ORIGINAL ARTICLE

Ca^{2+}-deposition in cell matrix correlates significantly with osteocalcin-expression in osteogenic differentiated ATSC: Even in a coculture system with HUVEC

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ABSTRACT

Background: Tissue engineering offers the means for replacing or repairing diseased organs within the patient's body. The current problem in its clinical use is sufficient and fast revascularization of the transplanted tissues. The idea of bone-reconstruction deals with three-dimensional bone equivalents that are composed of endothelial cells (ECs) and adipose tissue derived stromal cells (ATSCs) showing osteogenic differentiation. **Materials and Methods:** ATSC were isolated, cultivated until third passage and osteogenically differentiated by 1.25-dihydroxycholecalciferol. Coculture systems with human umbilical vein endothelial cells (HUVEC) were performed. Osteogenic differentiation was analyzed in FACS-analyses (n = 7), by the measurement of Ca^{2+}-deposition in the cell matrix (marker for osteogenic differentiation) and the expression of alkaline phosphatase (AP). **Results:** Ca^{2+}-deposition in the cell matrix and osteocalcin-expression correlated significantly (P = 0.030) during osteogenic differentiation (n = 7). The osteogenic cell differentiated ATSC in the coculture system (n = 6) even showed a clear increase of Ca^{2+}-deposition. The time of starting the coculture did not influence the differentiation. Measurement of the Ca^{2+}-deposition correlates significantly to the osteogenic differentiation and osteocalcin-expression. **Conclusion:** ATSC are a promising source for bone tissue engineering. They can be differentiated into osteoblasts in a coculture system with HUVEC without the loss of any differentiation capacity. For bone tissue-equivalent fabrication, this is an encouraging procedure that is feasible and provides fast revascularization of the bone-equivalents.

Key words: Cell matrix Ca^{2+}-deposition, co-culture with HUVEC, osteogenic differentiation of ATSC, screening method

INTRODUCTION

The aim of tissue engineering is to develop substitute tissues for replacing or otherwise restoring the function of damaged human tissues.[1] The basic idea of tissue engineering in the field of bone research is to generate "new bone" *in vitro*, *ex vivo*, or *in vivo* by combining osteo-conductive material, osteopotential cells with suitable growth factors. In the "classic way" of bone tissue engineering, there is a need for specific osteogenic cells (e.g. osteoblasts) or their progenitors (e.g. periosteal cells). These more or less differentiated cells are cultivated and seeded on biocompatible materials in order to create engineered bone equivalents. The problem with this approach remains in the low proliferation capacity of differentiated cells. The answer lies in adding progenitor or stem cells of high amplification rate with the potential to differentiate to lineages of mesenchymal tissues.

Bone marrow (BM) was the first source reported to contain mesenchymal stromal cells (MSCs) with a stem-cell-like character (bone marrow-derived stem cells, BMSCs). Several experimental approaches have been used to characterize the development and functional nature of these cells *in vivo* and their differentiation potential *in vitro*.[2] However, for clinical use, BM may be detrimental due to the highly invasive harvesting procedure and the decline in MSC number and differentiation potential with increasing age. In search of alternative sources to obtain MSCs the peripheral blood and

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the adipose tissue (AT) were found. Fat is easily accessible with minimal invasive techniques and represents an abundant reservoir of multipotent progenitor cells.[3,4]

In clinical use, the main problem in bone-tissue transplantations is high initial resorption rate of the transplanted equivalents, unless there is sufficient revascularization.[5,6] Therefore bone equivalents should contain both, osteoblastic and vascular cells, to accelerate the revascularization process of the bone tissue equivalents in the receptor area.

The osteogenic differentiation potential of adipose tissue derived stromal cells (ATSC), harvested according to a previously described cultivation method was analyzed in a coculture system with human vascular endothelial cells (HUVEC).[7] The measurement of the intracellular Ca^{2+}-deposition during the osteogenic differentiation was a reliable screening-method for osteogenic differentiation.

