Cell Culture Systems for Studies of Bone and Tooth Mineralization

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1. Introduction

The use of cell culture to characterize bone and tooth mineralization has blossomed, thanks to the development of genetically engineered animals with altered bone and tooth phenotypes,[1,2] the tissue engineering of bones, teeth, and cartilage,[3,4] and genetic profiling of mineralized tissue development.[5−8] There is little uniformity in the conditions used in these cultures, and where there is uniformity, the chemical basis for the reagents used is sometimes questionable. Moreover, while it is well established that histochemical staining is a poor substitute for physicochemical assays of the nature of the mineral formed,[9] the majority of both recent and classical papers in the literature rely on histochemistry to report the presence of bone- or tooth-like mineral. The goal of this review is to place cell culture methodologies in a chemical context, to review the different types of cell culture systems that have been used to study the deposition of mineral in bones and teeth, to discuss their limitations, and to suggest some guidelines for future studies.

1.1. Cells and Tissues of Bones and Teeth

Cell culture systems generally seek to recapitulate the events in the development of the tissue in situ. While some culture systems start with preformed substrates and others rely on the differentiation, proliferation, and maturation of cells, the ultimate goal is the formation of an analogue of the naturally occurring tissue. Questions that arise during these studies may include whether the cells in question have an altered ability to form this tissue, what the role of the genetic modification might be, or whether the material formed can be used to repair and replace native tissue. To understand the cell culture methodologies, it is necessary to first review the composition of these tissues and the cells involved in their formation.

1.1.1. The Mineral and Extracellular Matrix of Bones and Teeth

The mineral phase that is found in bones and teeth is a highly substituted analogue of the geologic mineral hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$).[10] In bone, cementum, and dentin, the platelike OH-deficient, CO_3-substituted apatite nanocrystals are oriented with their long axis parallel to the axis of the collagen fibrils. The insoluble fibrillar protein, collagen, is the major component of the organic matrix. Other noncollagenous proteins are important for the maintenance

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of the cell−matrix interactions, cell-signaling, regulation of cell metabolism, and control of the mineralization process.[11,12]

In mature enamel, the hydroxyapatite crystals are also carbonate-substituted, but they are larger, and there is little (<3%) organic matrix. There is no collagen in enamel; the major protein in mature enamel is amelogenin, but proteins account for a very small amount of the mature enamel's composition. During early enamel formation, there is a greater proportion of proteins, and these include enamel specific proteins, such as amelogenin, enamelin, tuftelins, and amelin, and some nonspecific proteins, such as phosphophoryn.[12,13]

1.1.2. Cells in Mineralized Tissues

Bone, dentin, cementum, and calcified cartilage contain cells that deposit the nonmineralized tissue, initiate and control tissue mineralization, and regulate tissue metabolism. In the case of bone, there are also cells that remove and remodel the tissue. In teeth, the remodeling process does not generally occur. Within the mineralized tissues, there are also neuroelements and vascular elements, but those cells are not of concern for this review.

The bone, calcified cartilage, and dentin forming cells are osteoblasts,[14] hypertrophic chondrocytes,[15] and odontoblasts,[16] respectively. These are the cells that produce the extracellular matrix and control the initial mineralization process. In bone, as osteoblasts become engulfed in mineral, they extend long processes to connect to one another. These cells, now called osteocytes,[17] are connected by this long canalicular (dendritic) network. Osteocytes have a different phenotype than osteoblasts, expressing different amounts of phenotypic markers,[18−21] some of which are osteocyte specific.[22]

In teeth, there are three major cell types, the odontoblasts that form dentin, the cementoblasts in cementum (a tissue intermediate between bone and dentin),[23] and the ameloblasts that form enamel.[24] Table 1 lists protein markers that are commonly used in immunohistochemistry, cell sorting, or related assays, to distinguish these different mineralized tissue-forming cell types. The mineralized tissue resorbing cells, osteoclasts and chondroclasts, whose functions are coupled with those of the osteoblasts and osteocytes, while often studied in culture, will not be discussed here, and the reader is referred to recent reviews for more information.[25−27]

2. Is the Mineral Formed in Culture Similar to "Physiologic Mineral"?

Before discussing how cell culture is performed in the different systems, it is important to describe methods that have been used to demonstrate that the mineral formed in culture is similar to that which occurs in nature. A concern in the analysis of mineral is the chemical treatments done prior to analysis, as some of these treatments can change the properties of the mineral. Often, the question of whether the mineral formed is at all like that present in the body is ignored, and authors show the presence of calcium and phosphate ions without showing whether the mineral is hydroxyapatite-like, that the mineral is deposited with the correct organization on the appropriate matrix (i.e., aligned with collagen for bone, dentin, and cementum; and associated

