KU LEUVEN

FACULTY OF MEDICINE

Bone tissue engineering

Different gene expression levels between tibial, maxillary and mandibular-derived periosteal cells

Lisanne GROENEVELDT

Promotor: Prof. Dr. C. Politis

In collaboration with: Dr. M. Maréchal

Prof. Dr. D. Huylebroeck

Prof. Dr. F.P. Luyten

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Lisanne Groeneveldt

Prof. Dr. C. Politis

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Abbreviations

AFF2	AF4/FMR2 family member 2
BARX1	BARX homeobox 1
BDKRB1	Bradykinin Receptor B1
BLAST	Primer-Basic Local Alignment Search Tool
BMP	Bone morphogenetic protein
cDNA	Complementary deoxyribonucleic acid
COL13A1	Collagen 13 alpha 1 chain
CPD	Cumulative population doublings
CRMO	Chronic recurrent multifocal osteomyelitis
CT	Computer tomography
DLX	Distal-less homeobox
DLX1	Distal-less homeobox 1
DLX5	Distal-less homeobox 5
DLX6	Distal-less homeobox 6
DLX6-AS1	DLX6 antisense RNA
DMEM	Dulbecco´s Modified Eagle Medium
DMEM-C	DMEM-complete
DPP4	Dipeptidyl peptidase-4
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPC3	Glypican 3
GPRC5C	G protein-coupled receptor class C group 5 member C
hBMSCs	Human bone marrow stromal cells
HC	Hierarchical clustering
HCN1	Hyperpolarization-activated cyclic nucleotide-gated potassium channel 1
HOMER2	Homer scaffolding protein 2
HOTAIR	HOX transcript antisense RNA
HOXA10	Homeobox A10
HOXA11	Homeobox A11
HOXA7	Homeobox A7
HOXC10	Homeobox C10
HOXC10sh	Homeobox C10 short hairpin RNA
HOXC9	Homeobox C9
IL	Interleukin
LHX8	Lim homeobox 8
LRRC15	Leucine-rich repeat containing 15
MAPK	Mitogen-activated protein kinases
MSCs	Mesenchymal stem cells
MSX	Msh homeobox
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nkx3.2	Nk3 homeobox 2
PALMD	Palmdelphin
PAX	Paired box
PAX1	Paired box 1
PBS	Phosphate Buffered Saline
PC	Principal component

PCR	Polymerase chain reaction
PD	Population doublings
PKC	Protein Kinase C
rHox	Reproductive homeobox X-linked
RNA	Ribonucleic acid
RPM	Rotations per minute
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RUNX2	Runt-related transcription factor
SD	Standard deviation
Shh	Sonic hedgehog
SLC1A7	Solute carrier family 1 member 7
SMAGP	Small cell adhesion glycoprotein
STAT4	Signal transducer and activator of transcription 4
TFAP2C	Transcription factor AP-2 gamma
TMEM150C	Transmembrane protein 150C
TMEM255B	Transmembrane protein 255B
TNF-a	Tumor necrosis factor alpha
VEGFB	Vascular endothelial growth factor B
VEGFR-1	Vascular endothelial growth factor receptor 1
Wnt	Wingless-related integration site

Samenvatting

Achtergrond: Bone tissue engineering vertegenwoordigt een veelbelovende alternatieve behandelingsoptie voor de huidige standaard van autologe bottransplantaties. Deze benadering bestaat uit drie componenten: donorcellen die zich kunnen vermenigvuldigen en differentiëren, groeifactoren om de cellen in de juiste richting te laten differentiëren en een draagstructuur om het weefsel een 3-dimensionale vorm te geven. Wij zullen ons richten op de cellen die worden gebruikt bij bone tissue engineering, de mesenchymale stamcellen (MSCs). MSC's kunnen worden verkregen uit meerdere bronnen, waaronder het periost. Ons doel is om de verschillen in genexpressieprofielen van menselijke periostale cellen van drie verschillende oorsprongen te onderzoeken; de tibia, maxilla en mandibula.
Resultaten: RNA-sequencing werd uitgevoerd op periostale MSC's verkregen uit tibia, maxilla en mandibula. De 30 meest verschillend tot expressie gebrachte genen werden gevalideerd door reverse transcription kwantitatieve polymerasekettingreactie (RT-qPCR). Verrassend genoeg zijn veel genen die betrokken zijn bij embryologische ontwikkeling, zoals de HOX-genen, DLX-genen, *BARX1* en *PAX1*, nog steeds actief in periostale cellen van 16-30-jarige mensen. Wij vonden dat *HOXA11*, *HOXC10*, *HOXA10*, *HOXA7* en *HOTAIR* meer tot uiting komen in MSCs van de tibia, terwijl *DLX1* meer tot expressie kwam in maxillaire MSC's en *DLX5* meer in mandibulaire MSC's. Bovendien vonden wij een trend in de richting van hogere een expressie van *DLX6* in mandibulaire MSC's, bevestigd met duidelijke downregulatie van *DLX6-AS1* in deze cellen. *BARX1* werd meer tot uiting gebracht door craniofaciale MSC's, terwijl expressie van *PAX1* minder was in van mandibula afkomstige periostale cellen. Wij vonden ook een hogere expressie van *VEGFB* in maxillaire MSCs en een lagere expressie van *DPP4*.
Conclusies: Met behulp van literatuur suggereerden we een verband tussen *DPP4* en botmetabolisme. Op basis van de genexpressieprofielen in combinatie met de informatie over geneigenschappen die momenteel beschikbaar zijn, tonen periostale MSC's die van craniofaciale oorsprong zijn enkele voordelen, in vergelijking met periostale MSC's van de tibia voor bone tissue engineering.

Summary

Background: Bone tissue engineering represents a promising alternative treatment option for the current standard of autologous bone transplantation. This approach typically consists of three components: donor cells that are able to proliferate and differentiate, growth factors to direct the differentiation and a 3D structure. We will focus on the cells used for bone tissue engineering approaches, which are typically mesenchymal stem cells (MSCs). MSCs can be obtained from multiple sources, including the periost. Our goal is to investigate the differences in gene expression profiles of human periosteal cells of three different origins; the tibia, maxilla, and mandible.
Results: RNA-sequencing was performed on periosteal MSCs obtained from tibia maxilla and mandible. The 30 top differentially expressed genes were validated by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Surprisingly, a lot of genes involved in embryological development, such as the HOX-genes, DLX-genes, *BARX1*, and *PAX1*, are still active in periosteal cells from 16-30-year-old people. We found *HOXA11, HOXC10, HOXA10, HOXA7* and *HOTAIR* to be more expressed by tibial-derived MSCs, while *DLX1* was more expressed in maxillary-derived MSCs and *DLX5* more by mandibular-derived MSCs. In addition, we found a trend towards a higher expression of *DLX6* in mandibular-derived MSCs, confirmed with a clear downregulation of *DLX6-AS1* in those cells. *BARX1* was expressed more by craniofacial-derived MSCs, while expression of *PAX1* was less in mandibular-derived periosteal cells. We also found a higher expression of *VEGFB* in maxillary-derived cells and a lower expression of *DPP4*.
Conclusions: Using literature, we suggested a link between *DPP4* and bone metabolism. Based on the gene expression profiles combined with the information of gene properties currently available, craniofacial-derived periosteal MSCs show some advantages in comparison to tibial-derived periosteal MSCs for bone tissue engineering purposes.

Content

Introduction

Currently, treatments of big bone defects due to comminuted fractures, congenital or acquired craniofacial malformations, defects after oncologic resection, infections or osteonecrosis are still challenging. In order to treat those bone defects, materials such as autologous bone tissue, allogeneic bone tissue, xenografts like Bio-Oss, metals or bioceramics are used to treat the bone defects.

At this moment, the standard treatment includes the use of autologous bone. In order to obtain the bone graft, a second surgical site is generated. This goes along with donor site morbidity, such as post-operative pain, scar formation and the possibility of complications such as a post-operative infection. Infection rates of 0-12% are reported, including deep infections. Dural leaks were reported in 0.3-0.6% of patients when the cranial bone was used and pleural perforation in 0.9% in cases with rib grafts [1]. In case the iliac crest was used, herniation of abdominal contents was reported in 1.8% and cutaneous nerve injuries resulting in an altered sensation or even meralgia paresthetica in 14.3% of the cases [2]. Also, the amount of bone tissue which can be used from these donor sites such as the skull, mandibular ramus, chin, rib, iliac crest or fibula is limited.

The use of allogeneic materials goes along with a risk for transmission of infectious diseases or host-versus-graft reactions [3]. In order to reduce these risks, allogeneic materials are often freeze-dried, irradiated or chemically treated. However, the research groups of An and Thalgott have shown that freeze-dried tissues had a higher likelihood of failure, with pseudo-arthrosis in 6 out of 19 patients [4-6]. Sorger et al reported failure rates up to 30% mainly due to poor integration [7]. Also, osteoinductive and osteogenic properties can be impaired of allografts that are treated to minimize the risk for transmission of infectious diseases or host-versus-graft immunologic reactions [6, 8].

Xenografts like Bio-Oss are regularly used in implantology, usage of these grafts though could result in foreign body reactions, resulting in fibrous connective tissue matrix instead of bone tissue [9]. Rohner et al found only 26% more bone regeneration when Bio-Oss was used during LeFort 1 osteotomies compared to no use of bone substitutes [10]. Also, hydroxyapatite is used in the clinic, often coated with growth-enhancing gels since it does not contain osteoinductive properties of itself [11]. This means the surrounding bone tissue is needed to replace the hydroxyapatite by mineralized bone tissue, which becomes more difficult as the size of the defect increases. The use of metals like titanium to replace bone defects is associated with a higher risk for infections of about 16% when used for cranioplasties [12].

Bone tissue engineering represents a promising alternative treatment option. This approach typically consists of three components: donor cells that are able to proliferate and differentiate, growth factors to direct the differentiation and a 3D structure [13].

In bone tissue engineering, stem cells are often used because of their capabilities to proliferate substantially and to differentiate after priming towards osteoblasts or chondrocytes. In particular mesenchymal stem cells (MSCs) are used often in bone tissue engineering since they are better available compared to embryonic stem cells, they still have the capability to proliferate and are able to differentiate towards multiple mesenchymal lineages such as adipogenic cells, chondrogenic cells, osteogenic cells, skeletal myoblasts and smooth muscle cells [14-17]. MSCs were originally often obtained from bone marrow. Later, it became clear multipotent mesenchymal cells can be obtained from several other tissues like adipose tissues, gingiva, dental pulp, the periodontal ligament or periosteum. These alternative sources for multipotent MSCs are used more and more since deprival of these tissue results in less morbidity and an increased availability and quantity of these new sources for bone tissue engineering [18].

Bone tissue engineered constructs are already used in clinics: Trautvetter and colleagues reported in 2011 the successful use of periosteal-derived cells for augmentation of the maxillary sinus during insertion of Branemark implants [19]. Schimming and Schmelzeisen found promising results using the periosteal-derived tissue-engineering bone for augmentation of the maxillary sinus [20]. This team, however, reported six years later the use of tissue-engineered bone only was successful in a limited range of indications [21]. To overcome these limitations, researchers still are improving their technics using more sophisticated scaffolds to give a 3D structure to the tissue construct and coating them with angiogenic or osteogenic factors to improve vascularization or osteogenesis. Also, different methods of *in vitro* culturing and/or priming are investigated to improve their *in vivo* efficiency in bone tissue engineering [22-28]. However, these techniques frequently complicate their clinical feasibility since these techniques are difficult to scale up or their use of high levels of growth factors like bone morphogenetic proteins (BMPs) [23, 29].

