Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 362 (2007) 17–24
BBRC
www.elsevier.com/locate/ybbrc

Osteo-maturation of adipose-derived stem cells required the combined action of vitamin D3, β-glycerophosphate, and ascorbic acid

Gupta Anurag [a], Leong David Tai [b], Bai Hui Fen [c], Singh Shiv Brat [a,d], Lim Thiam-Chye [e], Hutmacher Dietmar Werner [c,f,g,*]

[a] School of Medical Science and Technology, Indian Institute of Technology, Kharagpur, India
[b] Department of Biological Sciences, National University of Singapore, Republic of Singapore
[c] Division of Bioengineering, National University of Singapore, Republic of Singapore
[d] Department of Materials and Metallurgical Engineering, Faculty of School of Medical Science and Technology, Indian Institute of Technology, Kharagpur, India
[e] Division of Plastic Surgery, National University Hospital, Republic of Singapore
[f] Department of Orthopedic Surgery, National University of Singapore, Republic of Singapore
[g] Institute of Health and Biomedical Innovation, Queensland University of Technology, Australia

Received 10 July 2007
Available online 1 August 2007

Abstract

This study investigated the effects of various components [vitamin D3 (VD3), β-glycerophosphate (BGP), and ascorbic acid (AA)] on the potential of human adipose-derived progenitor cells (ADPCs) to transdifferentiate into osteoblast-like cells. ADPCs were induced under four different supplement groups: (1) VD3 + BGP + AA, (2) VD3 alone, (3) BGP + AA, and (4) no VD3, BGP or AA. Mineralization studies and presence of bone matrix-related proteins by immunostaining showed that the Group 1 ADPCs showed their ability to undergo osteoblastic differentiation. Further evaluation was made by estimation of levels of RUNX-2 and TAZ genes. Group 1 ADPCs showed the consistent expression of RUNX-2 and TAZ levels over the study period of 28 days. The study showed good correlation among various parameters evaluated to conclude that ADPCs could be an alternative source for generating osteoblast-like cells.

Keywords: Adipose-derived stem cells; Vitamin D3; Osteoblasts; Differentiation; Induction

The bone marrow mesenchymal stem cell is one of the most well-characterized adult stem cell types. These mesenchymal stem cells are capable of differentiating into osteoblast-like cells. In recent years, descriptions of other possible adult stem cell sources are emerging amongst the stem cells community. Adipose-derived stem or progenitor cells (ADPC) might be a promising alternative to bone marrow mesenchymal cells [1] as studies have indicated that the yield of ADPCs is approximately 5000 CFU-F/g adipose tissue. This compares with estimates of approximately 100–1000 CFU-F/ml bone marrow [2]. Hence, it is argued from a tissue engineering perspective that ADPCs could give an improved yield of the order of 10. We and others have demonstrated the ability of ADPCs to undergo differentiation along osteogenic lineage in cell culture [3–5]. However, more recent data demonstrated a large variation of osteogenic potential among donors and that vitamin D—1α,25-dihydroxycholecalciferol (VD3) might be used instead of dexamethasone. However, the underlying etiology remains unknown.

It has been studied that the progenitor cells for adipocytes and osteoblasts share some of the cell markers and properties in their earlier part of development [6,7]. Therefore, it is possible that precursor cells developed from a more primitive progenitor/stem cell. These precursor cells

* Corresponding author. Address: Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove Campus, Qld 4059, Australia. Fax: +61 7 313 86030.
E-mail address: Dietmar.Hutmacher@qut.edu.au (D.W. Hutmacher).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.07.112

then shared a common path along their differentiation progress and diverge from a common point forming either adipocytes or osteoblasts depending upon the requirements of the body and local environmental stimuli like, Runt-related transcriptional factors, Smad proteins, BMP-2, and collagen type 1 [6,7]. This cascade of factors eventually leads to the expression of important bone matrix proteins like osteocalcin, osteopontin, osteonectin, alkaline phosphatase, and a mineralized matrix [8].

VD3 plays an important physiological role in bone formation and its maturation. Importance and requirement of VD3 in bone biology starts from the very beginning, affecting proliferation and differentiation of early members of osteoblastic lineage in addition to enhancing matrix mineralization for mature bone formation. It helps in maturing bone synthesis by increasing Ca^{2+} concentration at the mineralization site in the bone. Moreover, it has been reported that it increases gene expressions of genes which help in matrix formation and mineralization of bone. It is also reported that VD3 promotes the early differentiation of progenitor cells to osteoblastic lineage and it decreases apoptosis in osteoblast-like cells [9]. Cross-talk of the osteogenic signals of RUNX-2 and VD3 at bone-specific promoters may represent an important component of the mechanisms mediating tissue-specific expression of osteoblast phenotypic genes [10]. However, not much is known of the specific impact of VD3 on multipotent precursor cells from human adipose tissue.