MATERIALS AND METHODS

Cells

ATSC

Preparation and cultivation of ATSC is according to methods described before.[6,7] Small pieces of subcutaneous AT (<0.5 cm^3) from the lateral thigh of seven different donors (n = 7) were acquired during elective surgery (Department of Oral and Maxillofacial Surgery,). Informed consent was obtained. The AT was minced with sterile scissors and subjected to collagenase digestion (collagenase type II, Boehringer, Mannheim, Germany). The suspension was centrifuged (300 g/10 min) and plated in tissue culture flasks (Greiner, Frickenhausen, Germany). Cells were cultured in 5% humidified CO_2 atmosphere at 37°C. "Standard" culture medium (Iscove's modified Dulbecco's medium IMDM/HAM F12 1:1) supplemented with 10% NCS (neonatal calf serum; all from Life Technology, Paisley, Scotland). It was changed every second day and used as control. After cell-colonization, the complete surface of the first culture flask were brought into suspension by trypsination (0.25% trypsin, 1 mM EDTA) and distributed in four new flasks (1st passage). Subsequently, they were split (1:4/5 ratio) and amplified up to the 3rd passage. The undifferentiated cells were negative for osteocalcin [Figure 1a].

HUVEC

HUVEC were isolated from umbilical vein vascular wall (informed consent was obtained) according to the technique followed by Jaffe *et al*.[8] Then they were seeded on fibronectin-coated plates and cultured in a Dulbecco's Modified Eagle Medium (Invitrogen, Karlsruhe, Germany) with Earles' salts (Invitrogen, Karlsruhe, Germany) and 10% NCS for 7 days in a incubator (37°C, 5% CO_2 atmosphere). Cell confluence was monitored by phase-contrast microscopy. [Figure 1b].

Coculture system

For the osteogenic differentiation in the coculture a two-dimensional dish system was used (n = 6). The cells were maintained in "standard" medium until they showed a confluence of 80% in the 3rd passage. After

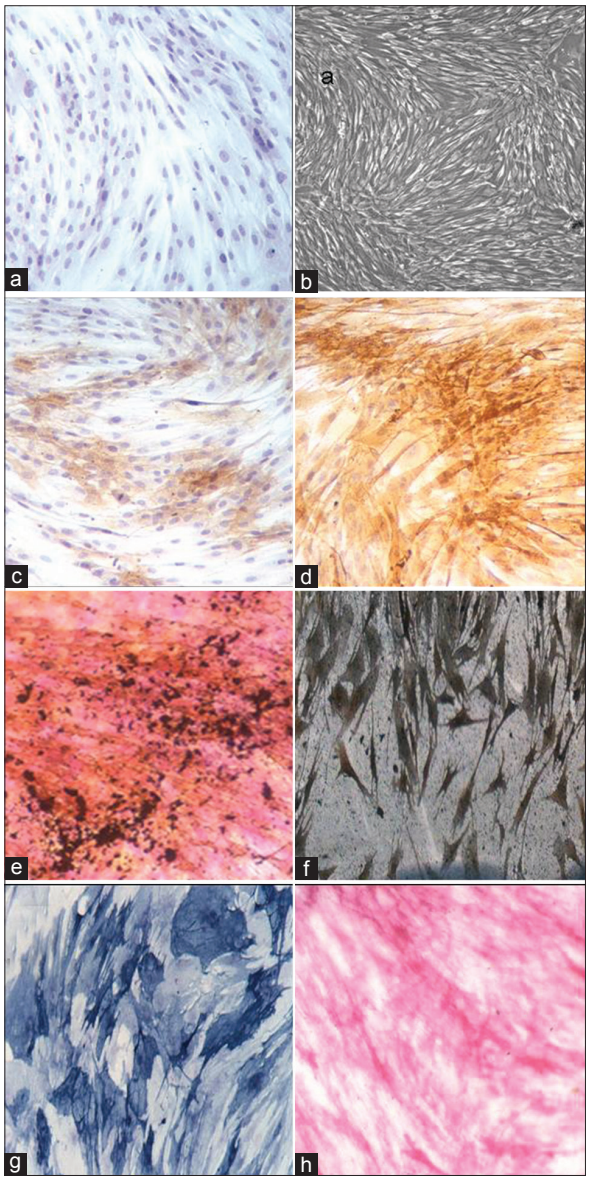

Figure 1: (a) Negative staining for osteocalcin in undifferentiated adipose tissue derived mesenchymal stromal cells (ATSC) (IHC stain, ×200), (b) Cell confluence of human umbilical vein endothelial cells (HUVEC) monitored by phase-contrast microscopy (x100). There was a positive expression of stem cell specific marker SH2 (c) (IHC stain, ×100) and SH3 (d) (IHC stain, ×100) in these undifferentiated ATSC. Osteogenic differentiation showed a positive reaction to Von Kossa (e) (Von Kossa stain, ×100), (f) silver staining (silver nitrate, ×100), (g) osteocalcin protein (IHC stain, ×100) and a positive reaction to enzyme alkaline phosphatase (h) (colorimetric enzyme assay, ×100)