Another way to improve bone tissue engineering for a wider range of applications is to mimic the embryological development with the best cells we have available.

The development of bone tissue occurs via two ways: by intramembranous ossification and through endochondral ossification. Flat bones such as the maxilla are formed by intramembranous ossification. In this process, MSCs differentiate towards osteoblast, depositing later on bone matrix. Long bones such as the tibia and the areas of the symphysis and condyles of the mandible are forms by endochondral ossification. In this process, MSCs first differentiate towards chondrocytes. These chondrocytes become hypertrophic, die and will leave cavities that will later become invaded by osteoprogenitor cells which are delivered by the blood flow in the already vascularized matrix. The osteoprogenitor cells differentiate subsequently towards osteoblasts, producing the bone matrix [30]. Also, fracture healing occurs via endochondral ossification, for which the MSCs are delivered by the periosteum [31].

In the past, many researchers focused on bone tissue engineering via the intramembranous ossification pathway. Methods of culturing, methods of priming and different scaffolds, creating a three-dimensional construct, were explored. However, these constructs failed when upscaled since vascularization lacked, resulting in tissue necrosis [32]. Another way is to engineer bone tissue by using the endochondral ossification pathway in which first a cartilage intermediate tissue is formed. Chondrocytes are known to live in a hypoxic environment. When these chondrocytes become hypertrophic, they will attract blood vessels and ultimately osteoblasts are deposed in the created vacuoles, creating a mineralized tissue. Bone tissue engineering via endochondral ossification is known to give eight times higher bone volume construct *in vivo* compared to bone tissue engineering via intramembranous ossification [33].

Recently, the focus in bone tissue engineering is more and more towards mimicking the embryological development of bone tissue [23, 34-36]. More and more methods for culturing of MSCs are explored, such as culturing in bioreactors in which oxygen tension and flow are regulated very strictly [29, 37, 38]. Colnot and others recommended using periosteal-derived cells for bone tissue engineering since those cells are involved in fracture healing processes and so may be a more clinically relevant cell source for engineering purposes [39-41]. De Bari, Nakahara and van Gastel reported successful isolation of multipotent cells from mouse and human periosteum [42-44]. Bone tissue engineering mimicking the embryological development via endochondral ossification using periosteal-derived MSCs, based on the fracture healing process, is getting better and better [23, 45-47]. Especially the research team of Luyten has done a lot of research in improving bone tissue engineering using those cells [22, 23, 42, 44, 48-52]. They found that periosteal-derived cells organize *in vivo* into a cartilage template when primed with Fibroblast Growth Factor 2 (FGF2), while FGF2-primed bone marrow stromal cells produce bone tissue exclusively via intramembranous ossification, resulting in less bone tissue. The endochondral ossification of FGF2-primed periosteal-derived cells is found to be driven by an increased production of bone

morphogenetic protein 2 (BMP2) [53]. This means that BMP2 expression could be used to select the bone-forming cells. Johanna Bolander published even about successful bone tissue engineering using serum-free *in vitro* priming of endochondral-derived multipotent cells [23]. However, these periosteal cells are derived from the tibia.

Craniofacial bone tissue arises from the neural crest cells instead of the paraxial mesodermal cells of which long bone such as the tibia develops. Those neural crest cells arise from the ectodermal cells, at the border of the neural plate which will converge to become the neural tube under influence of wnt-signaling, BMPs, fibroblast growth factors, Msh homeobox 1 (MSX1), MSX2, distal-less homeobox 5 (DLX5) and paired box (PAX) genes [54-57]. During merging of both ends of the neural tube, the neural crest cells obtain also properties of mesenchymal cells and are released from the edges of the neural plate. From this moment they are also known as ectomesenchymal cells. Those cells migrate cranially and differentiate in the cranial region towards osteoblastic, adipogenic, chondrogenic and muscle cells, but also towards neural cells such as neurons and glia and even melanocytes [57]. This could result in different properties of the bone tissue itself and the bone forming cells.

We already know that craniofacial bone tissue has a higher turnover rate, "ages" more slowly and has a higher expression of osteoblastic markers compared to skeletal bone tissue such as the tibia [58]. Maxillary bone tissue arises by intramembranous ossification and consists of trabecular bone tissue surrounded by a thin cortical layer, a low bone density and mineral content and a good vascularization. Mandibular bone tissue, on the other hand, develops by both endochondral ossification and intramembranous ossification and consists of lamellar bone tissue surrounded by a thick cortical layer with a high mineral content and bone density [59-62].

In our previous research [63], we compared the proliferation capacity of periosteal-derived stem cells from the tibia, maxilla, and mandible. We found a trend towards a higher proliferation rate for mandibular-derived periosteal cells compared to periosteal cells derived from the tibia. This difference in proliferation rate for craniofacial tissues versus skeletal tissues is also shown by Aghaloo and Alge and co-workers for respectively skeletal tissues and dental pulp stem cells in comparison to bone marrow-derived MSCs [58, 64]. Also, an upregulation of the osteogenic genes alkaline phosphatase and osteocalcin and the angiogenic vascular endothelial growth factor receptor 1 was found in maxillary-derived MSCs compared to tibial-derived periosteal cells. Alge and researchers also found more mineralization in bone tissue engineered using dental pulp stem cells in comparison to bone tissue developed by MSCs obtained from bone marrow [64].

From currently available data, we learned bone tissue engineering has great opportunities when we mimic the embryological development [23, 35, 36]. Since we know that craniofacial bone tissue has different properties in comparison to skeletal bone tissue, while they also differ in embryological development, we would define the differences between craniofacial-derived periosteal cells and cells obtained from the periosteum of long bones such as the tibia. We already know there are differences in proliferation rate and expression of osteogenic markers after priming of those cells. In this research project, we will explore whether there are still differences in the expression of developmental genes such as the BMPs, FGFs, Wnt-related genes, MSX1, MSX2, DLX5, and PAX-genes.

Materials and methods

MSC isolation

Periosteal samples of 5 x 10 mm were obtained from the posterior areas of the maxilla and mandible on the right side by maxillofacial surgeons C.P. and B.G of 16-30-year-old healthy patients who underwent bimaxillary orthognathic surgery after informed consent was obtained (Belgian registration number B322201731127). The periosteal samples of 5 by 10 mm from the proximal anterior part of the tibia were assembled by orthopedic surgeons. The samples were collected in tubes with Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, USA) in addition of 10% Fetal Bovine Serum (FBS; Thermo Fisher Scientific, Waltham, USA), 1% (w/v) sodium pyruvate (Thermo Fisher Scientific) and antibiotics-antimycotics solution (200 units penicillin/ml, 200 µg streptomycin/ml and 0.50 µg amphotericin-B/ml; Invitrogen) and stored at 4 °C till processing. Cells were rinsed in Hanks Balanced Salt Solutions (HBSS; Invitrogen) twice, cut into small pieces and digested in 4,400 units of collagenase type-IV, dissolved in DMEM for 16 hours. After digestion, cells were separated from the digestion fluid by centrifugation at 1300 rotations per minute (RPM) for 10 minutes and rinsed in DMEM-complete (DMEM-C; DMEM + 10% FBS, 1% (w/v) sodium pyruvate, 100 units penicillin/ml, 100 µg streptomycin/ml and 0.25 µg amphotericin-B/ml). Next, cells were plated in 5 ml of DMEM-C in a well of 9.5 cm^2 and cultured in a humidified atmosphere at 5% CO_2 and 37 °C.

Cell proliferation

Cells were seeded in duplicate in CELLSTAR® flasks (Greiner Bio-One, Kremsmünster, Austria) with filter screw cap with a density of 5700 cells per cm^2 in DMEM-C and cultured in a humidified atmosphere at 5% CO_2 and 37 °C. The medium was replaced twice a week by new DMEM-C in order to supply nutritional factors and to remove waste and non-adherent cells. When 90-95% confluency was reached, cells were washed twice in Phosphate Buffered Saline (PBS; Thermo Fisher Scientific) and detached from the flask with TrypLE Express (Thermo Fisher Scientific) for 10 minutes. TrypLE was inactivated by addition of DMEM-C twice as much as the volume of TrypLE and washed away using DMEM-C and subsequent centrifugation at 1300 RPM for 10 minutes. Next, the cell pellet was dissolved in a limited volume of DMEM-C in order to define the cell density. From this well-mixed solution, 10 µl was taken, mixed with 10 µl trypan blue staining solution (Thermo Fisher Scientific) and counted using a hemocytometer. The cell density in 10 µl was calculated from these data and the cell-solution was diluted by addition of more DMEM-C in order to plate 5700 cells/cm^2 in a density of 28.500 or 40.000 cells/ml, depending on the size of the flask that was used.

Population doublings (PD) were calculated during each trypsinization by using the formula PD = log (n_2) – log (n_1). In this formula, n_1 represents the number of cells seeded in the flask and n_2 represents the number of cells obtained by trypsinization of cells in the same flask as used for n_1. For the cumulative population doublings (CPD), the PD from all trypsinization moments of the same cells were summed together from the first moment of seeding in flasks after cell counting till the last moment of trypsinization.

RNA isolation

RNA was isolated when a CPD of 6-7 was reached. Cells were trypsinized and counted as described before. 1 Million of these cells were rinsed twice in PBS and centrifugated at 1300 RPM for 10 minutes. Next, 350 µl RLT buffer (Qiagen, Hilden, Germany) and 3,5 µl β-mercaptoethanol (Sigma Aldrich, Saint Louis, USA) was added to lyse the cells and homogenize them. Samples were directly placed on ice and frozen at -80 °C till further processing. For further processing, samples were thawed on ice and manufacturer's instructions were followed using the RNeasy mini kit (Qiagen). Complementary deoxyribonucleic acid (cDNA) was obtained by reverse transcription of 500ng of

the total ribonucleic acid (RNA), using 1 mM oligo-dT20 and random hexamer primers (Thermo Scientific).

RNA sequencing

RNA sequencing was performed on samples in duplicate by the Erasmus Biomics Center (Rotterdam, the Netherlands). RNA was isolated as described before and sent on ice to the center. Further processing of the samples in Rotterdam was done using the RNA-sequencing protocol and RNA-sequencing kit from Illumina (San Diego, USA). The samples were single-read sequenced using the Illumina Genome Analyzer II generating 76bp reads. The reads were trimmed to remove the Illumina adapter sequences and mapped against human genome h18 and h19. Data were analyzed by E.M., a specialist in bioinformatics generated by RNA sequencing.

Quantitative PCR

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed on the top 30 genes that differed in expression levels between the three origins. Samples were processed and measured in duplicates on a Step-One-Plus PCR machine (Applied Biosystems, Foster City, USA) using a Sybr® Green detection system (Life Technologies, Carlsbad, United States) according to the manufacturer's instructions with a 2-min holding step at 45 °C. The two-step reaction consisting of 95 °C for 15 seconds and 60 °C for 60 seconds, was cycled 40 times and relative gene expression was calculated using the $2^{-\Delta CT}$ method by normalizing to the measured transcript levels of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) [65]. The human primer sequences were designed by using the National Center for Biotechnology Information (NCBI) nucleotide database and Primer- Basic Local Alignment Search Tool (BLAST) tool from NCBI. The respective primer sequences are listed in the Supplementary Table.

Statistical analysis

Statistical analysis was carried out using student's t-test using a statistical software package (Prism 5.00, GraphPad Software). Results were considered statistically significant at $p < 0.05$. Data are presented as mean values ± standard deviation (SD).

