Original Article

EFFECT OF EQUINE XENOGRAFT ON GENE EXPRESSION OF DENTAL PULP STEM CELLS IN VITRO

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ABSTRACT

Bone grafting is a surgical procedure used to repair complex bone deficit. The damaged bone is replaced with a graft that supports bone regeneration and healing of bone defects. This technique is vital in orthopedics, dentistry, and trauma surgery, addressing the conditions that traditional methods cannot effectively treat. Bone grafts can originate from various sources, including the patient's body, donors, or synthetic materials, each with distinct advantages and challenges. In this study, we cultured dental pulp stem cells to determine whether equine xenograft (EQX) bones can promote osteoblast differentiation. The gene expression of a panel of 15 differentiation markers was analyzed at two-time points. After 24 h of treatment, FOSL1, SPP1, and MMP14 expression were upregulated. After four days, increased expression of SP7, COL1A1, and COL4A1 was observed. This study indicates that EQX could be considered a material that favors bone regeneration by promoting osteoblast differentiation.

KEYWORDS: *graft, bone, osteoblasts, stem, expression*

INTRODUCTION

Bone grafting is a surgical procedure that replaces missing or damaged bone to repair complex bone fractures, support bone regeneration, and facilitate the healing of bone defects (1). This technique is fundamental in orthopedics, dentistry, and trauma surgery, addressing conditions that traditional methods cannot effectively treat. Bone grafts facilitate healing through three primary mechanisms: osteogenesis, i.e., the process of new bone formation by osteoblasts contained within the graft material; osteoinduction, i.e., the recruitment and differentiation of progenitor cells into osteoblasts, promoted by growth factors such as bone morphogenetic proteins or osteoinductive materials, and osteoconduction, i.e., the process where bone tissue grows onto a surface, providing a scaffold for new bone formation.

Osteoconductive materials can act as a framework for bone ingrowth and support the migration and proliferation of bone-forming cells, facilitating bone regeneration.

Bone grafts can originate from various sources, including the patient's body, donors, or synthetic materials, each with distinct advantages and challenges (2). Autografts involve the patient's bone, typically harvested from the iliac crest, tibia, or ribs. This method is considered the gold standard due to its biocompatibility, osteogenic potential, and minimal

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risk of immune rejection. Autografts support bone healing through osteogenesis, osteoinduction, and osteoconduction. However, drawbacks include limited availability, potential donor site morbidity, and increased surgical time.

Allografts use processed bone derived from cadaveric donors to ensure sterility and reduce immunogenicity. These grafts provide a structural framework for new bone growth and can be stored in tissue banks for future use. Despite their convenience and availability, allografts carry risks of disease transmission and immune response and lack osteogenic properties, relying solely on osteoconductive and osteoinductive capabilities. Xenografts use bone from other species, commonly bovine. These grafts undergo extensive processing to remove all cellular components and reduce immunogenicity, leaving behind a mineralized matrix that supports bone ingrowth. Xenografts are readily available and avoid donor site complications (2). Synthetic bone substitutes include hydroxyapatite, tricalcium phosphate, and bioactive glass (3). These grafts are designed to mimic natural bone's physical and chemical properties, promoting osteoconduction and, in some cases, osteoinduction.

Synthetic materials eliminate risks of disease transmission and donor site morbidity, offering a customizable and readily available alternative.

In the present investigation, an in vitro model was used to test the effect of a xenograft material on undifferentiated cells. Osteoplant (Bioteck SRL, Vicenza, Italy) is an equine xenograft (EQX) of cortical and spongy bone tissue used to fill bone defects in orthopedic, maxillofacial, and oral surgery (4-6). To verify how EQX acts on stem cells to induce bone formation, we treated dental pulp stem cells (DPSCs) with Osteoplant to analyze gene expression.

MATERIALS AND METHODS

Dental Pulp Stem Cells (DPSCs) Isolation

Dental pulp was extracted from the third molars of healthy subjects and digested for 1 h at 37°C in a solution containing 1 mg/ml collagenase type I and 1 mg/ml dispase, dissolved in phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 500 µg/ml clarithromycin. The solution was then filtered using 70 µm Falcon strainers (Sigma Aldrich, St Louis, Mo, U.S.A.) to separate mesenchymal stem cells from fibroblasts. Stem cells were cultivated in α-MEM culture medium (Sigma Aldrich, St Louis, Mo, U.S.A.) supplemented with 20% Fetal Bovine Serum (FBS), 100 µM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma Aldrich, St Louis, Mo, U.S.A.). The flasks were incubated at 37°C and 5% CO2, and the medium was changed twice weekly.