seeding the ATSC in 25 cm^2 cell-culture flasks (Thermo Electron LED GmbH, Langensel bold, Germany), by splitting 1:3-1:5 (ca. 80.000-100.000 cells/25 cm^2), the HUVEC (ca. 40.000 cells/25 cm^2) were added to the ATSC at different times [Table 1]. Osteogenic differentiation was mediated by "osteogenic" differentiation medium (IMDM/F12, 10% NCS, 10 mM dexamethasone, 10 mM β-glycerophosphate, 10 nM 1.25-dihydroxycholecalciferol) as described by Beresford *et al*.[9] Additional parallel running cultures received the "standard" medium as control. The medium was changed twice a week.

Flow cytometry (FACS-analyses)

Osteogenic differentiated ATSC were analyzed at different times [Table 2]. Therefore cell-cultures (n = 7) were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS). FACS-analyses were done with the stem-cell specific markers SH2 (CD105) [Figure 1c], SH3 [Figure 1d] (CD73, both provided by Vita34, Leipzig, Germany) and the fibroblastic-marker AS02 (CD91) (Dianova GmbH, Hamburg, Germany) and the monoclonal rabbit osteocalcin antibody (Biotrend, Köln, Germany).[10] Negative control was IgG1-isotype (Dako, Denmark).

Matrix mineralization (Ca^{2+}-deposition) and alkaline phosphatase (AP) activity

Osteogenic differentiation of ATSC was measured by the extent of matrix mineralization (calcium-deposition) during the differentiation process. Therefore 25 cm^2-culture flasks (Greiner, Germany) were harvested prior to differentiation (week 0, undifferentiated), in the 1st week, the 3rd and the 5th week after osteogenic differentiation. Cell cultures receiving standard medium were analyzed as control. After rinsing, the bottom of the flasks were shaken with 0.5 N HCl for 4 hours; the supernatant was centrifuged (1000 g/10 min) to remove cell remnants. Calcium concentration (Ca^{2+}) was measured by the o-cresolphthalein-complex-method at 570 nm in the photometer and the results were shown graphically (µmol/cm^2).[11] AP activityin the culture medium was measured by colorimetric enzyme assay in µkat/l

Table 1: Experimental protocol for the coculture system of ATSC and HUVEC in simultaneous cultivation

Group (n=6)	Differentiation	HUVEC-addition		1 Week (mean±SD)	3 Weeks (mean±SD)	5 Weeks (mean±SD)
1 control	None	Non-addition	Ca^{2+}	0±0	0±0	0±0
			AP	0.35±0.03	0.34±0.03	0.36±0.05
2	Non	Simultaneous addition	Ca^{2+}	0±0	0±0	0±0
			AP	0.33±0.03	0.34±0.05	0.46±0.09
3	Osteogenic	Non	Ca^{2+}	0.03±0.04	0.15±0.04	0.87±0.23
			AP	0.3±0.06	0.28±0.05	0.65±0.14
4	Osteogenic	Simultaneous addition	Ca^{2+}	0.03±0.04	0.15±0.04	0.86±0.22
			AP	0.33±0.03	0.37±0.06	0.43±0.05
5	Osteogenic	Simultaneous and at 2nd medium change	Ca^{2+}	0.03±0.05	0.16±0.08	0.76±0.06
			AP	0.34±0.03	0.37±0.06	0.43±0.05

HUVEG: Human umbilical vein endothelial cells; ATSC: Adipose tissue derived stromal cells; SD: Standard deviation; Ca^{2+}-deposition in the coculture system (mean±SD) showed a significant increase in osteogenic differentiated cocultures (n=6)

Table 2: FACS-analyses of the time-depended marker expression (mean±SD) during osteogenic differentiation (n=7) of ATSC, isolated from seven different donors