Results

RNA isolation

Periosteal samples from tibia, maxilla, and mandible were obtained from respectively 3, 4 and 4 donors. Maxillary and mandibular samples could be obtained from the same donor in 3 cases since bimaxillary surgery was performed on them. The fourth periosteal sample originating from the maxilla failed during digestion of the sample, the digestion of the other samples and proliferation of those achieved cells was successful. When the cells had reached 10 population doublings, cells were harvested for RNA extraction. RNA extraction was successful in all samples.

Quality check RNA sequencing

RNA sequencing was performed on 3 samples obtained from mesenchymal stem cells from the periosteum of the tibia, 3 maxillary samples, and 4 mandibular samples, all in duplicate. 19-21 million reads were performed on each sample with a base pair length of 50. An alignment of >90% was reached and quality check of the samples marked them as good quality.

Principal component (PC) analysis shows that the duplicates (a and b) are clustered together, but the samples are also clustered by origin (Figure 1). Hierarchical clustering (HC) plots confirm this in the cluster dendrogram (Figure 2).

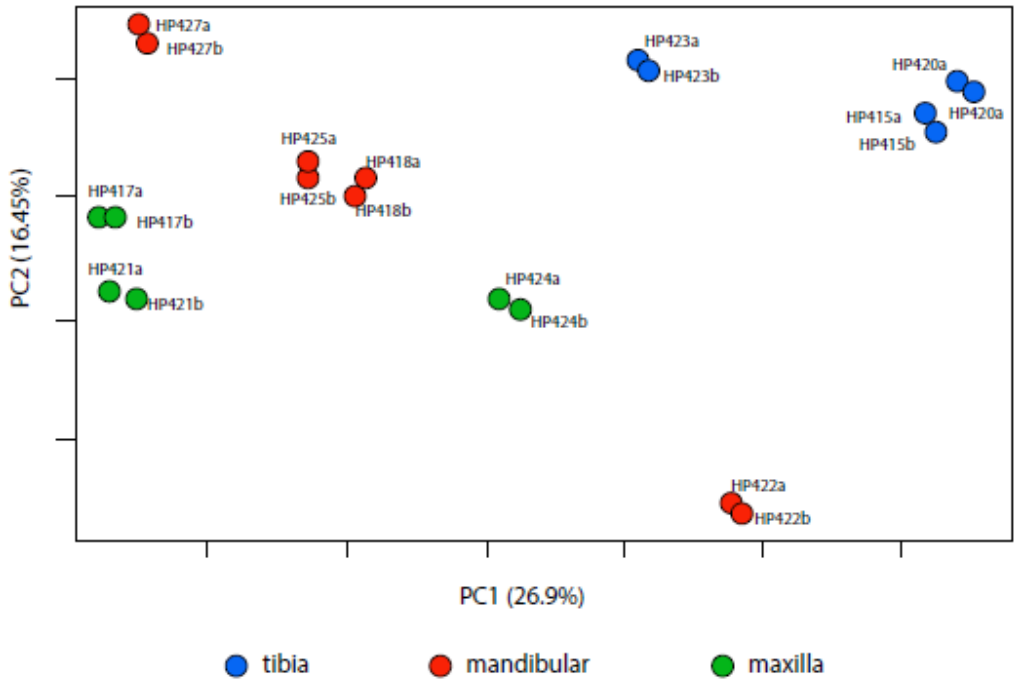

Figure 1: Principal component analysis

Principal component analysis performed on periosteal-derived mesenchymal stem cells, obtained from the tibia (n=3, blue spots), mandible (n=4, red spots) and maxilla (n=3, green spots). On each sample, 19-21 million reads were performed.

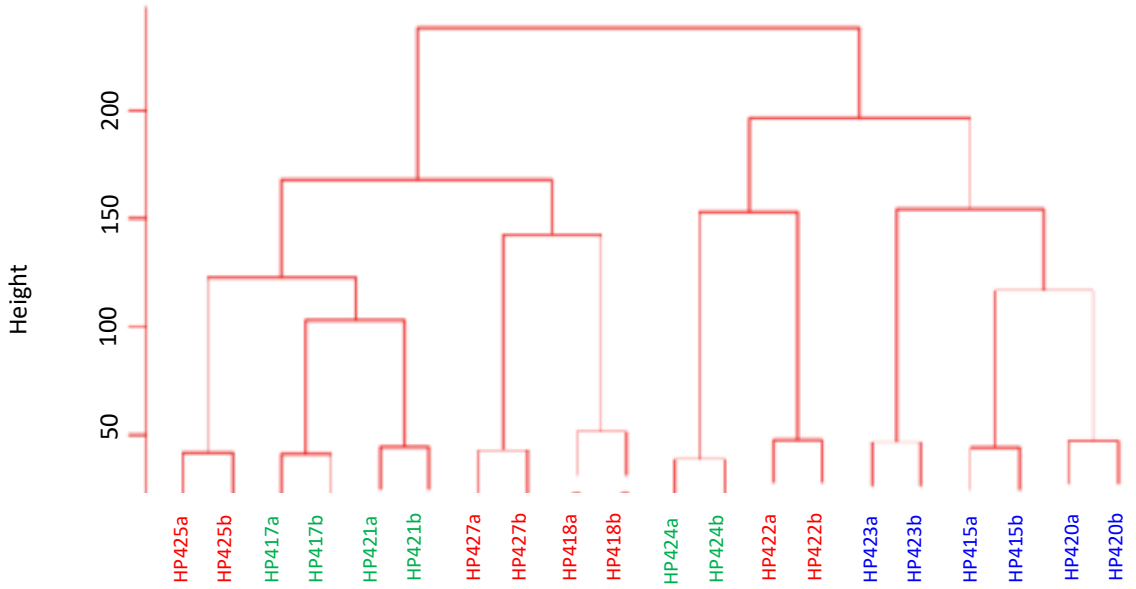

Figure 2: Cluster dendrogram

Cluster dendrogram showing the duplicates contain the highest degree of similarities. Also, the samples from obtained from the tibia (blue) are clustered together to the right side of the dendrogram. Samples from maxillary-derived MSCs are shown in green, those of mandibular-derived MSCs in red.

Differentially expressed genes

We visualized the genes with a statistically different expression between tibial-derived MSCs and maxillary-derived MSCs (Figure 3A), tibial-derived MSCs and mandibular-derived MSC (Figure 3B) and maxillary versus mandibular-derived cells (Figure 3C) using an interactive HC plot.

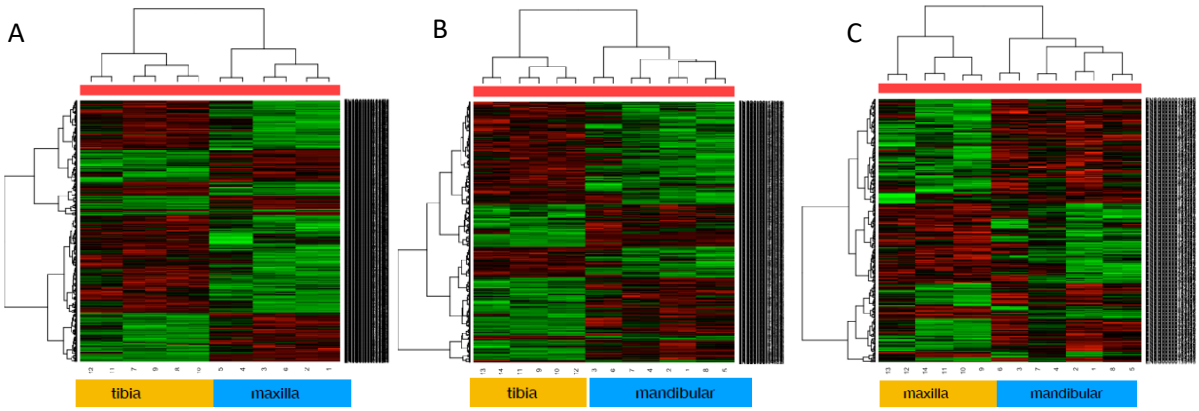

Figure 3: Hierarchical clustering maps

Clustering maps of the statistically significant ($p<0.05$) different gene expression numbers, based on the counts per million reads. Those reads were log transformed and z-score normalized across samples. Green indicates low expression and red high. Presented are the differentially expressed genes between tibial-derived mesenchymal cells and maxillary-derived mesenchymal cells (A), tibial versus mandibular-derived MSCs (B) and maxillary MSCs compared to mandibular-derived MSCs (C).

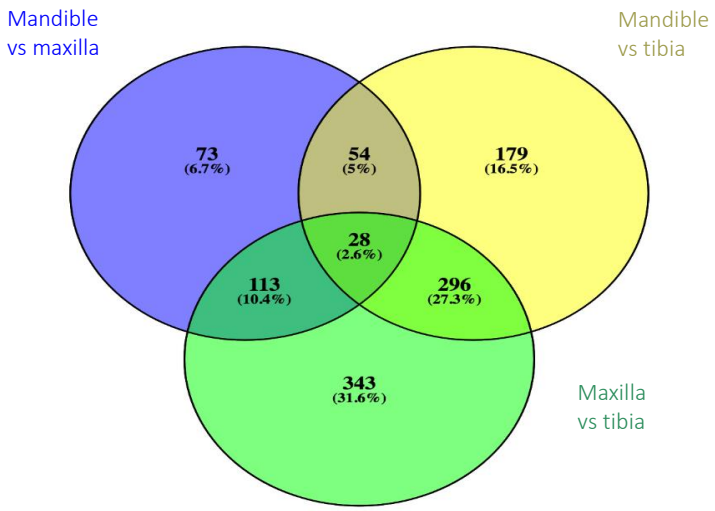

Figure 4: Differential expression between different samples

Given are the numbers of differentially expressed genes for maxillary-derived MSCs compared to tibial-derived MSCs (in green) and to mandibular-derived MSCs (in blue). Also, the number of genes that significantly differ in expression level between mandibular- and tibial-derived periosteal cells are shown (in yellow). An overlap between the circles represents identical genes.

In the end, we observed 780 genes that are statistically significantly differentially expressed by tibial-derived MSCs versus maxillary-derived MSCs. Between tibial-derived MSCs and mandibular-derived MSCs, we found 557 genes that were expressed differently statistically significant. Of these 557 genes, 113 genes also varied between de maxillary- and tibial-derived cells. Between the mandibular- and maxillary-derived MSCs, only 268 genes where regulated statistically different (Figure 4).

Validation by quantitative PCR

The top 30 differentially expressed genes found by RNA sequencing were tested by quantitative-polymerase chain reaction. These genes are Homeobox C10 *(HOXC10),* Homeobox A10 *(HOXA10),* Homeobox A11 *(HOXA11),* Homeobox C9 *(HOXC9),* Homeobox A7 *(HOXA7),* HOX transcript antisense RNA *(HOTAIR),* transmembrane protein 255B *(TMEM255B),* Dipeptidyl peptidase-4 *(DPP4),* Distal-less homeobox 5 *(DLX5),* Distal-less homeobox 6 *(DLX6),* DLX6 antisense RNA *(DLX6-AS1),* Bradykinin Receptor B1 *(BDKRB1),* Leuchine rich repeat containing 15 *(LRRC15),* AF4/FMR2 family member 2 *(AFF2),* Collagen 13 alpha 1 chain *(COL13A1),* Signal transducer and activator of transcription 4 *(STAT4),* Distal-less homeobox 1 *(DLX1),* Transcription factor AP-2 gamma *(TFAP2C),* Small cell adhesion glycoprotein *(SMAGP),* Homer scaffolding protein 2 *(HOMER2),* Vascular endothelial growth factor B *(VEGFB),* BARX homeobox 1 *(BARX1),* Lim homeobox 8 *(LHX8),* Paired box 1 *(PAX1),* hyperpolarization activated cyclic nucleotide gated potassium channel 1 *(HCN1),* Solute carrier family 1 member 7 *(SLC1A7),* Palmdelphin *(PALMD),* Glypican 3 *(GPC3),* G protein-coupled receptor class C group 5 member C *(GPRC5C),* and Transmembrane protein 150C *(TMEM150C).*

Tibial, maxillary and mandibular periosteal samples were obtained from 3 donors. Since 3 donors underwent bimaxillary surgery, the maxillary and mandibular samples are obtained from the same patients. Periosteal samples were digested and obtained MSCs were proliferated successfully till 10 population doublings, as well as the RNA isolation and cDNA production.