DPSCs were characterized by immunofluorescence for the positive mesenchymal stem cell marker, CD105, CD90, and CD73, and negative marker CD34, as described in Sollazzo et al. (7).

Cell treatment

DPSCs were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The cells were seeded at a concentration of 1.0×10^5 cells/ml with Osteoplant (Bioteck SRL, Vicenza, Italy) at the concentration of 1 mg/ml in 9 cm^2 (3 ml) wells containing DMEM supplemented with 10% serum and antibiotics. Another set of wells containing untreated cells was used as a control. The treatment was carried out at two time points: 24 h and 4 days. At the end of the treatment period, the cells were lysed and processed for total RNA extraction.

RNA isolation, reverse transcription, and quantitative real-time RT-PCR

According to the manufacturer's instructions, total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, Hilden, Germany). The pure RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

cDNA synthesis was performed starting from 500 ng of total RNA using the PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Japan). The reaction mixture was incubated at 37 °C for 15 min and inactivated by heating at 70 °C for 10 s. cDNA was amplified by real-time quantitative PCR using an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). All PCR reactions were performed in a 20 µL volume. Each reaction contained 10 µl of 2x qPCRBIO SYGreen Mix Lo-ROX (PCR Biosystems, Ltd., London, UK), 400 nM of each primer, and cDNA.

Custom primers belonging to the “extracellular matrix, adhesion molecule” pathway, “osteoblast differentiation,” and “inflammation” pathway were purchased from Sigma Aldrich. The selected genes grouped by functional pathways are listed in Table I.

All experiments were performed using non-template controls to exclude reagent contamination. PCR was performed using two analytical replicates. The amplification profile started with 10 min at 95°C, followed by a two-step

amplification for 15” at 95°C and 60” at 60°C for 40 cycles. In the final step, a melting curve dissociation analysis was performed.

Table I. *Selected genes used in Real-Time PCR grouped by functional pathway.*

Pathway	**Gene**
Osteoblast differentiation	RUNX2 (Runt-related transcription factor 2) ALP (Alkaline phosphatase) FOSL1 (FOS-like antigen 1) SP7 (Osterix) ENG (Endoglin) SPP1 (Osteopontin) SPARC (Osteonectin
Extracellular matrix, adhesion molecule	COL1A1 (Collagen type I alpha1) COL3A1 (Collagen type III alpha 1) COL4A1 (Collagen type IV alpha 1) MMP VII (Matrix Metallopeptidase 7) MMP XII (Matrix Metallopeptidase 12) MMP XIV (Matrix Metallopeptidase 14)
Inflammation	IL1A (Interleukin 1 Alpha) IL1R (Interleukin 1 Receptor Type 1) IL6 (Interleukin 6)
Reference gene	RPL13 (Ribosomal protein L13)

Statistical analysis

Quantification was performed using the delta-delta Ct method. The gene expression levels were normalized to the expression of the reference gene (RPL13) and expressed as fold changes relative to the expression in untreated cells.

RESULTS

The DPCSs were phenotypically characterized using immunofluorescence. Fig. 1a shows cytoskeletal filaments stained with vimentin. The cell surfaces were positive for mesenchymal stem cell markers CD90 (Fig. 1b) and CD73 (Fig. 1c) and negative for markers of hematopoietic origin CD34 (Fig. 1d).