This study investigated the efficacy of VD3 on osteogenic induction of progenitor cells from human adipose tissue with or without supplementation of ascorbic acid (L-ascorbic acid-2-phosphate) and an organic phosphate source (β-glycerophosphate) with the aim of a better understanding of *in vitro* osteogenesis of ADPC which can allow a better refinement to the current induction regime and its application in bone engineering.

Materials and methods

Samples were collected from the abdominal region with approval from the Institutional Review Board of National University Hospital, Singapore, and written informed consent from donors (healthy females, mean age = 42 ± 7.5 years undergoing elective liposuction). Aspirated adipose tissue samples were washed with sterile PBS and digested with Collagenase I (Gibco) at 37 °C with shaking for 1 h. After centrifugation at 1000*g*, the cell pellet was resuspended and plated in Dulbecco's modified Eagle's medium (Sigma, D 1152) with 10% fetal bovine serum (Hyclone) and cultured. Passaging of cultures took place when 90% confluence was reached.

Experimental cell cultures and induction regimes. Cells were cultured at a density of 3000 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. For calcified matrix staining and immunostaining for the bone matrix proteins, cells were plated in 48-well plates (Nunc) and for collecting RNA samples, cells were plated in 60-mm cell culture dishes (Nunc). For every experimental procedure, cells were plated in four groups according to four different induction regimes (Table 1). Samples were assayed at Day 0, 7, and 28 of induction under the four induction regimes.

Alizarin red S staining for calcified matrix. For each time point, the culture medium was removed and washed twice with PBS. Cells were fixed in 10% formalin solution for 10 min and stained with 200 μl 2% Alizarin red S (Sigma, A5533) in each well for 5 min. The dye from the wells was washed thoroughly with distilled water. Samples were evaluated under microscope for identification of calcified nodules and were documented with Leica DMIRB, Wetzlar, Germany.

Immunostaining for bone matrix-related proteins. For each time point, the culture medium was removed completely and washed twice with PBS. Samples were fixed in −20 °C methanol for 10 min and blocked with 10% goat serum (Dako, X0907) for 1 h. Respective wells were incubated with 150 μl primary antibody dilutions for Collagen type 1 (1:500, Chemicon AB0745), Osteopontin (1:500, Chemicon AB1870), Osteonectin (1:1000, Chemicon AB1858), and Osteocalcin (1:500, Chemicon AB1857) for 90 min on a shaker. The primary antibodies were washed thrice with PBS, 5 min between washes. One hundred and fifty microliters secondary antibodies (provided with Dako EnVision kit, K4011) was added for 1 h. DAB solution was added to each well and incubated for 5 min and then washed with water. Each sample was treated with hematoxylin for nuclear staining.

Real-time PCR for absolute quantification of genes. RNA extraction, deoxyribonuclease I (DNase I) treatment, and reverse transcription, PCR standards preparation, quantitative real-time PCR were done as per the established protocol by our group [11].

Primer design. Primers for genes of interest—RUNX-2 (5′ TCTTCCC AAAGCCAGAGTG; CATGGGAAACTGATAGGATCC 3′) and TAZ (5′ CGTGAAGTGGCCGTTCCC; AGGTGGTTCATGTACTTGGTCC 3′) were designed and selected the pairs which were found unique after "blastn" (http://www.ncbi.nlm.nih.gov/BLAST/). Moreover, the unique amplified products were confirmed on 3% agarose gel.

Results

VD3, BGP, and AA are essential for mineralization

The Alizarin red S staining of the ADPC cultures at Day 0 and Day 7 of induction showed no focus of mineralization (Fig. 1A and insets, respectively). Cells cultured with combination of VD3 along with BGP and AA (Group 1), clustered and formed distinct mineralization foci after 28 days of induction (Fig. 1B) whereas cultures induced alone with VD3 or with combination of BGP and AA did not show any focus of mineralization (Fig. 1C and D, respectively). Cells cultured with VD3 alone (Fig. 1C) or those with only BGP and AA (Fig. 1D) also showed some tendency towards cell clustering but failed to present mineralization.

Collagen type 1 secretion by ADPC is upregulated by BGP and AA

Immunostaining at Day 0 for Collagen type 1 showed it to be localized in the intracellular regions of ADPC (Fig. 2A). Collagen type 1 was observed in all the Day 7 cultures with the highest expression in the $VD3^-/BGP^+/AA^+$ Group (Fig. 2D, inset). However, at the Day 28 cultures, both $VD3^+/BGP^+/AA^+$ and $VD3^-/BGP^+/AA^+$ groups showed comparable amount of collagen type 1 expression (Fig. 2B and D). Collagen type I was also localized at the extracellular regions of the cell clusters.