	Undifferentiated Control (mean±SD)	Osteogenic Control (mean±SD)	Undifferentiated 3 Weeks (mean±SD)	Osteogenic 3 Weeks (mean±SD)	Undifferentiated 4 Weeks (mean±SD)	Osteogenic 4 Weeks (mean±SD)	Undifferentiated 5 Weeks (mean±SD)	Osteogenic 5 Weeks (mean±SD)
SH2	36.0 ± 7.7	23.9 ± 14.5	19.7 ± 10.0	27.1 ±15.2	15.0 ± 7.5	25.8 ± 15.8	9.3 ± 3.0	16.5 ± 12.6
SH3	69.3 ± 19.2	45.3 ± 17.1	35.0 ±17.5	34.8 ± 19.1	27.0 ± 13.3	41.4 ± 20.6	9.2 ± 5.6	29.7 ± 14.4
OC	0.5 ± 0.3	0.1 ± 0.1	40.1 ±	0.12 ± 0.2	55.8 ± 17.7	0.3 ± 0.3	45.0 ± 16.9	0.56 ± 0.6
AS02	36.0 ± 13.9	34.5 ± 7.8	33.9 ± 21.0	32.86 ± 5.9	16.7 ± 9.4	35.8 ± 17.9	9.7 ± 4.1	60.2 ± 13.3
Control	0.4 ± 0.05	0.4 ± 0.2	0.6 ± 0.2	1.54 ± 2.0	1.7 ± 2.3	1.1 ± 1.2	1.2 ± 1.4	1.2 ± 1.0
Ca^{2+}-deposition (µmol/cm^2)	0.02 ± 0.0	0 ± 0	0.0 ± 0.0	0.06 ± 0.04	0.01 ± 0.0	0.28 ± 0.09	0.0 ± 0.0	0.78 ± 0.1
AP activity (µkat/l)	0.29 ± 0.03	0.14 ± 0.06	0.30 ± 0.02	0.3 ± 0.04	0.42 ± 0.11	0.58 ± 0.03	0.42 ± 0.16	0.98 ± 0.09

FACS: Fluorescence activated cell sorter; SH: Stem cell specific marker ; AS: Fiborblastic marker; OC: Osteocalcin; SD: Standard deviation; ATSC: Adipose tissue derived stromal cells; ACS: Analysis of osteogenic differentiated ATSC (n=7) compared with the intracellular Ca^{2+}-deposition and the AP-activity. The calcium-deposition in the cell matrix significantly corresponded to the osteocalcin-expression (P=0.03). The undifferentiated ATSC showed no ostoecalcin-expression and calcium-deposition of the cells (P=0.031)

Statistical evaluation

All results were analyzed statistically (SPSS, version 12) using the *t*-test. Statistical significance was accepted when the probability $P \leq 0.5$.

RESULTS

Osteogenic differentiation of ATSC

ATSC of seven different donors ($n = 7$) showed an increasing osteocalcin-expression [Figure 1g] (FACS-analyses) and a significant corresponding increase of the Ca^{2+}-deposition in the cell matrix ($P = 0.03$) during osteogenic differentiation [Figure 1e and f]. The stem-cell specific markers SH2 (CD105, $P = 0.01$), SH3 (CD73, $P = 0.06$) and the fibroblastic marker AS02 (CD90, $p_{AS02} = 0.06$) decreased significantly during the differentiation-period [Figure 2a].

The nondifferentiated ATSC (control) showed no osteocalcin-expression. There was a slow, but significant decrease of the stem cell specific marker SH2 and SH3 ($p_{SH2} = 0.01$, $p_{SH3} = 0.005$) during cultivation. These findings were accompanied by an increase in non-specific fibroblastic marker $AS0_2$, caused by an imbrutement of the cell culture [Table 2, Figure 2b] during *in vitro* cultivation. There was no intracellular calcium deposition as a marker for the matrix mineralization in the control group.

Time-depended matrix mineralization and alkaline phosphatase activity

Ca^{2+}-deposition in the cell-matrix [Table 2, Figure 3a] showed a clear increase in the osteogenic differentiated cells from the 3rd week (mean: 0.28 µmol/cm², SE: 0.09) to the 5th week (mean: 0.78 µmol/cm², SE: 0.1).

AP increased consecutively, but was less distinct after the 3rd (mean: 0.58, SE: 0.03) to the 5th week (mean: 0.98, SE: 0.09, [Table 2, Figure 3b. Figure 1h].