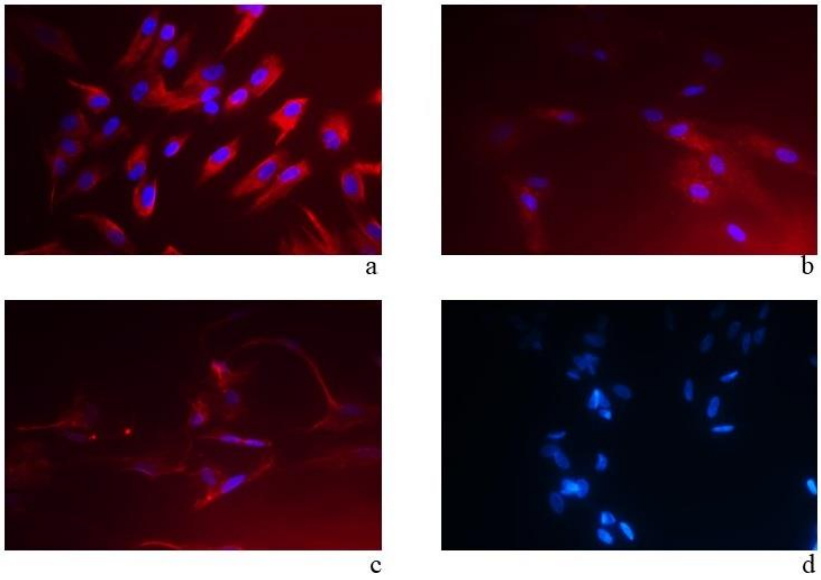

Fig. 1. *DPCSs by indirect immunofluorescence (Rhodamine). Immunofluorescence staining of vimentin (**a**), mesenchymal stem cell marker CD73 (**b**), CD90 (**c**), and hematopoietic markers CD34 (**d**). Nuclei were stained with DAPI. Original magnification x40.*

The effect of EQX treatment in the modulation of gene expression was analyzed by quantitative real-time RT-PCR; specifically, the expression levels of osteoblast-related genes, extracellular matrix, and inflammation pathways were measured. Table II reports the gene expression level variation obtained after 24 h and 4 days of cell treatment.

Following EQX treatment, several genes exhibited a significant increase in expression levels, exceeding a two-fold change compared to the untreated cells (Table II). Specifically, FOSL1, SPP1, and MMP14 were up-regulated shortly after only 24 h of treatment, while SP7, COL1A1, and COL4A1 were up-regulated after 4 days of treatment.

Table II. *Gene expression in ADSCs after 24h and 4 days of treatment. Numbers express the fold changes of the de-regulated genes in treated cells vs. untreated cells. ND – not determined. In bold significant gene expression level.*

Gene	Protein	24 h	4 days
RUNX2	Runt-related transcription factor 2	1.7	1.0
ALP	Alkaline phosphatase	1.7	1.7
FOSL1	FOS-like antigen 1	**2.9**	1.5
SP7	Osterix	0.7	**2.2**
ENG	Endoglin	1.9	1.2
SPP1	Osteopontin	**3.3**	nd
SPARC	Osteonectin	1.6	1.2
COL1A1	Collagen type I alpha1	1.5	**2.4**
COL3A1	Collagen type III alpha 1	1.7	1.2
COL4A1	Collagen type IV alpha 1	1.7	**2.5**
MMP7	Matrix Metalloproteinase 7	0.9	1.7
MMP12	Matrix Metalloproteinase 12	0.1	1.2
MMP14	Matrix Metalloproteinase 14	**5.1**	1.6
IL1A	Interleukin 1 Alpha	1.3	1.1
IL1R1	Interleukin 1 Receptor type 1	1.0	1.0
IL6	Interleukin 6	1.5	0.6
IL6R	Interleukin 6 Receptor	2.0	1.2

DISCUSSION

DPSCs are a type of mesenchymal stem cell found within the dental pulp (8). DPSCs have gained attention for their remarkable regenerative capabilities, including the potential to differentiate into various cell types (9). For this reason, the DPSCs were considered a promising candidate for regenerative medicine and tissue engineering applications.

These cells are accessible, ethically non-controversial, and possess unique properties that distinguish them from other stem cells.

DPSCs are typically harvested from the pulp of extracted teeth, such as third molars (wisdom teeth) or deciduous teeth. The dental pulp tissue is subjected to enzymatic digestion or explant culture techniques to obtain the stem cells.

The isolation process is relatively straightforward and minimally invasive, making DPSCs a readily available source of stem cells compared to other MSCs derived from bone marrow or adipose tissue.

DPSCs exhibit several key properties that make them valuable for regenerative therapies like self-renewal and multipotency since they can differentiate into various cell types, including odontoblasts, osteoblasts, chondrocytes, adipocytes, and even neurons and myocytes, as well as immunomodulatory effects, i.e., the ability to modulate immune responses, reducing inflammation and promoting tissue repair (10).