VD3 resulted in osteonectin, osteopontin, and osteocalcin expression

After 7 days of induction, osteonectin (Fig. 3 insets), osteopontin (Supplementary Fig. 1 insets), osteocalcin

Table 1
Induction regimes

	0.01 mM 1α,25-dihydroxy-vitamin D3 (VD3)	50 mM ascorbate-2-phosphate (AA) + 10 mM β-glycerophosphate (BGP)
Group-1	+	+
Group-2	+	–
Group-3	–	+
Group-4	–	–

(Supplementary Fig. 2 insets) proteins were not detected by immunostaining in any of the four groups. However, after 28 days of induction, groups with $VD3^+$ in the induction cocktail showed the presence of osteonectin in significant amount (Fig. 3B and C) while negative osteonectin staining was observed in the groups without VD3. The staining of osteonectin, however appeared to be intracellular in the case of $VD3^+/BGP^-/AA^-$ (Fig. 3C) compared to the staining of the matrix in the $VD3^+/BGP^+/AA^+$ Group (Fig. 3B). Osteopontin and osteocalcin were expressed in significant amount only in the $VD3^+/BGP^+/AA^+$ Group at Day 28 (Supplementary Figs. 1B and 2B, respectively) and not in the other three groups (Supplementary Figs. 1C–E and 2C–E, respectively). A summary table for Alizarin red S staining and immunostaining of various bone matrix protein could be found in Table 2.

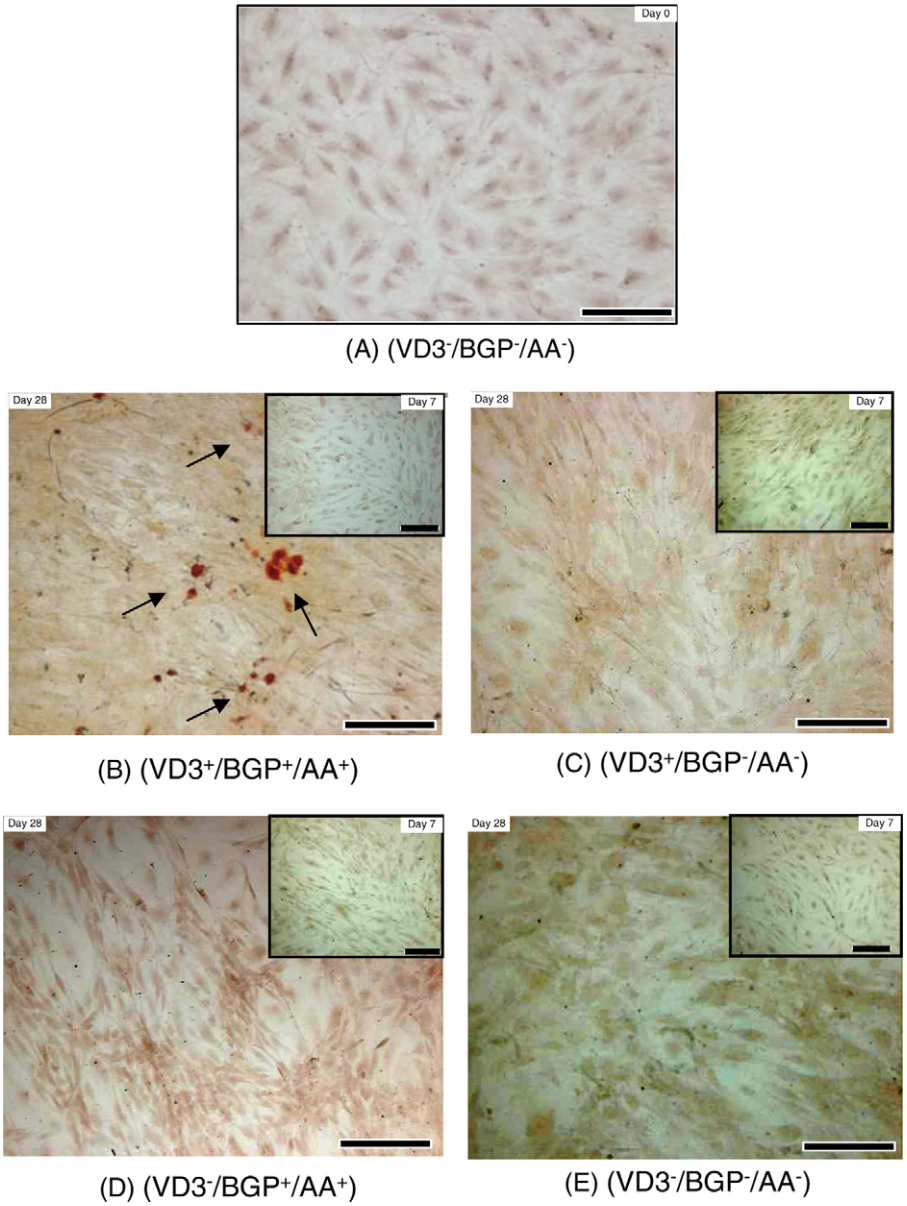

Fig. 1. Alizarin red S staining of ADPC cultures subjected to different induction regimes. (A) Uninduced Day 0 cultures. (B–E) Cultures of various induction regime at Day 28 and Day 7 (corresponding insets). Staining showed no foci of mineralization at Day7 in any of the groups (insets). Cells acquired fibroblastic morphology over the course of induction (contrast B–D with A and E). The $VD3^+/BGP^+/AA^+$ Group showed several foci of mineralization at Day 28 with cell aggregations around the foci (arrows). Scale bar represented 200 μm.