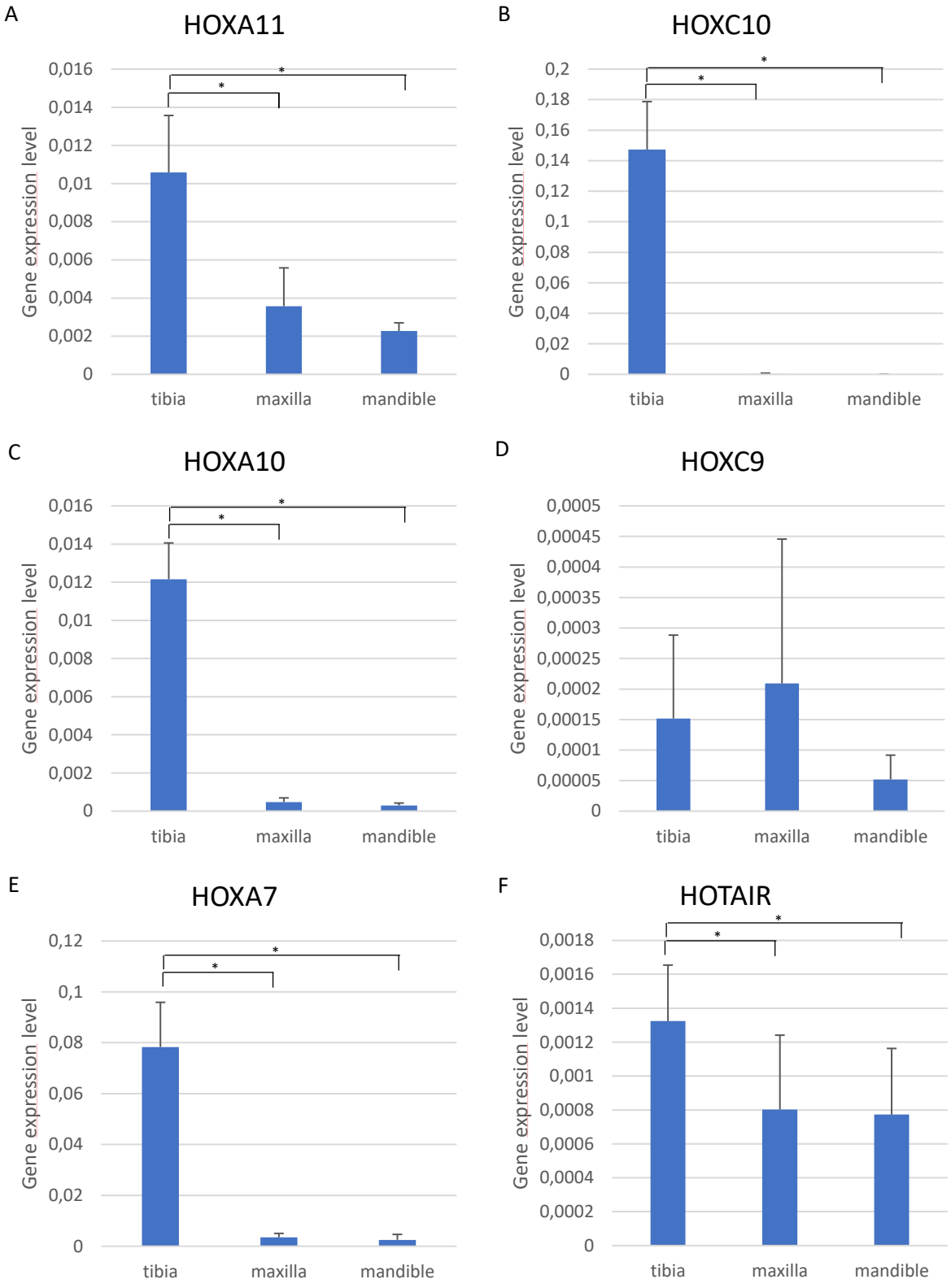

Figure 5: RT-qPCR data for HOX genes.

Tibial, maxillary and mandibular-derived cells were compared for their expression of HOXA11 (A), HOXC10 (B), HOXA10 (C), HOXC9 (D), HOXA7 (E) and HOTAIR (F), corrected for GAPDH. * means $p<0.05$.

HOX genes

Expression of *HOXA11*, a transcription factor which is part of a developmental regulatory system that provides cells with specific positional identities on the anterior-posterior axis [66-68], is elevated in tibial-derived periosteal MSCs compared to maxillary- and mandibular-derived MSCs, with a level of significance of respectively 0.00054 and 0.00046 (Figure 5A). *HOXC10,* a transcription factor which also provides cells with specific identities on the anterior-posterior axis, but especially in the hindlimbs [69, 70], is expressed more in tibial-derived periosteal cells compared to those obtained from maxilla (p=0.000044) or mandible (p=0.000044; Figure 5B). The expression of *HOXA10*, coding for a transcription factor resulting in providing cells specific information about the anterior-posterior axis [71], is expressed more by tibial-derived periosteal cells compared to those from maxillary (p=0.000009) or mandibular origins (p=0.00001; Figure 5C). For *HOXC9,* coding for a transcription factor giving an anteroposterior identity to cells especially in the hind limbs [72, 73], no statistically significant differences could be found (Figure 5D). *HOXA7,* a transcription factor found especially in the upper limbs [74], was expressed significantly more by tibial-derived cells compared to maxillary-derived MSCs (p=0.000054) and mandibular-derived MSCs (p=0.000054). *HOTAIR* regulates the chromatin state in chromosome 2 in order to silence the *HOXD*-cluster, another cluster of genes responsible for the identity of cells on the anteroposterior axis [75]. *HOTAIR* is expressed more by periosteal obtained from the tibia compared to those obtained from the maxilla (p=0.022) and mandible (0.012). This results in the conclusion that tibial-derived periosteal cells still express genes that are involved in the anteroposterior patterning during the embryological phase.

DLX genes

DLX1, a transcriptional regulator of signals from multiple transforming growth factor beta (TGF-β) superfamily members [76], is expressed more by mandibular-derived MSCs compared to tibial-derived MSCs with a p-value of 0.0021 (Figure 6A). *DLX1* is also known as having an association with cancer and oral clefts in case a certain variant of the gene is expressed [77]. *DLX5* is a gene involved in bone development and fracture healing, acting as an early BMP-responsive transcriptional activator resulting in osteoblast differentiation. It works via pathways in neural crest differentiation and signaling pathways that regulate pluripotency of stem cells [78]. *DLX5* is expressed more by mandibular-derived cells compared to tibial- (p=0.0054) and maxillary-derived cells (p=0.0249; Figure 6B). For *DLX6*, a gene involved in the development of the forebrain and craniofacial tissues [79, 80], acting as a paralog of *DLX1*, no statistically significant differences were found (Figure 6C). However, for the antisense RNA of DLX6 (DLX6-AS1), which is also linked to the sonic hedgehog (Shh) and DLX family, involved in forebrain and craniofacial development [81], a downregulation is seen in mandibular-derived MSCs compared to MSCs obtained from the tibia (0.0294; Figure 6D). This is in accordance with the trend towards a higher expression of *DLX6* in mandibular-derived periosteal cells.

Genes known to be involved in craniofacial development

Expression of *BARX1*, a bar subclass of the homeobox transcription factors which plays a role in tooth development and the craniofacial mesenchymal tissues originating from the neural crest, was higher in craniofacial-derived MSCs compared to those arrived from the tibia [82]. For maxillary-derived MSCs, a p-value of 0.0253 was found, for mandibular-derived MSCs a p-value of 0.00060 was found (Figure 7A). For *LHX8*, a transcription factor that plays a role in tooth morphogenesis, oogenesis and neuronal differentiation [83, 84], a higher expression was found in maxillary-derived MSCs compared to tibial-derived MSCs (p= 0.0036) and mandibular-derived MSCs (p=0.0121; Figure 7B). This gene is also known to be a candidate gene for cleft palate and odontoma formation [83]. *TFAP2C* is a sequence-specific DNA binding transcription factor involved in the activation of

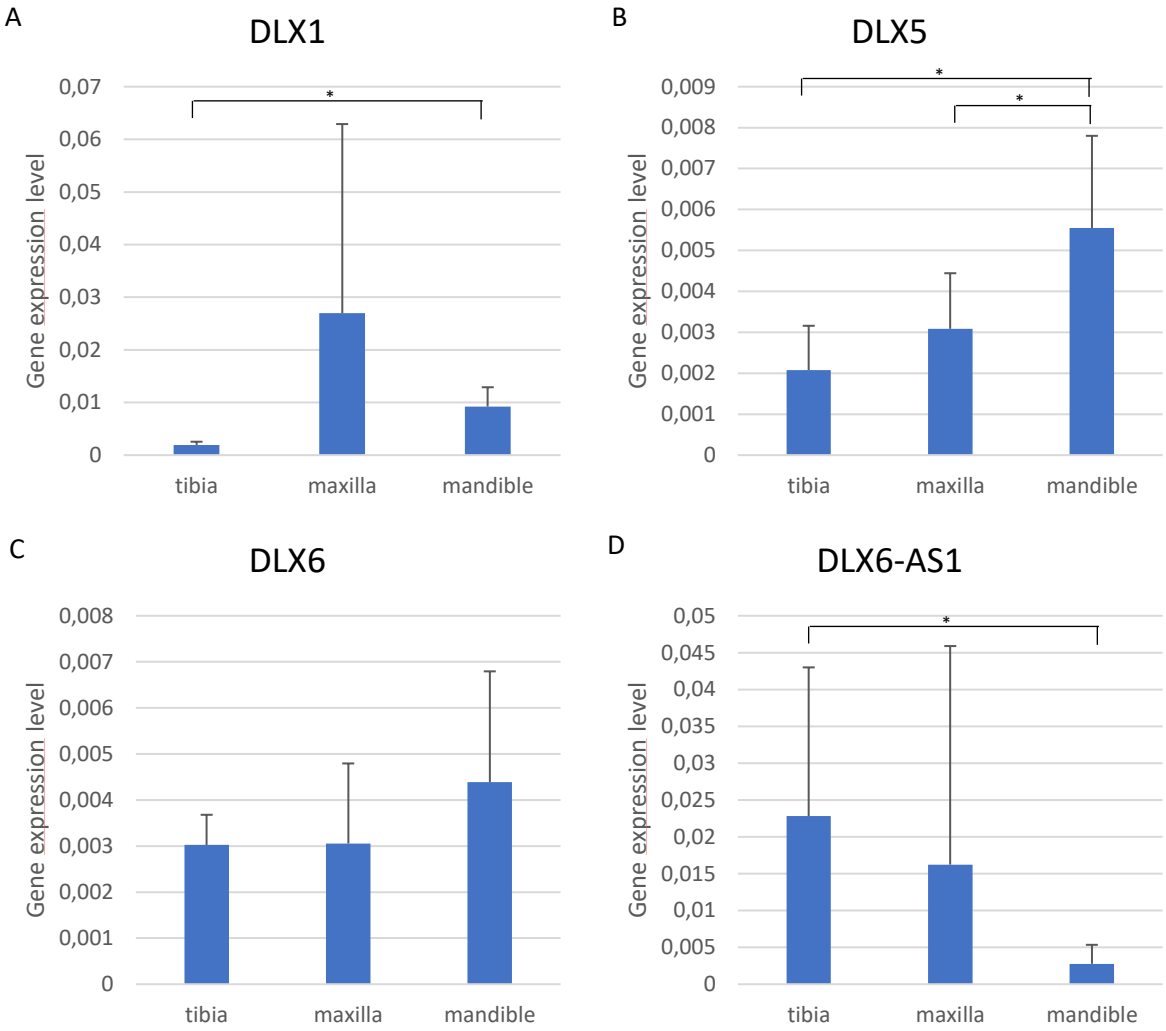

Figure 6: **RT-qPCR data for DLX genes.**

Tibial, maxillary and mandibular-derived cells were compared for their expression of DLX1 (A), DLX5 (B), DLX6 (C) and DLX6-AS1 (D), corrected for GAPDH. * means $p<0.05$.