Here, we investigated if EQX can induce DPSCs differentiation toward osteoblast lineage. Some genes related to bone formation are activated, including transcription factors FOS-like antigen 1 and Osterix, matrix proteins Osteopontin, Collagen type I and IV, and matrix the remodeling protein Matrix Metalloproteinase 14.

FOSL1, a member of the FOS family of transcription factors, also known as FRA1, plays a significant role in regulating cellular processes such as proliferation, differentiation, and apoptosis (11). Its involvement in osteogenesis, the process of bone formation, has emerged in recent years (12).

FOSL1 and the other FOS family members form heterodimers with members of the Jun family, constituting the AP-1 transcription complex (13). Its transcriptional activity is modulated by various signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway and the Wnt/β-catenin pathway, which regulate FOSL1 expression and activity during osteogenesis.

Several loss- and gain-of-function studies in mice have demonstrated that Fos family members play specific roles in osteogenesis and bone remodeling. Transgenic mice overexpressing Fra-1 display severe osteosclerosis, a bone disorder characterized by increased bone mass caused by increased osteoblast differentiation and function (14). Mice lacking Fra-1 develop osteopenia, a low bone mass disease, proving that Fra1 is an important regulator of bone mass by affecting bone matrix production and maintaining osteoblast activity (15).

FOSL1 plays diverse roles in osteogenesis, influencing osteoblast differentiation and modulating gene expression in extracellular matrix synthesis and remodeling.

SP7, also known as osterix, is a zinc finger-containing transcription factor that plays a critical role in bone formation and osteoblast differentiation (16, 17). Osterix is required to induce osteoblast-specific genes, such as osteonectin, osteopontin, osteocalcin, and alkaline phosphatase, crucial for osteoblast differentiation and bone mineralization (18, 19).

Identified as a key regulator of the genetic network controlling osteogenesis, SP7 functions downstream of Runx2, another essential transcription factor in bone development (19, 20). Other findings suggest that Osterix is regulated via both Runx2-dependent and -independent mechanisms and that Osterix controls osteoblast differentiation, at least in part, by regulating the expression of genes not controlled by Runx2 (21).

Given its pivotal role in bone formation, mutations in or dysregulation of SP7 are associated with various bone disorders. Common SP7 polymorphisms are associated with bone mineral density variation and fracture risk, rare SP7 mutations cause skeletal dysplasia, and SP7 may contribute to bone metastasis (22).

Osteogenesis imperfecta, a genetic disorder characterized by brittle bones, has been linked to mutations in SP7 (23). Patients with these mutations exhibit symptoms such as frequent fractures, bone deformities, and growth deficiencies, reflecting impaired osteoblast function and bone matrix production (24).

Osteoporosis is a condition characterized by reduced bone mass and increased fracture risk. Dysregulation of SP7 expression or activity can disrupt the balance between bone formation and resorption, contributing to the development of osteoporosis (22).

In addition to its role in osteoblasts, SP7 also influences the differentiation of chondrocytes, which are responsible for cartilage formation. Although primarily known for its osteogenic functions, emerging evidence suggests that SP7 may play a role in regulating the balance between osteogenesis and chondrogenesis, which is crucial for endochondral ossification, a process by which long bones are formed (25).

SPP1 gene codes for Osteopontin, a prominent bone matrix protein expressed by preosteoblastic cells early in bone formation. Still, the highest expression is observed in mature osteoblasts at sites of bone remodeling (26). It plays a crucial role in bone mineralization and in the attachment of osteoclasts to the mineral matrix. Osteopontin is involved in various physiological and pathological processes, including bone remodeling, immune response, and inflammation (27).

Collagen Type I is the main structural protein in bone extracellular matrix. Although the production of type I collagen is not exclusive to the differentiating osteoblast but is also produced by fibroblasts, type I collagen is considered a useful osteoblast differentiation marker when expressed with other bone markers (28).