Table 1. Mineralized Tissue Cell Types and their Cell Specific[a] Protein Markers

cell	marker	also used as markers[b]
hypertrophic chondrocyte	type X collagen[257]	types II and IX collagen
	MMP-9[258]	DMP1
osteoblast	osteocalcin[259]	type I collagen
	periostin[260]	alkaline phosphatase
	bone sialoprotein (BSP)[261]	Runx2[264,265]
	MEPE/OF45[262]	osterix[266]
	PHEX/Pex[263]	response to PTH[267]
osteocyte	sclerostin (SOST)[268,269]	osteocalcin
	DMP1[19,270]	type I collagen
	actin-binding proteins[271]	alkaline phosphatase
	fimbrin[271]	
	podoplanin/E11[21]	
	MEPE/OF45[262]	
	PHEX/Pex[272]	
odontoblast	Dspp (gene)[156,273,274]	type I collagen
	-phosphophoryn[156]	alkaline phosphotase[276]
	-dentin sialoprotein[156,274]	MEPE[276]
	DMP4[275]	
cementoblast	cementum protein 23[277]	bone matrix proteins
ameloblast	amelogenin[207]	
	ameloblastin[207,278]	
	tuftelin[279]	
	enamelin[280]	
	amelotin[281]	
	cytokeratin 14[282]	

[a] Some of these proteins are found at low levels in other tissues. [b] Many of the protein markers listed here are expressed at high levels by two or more of these cell types but are frequently designated as markers.

with amelogenin nanospheres for initial enamel deposition), and that the crystals are of the size of physiologic crystals.

2.1. X-ray Diffraction

The gold-standard method for identifying the mineral phase present in any material is X-ray diffraction.[28] X-ray diffraction can also be used to measure and calculate the average crystal size and orientation of the mineral crystals in the sample. In the case of calcified tissue analysis, this requires homogenization of the tissue (or culture), which in turn mandates dehydration (lyophilization) and grinding; procedures that may prevent the observation of precursor phases.[29,30] Further, in the case of cultures where the yield of mineral is small, it is usually necessary to perform some type of microdiffraction or to pool material from multiple cultures to determine if the mineral phase present is hydroxyapatite or some other calcium phosphate. Although X-ray diffraction is the most definitive way to characterize the mineral formed in culture, there are a few reports where X-ray or the related electron- diffraction has been used to identify mineral in culture. Some examples are listed in Table 2.

2.2. SEM and TEM and Related Techniques

Selected area diffraction, performed under the transmission electron microscope (TEM), and/or dark field evaluation of mineral crystallites, enables evaluation of the individual crystals formed in a tissue or in culture. To perform these analyses, the cultures are often fixed, embedded, and sectioned by methods described elsewhere;[31] some of these methods can cause dissolution of the mineral crystals, yielding crystals that are different in size or composition than those initially present. Alternatively, use of nonaqueous fixation in ethylene glycol has long been known to prevent these changes;[32] however, nonaqueous fixation rarely has been used to prepare cultures for mineral analyses.[33,34]

Selected area electron diffraction is a more difficult procedure than X-ray diffraction, requiring alignment of the electron microscope aperture with the crystals in question. Additionally, calibration of the diffraction pattern obtained with a standard run under the same experimental conditions is necessary. There are, however, a few culture studies where electron diffraction has been used to identify the presence of hydroxyapatite: for example, studies showing the need for 1−2 mM inorganic phosphate for chondrocyte mediated mineralization in both monolayer and agarose-suspension cultures,[35] and a study of high density suspension cultures of chondrocytes in which TEM was used to identify matrix vesicles, and then selected area diffraction was used to confirm the presence of hydroxyapatite.[36] While there are a few studies that use selected area diffraction to verify the presence of hydroxyapatite, TEM and SEM are widely used to show that the mineral crystals are aligned with respect to the collagen axis, as is typical of in situ calcification (Figure 1). Sizes of mineral aggregates, or in the case of dark field analyses, of individual crystals, can be meaasured by TEM. Most importantly, using TEM, the orientation of the mineral on the collagen or amelogenin substrates can readily be demonstrated.[37−43]

At the same resolution, using an electron microscope, backscatter electron imaging and energy dispersive X-ray analysis (EDX) can be used to provide insight into the size distribution of the crystals and the chemical composition of the mineral deposited in culture. There are but a few examples, however, where these techniques have been used to analyze mineral formed in culture.[43−46] One interesting illustration of the combined power of these quantitative techniques is a report of a chick bone marrow stromal cell culture system. In these cultures, mineralization occurred in the presence of 10 mM BGP (β-glycerophosphate) supplemented DMEM, with vitamin D and BMP-2 to stimulate differentiation. While no mineralization occurred in BMP-free cultures under the same conditions, in this study TEM and SEM as well as EDX were used to quantify the distribution of mineral ions as well as crystal shape, X-ray diffraction, and quantitative infrared to show the formation of small crystals in vacuoles which spread to the collagen matrix and matured into aligned hydroxyapatite crystals.[47]

