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In vitro osteogenesis assays: Influence of the primary cell source on alkaline phosphatase activity and mineralization

Essais ostéogéniques in vitro : l'influence de la source des cellules primaires sur l'activité phosphatase alcaline et la minéralisation

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Abstract

In trabecular bone fracture repair in vivo, osteogenesis occurs through endochondral ossification under hypoxic conditions, or through woven bone deposition in the vicinity of blood vessels. In vitro osteogenesis assays are routinely used to test osteoblastic responses to drugs, hormones, and biomaterials for bone and cartilage repair applications. These cell culture models recapitulate events that occur in woven bone synthesis, and are carried out using primary osteoblasts, osteoblast precursors such as bone marrow-derived mesenchymal stromal cells (BMSCs), or various osteoblast cell lines. With time in culture, cell differentiation is typically assessed by examining levels of alkaline phosphatase activity (an early osteoblast marker) and by evaluating the assembly of a collagen (type I)-containing fibrillar extracellular matrix that mineralizes. In this review, we have made a comparative analysis of published osteogenic assays using calvarial cells, calvaria-derived cell lines, and bone marrow stromal cells. In all of these cell types, alkaline phosphatase activity shows similar progression over time using a variety of osteogenic and mineralizing media conditions; however, levels of alkaline phosphatase activity are not proportional to observed mineralization levels.

Résumé

Suite à des fractures osseuses in vivo, la réparation des os se produit soit par le processus d'ossification endochondrale dans des conditions hypoxiques, soit par le dépôt d'os à partir d'ostéoblastes près des vaisseaux sanguins. Les essais d'ostéogenèse in vitro ont été établis afin de tester la réponse des ostéoblastes aux médicaments, hormones, et biomatériaux utilisés pour la guérison de l'os et du cartilage. Ces modèles de culture cellulaire récapitulent les événements de la synthèse de nouvel os, et sont menés avec les cultures primaires d'ostéoblastes, les précurseurs d'ostéoblastes (cellules stromales mésenchymateuses de la moelle osseuse [BMSCs]), ou les lignées cellulaires ostéoblastiques. En culture, avec le temps, la différenciation cellulaire est dosée par le niveau d'activité de la phosphatase alcaline (un marqueur de l'ostéoblaste précoce), et par l'évaluation de l'assemblage d'une matrice fibrillaire de collagène (type I) minéralisée. Dans cette revue, nous avons comparé les essais cellulaires d'ostéogenèse qui utilisent une variété de cellules ostéogéniques et milieux de culture, et nous avons constaté que les niveaux d'activité de la phosphatase alcaline ne concordent pas avec les niveaux de minéralisation.

Keywords: In vitro osteogenesis; Bone cell culture; Bone marrow stromal cells; MC3T3-E1; Chitosan; Cartilage repair; Bone fracture repair; Alkaline phosphatase; Glycerol phosphate; Dexamethasone; Mineralization

Mots clés : Ostéogénèse in vitro ; Cultures ostéoblastes ; Cellules de la moelle osseuse stromales ; MC3T3-E1 ; Chitosan ; Réparation du cartilage articulaire ; Réparation d'os ; Phosphatase alcaline ; Glycérol phosphate ; Dexamethasone ; Minéralisation

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1. Osteoblast cell origin and culture media

Osteogenesis in vivo in repairing trabecular bone defects below the articular cartilage layer, can occur through an endochondral process involving the generation of chondrogenic foci (Fig. 1A), followed by cell hypertrophy, vascular invasion of the cartilage nodule, and mineralization much like the growth plate [1]. In trabecular subchondral defects that develop a more vascularized granulation tissue [2], new bone can be generated through direct woven bone synthesis by osteoblasts (Fig. 1B). To improve our understanding of fracture repair in the context of cartilage repair surgical procedures involving microfracture or drilling of subchondral trabecular bone [3-6], we undertook a review of in vitro osteogenesis assays developed over the past few decades that provide models for osteoblast differentiation leading to alkaline phosphatase expression and matrix mineralization.