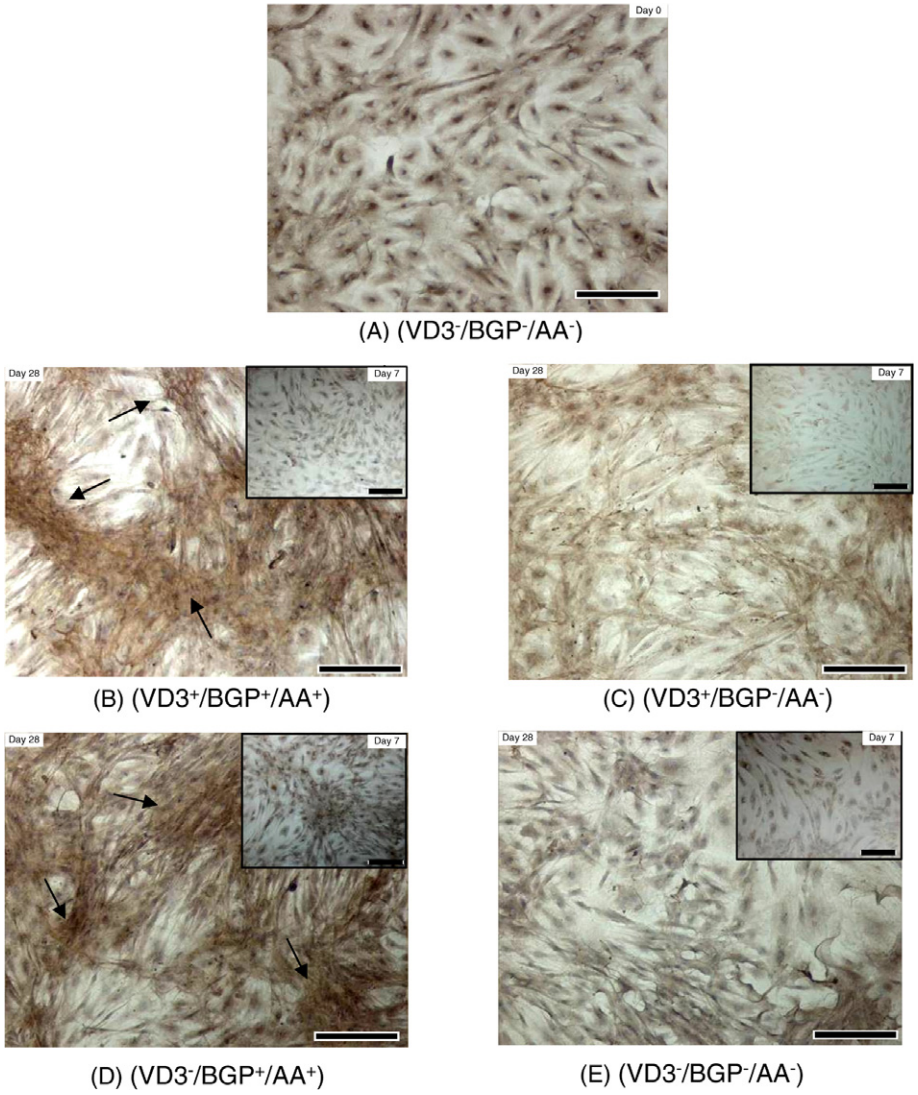

Fig. 2. Immunostaining of ADPC culture for collagen type 1. (A) Uninduced Day 0 cultures. (B–E) Cultures of various induction regime at Day 28 and Day 7 (corresponding insets). At Day 7, the $VD3^{-}/BGP^{+}/AA^{+}$ Group (B) showed the highest amount of collagen type 1 (D, inset) amongst the various regimes. However, at Day 28, amount of collagen type 1 present in Group $VD3^{+}/BGP^{+}/AA^{+}$ (B) and $VD3^{-}/BGP^{+}/AA^{+}$ (D) showed higher and dense clusters (arrows) of collagen type 1 expression compared to the other two groups. Collagen type 1 was observed to be localized at the clusters of cells (B,D). Scale bar represented 200 μm.

VD3, BGP, and AA synergistically sustained osteoblastic transcriptional gene expression

Each induced sample group was normalized to their corresponding uninduced sample to allow comparison of the effects of different induction combinations. After 7 days of induction, comparable and increased levels (>100% of uninduced levels) of RUNX-2 was observed. However, both Group $VD3^{+}/BGP^{-}/AA^{-}$ and $VD3^{-}/BGP^{+}/AA^{+}$ had decreased levels of RUNX-2 (<100% of uninduced levels) by Day 28, while only for Group $VD3^{+}/BGP^{+}/AA^{+}$, there was sustained RUNX-2 expression (>100% of uninduced levels) (Fig. 4A).