2.3. Atomic Force Microscopy

The atomic force microscope, also called the scanning force microscope,[48] has been used to visualize living cells (osteoblasts, osteocytes, odontoblasts, ameloblasts) and protein surfaces, as well as the properties of the crystals formed on the matrix produced by these cells. Certain cautions apply, as the force used to "tap" the cells must be limited to prevent compression of the cell or induction of cell death; none-the-less, 50 nm resolution has been reported for osteoblasts.[49] At this high resolution, atomic force microscopy (AFM) has been used to characterize the adherence of osteoblast-like cells to different matrices,[50] including different size hydroxyapatite crystals;[51] to characterize the interaction of both fibroblasts and osteoblasts with amelogenin;[52] and to characterize the growth of hydroxyapatite crystals into enamel prisms in the absence of cells.[53−55] But to the best of this reviewer's knowledge, AFM has only been used twice to study mineralization in culture. AFM was used to characterize mineral mechanical

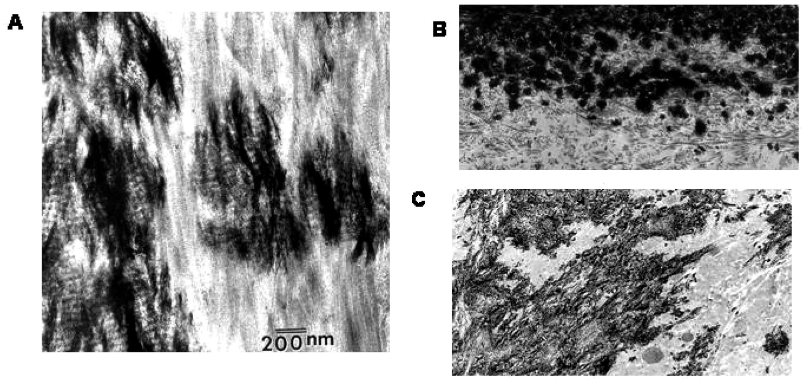

Figure 1. Transmission electron microscopy (TEM) reveals the orientation of the mineral crystals relative to collagen fibrils. (A) High resolution image of a bone specimen. Note the electron dense mineral is aligned parallel to the collagen fibrils. (B) Lower resolution image of an osteoblast culture, mineralized in the presence of 5 mM BGP; clumps of mineral crystals are associated with the collagen fibrils, but the crystals do not appear to be aligned. (C) Low resolution image of a differentiating mesenchymal cell micromass culture at 23 days showing the electron dense mineral associated with collagen fibrils; because the cells are chondrocytes, they make type II collagen, yet the mineral is associated with the collagen fibrils. (Photomicrographs were provided from Dr. S. B. Doty, Hospital for Special Surgery, New York, NY.)

Table 2. Applications of X-ray and Electron Diffraction and Vibrational Spectroscopy, To Identify Mineral Formed in Culture

culture type	diffraction used	vibrational spectroscopy used	notes	ref
primary osteoblast	X		no BGP	283
		X	±BGP	65, 284
		osteoblast cell lines		
MC3T3-E1	X	X	+BGP	137, 138
UMR106	X	X	+BGP	79
marrow stromal cells	X	X	±BGP	47, 267, 285−287
osteoblasts on scaffolds				
chitosan	X			288
collagen fibrils	X			289
hydrogels	X			289
collagen honeycombs	X			290
		other cell types		
odontoblasts	X	X	pulp cells and organ culture	291−293
chondrocytes	X	X	4 mM P	71, 191
ameloblasts	X		organ culture without serum	291, 292
ATDC5 cells		X	no BGP	167

properties in an orthotopic transplantation model where precultured human bone marrow stromal cells were implanted in a mouse calvaria[56] and recently to characterize the spherical bodies with which mineral is associated in the MLO-A5 late osteoblast/early osteocyte cell line (Figure 2).[57]

2.4. Light Microscopy

Observation of tissues or cultures under the microscope usually requires fixation and application of chemical stains. Cultures, of course, can simply be examined under a microscope, and the types of cells present and their arrangement can be identified. Dallas's group[58] has coupled a time-lapse imaging technique to monitor how the cells move, showing that osteoblasts and osteocytes are motile cells, even when engulfed in the mineralized matrix they form. That sort of analysis is more difficult to perform in tissues, and thus comparisons to what happens in the body are not yet available. In other cases, the tissue is often dehydrated to allow staining, a process that could cause the artifactual/spontaneous deposition of mineral, or fixed with a number of different materials that are known to change the solubility of apatite, to change the cross-links within the matrix, and/or to cause solubilization and redeposition of mineral crystals.[31] Keeping the pH of the fixative and staining solution physiologic (pH > 7.4) minimizes dissolution and reprecipitation of mineral phases.