several developmental genes, induced during retinoic-acid mediated differentiation. It plays a role in the development of eyes, face, body wall, limbs and neural tube [85, 86]. For this gene, no statistically significant differences were found in the expression levels between tibial-, maxillary- and mandibular-derived MSCs (Figure 7C). *PAX1* was downregulated in MSCs originating from the mandible, compared to those originating from the tibia ($p=0.0258$; Figure 7D). This gene is known to be involved in pattern formation, the development of the vertebral column and chondrogenesis. It is associated with vertebral malformation, but also with the otofaciocervical syndrome [87-89].

Collagen-associated genes

COL13A1, a gene found in nonfibrillar collagens, having a function in endochondral ossification [90], is downregulated in MSCs obtained from the mandible, compared to those obtained from the tibia ($p= 0.0383$; Figure 8A). *LRRC15* is a protein kinase inhibitor that binds fibronectin, collagen, and laminin [91]. Expression of this gene was not statistically significantly different between the MSCs obtained from tibia, maxilla, and mandible (Figure 8B).

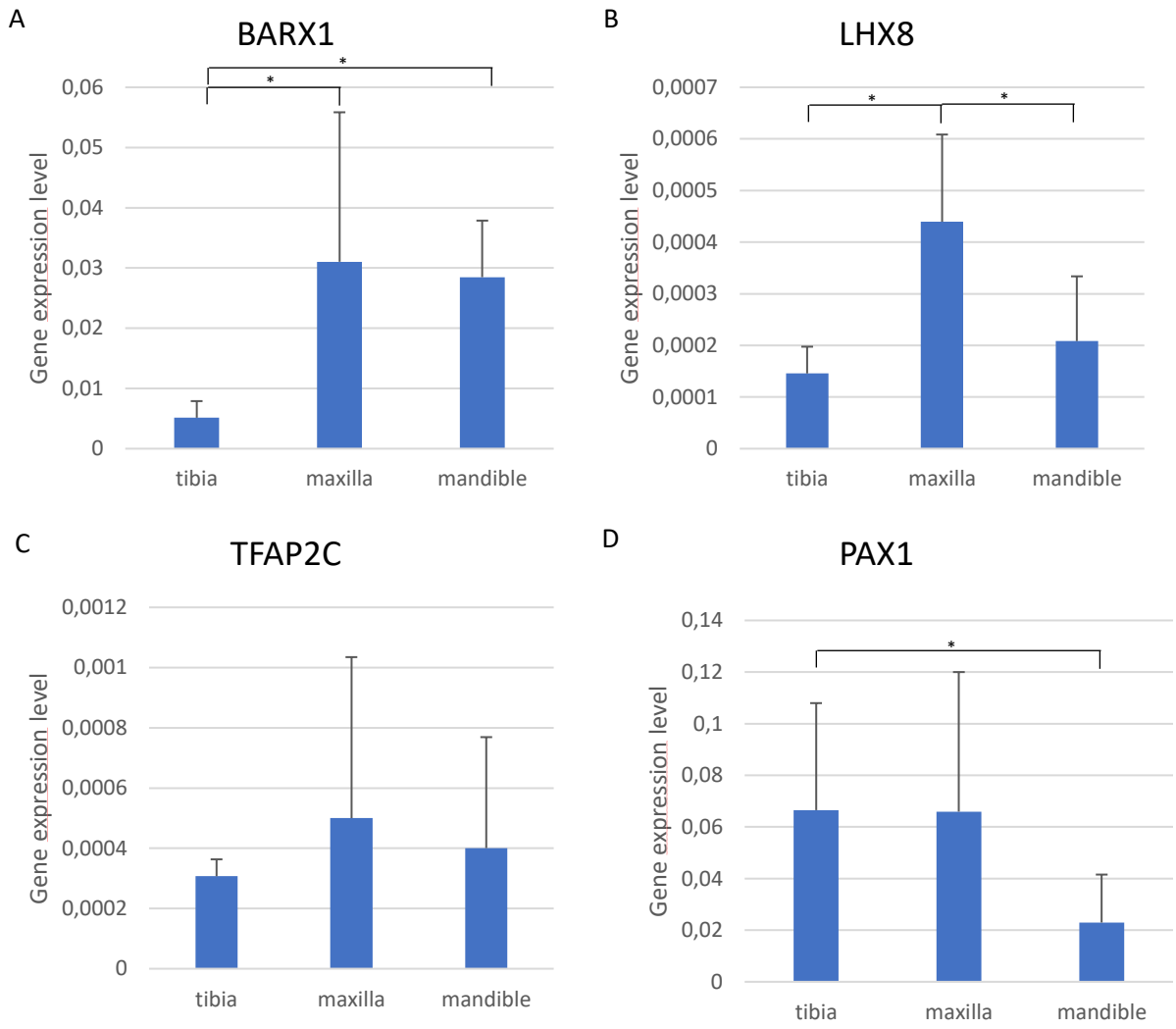

Figure 7: RT-qPCR data for genes known to be involved in craniofacial development.

Tibial, maxillary and mandibular-derived cells were compared for their expression of BARX1 (A), LHX8 (B), TFAP2C (C) and PAX1 (D), corrected for GAPDH. * means $p<0.05$.

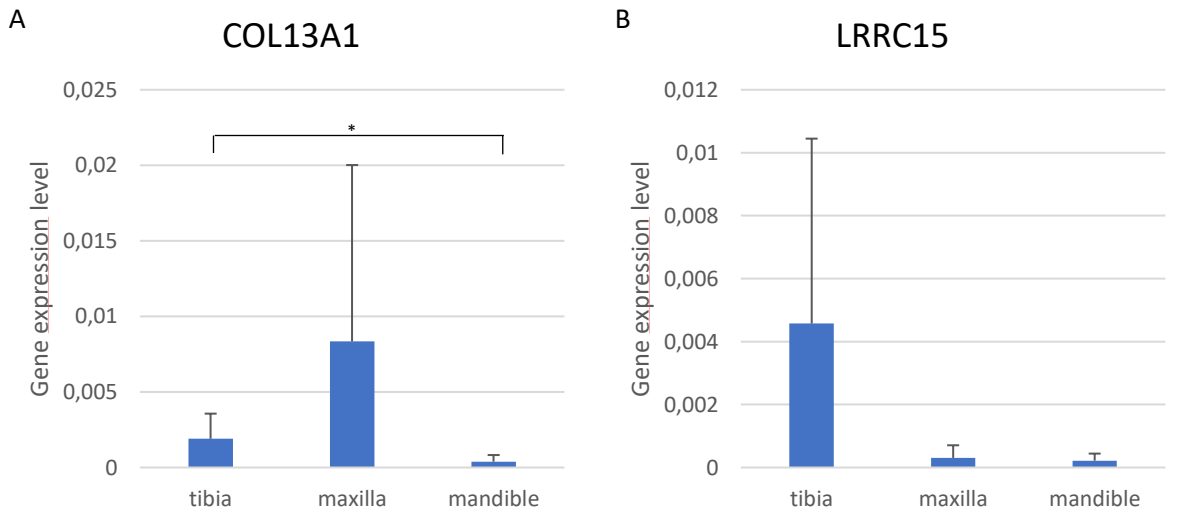

Figure 8: RT-qPCR data for collagen-associated genes.

Tibial, maxillary and mandibular-derived cells were compared for their expression of COL13A1 (A) and LRRC15 (B), corrected for GAPDH. * means $p<0.05$.

Genes known to be involved in neuronal systems

Also *SLC1A7*, *HCN1*, and *HOMER2* came up in the list of top 30 differentially expressed genes of our RNA sequencing study. *SLC1A7* is a glutamate transporter, known to be involved in the circadian entrainment and transmission across chemical synapses [92]. *HCN1* is known to code for a channel involved in native pacemaker current in heart and neurons [93]. *HOMER2* codes for a protein that regulates the glutamate receptor function [94]. Validation using q-PCR technique did not confirm these results. For none of these genes, a statistically significant difference in expression level was found (Figure 9).