There were higher expression of TAZ in the $VD3^{+}/BGP^{-}/AA^{-}$ and $VD3^{-}/BGP^{+}/AA^{+}$ Group compared to the $VD3^{+}/BGP^{+}/AA^{+}$ Group at Day 7. However, a similar trend as observed in the RUNX-2 transcript levels expression at Day 28, the TAZ expression level was sustained in the $VD3^{+}/BGP^{+}/AA^{+}$ Group while that of the $VD3^{+}/BGP^{-}/AA^{-}$ and $VD3^{-}/BGP^{+}/AA^{+}$ declined steeply (Fig. 4B).

Discussion

One of the main factors that determine the achievement of *in vitro* osteogenesis by osteoblast-like cells is the secretion of bone matrix-related proteins like osteonectin (ON), osteopontin (OP), osteocalcin (OC) in addition to collagen type 1 prior to and during the mineralization phase. The cavities of the staggered structure of collagen type 1 are the sites where hydroxyapatite crystallization starts [12]. Initially, the above mentioned non-collagenous proteins

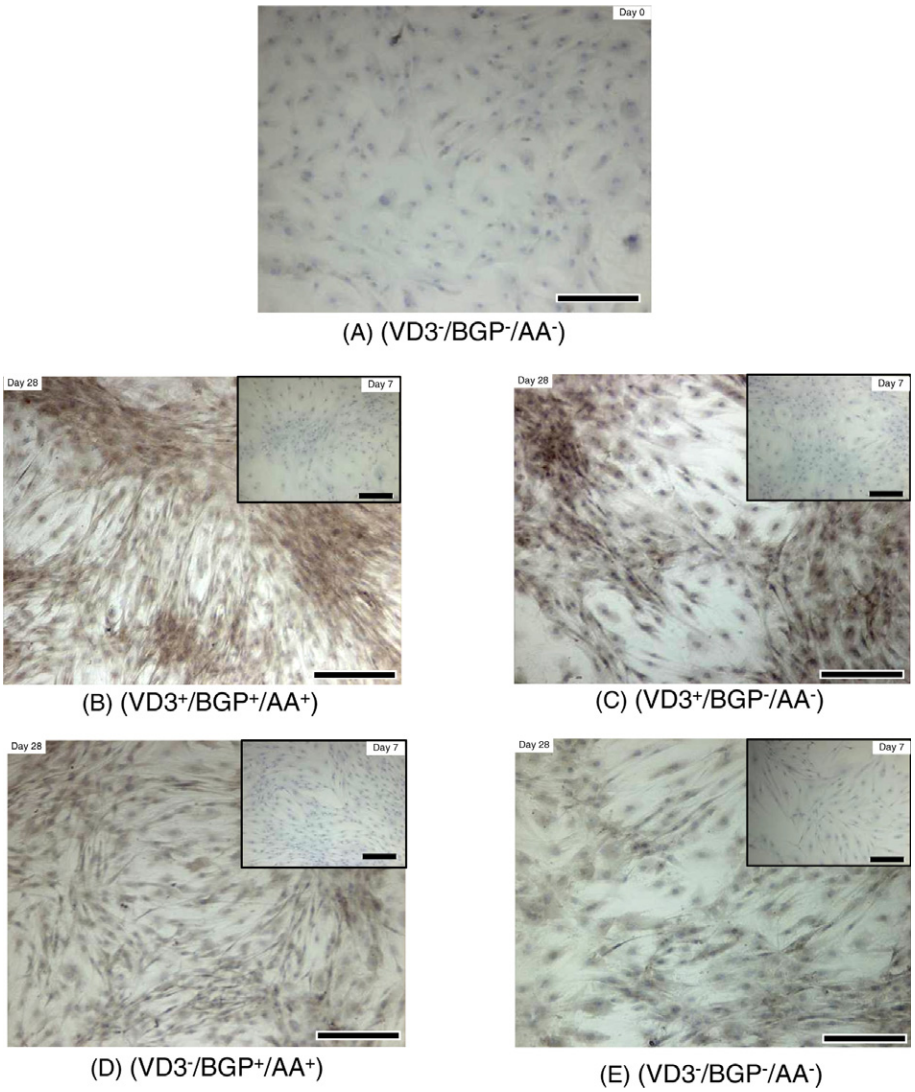

Fig. 3. Immunostaining of ADSC cultures for osteonectin. (A) Uninduced Day 0 cultures. (B–E) Cultures of various induction regime at Day 28 and Day 7 (corresponding insets). At Day 7, all groups did not show positive osteonectin immunostain. However, after 28 days of induction, the $VD3^{+}/BGP^{+}/AA^{+}$ (B) and the $VD3^{+}/BGP^{-}/AA^{-}$ (C) groups showed high expression of osteonectin. Osteonectin expression was observed to be localized with the cell clusters (B,C). Scale bar represented 200 μm.

act as nucleation cores on which hydroxyapatite (HA) crystallization grows and finally these proteins surround the HA crystals thereby controlling their final size and shape. Our results (summarized in Table 2) showed that ADPCs are observed to express genes and proteins associated with an osteoblasts phenotype, only if cultured with all three supplements ($VD3^{+}/BGP^{+}/AA^{+}$).