In vitro osteogenic assays using bone-derived cells are typically carried out using three different primary cell sources: (1) fetal calvarial cells (from enzymatic digests of rat or mouse cranial bones), (2) bone marrow-derived mesenchymal stromal cells (BMSCs) (from bone marrow iliac crest aspirates or from marrow in femoral shafts, rib, surgical waste, and vertebrae biopsy), or (3) cells migrating from trabecular bone explants. Prior to initiating the osteogenesis assay, cells are sparsely seeded in petri dishes (96-well plates to 100 mm dishes), and allowed to proliferate to confluency. During the assay, cultures are typically fed twice weekly over a 2–3 week period, with ''Complete Culture Media'' (CCM, see Table 1) which contains basal media, fetal bovine serum to promote cell survival, division, and metabolism, ascorbate to permit collagen type I fibril assembly, and an exogenous source of phosphate to promote mineralization of collagen fibrils [7,8]. Ascorbate is present in αMEM but not in DMEM, and in some assays an ascorbate analog that resists hydrolysis (ascorbate-2-phosphate) is used. Calvarial cells and calvaria-derived MC3T3-E1 cells will mineralize in CCM over a 2–3 week period. Less alkaline phosphatase (ALP) activity is obtained in MC3T3-E1 cultures, when carried out in charcoal-stripped fetal bovine serum which is depleted of steroid hormones (see Table 1, [9]).

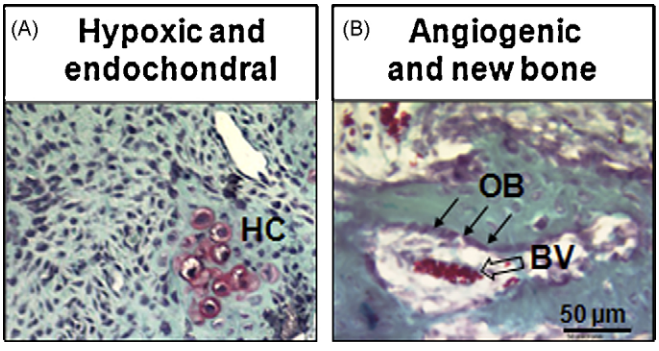

Fig. 1. Drilled trabecular bone undergoes endochondral or woven bone repair. Paraffin sections were obtained from decalcified rabbit trochlea which received full-thickness chondral defects and four, 0.9 mm-diameter microdrill holes [3] followed by 3 weeks of repair, with section staining by Safranin O/fast green (A) or Gomori trichrome (B). In areas of low vascularity, endochondral ossification is typically seen (A). The development of vascularized granulation tissue is accompanied by new bone formation (B). HC, hypertrophic chondrocytes within chondrogenic foci stained with Safranin O. BV, blood vessel; OB, osteoblast.

2. Alkaline phosphatase activity

BMSCs cultured for three weeks in CCM exhibit a spindle-shaped morphology with oblong nuclei (Fig. 2) and develop a collagen matrix that most frequently fails to mineralize (Fig. 3A–D). BMSCs cultured in CCM express sporadic low levels of alkaline phosphatase (ALP) (0.1 nmol/min/µg protein or 0.5 nmol/min/10,000 cells, Table 1) that increases several-fold over a 3-week culture in parallel with a modest doubling or tripling in cell number (Table 1) [14,20]. Treatment of BMSCs with dexamethasone, BMP-2 or vitamin D3 stimulates a 2–6-fold increase in ALP activity relative to parallel control cultures in CCM, and mineralization (Table 1, Fig. 3) [12,17,18]. The MC3T3-E1 mouse calvarial cell line develops similar levels of ALP activity after 2 weeks of culture in CCM as human BMSCs stimulated with 100 nM dexamethasone or with vitamin D3 (compare Chung, with Chang and Liu, Table 1, Fig. 4) [13,14,18].

Different laboratories use either 10 or 100 nM dexamethasone to stimulate osteogenesis in BMSC cultures, and during the osteogenesis assay, human BMSCs constitutively express the glucocorticoid receptor [14]. The continuous presence of dexamethasone, a glucocorticoid receptor agonist, added over two weeks generates a more cuboidal BMSC cell shape, stimulates ALP mRNA, and suppresses bone sialoprotein (BSP) [10,15]. Treatment of BMSCs with either 10 nM or 100 nM dexamethasone reproducibly stimulates ALP activity in the first week of culture [20], however after three weeks of culture, 100 nM dexamethasone suppresses ALP activity (Fig. 3G *versus* H) [19,21].

