regulated by the *RANKL-RANK* signaling pathway, which is one of the important pathways for osteoclastogenesis. Activation of this signaling pathway in osteoclast precursors enhances the pro-osteoclastogenesis transcriptional factor, *NFATc1* which allows the initiation of *CTSK* transcription to occur (Fig **2**) (Dai et al. 2020). In addition, an increase in lamin genes (*LMNA*) and upregulation of the *MIRLET7A2* gene enhanced osteogenesis and impaired adipogenesis (Bradamante et al. 2018).

Conclusion

qPCR was the most used technique in recent studies and is suggested as a standard approach for osteoblast and osteoclast detection as it provides qualitative information on the gene expression profiles. *RANKL* has been widely used as an osteogenic marker followed by *CTSK*

as a suggested osteoclast marker and finally both *Runx2* and *Col1a* genes as osteoblast markers. This review has been able to provide useful insights on gene expression profiles to future researchers involved in human osteoblast and/or osteoclast stem cells studies. Identification of these gene markers will assist in future osteogenic research which ultimately generates better therapies and medications.

List of Abbreviations

ALP	Alkaline phosphatase
BMP	Bone morphogenic proteins
BSP	Bone sialoprotein
CHRDL1	Chordin Like 1
CTSK	Cathepsin K
Col1	Type 1 collagen
ECM	Extracellular matrix
FHL2	Four and half LIM domains 2
hBPMC	Human blood peripheral mononuclear cells
hMSC	Human mesenchymal stem cells
HSC	Hematopoietic stem cells
LMNA	Lamin A/C
M-CSF	Macrophage colony-stimulating factor
MMPs	Matrix metalloproteinases
MSC	Mesenchymal stem cell
NFAT	Nuclear factor of activated T
NGS	Next Generation Sequencing
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
qPCR	Quantitative polymerase chain reaction
RANKL	Nuclear factor-κB ligand
RNA	Ribonucleic acid
SPARC	Osteonectin
SPP1	Secreted phosphprotein 1

REFERENCES

Ansari, S., Ito, K. & Hofmann, S. 2021. Cell Sources for Human In vitro Bone Models. *Current Osteoporosis Reports* 19(1): 88–100.

Bhat, S., Viswanathan, P., Chandanala, S., Prasanna, S.J. & Seetharam, R.N. 2021. Expansion and characterization of bone marrow derived human mesenchymal stromal cells in serum-

434 free conditions. *Scientific Reports* 11(1): 1–18.

435 Bisgin, H., Gong, B., Wang, Y. & Tong, W. 2018. Evaluation of bioinformatics approaches for
436 next-generation sequencing analysis of microRNAs with a toxicogenomics study design.
437 *Frontiers in Genetics* 9(FEB): 1–12.

438 Bradamante, S., Rivero, D., Barenghi, L., Balsamo, M., Minardi, S.P., Vitali, F. & Cavalieri, D.
439 2018. SCD – Stem Cell Differentiation Toward Osteoblast Onboard the International Space
440 Station. *Microgravity Science and Technology* 30(5): 713–729.

441 Brown, T. 2012. Introduction to Genetics : A Molecular Approach. E. Owen (Pnyt.) hlm. Edisi
442 ke-1. Garland Science, Taylor & Francis Group: New York & London.

443 Dai, R., Wu, Z., Chu, H.Y., Lu, J., Lyu, A., Liu, J. & Zhang, G. 2020. Cathepsin K: The Action
444 in and Beyond Bone. *Frontiers in Cell and Developmental Biology* 8.

445 Han, Y., Kim, C.Y., Cheong, H. & Lee, K.Y. 2016. Osterix represses adipogenesis by negatively
446 regulating PPARγ transcriptional activity. *Scientific Reports* 6(1): 35655.

447 Hashimoto, K., Ochi, H., Sunamura, S., Kosaka, N., Mabuchi, Y., Fukuda, T., Yao, K., Kanda,
448 H., Ae, K., Okawa, A., Akazawa, C., Ochiya, T., Futakuchi, M., Takeda, S. & Sato, S.
449 2018. Cancer-secreted hsa-miR-940 induces an osteoblastic phenotype in the bone
450 metastatic microenvironment via targeting ARHGAP1 and FAM134A. *Proceedings of the*
451 *National Academy of Sciences of the United States of America* 115(9): 2204–2209.