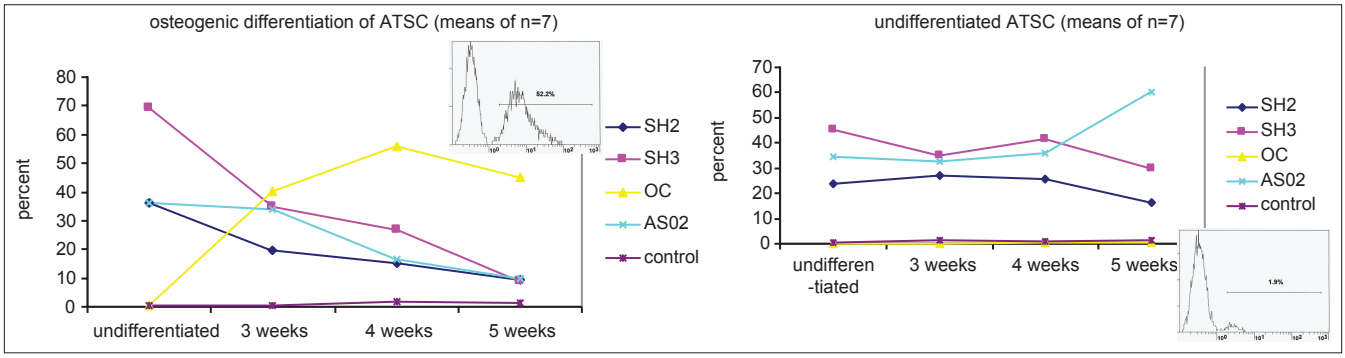

Figure 2: FACS-analysis of the marker expression during osteogenic differentiation of ATSC (n = 7). All stem-cell specific markers (SH2, SH3) decreased during osteogenic differentiation, while the specific osteogenic marker osteocalcin increased. ATSC of the control group (showed a light decrease of the stem-cell specific markers over the cultivation period and an increase of the unspecific fibroblast marker AS02 caused by imbrutement of the cell cultures. There was no expression of osteocalcin in undifferentiated cells

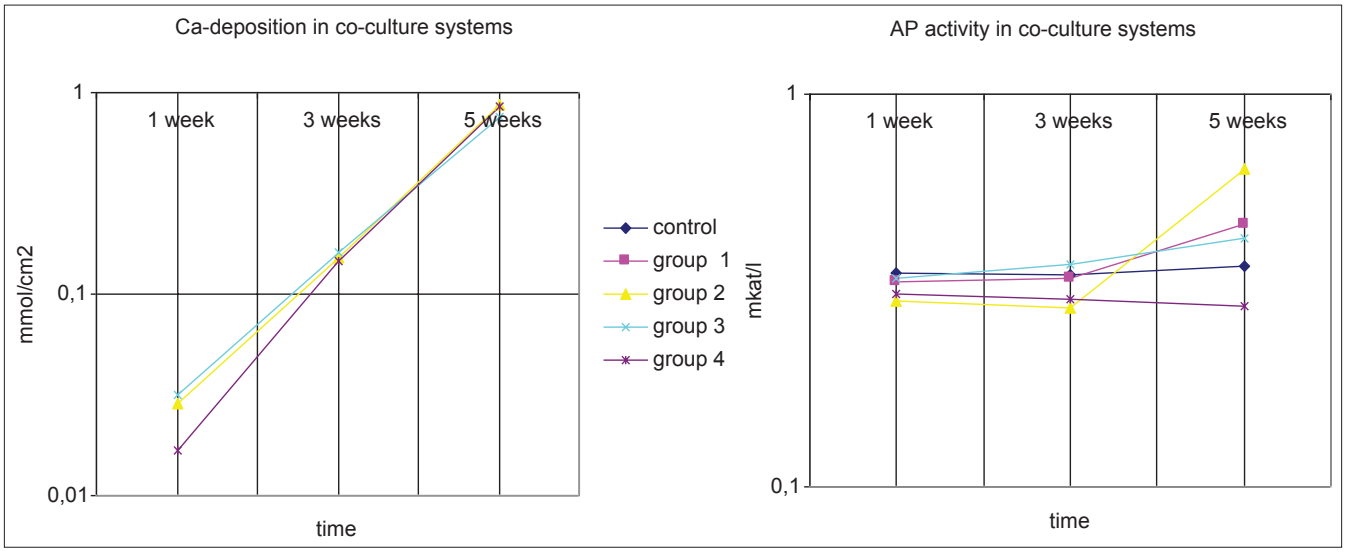

Figure 3: Graphical description of the Ca^{2+}-deposition and AP expression in a coculture system over 5 weeks of osteogenic differentiation. There is a clear increase of the Ca^{2+}-deposition during differentiation time, independent of time of starting the co-culture

Coculture system of osteogenic differentiated ATSC and HUVEC

HUVEC were added to ATSC during osteogenic differentiation at three different times [Table 1]. Group 1 was the first control group of ATSC that were not osteogenically differentiated and received no addition of HUVEC (control 1). The second group was another control group. This was to evaluate how far there was any influence of HUVEC on the calcium-deposition or the AP-activity in the coculture system (HUVEC-control). The third group was to analyze the calcium-deposition or the AP-activity in osteogenic differentiated ATSC without any addition of other cells (osteogenic control). In groups 4 and 5, HUVEC were added to the osteogenic differentiated ATSC at two different times (simultaneous and at 2nd medium change (2 times), to analyze any potential influence of HUVEC on the osteogenic differentiation of ATSC.