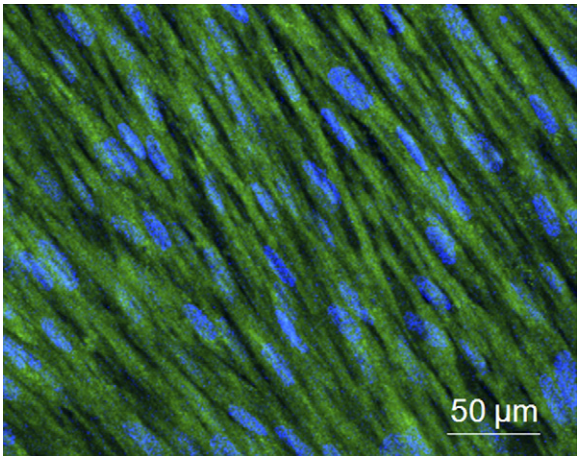

Fig. 2. Typical cell morphology of passage 3 human BMSCs (Tulane Center for Gene Therapy, New Orleans, LA, USA) after three weeks of culture in Complete Culture Medium (CCM: αMEM, 16% Fetal Bovine Serum from Atlanta Biologics, GA, USA, 5 mM βGP, 30 µg/mL ascorbate-2-phosphate, Penicillin-Streptomycin). Cells were stained with calcein AM (green) and Hoechst 33342 for nuclei (blue) (Invitrogen Molecular Probes, Mississauga ON, Canada), and imaged by confocal 2-photon microscopy (Carl Zeiss, Germany).

Table 1
In vitro development of alkaline phosphatase activity and mineralization

Rat primary bone marrow stromal cells				
Citation	Cell source "Osteogenic Media"	Alkaline Phosphatase activity		Units
		CCM[a]	OSM[b]	
Evans et al., 2000 [10]	Trabecular bone 8 wk ± HX[c] 10 nM dex, CCM	3.5 (day 8) 2.5 (HX, day 8) 4.5 (day 15) 2.0 (HX, day 15)	7.0 (day 8) 4.5 (HX, day 8) 4.4 (day 15)[e] 4.5 (HX, day 15)[e(HX: less Ca++)]	nmol/min/10,000 cells[d]
Rickard et al., 1994 [11]	Whole marrow, 4–5 wk Wistar♀ 10 nM dex, CCM (15 % FBS), P-S	0.16 (day 7) 0.5 (day 10)	1.1 (day 7) 4.6 (day 10)	nmol/min/10,000 cells[d]
Radin, 2005 [12]	Femora, 4–5 wk Wistar, P1 ± BG 100 nM dex, CCM (100 µg/mL asc), P-S		0.5 (plastic) vs 1.8 (BG, day 7) 0.5 (plastic) vs 6 (BG + BMP-2[g], day 7)	(nM)/MTS × 100
Rat and murine cell lines[a]		CCM		
Quarles et al., 1992 [7]	MC3T3-E1, CCM1	0.01 (day 3); 0.13 (day 15); 0.35 (day 31)[e]		nmol/min/10,000 cells[d]
Yohay et al., 1994 [9]	MC3T3-E1, CCM1 with αMEM CCM1 with αMEM 10% charcoal-stripped FBS	1.5 (day 17) 0.2 (day 17)		nmol/min/10,000 cells[d] nmol/min/10,000 cells[d]
Chung et al., 1992 [13]	MC3T3-E1	0.5 (day 16)[e]		nmol/min/µg protein
Chung et al., 1992 [13]	ROS 17/2.8, CCM2	1.3 (day 8)[e]		nmol/min/µg protein
Human primary bone marrow stromal cells		CCM	OSM	
Chang et al., 2006 [14]	hBMSC fetal and adult, P2[f] 100 nM dex, CCM3, P-S-A	0.2 (day 9, fetal)[e: at day 30] 0.4 (day 9, adult)	0.5 (day 9, fetal)[e: at day 25] 1.2 (day 9, adult)	nmol/min/10,000 cells
Cheng et al., 1996 [15]	hBMSC all ages[f] 100 nM dex, CCM4 10 % HiFBS	0.03 (day 5) 0.03 (day 12) 0.06 (day 23)	0.1 (day 5) 0.14 (day 12) 0.33 (day 23)[e]	nmol/min/µg protein[d]
Coelho et al., 2000 [16]	Surgical waste, 96-well plate 10 nM dex, CCM5, P-S-F	0.2 (day 3) 0.6 (day14) 1.5 (day 21)	0.4 (day 3) 1.5 (day 14) 2.2 (day 21)[e]	nmol/min/µg protein
Fromigué et al., 1998 [17]	Trabecular bone fragments (10 nM dex, pre-confluent only) CCM5	0.09 (day 2) 0.07 (day 7) 0.02 (day 21)	0.16 (BMP-2[g], day 2) 0.25 (BMP-2[g], day 7) 0.04 (BMP-2[g], day 21)[e]	nmol/min/µg protein[d]
Liu et al., 1999 [18]	Human rib marrow. Vit D, CCM, 2% FBS	0.08 (day 8)	0.5 (50 nM Vit D, day 8)	nmol/min/µg protein[d]
Schecroun and Delloye 2003 [19]	hBMSC iliac crest, P1[f] 10 nM dex, CCM5 with HiFBS	1.0 (day 0) 1.8 (7 days)	2.0 (7 days), 5.5 (14 days)[e]	nmol/min/µg DNA