At the light microscopic level, the stains used to identify the hydroxyapatite mineral in culture are alizarin red (which chelates calcium) and von Kossa (which is a silver stain that causes silver phosphate to precipitate; the silver is then oxidized, leaving a black precipitate (Figure 3)). The alizarin red stain is often solubilizied and quantified spectrophotometrically. Newer fluorescent dyes such as xylenol orange or calcein blue can also be used to illustrate the distribution of calcium in the culture matrix without affecting cell viability and to associate the mineral with the presence of matrix proteins.[59,60]

2.5. Vibrational Spectroscopy and Vibrational Spectroscopic Imaging

Infrared and Raman spectroscopies provide information on the local environment of ions with asymmetric and symmetric vibrations, respectively.[61] Several investigators have used these techniques to analyze the mineral phase formed in homogenized cultures and the relative amounts of carbonate substitution in precipitated apatites.[44,62−68] Of interest, in light of the concerns expressed below concerning the use of BGP, a study of marrow stromal cells treated with basic fibroblast growth factor (bFGF) was exposed to 1 or 3 mM BGP for up to four weeks, and the mineral content (measured as the ratio of the area of the phosphate peak to the amide I peak as determined by FTIR) increased with

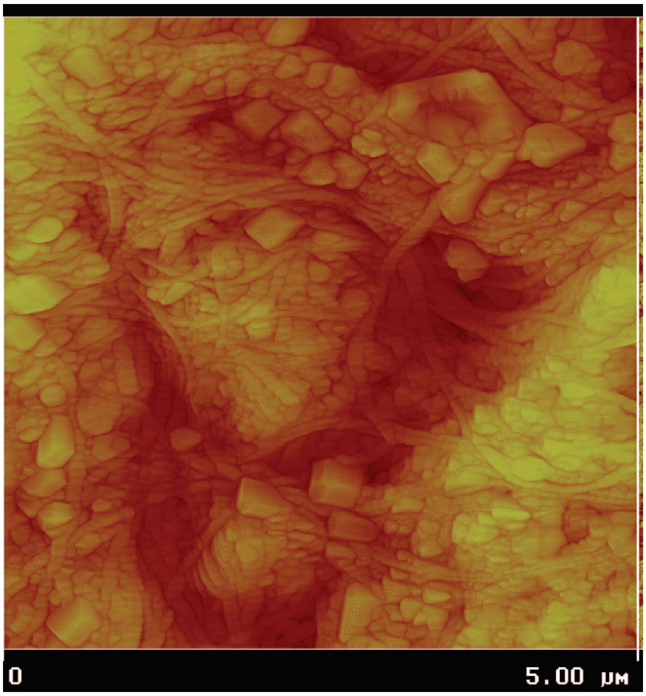

Figure 2. AFM of mineral in culture. AFM height map of MLO-A5 osteocytes at 12 days in culture showing multiple spherical structures intercalated between collagen fibers. The MLO-A5 cell culture figure was provided by Dr. Cielo Barragan-Adjemian and Dr. Lynda Bonewald, UMKC, Kansas City, MO, and analyzed with AFM by Dr. Dan Nicollela, Southwest Institute, San Antonio, TX. For details of the mineral-containing spherical structures, see ref 57.

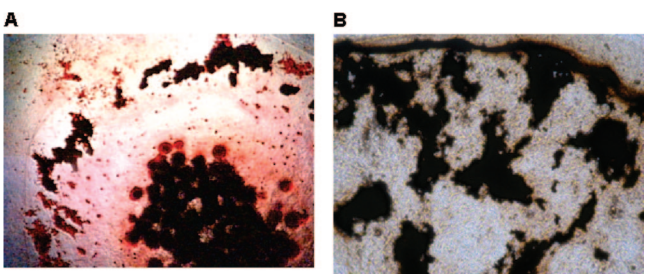

Figure 3. Histochemical analysis of mineral formed in chondrocyte micromass cultures using the silver-stain (von Kossa) technique. (A) Primary chick limb-bud mesenchymal cells that differentiated into chondrocytes shown at day 24. Micromass cultures were maintained in DMEM containing 10% fetal bovine serum with 4 mM inorganic phosphate, 50 μg/mL ascorbate, and antibiotics. (B) ATDC5 cells maintained in culture for 35 days in the presence of the same additives plus 100 ng/mL BMP-2. Cultures counter stained with neutral red. Black deposits in the center of the culture dish and around the periphery are the von Kossa positive material.

increasing BGP concentration, along with the mechanical properties of the mineralized matrix formed in the cultures, but the crystallinity and carbonate/phosphate ratio were not altered by BGP treatment.[62]