Collagen Type IV is a structural component of the extracellular matrix and a major basement membrane component that separates epithelial and endothelial cells from the connective tissue (29). Studies have shown that Collagen Type IV is not only a structural protein but is also involved in tissue genesis, differentiation, homeostasis, and remodeling (30). This role appears to be not limited to epithelial cells but involves additional cell types, including mesenchymal stem cells. Collagen Type IV seems to play a significant role in the differentiation of stem cells towards osteoblasts and adipoblasts. Indeed, Li et al. (31) demonstrated that the inhibition of miR-214-5p promotes the cell survival of osteoblasts and extracellular matrix production by targeting COL4A1. Later, it was demonstrated that miR-214-5p may weaken osteogenic differentiation of bone marrow stem cells by downregulating COL4A1. Indeed, miR-214-5p may promote adipogenic differentiation downregulating the TGF-β/Smad2/COL4A1 signaling pathway (32). A genome-wide linkage scan found a genomic region at 13q34, including COL4A1 and COL4A2 (collagen type IV alpha-1 and alpha-2 subunits) significantly linked with forearm bone mineral density (33). A significant COL4A1 gene

expression level was found in human osteoporosis fracture bone, compared with bone from individuals with osteoarthritis and individuals without bone pathology (34).

The matrix metalloproteinase protein (MMP) family is involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development and tissue remodeling. Although MMP14 is not considered a classic marker of osteoblast differentiation, MMP14 appears to play a multifaceted role, influencing various signaling pathways and cell fate decisions critical for bone formation and remodeling (35). Deletion of the membrane-anchored Mmp14 in mesenchymal progenitors, but not in committed osteoblasts, redirects cells' fate decisions from osteogenesis to adipo- and chondrogenesis (36).

The collagenolytic activity of MMP14 also regulates the differentiation of mesenchymal stem cells into bone-producing osteoblasts in 3-dimensional (3D) collagen matrices (37). Furthermore, MMP14 is essential for osteoblast survival during the osteoblast/osteocyte transition and is required for proper lacunae formation in osteocytes (38). The parathyroid hormone stimulates osteocyte proliferation by activating the Wnt pathway and increasing the MMP14 expression level, which appears to control bone resorption by regulating soluble RANKL production (39). Furthermore, MMP14 has been implicated in osteoclastogenesis regulation. Indeed, the suppression of MMP14 in osteoblasts increased osteoclastogenesis (39, 40).

CONCLUSIONS

Bone grafting is a crucial technique in modern medicine, offering solutions for complex bone defects and promoting effective healing. Continued advancements in biomaterials, biological enhancements, and regenerative medicine are poised to overcome current limitations, expanding potential applications and success rates of bone grafts.

Through ongoing research and innovation, bone grafting will continue to evolve, enhancing patient care and surgical outcomes in orthopedic and reconstructive procedures. EQX is currently used in dental practice. DPSCs represent a promising frontier in regenerative medicine, offering potential solutions for various dental and orthopedic conditions. Their ease of access, multipotency, and immunomodulatory properties position them as a valuable resource for developing innovative therapies. We demonstrated that EQX could stimulate DPSCs to differentiate into the osteoblast lineage. We understand that further research is necessary to comprehend the mechanism by which EQX influences stem cells fully.

REFERENCES

1. Fillingham Y, Jacobs J. Bone grafts and their substitutes. *Bone Joint J.* 2016;98-B(1 Suppl A):6-9. doi:https://doi.org/10.1302/0301-620X.98B.36350
2. Shibuya N, Jupiter DC. Bone graft substitute: allograft and xenograft. *Clin Podiatr Med Surg.* 2015;32(1):21-34. doi:https://doi.org/10.1016/j.cpm.2014.09.011
3. Valtanen RS, Yang YP, Gurtner GC, Maloney WJ, Lowenberg DW. Synthetic and Bone tissue engineering graft substitutes: What is the future? *Injury.* 2021;52 Suppl 2(S72-S77. doi:https://doi.org/10.1016/j.injury.2020.07.040
4. Zollino I, Girardi A, Palmieri A, et al. Anatase-based implants nanocoating on stem cells derived from adipose tissue. *Implant Dentistry.* 2012;21(2):118-123. doi:https://doi.org/10.1097/ID.0b013e31824bc948
5. Santini S, Barbera P, Modena M, Schiavon R, Bonato M. Equine-derived bone substitutes in orthopedics and traumatology: authors' experience. *Minerva Chir.* 2011;66(1):63-72.
6. Nevins M, Heinemann F, Janke UW, et al. Equine-derived bone mineral matrix for maxillary sinus floor augmentation: a clinical, radiographic, histologic, and histomorphometric case series. *Int J Periodontics Restorative Dent.* 2013;33(4):483-489. doi:https://doi.org/10.11607/prd.1728
7. Sollazzo V, Palmieri A, Girardi A, et al. Osteoplant acts on stem cells derived from peripheral blood. *J Indian Soc Periodontol.* 2010;14(1):12-17. doi:https://doi.org/10.4103/0972-124X.65429
8. Liu H, Gronthos S, Shi S. Dental pulp stem cells. *Methods Enzymol.* 2006;419(99-113. doi:https://doi.org/10.1016/S0076-6879(06)19005-9
9. Masuda K, Han X, Kato H, et al. Dental Pulp-Derived Mesenchymal Stem Cells for Modeling Genetic Disorders. *Int J Mol Sci.* 2021;22(5):doi:https://doi.org/10.3390/ijms22052269
10. Hossein-Khannazer N, Hashemi SM, Namaki S, Ghanbarian H, Sattari M, Khojasteh A. Study of the immunomodulatory effects of osteogenic differentiated human dental pulp stem cells. *Life Sci.* 2019;216(111-118. doi:https://doi.org/10.1016/j.lfs.2018.11.040
11. Sobolev VV, Khashukoeva AZ, Evina OE, et al. Role of the Transcription Factor FOSL1 in Organ Development and Tumorigenesis. *Int J Mol Sci.* 2022;23(3):doi:https://doi.org/10.3390/ijms23031521
12. Luther J, Driessler F, Megges M, et al. Elevated Fra-1 expression causes severe lipodystrophy. *J Cell Sci.* 2011;124(Pt 9):1465-1476. doi:https://doi.org/10.1242/jcs.079855