BG: bioactive glass; CCM: αMEM, 10% Fetal bovine serum (FBS), 10 mM GP, 50 µg/mL ascorbate, antibiotics; CCM1: DMEM + F12, 10 % FBS, 5 mM GP, 25 µg/mL ascorbate; CCM2: DMEM, 10 % FBS (Hyclone), 10 mM GP, 50 µg/mL ascorbate, antibiotics; CCM3: αMEM, 10 % Heat-inactivated FBS (HiFBS, Hyclone), 10 mM GP, 50 µM ascorbate-2-phosphate; CCM4: αMEM, 10 % Heat-inactivated Fetal bovine serum (HiFBS), 10 mM GP, 50 µg/mL ascorbate, antibiotics; CCM5: DMEM, 10 % FBS, 3 mM NaH_2PO_4, 50 µg/mL ascorbate, P-S-F-A: penicillin, streptomycin, fungizone, amphotericin.

[a] Complete culture medium (CCM) has no dexamethasone.
[b] Dexamethasone was used at 10 nM or 100 nM as indicated.
[c] HX: hypophysectomized Sprague Dawley.
[d] Adjusted from published data.
[e] Ca/Pi deposition seen.
[f] 24-well plate.
[g] 100 ng/mL.

As shown in Table 1, BMSCs from different species and laboratories consistently produce higher ALP when cultured in 10 nM dexamethasone (Table 1, [10-11,16]) compared to 100 nM dexamethasone (Table 1, [14-15]). Osteopontin (OPN) expression is also sensitive to different levels of dexamethasone; at 10 nM dexamethasone, rat OPN mRNA levels were stimulated along with higher osteocalcin (OC) (Table 1, [10]), while at 100 nM dexamethasone, human OPN was suppressed after two weeks (Table 1, [14]). These data could be partly explained by the tendency of higher dexamethasone concentrations to stimulate adipogenesis (Fig. 3E, inset). Low-level adipogenesis of human and murine BMSCS cultured in 100 nM to induce osteogenesis was previously noted by others [21,22]. Stronger induction of adipogenesis is achieved in BMSCs and fat-derived stromal cells in CCM containing 1000 nM dexamethasone, and other supplements including insulin (1 µg/mL), and either T3 [23] or indomethacin and 3-isobutyl-1-methylxanthine [22].

3. Mineralization

In studies using osteogenic cultures, mineralization is considered a functional in vitro endpoint reflecting advanced cell differentiation. Alizarin red staining is commonly used to