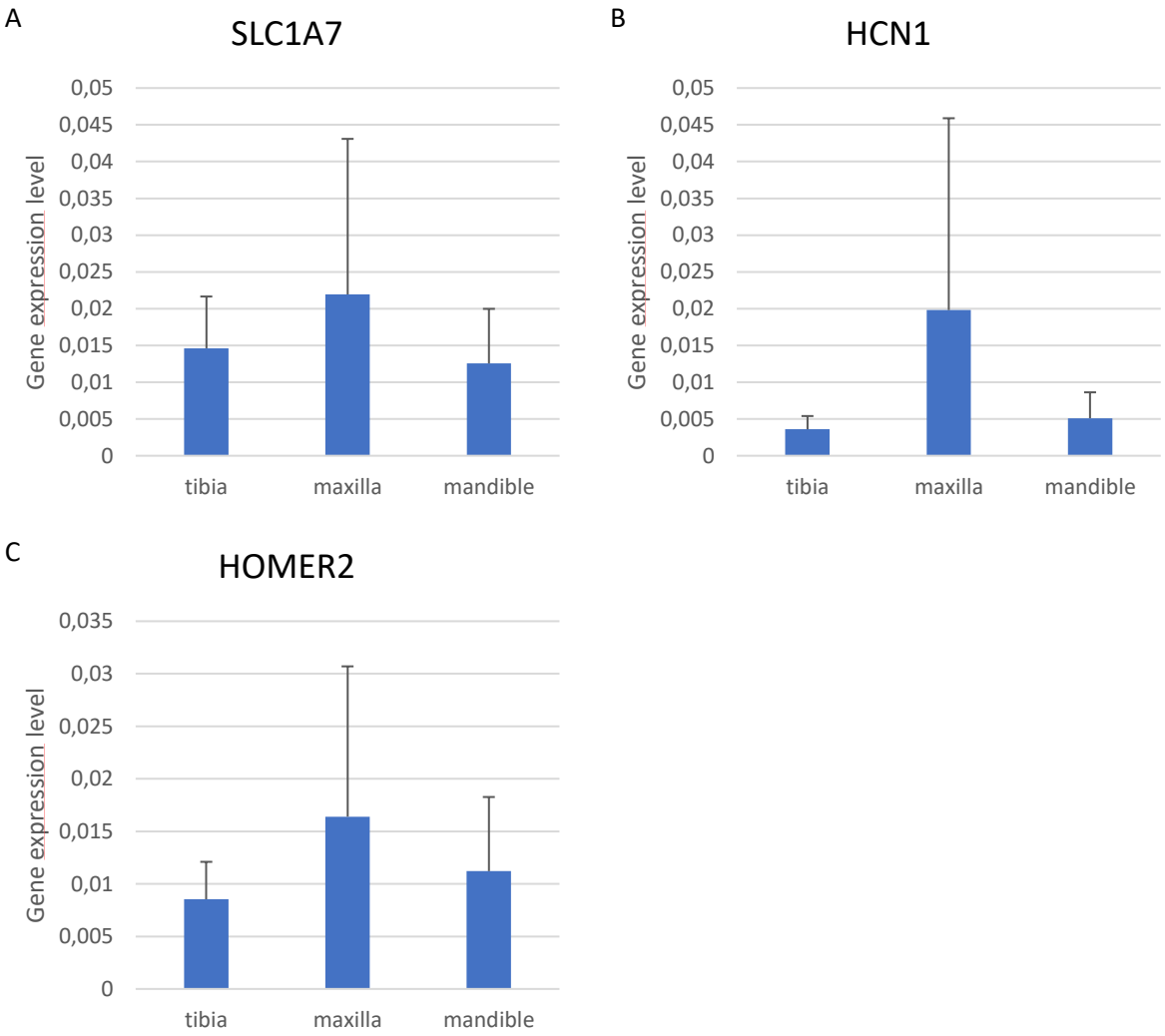

Figure 9: RT-qPCR data for genes known to be involved in neuronal systems

Tibial, maxillary and mandibular-derived cells were compared for their expression of SLC1A7 (A), HCN (B) and HOMER2 (C), corrected for GAPDH. * means $p<0.05$.

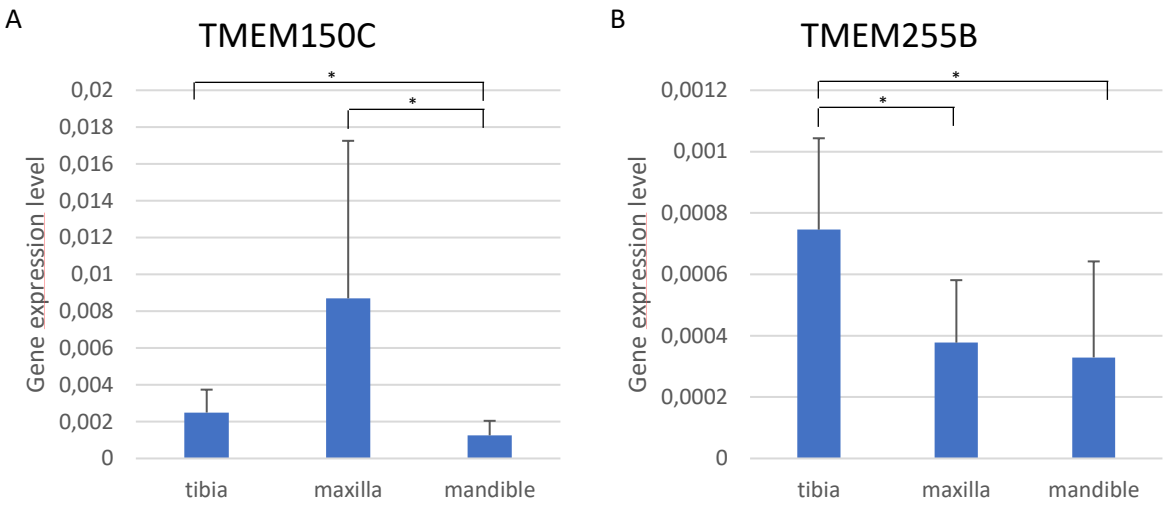

Figure 10: RT-qPCR data for genes of transmembrane proteins.

Tibial, maxillary and mandibular-derived cells were compared for their expression of TMEM150C (A) and TMEM255B (B), corrected for GAPDH. * means $p<0.05$

Genes of transmembrane proteins

TMEM150C and *TMEM255B* are genes coding for transmembrane proteins. *TMEM150C* codes for a slow-adapting part of a mechanosensitive channel, found in channels associated with hearing, touch, pain and blood pressure [95, 96]. This gene is downregulated in mandibular-derived MSCs compared to those obtained from the tibia ($p= 0.0356$) and maxilla ($p=0.0432$; Figure 10A). *TMEM255B* is coding for a relatively unknown transmembrane protein. Expression of this gene is upregulated in tibial-derived MSCs compared to those obtained from maxilla ($p=0.0172$) and mandible ($p=0.0200$; Figure 10B).

Genes with other functions

DPP4 is a gene involved in immune regulation, signal transduction, apoptosis, and insulin secretion [97-99]. This gene is expressed less by maxillary-derived MSCs compared to tibial- ($p=0.0374$) and mandibular-derived MSCs ($p=0.0130$; Figure 11A). *GPC3* is counteracting *DPP4* [100]. It is involved in cell division, growth regulation, and apoptosis. It is also known to be involved in limb patterning and skeletal development via BMP4 [101]. Both *DPP4* as *GPC3* are known to cause the Simpson dysmorphia syndrome in case of certain mutations [102]. No statistically significant differences were found in the expression of this gene between tibial-, maxillary- and mandibular-derived MSCs, however, they are showing counteracting trends compared to *DPP4* (Figure 11B)

STAT4 is a transcription factor involved in the T-helper 2 differentiation pathway and Akt signaling, which is involved in the production of proteins such as VEGFB and BMP [103, 104]. Expression of this gene was not statistically significantly different between the MSCs obtained from tibia, maxilla, and mandible (Figure 11C). Expression of *VEGFB*, a gene regulating the formation of blood vessels and involved in endothelial cell physiology [105], is upregulated in maxillary-derived MSCs compared to those from the tibia ($p=0.0337$; Figure 11D). *BDKRB1* is a gene linked to calcium-signaling due to inflammation, trauma, wounds, shock or allergic reactions [106]. No statistically significant differences were found in expression levels of this gene between periosteal cells obtained from tibia, maxilla, and mandible (Figure 11E).

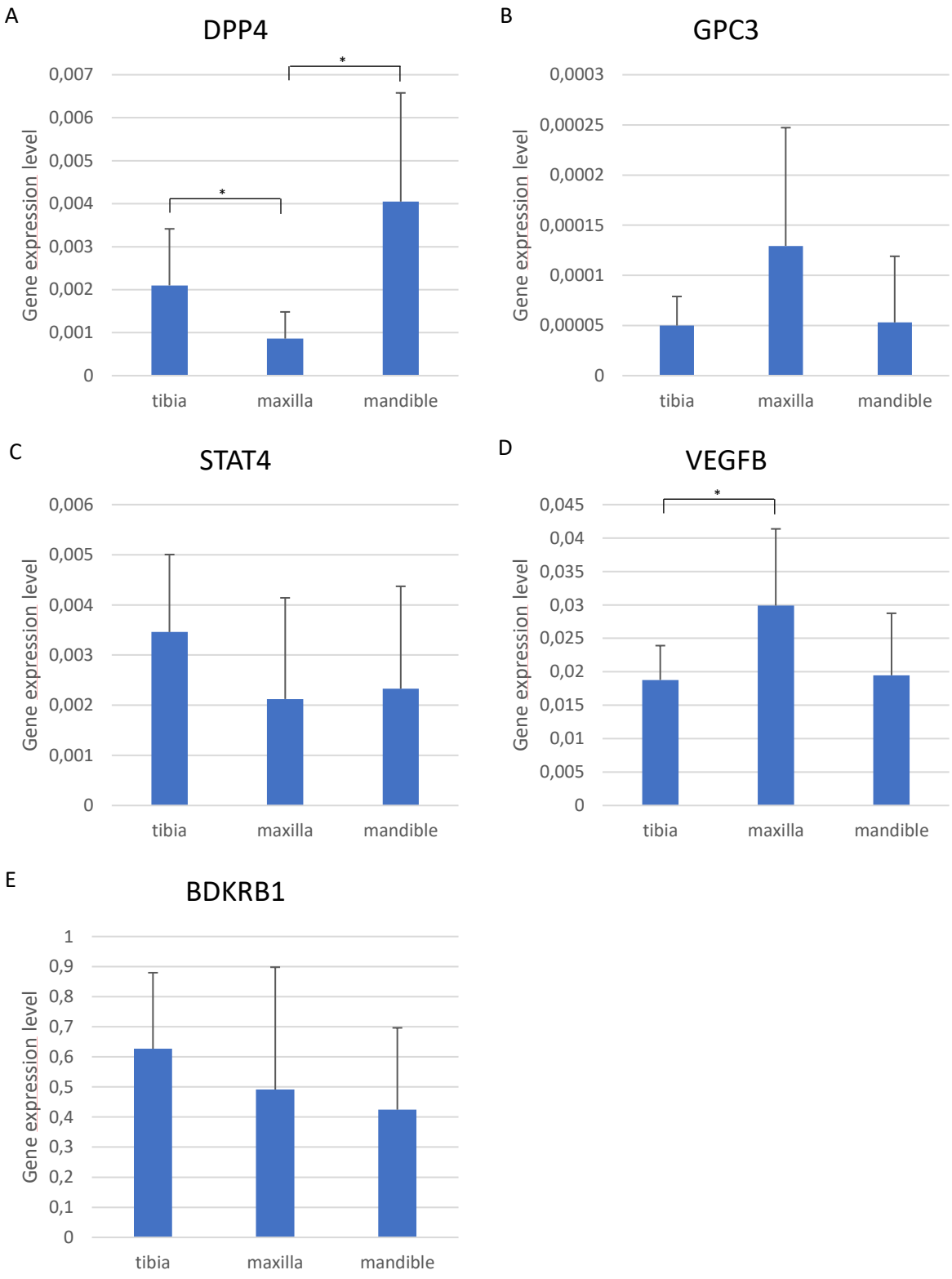

Figure 11: RT-qPCR data for genes with other functions.

Tibial, maxillary and mandibular-derived cells were compared for their expression of DPP4 (A), GPC3 (B), STAT4 (C), VEGFB (D) and BDKRB1 (E), corrected for GAPDH. * means $p<0.05$

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Genes with unknown functions

AFF2 is a putative transcriptional factor, is known to be associated with fragile X syndrome [107]. *AFF2* is downregulated in mandibular-derived MSCs compared to tibial-derived MSCs (p=0.0462, Figure 12A). *SMAGP* is a gene which may play a role in epithelial cell-cell contacts [108]. It was found as a differentially expressed gene in RNA sequencing experiments, however, by qPCR, no altered expression levels could be found between periosteal cells from tibia, maxilla, and mandible (Figure 12B). *PALMD*, a gene which is a target of p53, controls cell death and is associated with vesicoureteral reflux and calcific aortic valve stenosis [109, 110]. A downregulation of this gene was found in tibial-derived MSCs compared to maxillary-derived MSCs (p=0.0117; Figure 12C). *GPRC5C* is a gene coding for a G-coupled receptor with 7 transmembrane domains, of which the function is still unknown [111]. *GPRC5C* is downregulated in tibial-derived MSCs compared to mandibular-derived MSCs (p=0.0120, Figure 12D).