13. Karsenty G. Transcriptional control of skeletogenesis. *Annu Rev Genomics Hum Genet.* 2008;9(183-196. doi:https://doi.org/10.1146/annurev.genom.9.081307.164437
14. Jochum W, David JP, Elliott C, et al. Increased bone formation and osteosclerosis in mice overexpressing the transcription factor Fra-1. *Nat Med.* 2000;6(9):980-984. doi:https://doi.org/10.1038/79676
15. Eferl R, Hoebertz A, Schilling AF, et al. The Fos-related antigen Fra-1 is an activator of bone matrix formation. *EMBO J.* 2004;23(14):2789-2799. doi:https://doi.org/10.1038/sj.emboj.7600282
16. Hojo H, Ohba S. Sp7 Action in the Skeleton: Its Mode of Action, Functions, and Relevance to Skeletal Diseases. *Int J Mol Sci.* 2022;23(10):doi:https://doi.org/10.3390/ijms23105647
17. Nakashima K, Zhou X, Kunkel G, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell.* 2002;108(1):17-29. doi:https://doi.org/10.1016/s0092-8674(01)00622-5
18. He S, Choi YH, Choi JK, Yeo CY, Chun C, Lee KY. Protein kinase A regulates the osteogenic activity of Osterix. *J Cell Biochem.* 2014;115(10):1808-1815. doi:https://doi.org/10.1002/jcb.24851
19. Yang D, Okamura H, Nakashima Y, Haneji T. Histone demethylase Jmjd3 regulates osteoblast differentiation via transcription factors Runx2 and osterix. *J Biol Chem.* 2013;288(47):33530-33541. doi:https://doi.org/10.1074/jbc.M113.497040
20. Yang D, Okamura H, Teramachi J, Haneji T. Histone demethylase Utx regulates differentiation and mineralization in osteoblasts. *J Cell Biochem.* 2015;116(11):2628-2636. doi:https://doi.org/10.1002/jcb.25210
21. Matsubara T, Kida K, Yamaguchi A, et al. BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J Biol Chem.* 2008;283(43):29119-29125. doi:https://doi.org/10.1074/jbc.M801774200
22. Ludwig K, Ward LM, Khan N, et al. Dominant osteogenesis imperfecta with low bone turnover caused by a heterozygous SP7 variant. *Bone*. 2022;160:116400. doi:10.1016/j.bone.2022.116400
23. Hamon F, Wirotius JM, Nadeau G. [The nurse in a rehabilitation service and the handicapped person]. *Soins.* 1985;451):7-10.
24. Wang JS, Kamath T, Mazur CM, et al. Control of osteocyte dendrite formation by Sp7 and its target gene osteocrin. *Nat Commun.* 2021;12(1):6271. doi:https://doi.org/10.1038/s41467-021-26571-7
25. Komori T. Runx2, an inducer of osteoblast and chondrocyte differentiation. *Histochem Cell Biol.* 2018;149(4):313-323. doi:https://doi.org/10.1007/s00418-018-1640-6
26. Sodek J, Chen J, Nagata T, et al. Regulation of osteopontin expression in osteoblasts. *Ann N Y Acad Sci.* 1995;760(223-241. doi:https://doi.org/10.1111/j.1749-6632.1995.tb44633.x
27. Icer MA, Gezmen-Karadag M. The multiple functions and mechanisms of osteopontin. *Clin Biochem.* 2018;59(17-24. doi:https://doi.org/10.1016/j.clinbiochem.2018.07.003