452 Höner, M., Lauria, I., Dabhi, C., Kant, S., Leube, R.E. & Fischer, H. 2018. Periodic
453 microstructures on bioactive glass surfaces enhance osteogenic differentiation of human
454 mesenchymal stromal cells and promote osteoclastogenesis in vitro. *Journal of biomedical*
455 *materials research. Part A* 106(7): 1965–1978.

456 Huang, W.-C., Xie, Z., Konaka, H., Sodek, J., Zhau, H.E. & Chung, L.W.K. 2005. Human
457 Osteocalcin and Bone Sialoprotein Mediating Osteomimicry of Prostate Cancer Cells: Role
458 of cAMP-Dependent Protein Kinase A Signaling Pathway. *Cancer Research* 65(6): 2303–
459 2313.

460 Iaquinta, M.R., Mazzoni, E., Bononi, I., Rotondo, J.C., Mazziotta, C., Montesi, M., Sprio, S.,
461 Tampieri, A., Tognon, M. & Martini, F. 2019. Adult Stem Cells for Bone Regeneration and
462 Repair. *Frontiers in Cell and Developmental Biology* 7(November): 1–15.

463 Intan, Z.A., Shahrul, H.Z.A., Zaidah, Z.A. & Rohaya, M.A.W. 2010. Keupayaan pembezaan tiga
464 Jenis sel primitif daripada hasil perbezaan tempoh proliferasi darah mencit. *Sains*
465 *Malaysiana* 39(2): 305–313.

466 Kang, J.H., Ko, H.M., Moon, J.S., Yoo, H.I., Jung, J.Y., Kim, M.S., Koh, J.T., Kim, W.J. &
467 Kim, S.H. 2014. Osteoprotegerin expressed by osteoclasts: An autoregulator of
468 osteoclastogenesis. *Journal of Dental Research* 93(11): 1116–1123.

469 Katagiri, T. & Takahashi, N. 2002. Regulatory mechanisms of osteoblast and osteoclast

differentiation. *Oral Diseases* 8: 47–159.

Kenkre, J.S. & Bassett, J.H.D. 2018. The bone remodelling cycle. *Annals of Clinical Biochemistry* 55(3): 308–327.

Koh, B., Sulaiman, N., Ismadi, S.N.S.W., Ramli, R., Yunus, S.S.M., Idrus, R.B.H., Ariffin, S.H.Z., Wahab, R.M.A. & Yazid, M.D. 2021. Mesenchymal stem cells: A comprehensive methods for odontoblastic induction. *Biological Procedures Online* 23(1): 18.

Komori, T. 2009. Regulation of Osteoblast Differentiation by Runx2. Dlm. Choi, Y. (Editor). *Osteoimmunology*, pg. 43–49. Springer US: Boston, MA.

Konukoğlu, D. 2019. Bone markers. *International Journal of Medical Biochemistry* 2(2): 65–78.

Lai, C.-F., Bai, S., Uthgenannt, B.A., Halstead, L.R., Mcloughlin, P., Schafer, B.W., Chu, P.-H., Chen, J., Otey, C.A., Cao, X. & Cheng, S.-L. 2006. Four and Half Lim Protein 2 (FHL2) Stimulates Osteoblast Differentiation. *Journal of Bone and Mineral Research* 21(1): 17–28.

Luo, Z., Shang, X., Zhang, H., Wang, G., Massey, P.A., Barton, S.R., Kevil, C.G. & Dong, Y. 2019. Notch Signaling in Osteogenesis, Osteoclastogenesis, and Angiogenesis. *The American Journal of Pathology* 189(8): 1495–1500.

Mohammad, T., Mohammed, M.H., Negin, A. & Sadegh, J. 2020. RANKL/RANK/OPG Pathway: A Mechanism Involved in Exercise-Induced Bone Remodeling. *BioMed Research International* 2020: 1–11.

Narayanan, A., Srinaath, N., Rohini, M. & Selvamurugan, N. 2019. Regulation of Runx2 by MicroRNAs in osteoblast differentiation. *Life Sciences* 232: 1–9.