As seen from the results obtained from staining for calcium deposition and immunostaining for bone matrix-related proteins, it can be observed that calcium deposition can only be seen in the cultures showing presence of all of the above mentioned proteins in significant amounts; *i.e.* in the cultures of Group 1 ($VD3^{+}/BGP^{+}/AA^{+}$) but not in the culture lacking any of these supplements. Thus, this confirmed that mineralization of matrix requires the interaction of ON, OP, and OC with collagen type 1. The latter fact was emphasized by our results that the $VD3^{-}/BGP^{+}/AA^{+}$ though showed strong collagen type 1 staining but failed to mineralize; similarly the cultures treated with VD3 expressed ON in significant amounts and OP and OC to an insignificant extent but also showed no mineralization.

Vital role of 1α,25-dihydroxycholecalciferol in osteoblastic differentiation and final maturation is well-documented [10,13]. Vitamin D3 not only cross-talk with RUNX-2 to help in osteoblastic differentiation but also induces synthesis and secretion of bone matrix-related proteins especially ON, OP, and OC to attain mineralized matrix [14]. It can be concluded from our results that the cultures treated with 1α,25-dihydroxycholecalciferol showed the expression of bone matrix-related proteins is independent of the presence or absence of L-ascorbic acid-2-phosphate or β-glycerophosphate but the bone matrix protein concentrations are significantly higher in

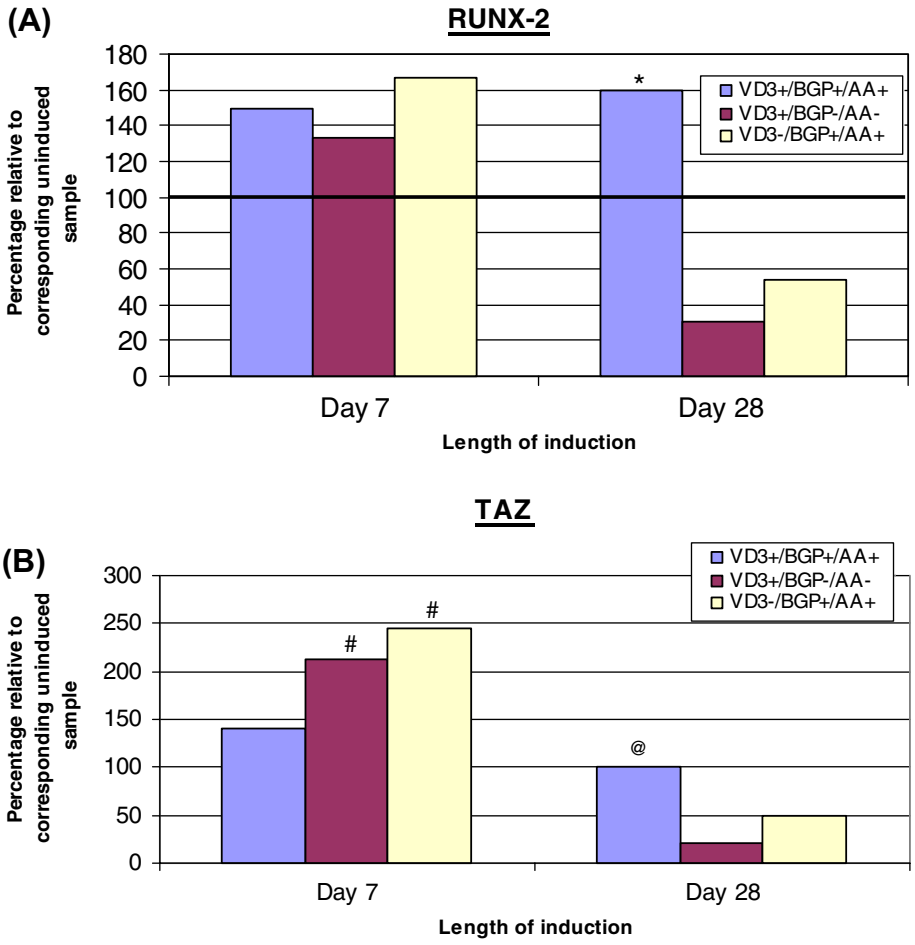

Fig. 4. Real-time RT-PCR analyses for (A) RUNX-2 and (B) TAZ. *y*-axis referred to % expression levels normalized to uninduced sample levels (Group $VD3^{-}/BGP^{-}/AA^{-}$). (A) After 7 days of induction, the levels of RUNX-2 expression were comparable across Group 1–3. After 28 days of induction, the level of RUNX-2 decreased for Group 2 and 3 while RUNX2 expression was sustained in Group 1. The levels observed in Group 2 and 3 were also lower than that of the uninduced group (<100% of uninduced levels). $^{*}p < 0.05$; compared to Day 28 samples of Group 2 and 3. (B) After 7 days of induction, the levels of TAZ expression of Group 2 and 3 were higher than that of Group 1. But there was sustained TAZ expression after 28 days of induction in Group 1 with a drastic drop of TAZ in Group 2 and 3 to below that observed in the uninduced group (<100% of uninduced levels). $^{\#}p < 0.05$, compared to Day 28 sample of Group 1; $^{@}p < 0.05$, compared to Day 28 samples of Group 2 and 3.