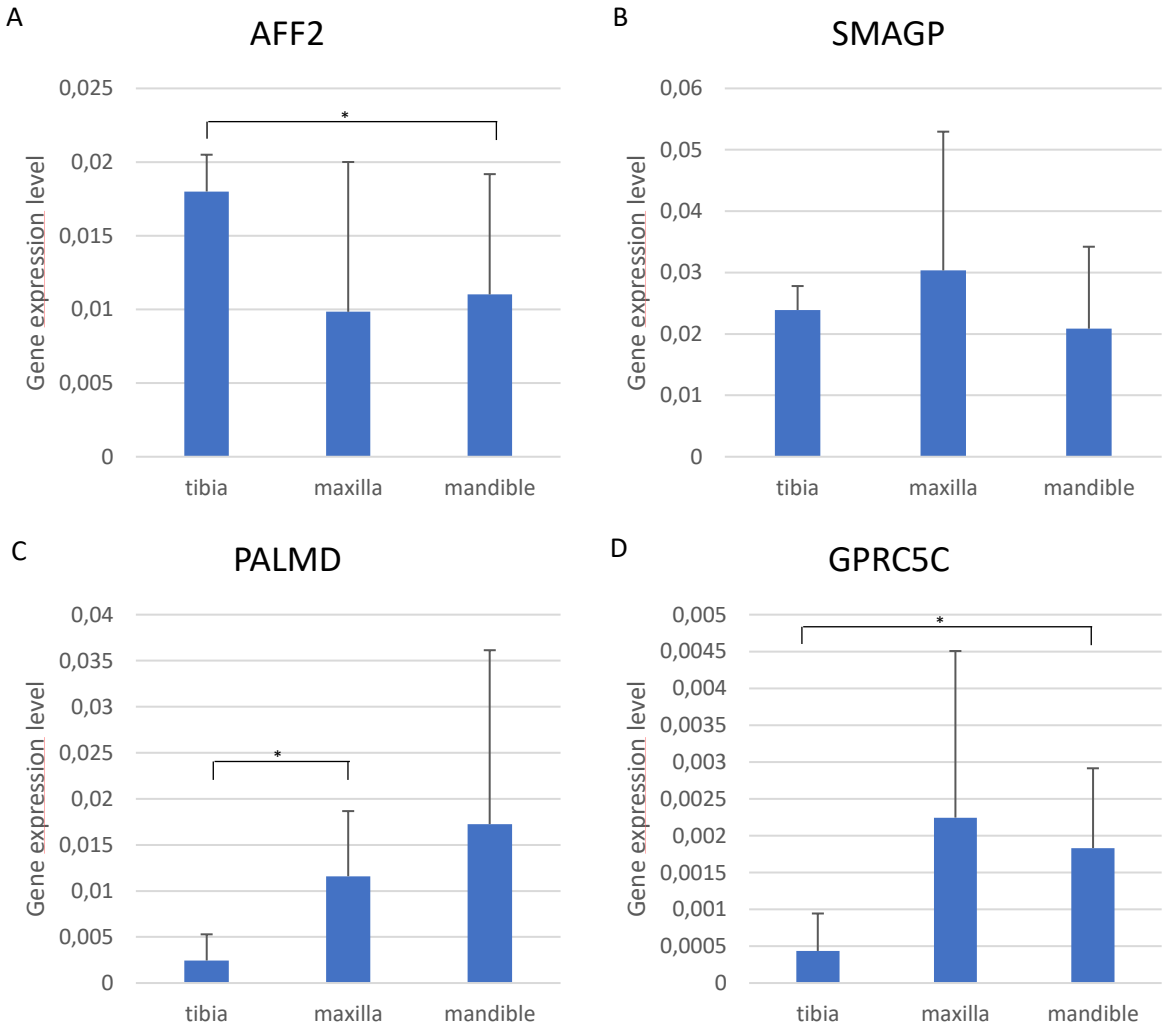

Figure 12: RT-qPCR data for genes with unknown functions.

Tibial, maxillary and mandibular-derived cells were compared for their expression of AFF2 (A), SMAGP (B), PALMD (C) and GPRC5C (D), corrected for GAPDH. * means p<0.05

Discussion

Treatment of large bone defects in the clinic remains challenging. Therapeutic options like autologous bone, allogeneic derived bone tissue or bone substitutes are still subject to limitations and complications including tissue necrosis, limited availability, infection and graft-versus-host reaction. Bone tissue engineering represents a promising alternative treatment option. Most research in cell-based bone tissue engineering has turned towards using MSCs, since these cells are able to proliferate and differentiate into chondrocytes followed by endochondral ossification or to differentiate into osteoblast-like cells, resulting in intramembranous ossification. Possible resources for MSCs are diverse. Many teams focus on bone marrow-derived MSCs or on MSCs obtained from the tibial periosteum. They mimic the embryological development of bone tissue since fracture repair is essentially a recapitulation of bone formation during development.
We hypothesized that MSCs obtained from periosteum, but at different sites, may act different with differences in gene expression profiles. Sacchetti and group already showed that MSCs from different sources could show differences in differentiation capacities and gene expression profile [112]. In this master thesis, we evaluated the differences in gene expression profiles between periosteal MSCs derived from the tibia, maxilla, and mandible by RNA sequencing and validated this data by RT-qPCR.

Expression of genes involved in embryological development

We showed upregulation of HOX-genes *HOXA11*, *HOXC10*, *HOXA10*, *HOXA7*, and *HOTAIR* in MSCs obtained from the tibial periosteum. But also other genes that are known to be involved in embryological development, such as *BARX1*, *LHX8*, *TFAP2C*, and *PAX1* are still detectable in those cells and even upregulated in some locations. This means these genes are still active in periosteal cells after the developmental stage. Gersch et al showed in 2005 already reactivation of the expression of HOX-genes during bone regeneration in six-month-old rats [113]. They showed an upregulation of the expression of rHOX (reproductive homeobox X-linked), *HOXA2* and *HOXD9* in rats during fracture healing. In this research, we also showed expression of HOX-genes in periosteal-derived MSCs while no fracture was present before or during harvesting of the periost.
Presently, we do still not completely understand the regulation of HOX-genes and their function. A lot of research in development has been done in the Drosophila model since this firefly has a more simplified embryological development. In this model, we know that HOX-expression is regulated by the repressing proteins of the polycomb-group and trithorax-proteins which prevent the silencing of HOX-genes by polycomb-proteins [114, 115]. These two groups of proteins are known to change histones by methylation, phosphorylation, acetylation, or ubiquitination, resulting in alterations in the chromatin remodeling [116]. Normally HOX-genes are repressed during many cell divisions after developmental phase through trimethylation of the histone 3 complex at the 27th amino acid (H3K27me3) [117]. However, polycomb-proteins are still important in repressing the HOX-genes, induced by the polycomb group response elements (PREs) [116, 118]. Probably this is the reason why HOX genes still can be active at later stages although they are methylated. This raises the question whether HOX-activity is due to less polycomb-activity or due to other factors that are still unknown.
We did see a statistically significant upregulation of the HOX-genes *HOXA11*, *HOXC10*, *HOXA10*, *HOXA7* and *HOTAIR* in cells derived from tibial periosteum compared to those derived from maxilla or mandible. This means that those developmental genes are expressed differently between the three origins. Because these genes are known to be involved in skeletal development, it is likely bone tissue engineering using these three sources will give different properties of bone tissue.

HOXA11

Research by Pineault et al shows in *HOXA/D11*-compound mutant mice the growth rate and terminal length of the radial and ulnar bones are reduced [119]. This means *HOXA11* and *HOXD11* are probably also postnatally involved in the development of long bones, such as the tibia [119]. This is consistent with our finding that *HOXA11* is upregulated in tibial-derived MSCs compared to those from the craniofacial origins.

HOXC10

HOXC10 was upregulated too in the MSCs obtained from tibial periosteum, compared to those obtained from maxillary and mandibular periost. This is surprising since HOXC-cluster genes normally are only expressed during embryologic development [120]. Li and colleagues found recently by using adipose-derived MSCs infected with *HOXC10* short hairpin RNA (*HOXC10sh)* lentiviruses, *HOXC10* inhibits osteogenesis [121]. When *HOXC10* was depleted, more osteogenesis was present. Also, an upregulation of the gene involved by endochondral ossification, runt-related transcription factor *(RUNX2)*, was present [121]. Based on this data, we could argue the use of tibial-derived MSCs, since they show an upregulation of *HOXC10* while we want to engineer bone tissue by endochondral ossification.

HOXA10

However, Elsafadi et al published in 2016 about the osteogenic potential of 2 clonal cell populations [122]. They found that the population expressing *HOXA10* at a higher level differentiated easily to adipocytes, chondrocytes, and osteoblasts and produced more alkaline phosphatase, produced by osteoblasts. We found *HOXA10* to be more expressed by tibial-derived MSCs compared to those derived from maxilla or mandible. Though, Johanna Bolander and her team found more relevant factors to find the proper cells for bone tissue engineering, namely those involved in early BMP, Wingless-related integration site (Wnt) and Ca^{2+}/ protein kinase C (PKC) pathway activation [22].

HOTAIR

For *HOTAIR* it is known it inhibits the osteogenic potential of MSCs [123-125]. Since expression of *HOTAIR* was upregulated in tibial-derived periosteal cells compared to craniofacial-derived MSCS, we have another reason to limit the use of tibial-derived MSCs in bone tissue engineering and choose for craniofacial-derived MSCs.

BARX1

BARX1 is known to repress joint formation and to stimulate cartilage formation. We found a higher expression of this gene in craniofacial-derived periosteal MSCs compared to tibial-derived periosteal MSCs. Whether this is a positive feature or not, is still unknown. Chondrogenic differentiation of MSCs could be seen as a positive feature, however, it would be useless if in those tissues endochondral ossification does not occur [126].

PAX1

PAX1 is expressed at a lower level in mandibular-derived MSCs compared to MSCs obtained from tibial periost. Normally *PAX1* is upregulated to promote the early stages of chondrogenic differentiation, but has to be downregulated later on since it inhibits chondrocyte hypertrophy via activation of Nk3 homeobox 2 (Nkx3.2) and inhibition of *RUNX2* [89, 127]. We have already shown that mandibular-derived mesenchymal stem cells showed spontaneous differentiation towards chondrocytes without the addition of chondrogenic factors [63]. Because of this, we would have

expected a higher expression in mandibular-derived MSCs compared to periosteal cells obtained from other sources. Especially since the cells for RNA sequencing were harvested together with the cells on which fluorescence-activated cell sorting (FACS) analysis was performed for MSC-markers. However, in previous research we have also shown a trend towards a higher expression of *RUNX2,* stimulating chondrocyte maturation [63]. This makes us think MSCs were already differentiated partly towards chondrocytes, but still expressing the MSC-markers CD73, CD90 and CD105 as shown by FACS-analysis. If this is true, mandibular-derived MSCs would definitely have benefits in the use of bone tissue engineering.

The influence of DLX-genes on bone tissue engineering

It is already known *DLX1* and *DLX2* are crucial factors for the development of the upper jaw, while *DLX5* and *DLX6* regulate the development of the lower jaw [128-131]. *DLX1* and *DLX5* are upregulated by BMP2 and BMP7 [132]. Especially BMP2 is known as a relevant protein for bone tissue engineering [22]. Zhu and Bendall also demonstrated the importance of *DLX5* and *DLX6* for endochondral ossification [133]. Since the downstream factors of BMP2, *DLX1* and *DLX5,* are upregulated in craniofacial-derived periosteal cells, and the anti-sense of *DLX6* is downregulated in mandibular-derived MSCs these cells are more preferable in comparison to tibial-derived MSCs.