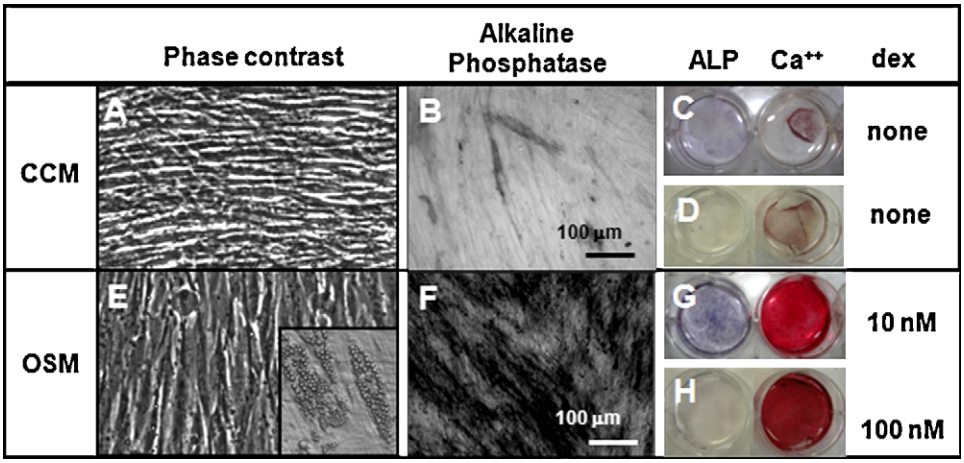

Fig. 3. Representative in vitro osteogenesis assays with passage three human BMSCs cultured for 3 weeks in CCM (A–D; $n = 5$) or CCM with dexamethasone (dex, osteogenic media, OSM) (E–G, $n = 3$; H, $n = 2$) followed by alkaline phosphatase enzyme staining and alizarin red staining for calcium/mineral as indicated. BMSCs were spindle-shaped in CCM (A) with sporadic ALP activity (B & C) and failed to mineralize (C & D). In osteogenic medium (OSM) with 10 nM dexamethasone, BMSCs were more cuboidal (E), demonstrated more uniform and higher levels of ALP activity (F & G), and the cultures mineralized (G). Calcium (Ca^{++}) deposition was revealed by alizarin red staining. In 100 nM dexamethasone-treated cultures, sporadic adipogenesis (E, inset), lower ALP levels and mineralization were observed (H).

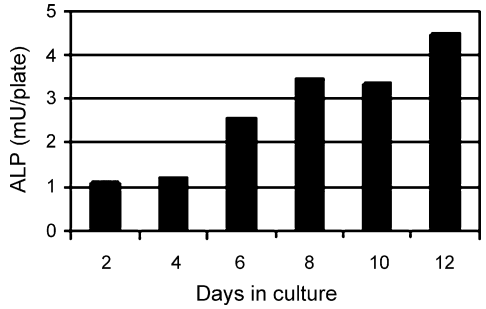

Fig. 4. Increase in alkaline phosphatase activity in confluent MC3T3-E1 cells during two weeks of culture without dexamethasone (based on published data [32]).

detect and quantify calcium, while von Kossa staining is used to visualize phosphate, within the deposited mineral. Confluent osteogenic cultures, including primary calvarial cells, MC3T3-E1 cells, and human BMSCs, follow a two-stage developmental process including a 1–2-week initiation phase during which cells slowly proliferate, express ALP activity and other bone-specific genes, and produce and assemble a collagen matrix. During a second maturation phase occurring in week 2 [24,25] or 3 [7,8,20], matrix mineralization is observed (Fig. 5). The mineral phase generally deposited in these osteoblast cultures is a calcium-phosphate, substituted hydroxyapatite similar to that seen in bone, cartilage and teeth [26]. In the presence of 2 mM calcium and 2 mM phosphate, however, osteoblast cell line cultures assemble a collagen matrix in vitro that generally is not mineralized [25]. Typical basal media (αMEM with 10% serum) provides ~2 mM inorganic phosphate (Pi) [8], however, in vitro mineralization is only initiated when the media is further supplemented with either 3 mM Pi or 5–10 mM disodium β-glycerol phosphate (βGP) [8]. Relatively low levels of ALP activity (0.05 nmol/min/µg protein [13]) are necessary and sufficient to rapidly convert βGP to Pi and glycerol in vitro [8,13]. Inhibition of ALP by levamisole or PPi also gives rise to cultures that accumulate non-mineralized collagen matrix [8,13,27–29]. Calvarial cell cultures having matured for two weeks in CCM without supplemental Pi are still competent to mineralize after further addition of βGP, even after the cells have been killed by freezing or light chemical

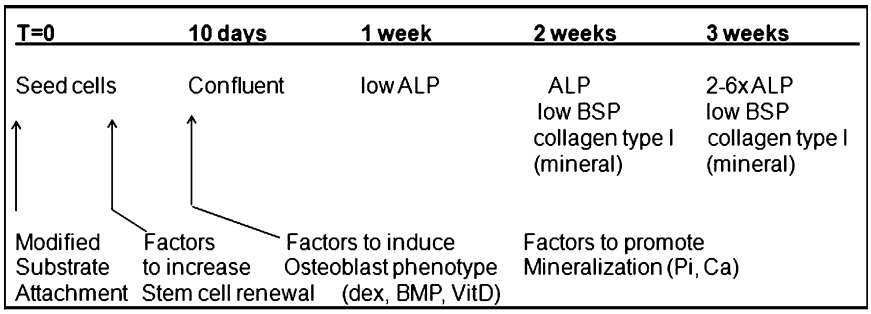

Fig. 5. Schema showing the progression of events in a typical osteogenesis assay, with strategic points in the assay where the culture conditions can be modified to influence ALP activity and mineralization. Abbreviations: BSP, bone sialoprotein; dex, dexamethasone; VitD, vitamin D; Pi, inorganic phosphate; Ca, calcium.

fixation which preserves trace ALP activity [25]. Therefore ALP activity in these cultures is necessary, but not sufficient, to produce mineralized matrix.