The calcium-deposition was measured after 1, 3 and 5 weeks of coculturing during osteogenic differentiation ($n = 6$).

ATSC showed the same potential of osteogenic differentiation by a clear and strong increase of Ca^{2+}-deposition in the cell matrix in the coculture system with HUVEC and without. This means there was no negative influence on the osteogenic differentiation potential by the addition of other cells. Time variation in starting a co-culture system did not have any adverse effect on the osteogenic differentiation potential.

DISCUSSION

ATSC are suitable and abundant source for tissue engineering of bone equivalents.[7,12] The vascularization of the transplanted tissue and its survival in the recipient area is still a problem, particularly when the recipient area is prestressed by radiation or former surgical approaches.

In maxillofacial and plastic aesthetic surgery, reconstruction of bone is very important for good aesthetic outcome. Til now, the transplantation of free bone equivalents and micro vascular anastomosed bone grafts is still challenging. The free bone equivalents require still a sufficient vascular supply and nutrition for a sufficient engraftment. Therefore different studies deal with the *in vitro* fabrication of functional blood vessels to provide a sufficient vascular supply to engineered bone grafts.[6]

In all cell cultures ($n = 7$, $n_{co\text{-}culuture} = 6$) the undifferentiated ATSC could be differentiated into matured osteoblasts, induced by 1.25-dihydroxycholecalciferol.[9] These differentiated osteoblasts secreted matrix rich in collagen I that calcified during the later stages of differentiation.[12,13] The mineralization and maturation of the osteogenic differentiated ATSC was accompanied by a significant increase in expression of the osteoblast-associated protein osteocalcin during the differentiation period.[10] All specific stem cell markers decreased during osteogenic differentiation, that can be explained by an imbrutement of the cells during *in vitro* cultivation.

The calcium-deposition in the cell matrix showed strong increase during the osteogenic differentiation, according to the findings in the FACS-analyses by the quantitative detection of osteocalcin, while the AP stayed almost the same.

In all cell culture systems the ostoegenic differentiation was reliable. HUVEC did not show any negative influence on the osteogenic differentiation of ATSC. This fact provides a possibility to fabricate ATSC tissue equivalents with other cell-types.

In summary, the measurements of the Ca^{2+}-deposition in the cell matrix showed a strong significant and reliable correlation of the osteocalcin-expression [Table 2, $P = 0.03$] in ATSC cells with osteogenic differentiation.

REFERENCES

1. Langer R, Vacanti JP. Tissue engineering. Science 1993;260:920-6.
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, *et al*. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-7.
3. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, *et al*. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13:4279-95.
4. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, *et al*. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001;7:211-28.
5. Blanton M, Hadad I, Johnstone BH, Mund JA, Rogers PI, Eppley BL, *et al*. Adipose stromal cells and platelet-rich plasma therapies synergistically increase revascularization during wound healing. Plast Reconstr Surg 2009;123:55-64.
6. Frerich B, Zückmantel K, Hemprich A. Microvascular engineering in perfusion culture: immunohistochemistry and CLSM findings. Head Face Med 2006;2:26.
7. Frerich B, Lindemann N, Kurtz-Hoffmann J, Oertel K. *In vitro* vascular stroma model for the engineering of vascularized tissues. Int J Oral Maxillofac Surg 2001;30:414-20.
8. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973;52:2745-56.
9. Beresford JN, Joyner CJ, Devlin C, Triffitt JT. The effects of dexamethasone and 1.25-dihydroxyvitamin D3 on osteogenic differentiation of human marrow stromal cells *in vitro*. Arch Oral Biol 1994;39:941-7.
10. Halvorsen YC, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, *et al*. Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng 2001;7:729-41.
11. Gosling P. Analytical reviews in clinical biochemistry: calcium measurement. Ann Clin Biochem 1986;23:146-56.

12. Weinzierl K, Hemprich A, Frerich B. Bone engineering with adipose tissue derived stromal cells. J Craniomaxillofac Surg 2006;34:466-71.
13. Gundberg CM, Hauschka PV, Lian JB, Gallop PM. Osteocalcin: isolation, characterization, and detection. Methods Enzymol 1984;107:516-54.

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