More recently, coupling of an array detector to an infrared or Raman spectrometer has enabled localized changes to be displayed with 10 or 1 μm spatial resolution, respectively.[69] Infrared spectroscopic imaging has been applied to characterize mineralization in differentiating chick limb-bud mesenchymal cell cultures,[70−73] in osteoblast cultures,[9,74,75] and, in one case, in odontoblast cultures[76] (Figure 4A). Typical FTIR images of calcifying cultures are seen in Figure 4B−D. Unlike for infrared imaging, for Raman microspectroscopy and imaging, the tissue does not need to be dehydrated and can be examined directly in the cell culture dish or on a slide. Infrared spectroscopic imaging requires thinner sections, and there is interference from water; thus, most commonly, the culture is removed from the dish and either air-dried or embedded and sectioned. Raman spectroscopic imaging has been used to study the mineral formed in calvarial organ cultures,[77] marrow stromal cell cultures,[44] and dental pulp cells.[68] Additional examples of the use of vibrational spectroscopy for the analysis of mineral formed in culture can be found in Table 2.

2.6. Radiographic and Related Methods

Radiographic methods detect changes in scattering elements and, thus, can distinguish the presence of calcium, usually as an increase in density. X-ray microcomputed tomography (XTM or micro-CT) was only recently introduced for the study of mineralization in cultures. Other techniques are related to nuclear magnetic resonance (NMR) and show the difference in the environments of elements with spin dipoles (^{1}H, ^{31}P), thereby giving insight into the changes in the phosphate distribution.

2.6.1. Magnetic Resonance Methods

Potter has pioneered the technique of magnetic resonance microscopy with or without manganese to characterize mineral formation in culture. She used this technique to monitor and quantify bone formation on scaffolds,[78] in bioreactors,[74] and in calcifying cartilage cultures,[39] monitoring relaxation times to obtain maps of mineral deposition.[74] Magnetic resonance microscopy has also been used to monitor the mineralization of tissue engineered constructs in vitro.[79] The lack of general availability of the equipment to do such studies has limited its broad applicability, but it provides new information not otherwise accessible on mineral and matrix without having to dehydrate the tissue.

2.6.2. Microcomputed Tomography (microCT) or X-ray Microtomography (XMT)

Changes in material density are routinely assessed by microCT for tissue samples. The same technique can be used with cell culture systems (Figure 5), providing insight into porosity and mineral deposition. Combining micro-MRI with microCT provides good visualization of water and mineral, although each technique has limitations.[80] The limitation of this method is that while the density of the matrix formed can be easily determined, along with its porosity, no information is provided on the chemical composition of the mineral or its crystalline phase.

In a recent study, Cowan et al.[81] compared the use of microCT with alizarin red staining and scanning electron microscopy to assess BMP-2 induced mineralization in MC3T3-E1 osteoblast-like cell loaded scaffolds. MicroCT has also been used to monitor scaffolds cultured in vitro and implanted in vivo.[82] The obvious advantage of microCT is that it provides a three-dimensional view of the culture, in contrast to the two-dimensional information provided by most other methodologies. Thus, it is likely that as microCT becomes more routinely available, there will be an increase in the applications to characterization of mineralization in culture; however, it must be noted that this technique provides no information about the nature of the mineral present.