COL13A1 has probably a limited function in bone tissue engineering

Ylönen et al demonstrated the role of *COL13A1* on bone mass and bone formation rate of particular long bones in transgenic mice [90]. *COL13A1* was expressed less in mandibular-derived periosteal cells. This could be a reason to rate those mandibular-derived cells as less preferable. However, we do not know whether *COL13A1* is expressed by MSCs or by other cells such as the more active osteoblasts or even other cells. Because of this, more research has to be done exploring the responsible cells for the high expression of *COL13A1* and its role in bone tissue engineering before mandibular-derived periosteal cells could be rated as less-preferable compared to other sources of MSCs.

The expression of *VEGFB* has a significant role in determining the best source

VEGFB-expression is an important factor in bone tissue engineering since *VEGFB* stimulates angiogenesis. Without vascularization, the bone tissue construct will fail *in vivo* [32, 33]. We showed a higher expression of *VEGFB* in maxillary-derived periosteal cells compared to those obtained from the tibia. We also have shown a higher expression of vascular endothelial growth factor receptor 1 (*VEGFR-1)* in cells obtained from the maxilla, compared to those obtained from tibia and mandible in previous work [63]. In comparison to periosteal cells derived from tibia or mandible, angiogenesis is more stimulated in maxillary cells. From these data, we can conclude maxillary-derived periosteal cells are probably, in case vascularization is the most predicting factor, preferable for use in bone tissue engineering. Although, we do need sufficient vascularization, but not an overstimulation of vascularization.

Differently expressed genes without known function in bone tissue development

The roles of *LHX8 and GPRC5C* in periosteal-derived MSCs are probably clinically non-significant due to their low expression levels, even in maxillary-derived MSCs.

The role of the genes for the transmembrane proteins TMEM150C and TMEM255B in bone development is not yet clear. Either the roles of *AFF,* and *PALMD* in bone development. It could be possible those genes are expressed more in particular sources due to other functions MSCs in periosteum also have to accomplish. However, it would be interesting to know the function of those

genes and their possible role in bone tissue development. Especially for *DPP4*, it would be useful to understand the interaction of this gene with bone metabolism, since overexpression of *DPP4* is associated with osteoporosis [134].

Chronic osteomyelitis gives cortical bone thickening via IL-10 upregulation

SAPHO-syndrome, a syndrome resulting in synovitis, acne, pustulosis, hyperostosis, and osteitis, is known to give cortical bone thickening. In addition, chronic recurrent multifocal osteomyelitis (CRMO) is known too for its hyperostosis, but without skin involvement [135, 136]. These diseases could give us information about the mechanisms how cortical bone tissue is formed. Previous research has shown the anti-inflammatory interleukins 1β (IL-1β) and IL-10, as well as the inflammatory interleukin IL-6 and tumor necrosis factor alpha (TNFα), are key factors in the pathogenesis of these disorders giving chronic non-bacterial osteomyelitis [136-138]. Probably those patients do not produce sufficient amounts of IL-10, resulting in overexpression of IL-6 and TNFα [138, 139]. The teams of Sun and Jang already showed an upregulation of IL-10 by inhibiting DPP4 using a DPP4-inhibitor [140, 141]. Van Vlasselaer et al found already in 1994 an inhibiting effect of IL-10 on TGF-β production, a protein that is needed for starting endochondral ossification [142]. Researchers in the team of Jung found IL-10 stimulates chondrogenic hypertrophy [143]. This step occurs after chondrogenic differentiation in the endochondral ossification pathway. Chen et al recently showed a concentration-dependent, dual interaction between IL-10 and the P38/mitogen-activated protein kinases (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways [144]. They influence in this way the osteogenic capacity of human bone marrow stromal cells (hBMSCs) [144]. Chen et al showed IL-10 stimulates hBMSC proliferation and a low dose of IL-10 stimulates the expression of *RUNX2*, a gene involved in endochondral ossification and maturation of osteoblasts. Also, the p38/MAPK pathway was stimulated by low dose IL-10 [144]. High expression of *IL-10* inhibits *RUNX2* expression and p38/MAPK signaling, but activates NF-κB signaling [144]. This NF-κB signaling acts as a mediator for the negative regulation of osteogenesis [144].

Future

For future research, it would be interesting to acquire more knowledge about the function of especially *DPP4* in bone tissue engineering. A lot of research has been done about *DPP4* and DPP4-inhibitors to improve glucose tolerance in diabetes [134]. We already know overexpression of *DPP4* results in osteoporosis and inhibiting *DPP4* leads to a higher bone mass, particularly at the distal end of long bones. Although, less is known about the mechanisms of *DPP4* acting on bone formation. We know there is an association between lowering levels of DPP4 and higher expression of IL-10, resulting in chronic non-bacterial osteomyelitis. We also know this symptom is frequently associated with hyperostosis. More information about the mechanism of *DPP4* interacting on bone metabolism could be acquired by defining the cells where *DPP4* is expressed. In order to investigate this, it would be useful to create sections of bone tissue including periost and perform in situ hybridization on them to find the cells that are expressing this gene. This could also be done by immunohistological staining on them since human primary antibodies are already available for DPP4 proteins [145]. Next question to be answered would be to determine the upstream factors of *DPP4*. These factors could be found by adding or blocking some potential upstream factors, concerning the involved pathways.

It would also be nice to perform more research to define the function of *TMEM150C* and *TMEM255B* with regard to bone tissue formation. We found an upregulation in respectively maxillary-derived MSCs and tibial-derived MSCs, while we don't have any information about their possibility to influence in bone tissue engineering. This could be done by creating knock-out mice for those genes and analyzing the bone volume by Nano-computer tomography (CT) scans, weight

and histological staining's. The cells expressing those genes could be found by immunohistological staining or in situ hybridization techniques on slides containing bone tissue and their surrounding tissues of knock-out mice. The downstream factors could be investigated by stimulation- and inhibition-studies while measuring gene expression levels of potentially involved genes. The upstream factors could be found, as described above, by adding or blocking some potential upstream factor.

The same techniques could also be used to acquire knowledge about possible functions of *AFF2* and *PALMD* in bone tissue metabolism or development.

In order to test whether the mandibular-derived MSCs where already differentiating towards chondrocytes, additional FACS analyses could be performed. FACS analysis was performed according to the MSC-criteria stated by Dominici and colleagues in Cryotherapy in 2006 [63, 146]. These markers include CD14, CD20, CD34, CD45, CD75, CD90 and CD105. CD14 is found on macrophages and monocytes at the surface, CD20 is found on B-lymphocytes. CD34 is a marker for hematopoietic progenitor cells and CD45 is a marker for hematopoietic stem cells. These markers were all expressed for less than 2%. CD75, CD90 and CD105 are markers for MSCs. These were expressed for more than 95% in mandibular-derived periosteal cells. Markers testing especially for cells that were differentiated towards chondrogenic lineages where not included. In future research, it would be nice to test for chondrogenic markers too, in order to answer this question.

Obviously, this is just a beginning of innovative research into bone tissue engineering. Bone tissue engineering implies a combination of selecting the proper cells, excellent *in vitro* priming techniques, selecting the best scaffolds to give the tissue a three-dimensional structure and perfect timing of the *in vivo* implantation. More research is needed to optimize the *in vitro* culture and priming of craniofacial-derived periosteal MSCs. Also, the proper scaffold still must be designed for three-dimensional bone tissue engineering using those cells. Lastly, *in vivo* animal research is required to increase the success rates and demonstrate the safety of this way of bone tissue engineering before these techniques can be tested in the clinic.

Conclusion

In this study, we have shown the importance of selecting the proper origin for cells to be used for bone tissue engineering. Surprisingly, a lot of genes involved in embryological development, such as the HOX-genes, DLX-genes, *BARX1,* and *PAX1,* are still active in periosteal cells from 16-30-year-old people. Based on the information of gene properties currently available, craniofacial-derived periosteal MSCs show some advantages in comparison to tibial-derived periosteal MSCs for bone tissue engineering since. With this research, we contributed to gathering information in order to select the proper cells for bone tissue engineering. A lot of work still has to be done before bone tissue engineering can be performed in clinical practice.

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Supplementary table

Gene	Forward code	Reverse code
HOXC10	CGGATAACGAAGCGAAAGAGGAG	GCGCTCTCGCGTCAAATACA
HOXA10	AGGATTCCCTGGGCAATTCCAAA	TTGTCTGTCCGTGAGGTGGA
HOXA11	TTTGATGAGCGTGGTCCCTG	AGTATGTCATTGGGCGCGAA
HOXC9	CCCCAGTAAGTTGGGAGCAAT	CCGACGGTCCCTGGTTAAAT
HOXA7	GGAGTTCCACTTCAACCGCT	CGGACCTTCGTCCTTATGCTC
HOTAIR	GCCAGTACCGACCTGGTAGA	GTCTGTGAGTGCCCGTCTTG
TMEM255B	CGTCCTCATAGTCACCGTCG	GCCACCAGCATTTGCCTTC
DPP4	GCTCGGCGCTCACTAATGTT	CACGGTGTCTTCATCGTCGG
DLX5	CACGGCTACTGCTCTCCTAC	CTTCTTTCTCTGGCTGGTTGGTG
DLX6	GAGGGGACGACACAGATCA	GTTCGGCTCTCTCTGGAAGG
DLX6-AS1	GGAGGATTCTGTGTGGGGTTG	ATGGGAGCACTCAGCCTACC
BDKRB1	GCCCCCTCTAGAGCTCCAAT	AAAGGTTCCCTAGGAGGCCG
LRRC15	GAGCTGCTGATGTGTCCCTAGC	GCCCACCAGCAAAAGGAGATAA
AFF2	TCTTGCGGGAGATGACCCATT	GACACTGACTTTGTAGAAGCTCTGG
COL13A1	CCACACCGGGAGTGCCTAAG	CCTGGAACGTCCGCCTTTTT
STAT4	TGGGTGGGAACTGACCCAAG	AGACATGCTAGCGCTCTCTCAG
DLX1	CGCTTCAATGGCAAGGGAAA	ATCTTGACCTGAGTCTGTGTG
TFAP2C	CTCCACGACATGCCTCACCA	TGGAAATGGGACCTTTGCGAAT
SMAGP	TTCAAAAGGACGCGCGGAG	GGGGTGGTCATCAGTTCTTCTCT
HOMER2	TGGGAGAACAGCCCATCT	TATGATCACCTTGGCTCCGTC
VEGFB	TATACTCGCGCTACCTGCCA	TGAGGATCTGCATCCGGACTT
BARX1	CAAAGCCAAGAAGGGGCGTC	ATCTATTCTGTCCGGCGTGGAA
LHX8	CTGGACCACTTGTGCTGGAGT	GGAAGCGTTTCCAGTCAAGCC
PAX1	TGCGCAAGGTCCTCCTCTG	GGAGGCTTCCTTCTCGGCT
HCN1	CTTTGGAGAGATTTGCCTGCT	AGTCGGTCAATGGCAACTGT
SLC1A7	GGCTCTCATGCGTGGAATGG	TAATTTCCTGTGGTGAGAGGCG
PALMD	AGACTCCAGGCCATCACAGATAA	TTCCTGTTCTTTTCCGCTGCT
GPC3	ACCACCACTGAAACTGAGAAGAA	TTGGCGTTGTTGAGAATGGGC
GPRC5C	TGCACAAAGTTCCGTCCGAAG	GTCCCACACGTAGGGGTTTCT
TMEM150C	AGATCCCACCCCTTCCTGA	CCATGCCTGTACCACTGCT