Nguyen, P.K., Nag, D. & Wu, J.C. 2010. Methods to assess stem cell lineage, fate and function. *Advanced Drug Delivery Reviews* 62(12): 1175–1186.

Ono, T., Hayashi, M., Sasaki, F. & Nakashima, T. 2020. RANKL biology: bone metabolism, the immune system, and beyond. *Inflammation and Regeneration* 40(2).

Ouryazdanpanah, N., Dabiri, S., Derakhshani, A., Vahidi, R. & Farsinejad, A. 2018. Peripheral Blood-Derived Mesenchymal Stem Cells: Growth Factor-Free Isolation, Molecular Characterization and Differentiation. *Iranian journal of pathology* 13(4): 461–466.

Page, M.J., McKenzie, J.E., Bossuyt, P.M., Boutron, I., Hoffmann, T.C., Mulrow, C.D., Shamseer, L., Tetzlaff, J.M., Akl, E.A., Brennan, S.E., Chou, R., Glanville, J., Grimshaw, J.M., Hróbjartsson, A., Lalu, M.M., Li, T., Loder, E.W., Mayo-Wilson, E., McDonald, S., McGuinness, L.A., Stewart, L.A., Thomas, J., Tricco, A.C., Welch, V.A., Whiting, P. & Moher, D. 2021. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ* 372(71).

Phan, T.C.A., Xu, J. & Zheng, M.H. 2004. Interaction between osteoblast and osteoclast: impact in bone disease. *Histology and histopathology* 19(4): 1325–1344.

505 Purnachandra Nagaraju, G., Dontula, R., El-Rayes, B.F. & Lakka, S.S. 2014. Molecular
506 mechanisms underlying the divergent roles of SPARC in human carcinogenesis.
507 *Carcinogenesis* 35(5): 967–973.

508 Roumeliotis, S., Roumeliotis, A., Dounousi, E., Eleftheriadis, T. & Liakopoulos, V. 2020.
509 Biomarkers of vascular calcification in serum. *Advances in Clinical Chemistry*, pg. 91–147.
510 Elsevier.:

511 Schroeder, T.M., Jensen, E.D. & Westendorf, J.J. 2005. Runx2: A master organizer of gene
512 transcription in developing and maturing osteoblasts. *Birth Defects Research Part C:*
513 *Embryo Today: Reviews* 75(3): 213–225.

514 Shahrul, H.Z.A., Intan, Z.Z.A., Yazid, M.D. & Wahab, R.M.A. 2010. Differentiation analyses of
515 adult suspension mononucleated peripheral blood cells of Mus musculus. *Cell*
516 *Communication and Signaling* 8.

517 Shahrul, H.Z.A., Nur, A.M.R., Rohaya, M.A.W., Sahidan, S., Intan, Z.Z.A. & Zaidah, Z.A.
518 2016. Analyses of basal media and serum for in vitro expansion of suspension peripheral
519 blood mononucleated stem cell. *Cytotechnology* 68(4): 675–686.

520 Shahrul Hisham, Z.A., Thanaletchumi, M., Intan Zarina, Z.A., Zaidah, Z.A. & Rohaya, M.A.W.
521 2019. Pengkulturan dan Pencirian Penanda Molekul Sel Stem Manusia daripada Pulpa Gigi
522 Susu (SHED) dan Gigi Kekal (DPSC). *Sains Malaysiana* 48(9): 1855–1865.

523 Srikanth, L., Sunitha, M.M., Kumar, P.S., Chandrasekhar, C., Vengamma, B. & Sarma, P.V.G.K.
524 2016. Gel based in vitro 3D model exploring the osteocytic potentiality of human CD34+
525 stem cells. *Molecular Biology Reports* 43(11): 1233–1242.

526 Vimalraj, S. 2020. Alkaline phosphatase: Structure, expression and its function in bone
527 mineralization. *Gene* 754.

528 Xie, Z., Xu, Y., Wei, X., An, G., Hao, M., Yu, Z., Qiu, L., Zheng, Y., Yang, J. & Miao, Y. 2021.
529 Four and a Half LIM Domains Protein 2 Mediates Bortezomib-Induced Osteogenic
530 Differentiation of Mesenchymal Stem Cells in Mult. *Oncol* 11: 729799.