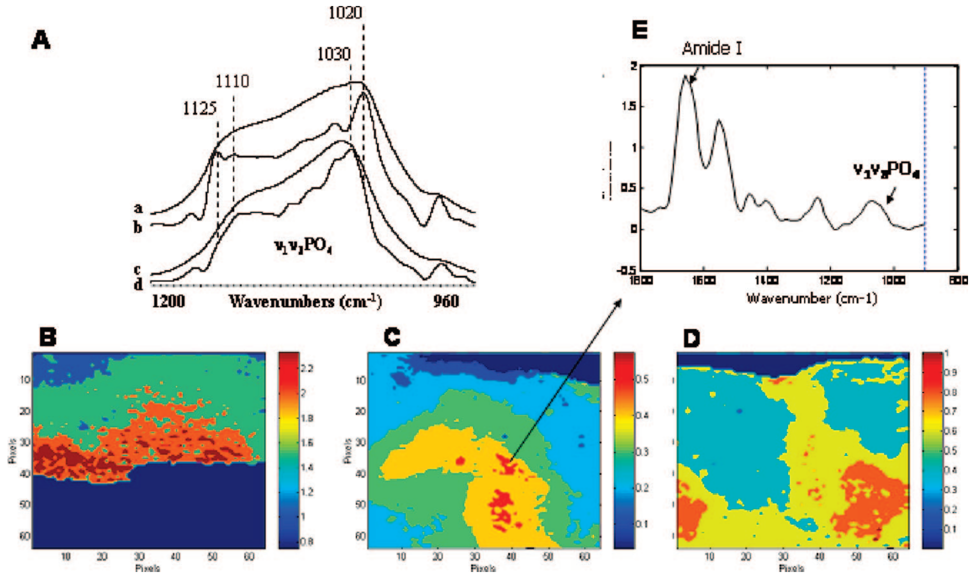

Figure 4. Fourier transform infrared analysis of mineral formed in culture. Infrared spectroscopy can be used to characterize the mineral formed in culture. (A) The phosphate absorption band observed in the odontoblast M2H4 cell line maintained in culture for days 8−21 with 10 ng of TGF-beta1, 100 ng/mL BMP-4, and 3 mM inorganic phosphate (a), when deconvoluted to reveal underlying peaks (b), resembles that obtained from a dentin slice (c) and its deconvoluted spectrum (d). The subbands at 1125 and 1020 cm^{-1} are characteristic of an immature hydroxyapatite rich in acid phosphate and carbonate substituents. Generously provided by Professor J. Guicheux and D. Magne. Details of the culture system are in ref 76. (B) By attaching an array detector to the infrared microscope, images corresponding to each of the parameters of interest can be obtained. FTIR spectroscopic imaging of the mineral/matrix ratio in a mineralizing chick limb-bud micromass culture at day 21 showing the distribution of mineral. These cells were maintained in DMEM with 1.4 mM calcium and 4 mM inorganic phosphate plus antibiotics and 40 uM ascorbate. (C) FTIR spectroscopic image of the mineral/matrix ratio in a "bone nodule" formed in an osteoblast culture at day 14. The cells were cultured with α-MEM containing ascorbate, vitamin D, and a total of 3 mM inorganic phosphate. Note the mineral/matrix ratio in the day 21 chondrocytes is higher than that in the 14 D osteoblast culture. (D) Image showing the distribution of crystal size (and perfection) (in the culture illustrated in part C). (E) Spectrum corresponding to the pixel indicated in part C. The amide I and phosphate bands are noted.

2.7. Chemical Analyses

Chemical analysis of the calcium and phosphate content of cultures or monitoring of the uptake of labeled calcium or phosphate during culture development is frequently used to measure rates of mineral accretion. Measurement of Ca/P ratios for mineral identification requires other techniques to verify that the mineral that forms is comparable to that in the tissue whose composition is being mimicked. Similarly, just showing that the calcium or phosphate contents of the culture are increased with time does not suffice, as many anionic matrix molecules can bind calcium, and the development of the culture generally involves changes in matrix protein phosphorylation.[9]

3. Systems for Studying Mineralization in Culture

While the conditions for forming bone, calcified cartilage, dentin, and cementum in culture are variable, they all share common features. First, there must be a source of cells; second, media that will support the growth of cells and allow mineralization to occur are required; finally, a substrate is needed upon which those cells will proliferate and grow. From a chemical point of view, the solution must be saturated with respect to hydroxyapatite (HA); hence, media are usually supplemented with calcium and phosphate and kept buffered at physiologic or slightly more basic pH. The cells must synthesize a matrix (collagen or the initial enamel matrix) upon which the mineral will deposit, and that matrix should include those proteins and peptides that support mineralization.[83] Factors that stimulate cell proliferation, differentiation, and maturation are generally added in the form of serum, although individual growth factors, hormones, and other exogenous regulators may be added.

3.1. Cell Sources

The cells used in cultures may be provided directly in the form of an immature intact matrix (organ culture), they may be released from tissues, (primary cultures) or they may have been immortalized or derived from tumor cells (cell lines), enabling standardized studies. There are also a variety of ways of distributing the cells in culture (plating the cells), and these will be discussed after the different types of cultures. The methodologies for releasing osteoblastic cells from different types of bone have recently been reviewed[84] and essentially consist of treatments that release the cells from both the mineral and matrix without disturbing the cellular membranes. Similar methods are used to release the other cell types from their matrices.

3.1.1. Organ Culture

Historically, the earliest bone and tooth cultures were performed with limb rudiments or tooth buds.[85−87] In general, the nonmineralized embryonic limbs, calvaria, or tooth organs are placed on grids and are maintained in tissue culture media for relatively short periods of time. The presence of mineral is usually determined histochemically or radiographically. These systems may also be used to study gene and protein expression during development and mineralization.[88] The rudiment system has been recapitulated recently by Price's group in a study of the effects of serum proteins on calcification.[89−91]

The advantage of the organ culture systems is that the tissue shape is maintained, formation and remodeling can be evaluated in the sample, and the system is less sensitive to media changes. On the other hand, the presence of small amounts of mineral already in the rudiments (which may be