Table 2
Summary table for Alizarin red S staining and immunostaining of various bone matrix proteins

Group	Mineralization	Collagen type	Osteonectin	Osteopontin	Osteocalcin
1	++	++++	+++	++	+
2	–	++	++	–	–
3	–	++++	–	–	–
4 (uninduced)	–	+	–	–	–

the cultures treated with all the three components. This also indicates the synergistic effect of VD3 with β-glycerophosphate and L-ascorbic acid-2-phosphate and shows the essentiality of all three components to achieve osteoblastic transformation and thereby assisting mineralization.

An indicator of mature bone is the presence of osteocalcin (OC) in the matrix. *In vitro* experiments proved a chemotactic activity of osteocalcin for monocytes and osteoclasts and an activation of initial adherence of preosteoclasts [15]. Data accumulated so far indicated that OC acts as a negative regulator of bone turnover and a suppressor of mineralization [16]. Although OC is not essential for mineralization, it is highly expressed during the last stage of bone formation [16]. According to our results, identification of OC in those cultures treated with VD3 with β-glycerophosphate and L-ascorbic acid-2-phosphate showed that the cells have attained mature osteoblastic characteristics with OC in the mineralized matrix.

The master conductor of the genetic orchestra regulating osteoblastic lineage commitment and differentiation,

RUNX-2 is also expressed by ADPCs in our study. RUNX-2 is essential for skeletal development during embryogenesis [17]. Interestingly, many of the vitamin D3-regulated genes are also under the transcriptional control of RUNX-2. Cross-talk of the osteogenic signals of RUNX-2 and VD3 at bone-specific promoters may represent an important component of the mechanisms mediating tissue-specific expression of osteoblast phenotypic genes [14]. One of the most important roles of RUNX-2 in osteogenesis is observed when pluripotent mesenchymal cells are committed to the bone cell lineage prior to expression of osteoblast phenotype [10]. This fact explains our observations of higher levels of RUNX-2 at day 0 in ADPCs, which further supports that ADPCs could be a good substitute for osteoblast-like cells. RUNX-2 mediated chromatin remodeling that facilitates promoter accessibility and integration of regulatory activities require VD3 [10] and thus cultures of Group 1 ($VD3^+/BGP^+/AA^+$) showed significant levels of bone matrix-related proteins in immunostaining studies also showed consistent expression of RUNX-2 over the study period. It was observed in all our cultures that consisted of VD3 and/or BGP and AA, that RUNX-2 was upregulated (relative to the uninduced group) at Day 7. However, there is no sustainable expression of RUNX-2 in the induction cultures with less than the complete list of VD3, BGP, and AA. It was arguable that the downregulation of RUNX-2 at Day 28 of the group lacking either one or two of the three induction components could be due to the possibility that RUNX-2 had completed transcriptional initiation of the osteoblastic differentiation mechanism. Counteracting that argument, since ADPCs are expected to be further away from mature osteoblasts than pre-osteoblasts, it is possible that ADPCs would require a prolonged effect of RUNX-2-mediated transcriptional process. It was perhaps to change the epigenetic control of ADPC's previous identity as they differentiated towards the osteoblastic lineage. This view is further supported by our observation in immunostaining assays that a distinct expression of osteocalcin, osteonectin, and osteopontin was only observed, not at Day 7 but at a prolonged time point at Day 28. Our real-time data also supported this second view. It is also noted that the $VD3^+/BGP^+/AA^+$ Group showed a sustained level of TAZ expression which is one of the proposed indicators of the degree of differentiation of MSCs towards the osteoblastic lineage [18]. TAZ acts downstream to RUNX-2 and assists in secretion of bone matrix-related proteins. This is arguable that the decrease in the absolute levels of RUNX-2 and TAZ at day 7 and day 28 could be due to conclusion of their role in differentiation of the cells in $VD3^+/BGP^-/AA^-$ and $VD3^-/BGP^+/AA^+$ Groups. However, this explanation is not supported by the lack of mineralization at Day 28 of induction of these two groups. Thus, the more probable explanation is that there is a synergistic relationship between 1-α,25-dihydroxycholecalciferol, β-glycerophosphate, and L-ascorbic acid-2-phosphate in the maintenance of relatively higher levels of RUNX-2 and TAZ mRNA over the time course of transdifferentiation towards osteoblastic lineage.

In conclusion, our study showed that VD3 plays important role in the expression of bone matrix-related proteins by ADPCs and that β-glycerophosphate and L-ascorbic acid-2-phosphate are important synergists to VD3 in attaining mineralized matrix.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.112.