531 Xu, S., Yang, F., Liu, R., Li, X., Fan, H., Liu, J., Wei, S., Chen, G., Chen, J. & Da, Y. 2018.
532 Serum microRNA-139-5p is downregulated in lung cancer patients with lytic bone
533 metastasis. *Oncology Reports* 39(5): 2376–2384.

534 Zakrzewski, W., Dobrzyński, M., Szymonowicz, M. & Rybak, Z. 2019. Stem cells: past, present,
535 and future. *Stem Cell Research & Therapy* 10(1).

536 Zhao, H., Chen, Q., Alam, A., Cui, J., Suen, K.C., Soo, A.P., Eguchi, S., Gu, J. & Ma, D. 2018.
537 The role of osteopontin in the progression of solid organ tumour. *Cell Death & Disease*
538 9(3).

Figure 1

Article selection process.

Article selection process is performed following the exclusion and inclusion criteria.

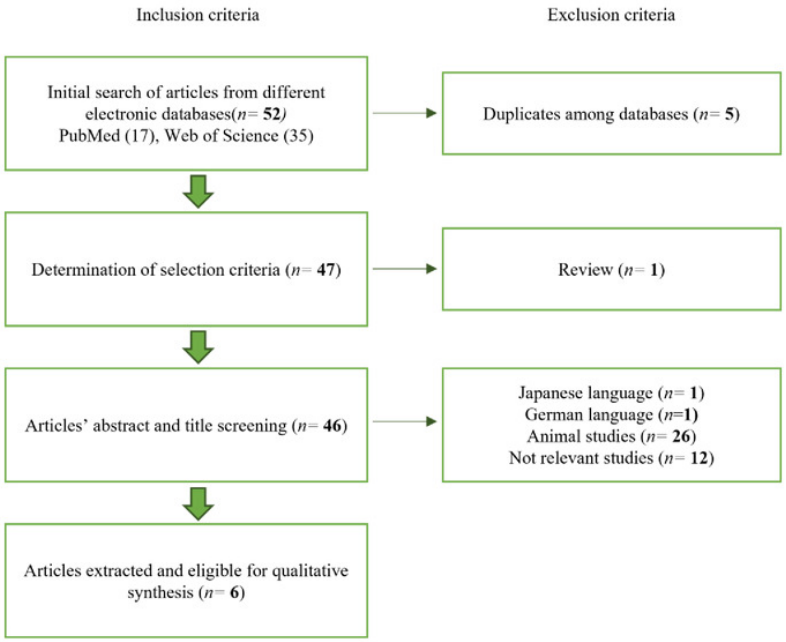

Figure 1: Article selection process.

Article selection process is performed following the exclusion and inclusion criteria.

Figure 2

RANKL/RANK interaction and CTSK expression.

Binding of RANKL, which is secreted by osteoblasts to its receptor, RANK embedded on osteoclasts results in bone resorption and bone loss. RANKL-RANK signalling pathway regulates the expression of CTSK through activation of NFATc1.