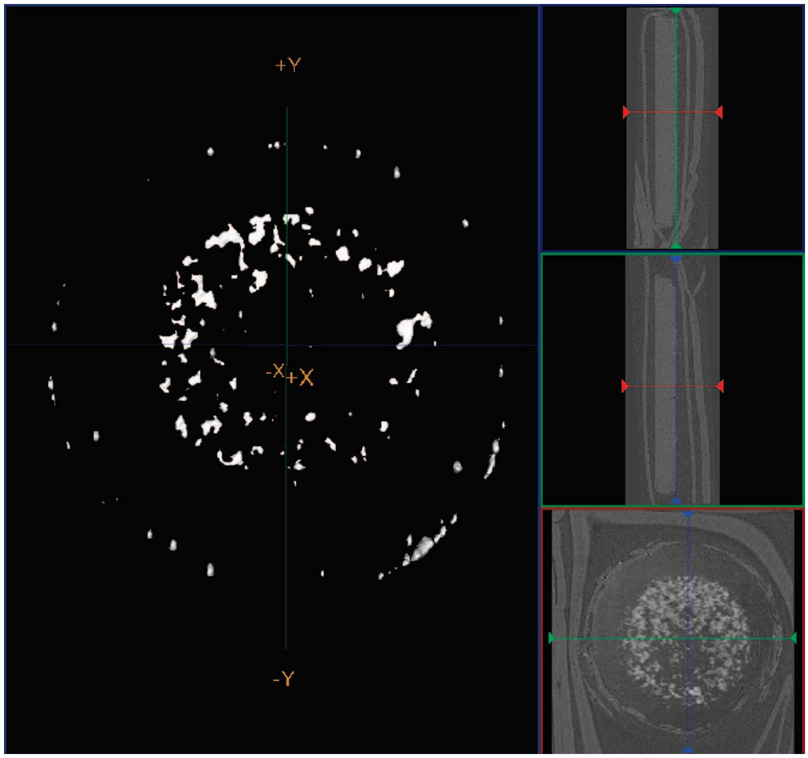

Figure 5. Microcomputed tomography (microCT) shows the increased density due to the mineral deposited in C3H10T1/2 cells grown in micromass culture at 35 days. These cultures were supplemented with ITS, 1% fetal bovine serum, ascorbate, and 4 mM phosphate. Figures on the right show lateral and bottom views of the dish and culture.

nondetectable) can easily serve as a nidus for further crystal proliferation, potentially invalidating the study. Cultured limb rudiments can be used for evaluation of both bone[89−92] and cartilage calcification.[93−95]

Fetal parietal (skull) bones will also mineralize in culture in the presence of 3 mM phosphate without any additional phosphate source.[96] In fact, when these bones were cultured in the presence of 6 mM inorganic phosphate, or 1−10 mM BGP, there was ectopic (unwanted/out of place) mineral deposition in areas of cell death and debris.[96]

Tooth bud organ cultures are frequently used to study both dentin and enamel development. Embryonic tooth buds, or early postnatal tooth buds, have been used. They are generally cultured in BGJb media (see Table 3) and mineralization is monitored by radioactive nuclide uptake ($^{45}CaCl_2$ and $^{32}PO_4$) and by light and electron microscopy.[97] Pulp organ cultures can also be used to generate both dentin and enamel.[98]

3.1.2. Primary Cells and Undifferentiated (Stem) Cells

3.1.2.1. Stem Cells. Osteoblasts are derived from osteoprogenitor cells that exist in the bone marrow stroma and are referred to interchangeably as mesenchymal stem cells or marrow stromal cells (MSCs). Marrow suspensions were first reported to differentiate into osteoblast-like cells when implanted in diffusion chambers.[99] Based on histochemistry, both calcified cartilage and bone were formed. Extension of this work[100] demonstrated that the mineral formed in confluent cultures of MSCs was hydroxyapatite. The marrow suspensions can also differentiate into osteoclasts,[101] cells of the macrophage lineage. Following the early reports, marrow stromal cells then became a standard for studying the effects of different agents on mineralization. However, marrow stromal cells and mesenchymal stem cells (same abbreviation) are not the same thing. Embryonic MSCs are distinct from embryonic stem cells, which, by definition, have the ability to differentiate into any cell type, but MSCs only make cells of the mesenchymal lineage. MSCs have been shown to form osteoblasts and make a mineralized matrix (based on histochemistry) and express early bone cell markers in culture with BGP, ascorbic acid, and 1,25-$(OH)_2$ vitamin D3.[102] The MSCs form fibroblasts, chondrocytes, adipocytes, hematopoetic cells, and osteoblasts,[103] in the presence of appropriate growth factors.[62,104,105]

Mesenchymal stem cells from limb buds as well as MSCs from marrow have been used as a source of cells for studying mineralization. Isolated from embryonic limb buds, they will differentiate into chondrocytes, osteoblasts, and adipocytes depending on the local environment and the nature of the growth factors present.[106]

Dental pulp stem cells differentiate into odontoblasts and deposit a mineralized matrix in the presence of 10% fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), dentin extract, and the mineralization supplements ascorbic acid and 10 mM BGP.[68] Raman microscopy was used to identify that these cells formed a hydroxyapatite containing mineralized matrix in culture.