References

[1] B.M. Strem, K.C. Hicok, M. Zhu, I. Wulur, Z. Alfonso, R.E. Schreiber, J.K. Fraser, M.H. Hedrick, Multipotential differentiation of adipose tissue-derived stem cells, Keio J. Med. 54 (2005) 132–141.
[2] Y. Xu, G. Balooch, M. Chiou, E. Bekerman, R.O. Ritchie, M.T. Longaker, Analysis of the material properties of early chondrogenic differentiated adipose-derived stromal cells (ASC) using an *in vitro* three-dimensional micromass culture system, Biochem. Biophys. Res. Commun. 359 (2007) 311–316.
[3] D.T. Leong, W.M. Khor, F.T. Chew, T.C. Lim, D.W. Hutmacher, Characterization of osteogenically induced adipose tissue-derived precursor cells in 2-dimensional and 3-dimensional environments, Cells Tissues Organs 182 (2006) 1–11.
[4] H. Li, K. Dai, T. Tang, X. Zhang, M. Yan, J. Lou, Bone regeneration by implantation of adipose-derived stromal cells expressing BMP-2, Biochem. Biophys. Res. Commun. 356 (2007) 836–842.
[5] M. Knippenberg, M.N. Helder, B. Zandieh Doulabi, P.I. Wuisman, J. Klein-Nulend, Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells, Biochem. Biophys. Res. Commun. 342 (2006) 902–908.
[6] J.M. Gimble, C.E. Robinson, X. Wu, K.A. Kelly, The function of adipocytes in bone marrow stroma: an update, Bone 19 (1996) 421–428.
[7] S.R. Park, R.O. Oreffo, J.T. Triffitt, Interconversion potential of cloned human adipocytes *in vitro*, Bone 24 (1999) 549–554.
[8] A. Jikko, S.E. Harris, D. Chen, D.L. Mendrick, C.H. Damsky, Collagen integrin receptors regulate early osteoblast differentiation induced by BMP-2, J. Bone Miner. Res. 14 (1999) 1075–1083.
[9] V. Shalhoub, F. Aslam, E. Breen, A. van Wijnen, R. Bortell, G.S. Stein, J.L. Stein, J.B. Lian, Multiple levels of steroid hormone-dependent control of osteocalcin during osteoblast differentiation: glucocorticoid regulation of basal and vitamin D stimulated gene expression, J. Cell. Biochem. 69 (1998) 154–168.
[10] G.S. Stein, J.B. Lian, A.J. van Wijnen, J.L. Stein, M. Montecino, A. Javed, S.K. Zaidi, D.W. Young, J.Y. Choi, S.M. Pockwinse, Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression, Oncogene 23 (2004) 4315–4329.
[11] D.T. Leong, A. Gupta, H.F. Bai, G. Wan, L.F. Yoong, H.P. Too, F.T. Chew, D.W. Hutmacher, Absolute quantification of gene expression in biomaterials research using real-time PCR, Biomaterials 28 (2007) 203–210.
[12] A.L. Arsenault, M.D. Grynpass, Crystals in calcified epiphyseal cartilage and cortical bone of the rat, Calcified Tissue Int. 43 (1998) 219–225.
[13] B.C. Heng, T. Cao, L.W. Stanton, P. Robson, B. Olsen, Strategies for directing the differentiation of stem cells into the osteogenic lineage *in vitro*, J. Bone Miner. Res. 19 (2004) 1379–1394.
[14] H. Drissi, A. Pouliot, C. Koolloos, J.L. Stein, J.B. Lian, G.S. Stein, A.J. van Wijnen, 1,25-$(OH)_2$-vitamin D3 suppresses the

bone-related Runx2/Cbfa1 gene promoter, Exp. Cell. Res. 274 (2002) 323–333.

[15] C. Chenu, S. Colucci, M. Grano, P. Zigrin, R. Barattol, G. Zamboni, N. Baldini, P. Vergnoud, P.D. Delmas, A.Z. Zallone, Osteocalcin induces chemotaxis, secretion of matrix proteins, and calcium-mediated intracellular signaling in human osteoclast-like cells, J. Cell. Biol. 127 (1994) 1149–1158.

[16] P. Ducy, C. Desbois, B. Boyce, G. Pinero, B. Story, C. Dunstan, E. Smith, J. Bonadio, S. Goldstein, C. Gundberg, A. Bradley, G. Karsenty, Increased bone formation in osteocalcin-deficient mice, Nature 382 (1996) 448–452.

[17] P. Ducy, R. Zhang, V. Geoffroy, A.L. Ridall, G. Karsenty, Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation, Cell. 89 (1997) 747–754.

[18] J.H. Hong, E.S. Hwang, M.T. McManus, A. Amsterdam, Y. Tian, R. Kalmukova, E. Mueller, T. Benjamin, B.M. Spiegelman, P.A. Sharp, N. Hopkins, M.B. Yaffe, TAZ, a transcriptional modulator of mesenchymal stem cell differentiation, Science 309 (2005) 1074–1078.