28. Carpio L, Gladu J, Goltzman D, Rabbani SA. Induction of osteoblast differentiation indexes by PTHrP in MG-63 cells involves multiple signaling pathways. *Am J Physiol Endocrinol Metab.* 2001;281(3):E489-499. doi:https://doi.org/10.1152/ajpendo.2001.281.3.E489
29. Sage H. Collagens of basement membranes. *J Invest Dermatol.* 1982;79 Suppl 1(51s-59s. doi:https://doi.org/10.1111/1523-1747.ep12545773
30. Boudko SP, Danylevych N, Hudson BG, Pedchenko VK. Basement membrane collagen IV: Isolation of functional domains. *Methods Cell Biol.* 2018;143(171-185. doi:https://doi.org/10.1016/bs.mcb.2017.08.010
31. Li QS, Meng FY, Zhao YH, Jin CL, Tian J, Yi XJ. Inhibition of microRNA-214-5p promotes cell survival and extracellular matrix formation by targeting collagen type IV alpha 1 in osteoblastic MC3T3-E1 cells. *Bone Joint Res.* 2017;6(8):464-471. doi:https://doi.org/10.1302/2046-3758.68.BJR-2016-0208.R2
32. Qiu J, Huang G, Na N, Chen L. MicroRNA-214-5p/TGF-beta/Smad2 signaling alters adipogenic differentiation of bone marrow stem cells in postmenopausal osteoporosis. *Mol Med Rep.* 2018;17(5):6301-6310. doi:https://doi.org/10.3892/mmr.2018.8713
33. Niu T, Chen C, Cordell H, et al. A genome-wide scan for loci linked to forearm bone mineral density. *Hum Genet.* 1999;104(3):226-233. doi:https://doi.org/10.1007/s004390050940
34. Hopwood B, Tsykin A, Findlay DM, Fazzalari NL. Gene expression profile of the bone microenvironment in human fragility fracture bone. *Bone.* 2009;44(1):87-101. doi:https://doi.org/10.1016/j.bone.2008.08.120
35. Paiva KBS, Granjeiro JM. Matrix Metalloproteinases in Bone Resorption, Remodeling, and Repair. *Prog Mol Biol Transl Sci.* 2017;148(203-303. doi:https://doi.org/10.1016/bs.pmbts.2017.05.001
36. Tang Y, Rowe RG, Botvinick EL, et al. MT1-MMP-dependent control of skeletal stem cell commitment via a beta1-integrin/YAP/TAZ signaling axis. *Dev Cell.* 2013;25(4):402-416. doi:https://doi.org/10.1016/j.devcel.2013.04.011
37. Egeblad M, Rasch MG, Weaver VM. The dynamic interplay between the collagen scaffold and tumor evolution. *Curr Opin Cell Biol.* 2010;22(5):697-706. doi:https://doi.org/10.1016/j.ceb.2010.08.015
38. Papadogiannis F, Batsali A, Klontzas ME, et al. Osteogenic differentiation of bone marrow mesenchymal stem cells on chitosan/gelatin scaffolds: gene expression profile and mechanical analysis. *Biomed Mater.* 2020;15(6):064101. doi:https://doi.org/10.1088/1748-605X/aba325

39. Delgado-Calle J, Hancock B, Likine EF, et al. MMP14 is a novel target of PTH signaling in osteocytes that controls resorption by regulating soluble RANKL production. *FASEB J.* 2018;32(5):2878-2890. doi:https://doi.org/10.1096/fj.201700919RRR
40. Hikita A, Yana I, Wakeyama H, et al. Negative regulation of osteoclastogenesis by ectodomain shedding of receptor activator of NF-kappaB ligand. *J Biol Chem.* 2006;281(48):36846-36855. doi:https://doi.org/10.1074/jbc.M606656200