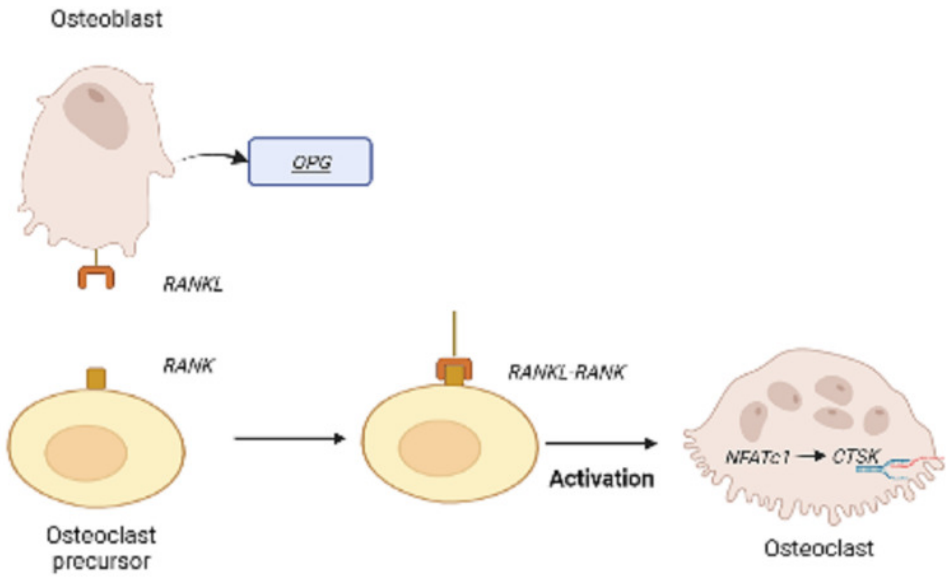

Figure 3

Gene expression profile of the osteoblastic cell.

Runx2 expression is upregulated throughout from osteoprogenitor to osteoblast. However, there is a downregulation in mature osteoblast. *Runx2* activates *osterix* to produce immature osteoblast and both *Runx2* and *osterix* induce osteoblastic cells. *Col1* and *ALP* genes are expressed in osteoprogenitor to immature osteoblast under different conditions. Other genes such as *OPG*, *SPARC*, *OCN*, and *OPN* are secreted and induced mature osteoblastic cells.

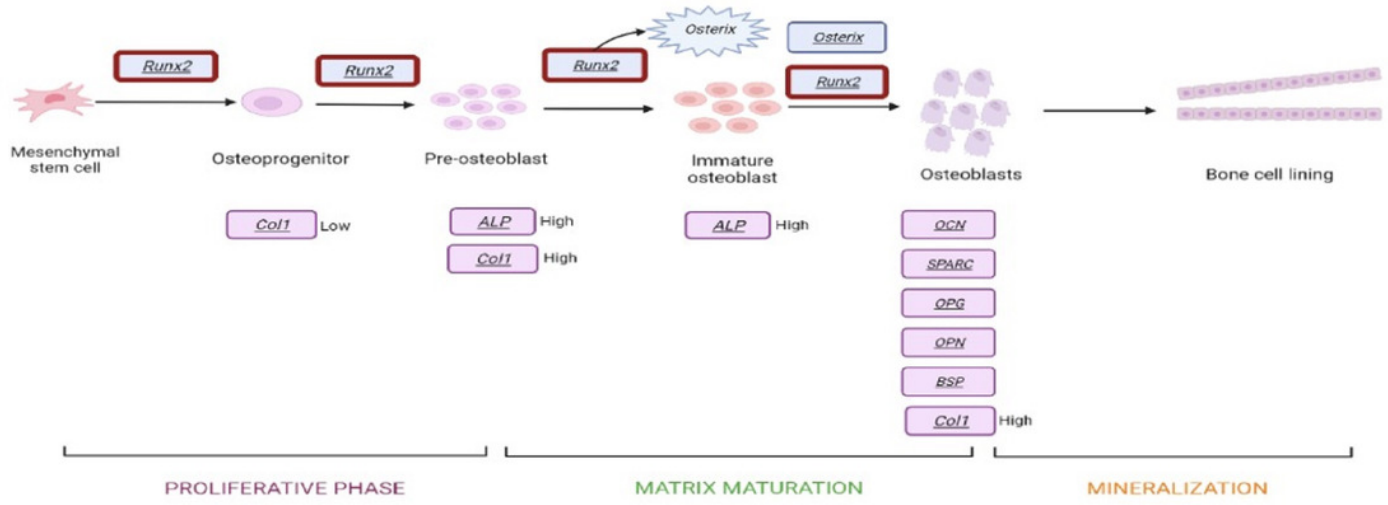

Table 1(on next page)

The combinations of the keywords used in the search.

1 **Table 1: The combinations of the keywords used in the search.**

Database	Search strategy
PubMed	{[((((molecular analysis) AND (stem cell)) AND (differentiation)) AND (osteoblast)] AND (osteoclast)} AND (human)
Web of Science (WOS)	ALL= (molecular analysis AND stem cell AND differentiation AND osteoblast AND osteoclast AND human)

2

Table 2(on next page)

Risk of Bias Assessment.