Although the type of mineral formed during in vitro osteogenesis studies is rarely characterized in any detail, it is generally considered to be a substituted hydroxyapatite – although another type of calcium-phosphate mineral has been described (see below). In some MC3T3-E1 and BMSC cultures, where 10 mM βGP is added, a hydroxyapatite-like mineral aligned with collagen fibrils and having an appropriate calcium/phosphate ratio has been reported [20,29,30]. Others have characterized the mineral formed in the presence of 10 mM βGP as being a non-hydroxyapatite, cell-associated precipitate with a higher calcium/phosphate ratio than collagen-associated mineral formed in cultures with 3 mM Pi [13,24,27]. Even skin fibroblast monolayer cultures can develop mineral deposits after weeks of culture with 6–10 mM Pi [13]. Thus, the diffuse mineral staining frequently observed for passaged BMSCs cultured in the presence of 10 mM βGP [14,16,20] may not always be representative of bone-like matrix mineralization, and its more detailed characterization and validation of its association with matrix components is thus warranted in most studies. The diffuse background accumulation of calcium in human BMSC cultures with 5 mM βGP without dexamethasone (Fig. 3C and D) is highly intensified by 5 mM βGP with 10 nM dexamethasone (Fig. 3E and F). These observations indicate that it is important to include the same concentration of βGP or Pi in the media of negative control cultures to demonstrate the effect of added factor(s), such as dexamethasone, on in vitro mineralization.

Osteoblasts in primary calvarial cell cultures assemble 3-dimensional, collagen matrix-rich nodules that mineralize and occlude a network of osteocyte-like cells. Both these cells are believed to arise from a local clonal expansion and differentiation of subconfluent osteoprogenitors. The number and surface area of mineralized foci is diminished with passage number [31] and hypophysectomy [10], suggesting that osteoblast precursors can be diluted and lost during cell passage, or diminished by donor health status. Addition of dexamethasone during cell passage and amplification of primary calvarial cells was found to enhance osteoblast stem cell renewal and/or survival in vitro, resulting in increased number and surface area of mineralized foci [17,31]. Compared to primary calvarial cells that contain a limited percentage of osteoblast precursors capable of forming mineralized foci [31], confluent MC3T3-E1 cells form a more continuous layer of cells and mineralized extracellular matrix [24,29,30].

First-passage BMSCs have been reported to form mineralized foci [8,10,19]. Beyond passage 2, however, mineralized foci are scarcely detected in human BMSC cultures although uniform mineral deposits can be detected using alizarin red staining (Fig. 3G and H). Mineralization is observed in BMSC cultures that achieve a minimal ALP activity (∼0.25 nmol/min/μg protein or 1.2 nmol/min/10,000 cells) at some point during the 2–3 week culture period (Table 1). Occasionally, BMSCs cultured in CCM without dexamethasone have been reported to achieve relatively high ALP activity (1.5 nmol/min/μg protein) and to mineralize, although with a one-week delay relative to dexamethasone-treated cultures (Table 1, [14,16]). In the study by Chang et al. [14], the authors speculated that the primary aspirates that mineralized without dexamethasone may have contained more primary osteoblasts. Finally, given that hypophysectomized rat BMSC cultures developed high levels of ALP activity while producing few mineralized foci relative to non-hypophysectomized rat BMSCs (see [10], Table 1), it is evident that some BMSC cultures can produce high levels of ALP in vitro without ever mineralizing [10].

4. Summary

ALP activity increases over time in confluent monolayer bone-derived cell cultures that are slowly dividing over three weeks. Primary calvarial osteoblast progenitors and the MC3T3-E1 preosteoblast cell line develop increasing ALP activity in the absence of dexamethasone, while human BMSCs generally require from 10 to 100 nM dexamethasone to stimulate a 2–6-fold increase in ALP activity. Cultures from different donors show variable levels of dexamethasone-dependent mineralization after three weeks of culture. Addition of 100 nM dexamethasone can lead to lower ALP activity than 10 nM dexamethasone after three weeks of culture, and at this concentration adipogenesis is also stimulated. The progression of events in a three-week osteogenesis assay can be promoted or inhibited by a variety of biomaterials, hormones, cytokines, or pharmacological agents at key developmental stages (Fig. 5).

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