1 **Table 2: Risk of Bias Assessment.**

Author (year)	1	2	3	4	5	6	7	8	9
Srikanth L., Sunitha, M., Kumar, P. et al. 2016	+	+	+	+	+	+	/	/	+
Bradamante, S., Rivero, D., Barenghi, L. et al. 2018	+	+	+	+	+	+	+	/	+
Xu, S., Yang, F., Liu, R. et al. 2018	+	+	+	+	+	+	+	+	+
Höner, M., Lauria, I., Dabhi, C. et al. 2018	+	+	+	+	+	+	/	/	+
Hashimoto, K., Ochi, H., Sunamura, S. et al. 2018	+	+	+	+	+	+	/	/	+
Xie, Z., Xu, Y., Wei, X. et al. 2021	+	+	+	+	+	+	+	+	+

2

Table 3(on next page)

Gene expression profile approach.

1 **Table 3: Gene expression profile approach.**

Reference	Cell variant	Technique(s) used	Upregulation/ Highly expressed		No expression	Downregulation/ Low expression	
			Osteoblast	**Osteoclast**	**Osteoclast**	**Osteoblast**	**Osteoclast**
Srikanth L., Sunitha, M., Kumar, P. et al. 2016	$CD34^+$ peripheral blood stem cell (HSC)	qPCR	*Runx2, Osterix, RANKL, SPARC*		*RANK, OSCAR, NFATc, CTSK*		
Bradamante, S., Rivero, D., Barenghi, L. et al. 2018	hBMSC	Microarray analysis	*OCN, CHRDL1, SPP1, CYP19A1, AKR1B1, HSD11B1, Runx2, COL1a, MIRLET7A2 , CYP24A1, LMNA*			*MIR31*	
		NGS	*BGLAP, CHRDL1, SPP1, CYP19A1, HSD11B*			*COL11A1, COL12A1, COL4A1, COL4A2, ITGA11, THBS1*	
		miRNA-seq analysis	miR-142-5p				

Xu, S., Yang, F., Liu, R. et al. 2018	Primary culture of hMSC from blood peripheral monocyte	qPCR	*BSP, COLA1, OPN, Runx2, miR-139-5p*		*Notch1, Hey1, Hes1*
Höner, M., Lauria, I., Dabhi, C. et al. 2018	hMSC	qPCR	*Runx2, OPN, Col1, OPG, ALP*		
Hashimoto, K., Ochi, H., Sunamura, S. et al. 2018	hMSC cell line from umbilical cord	qPCR	miR-940, *ALP*	*CTSK*	
Xie, Z., Xu, Y., Wei, X. et al. 2021	hMSC	qPCR	*FHL2, Runx2, ALP, Col1a*		

2

Table 4(on next page)

Summary of the gene markers for osteoblast and osteoclast cells.

1 **Table 4: Summary of the gene markers for osteoblast and osteoclast cells.**

Gene markers	Frequencies used	Indication
Nuclear factor κb ligand (*RANKL*)	1	Osteoblast & osteoclast differentiation
Runt-related transcription factor 2 *(Runx2*)	5	Osteoblast differentiation
Collagen type 1 *(COL1a*)	4	
Alkaline phosphatase (*ALP*)	2	
miR-142-5p	1	
Osterix	1	
Bone sialoprotein (*BSP*)	1	
Osteopontin (*OPN)*	1	
miR-139-5p	1	
Osteonectin (*SPARC*)	1	
Osteoprotegerin (*OPG*)	1	
Osteocalcin (*OCN*)	1	
Chordin like 1 (*CHRDL1*)	1	
Secreted phosphoprotein 1 (*SPP1*)	1	
Lamin A/C (*LMNA*)	1	
Metabolism of Steroid Hormones and Vitamins A and D Pathway *(CYP19A1, CYP24A1, HSD11B1, AKR1B1)*	1	
MIRLET7A12	1	
miR-940	1	
Four and a half LIM domains 2 *(FHL2* gene)	1	
Cathepsin K (*CTSK*)	2	Osteoclast differentiation
Notch signalling pathway *(Notch1*, *Hes1, Hey1)*	1	

2

3

4