3.1.2.2. Primary Cells. The most frequently used source of osteoblasts for studies of mineralization in culture is fetal calvarial cells. Primary osteoblasts can also be obtained from long bone and periosteum.[107] These cells are generally released by enzymatic digestion of the poorly mineralized calvaria but may be derived from explant cultures.[108] Cultures of primary osteoblasts were described as undergoing two stages of mineralization: a proliferation phase which was independent of BGP and a mineral deposition phase which required BGP and responded in a dose-dependent manner to 1−14 mM BGP. BGP in these cultures was almost

Table 3. Ca x P Concentrations in Media used for Mineralization Studies

media	culture system	Ca	P	Ca x P (mM^2)	other factors that could affect mineralization
α-MEM	all	$CaCl_2$ 1.8 mM	$NaH_2PO_4 \cdot H_2O$ 1.0 mM	1.80	
DMEM	all	$CaCl_2$ 1.8 mM	$NaH_2PO_4 \cdot H_2O$ 0.90 mM	1.62	$MgSO_4$ 0.8 mM
DMEM-F12	osteoblast	$CaCl_2$ 1.05 mM	$NaH_2PO_4 \cdot H_2O$ 0.45 mM	0.995	$MgSO_4$ 0.4 mM
			Na_2HPO_4 0.5 mM		$MgCl_2$ 0.3 mM
BGJb-Fitton Jackson modification[294]	osteoblast	Ca-lactate 2.54	$NaH_2PO_4 \cdot H_2O$ 4.065 mM	4.1	$MgSO_4$ 0.8 mM
		$CaCl_2$ 1.25 mM	KH_2PO_4 1.0 mM	5.0	
BGJb-Fitton Jackson modification[294]	osteoblast	Ca-lactate	$NaH_2PO_4 \cdot H_2O$ 0.65 mM	4.1	$MgSO_4$ 0.8 mM
		2.54 mM	KH_2PO_4 1.0 mM		
CMRL	osteoblast	$CaCl_2$ 1.8 mM	$NaH_2PO_4 \cdot H_2O$ 1.0 mM	1.8	$MgSO_4$ 0.8 mM
HAMS F-12	osteoblast	$CaCl_2$ 0.30 mM	$NaH_2PO_4 \cdot H_2O$ 0.86 mM	0.26	$MgCl_2$ 0.60 mM; $FeSO_4 \cdot 7H_2O$ 0.003 mM
OptiMem	all	$CaCl_2$ 0.91 mM	$NaH_2PO_4 \cdot H_2O$ 1.0 mM	0.91	insulin & transferin supplements
osteogenic media	osteoblast	$CaCl_2$ 1.8 mM	10 mM BGP	12.6[a]	nM dexamethasone
RPMI 1640[295]	odontoblast	$CaCl_2$ 0.8 mM	5 mM Pi	4.0	
low Ca high Pi media	chondrocyte	$CaCl_2$ 1.0 mM	4 mM Pi	4.0	
chondrocyte calcification media[295]	chondrocyte	$CaCl_2$ 1.8 mM	5 mM BGP	7.2[a]	1,25(OH)2D3 and 24,25(OH)2D3
LHC-9 Lechner and LaVeck medium-9[296]	ameloblast				
KGM-2 (low Ca)	ameloblast	$CaCl_2$ 0.15 mM	not specified	?	bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, epinephrine, transferrin,
high Ca		$CaCl_2$ 2 mM			
high glucose DMEM	mineralizing ameloblasts	2.5 mM	$NaH_2PO_4 \cdot H_2O$ 1.0 mM	2.5	10% FBS and 10 ng/mL EGF, 3 ng/mL TGF-β

If no reference provided data is available in the Invitrogen catalogue.
[a] assumes 60% of BGP is hydrolyzed in media with 1.0 mM Pi.

completely hydrolyzed in 8 h,[109] and mineralization could be blocked by inhibition of alkaline phosphatase during the first but not the second phase.[110] Mineralization could equally be initiated and maintained by 2−5 mM inorganic phosphate.[109]

The initial odontoblast primary cultures used methods derived from bone biology[111] to obtain cells from dental pulp. Pulp tissue from incisors of adult male rats was shown to mineralize in the presence of "osteogenic media" with a minimum of 5 mM BGP,[112] although, more recently, Balic and Mina produced mineralization in similar cultures with 4 mM BGP.[113] There are no reported studies of primary odontoblasts-mediated mineral deposition without dexamethasone and BGP.

For calcified cartilage formation, chondrocytes are frequently isolated from the growth plates of young animals[114] or noncalcified regions of the ribs.[115] In some cases, animals are made vitamin D deficient to inhibit cartilage calcification, and these rachitic animals provide the source of chondrocytes.[116] Culturing of these cells in medium with BGP or inorganic phosphate, and either retinoic acid (to induce alkaline phosphatase expression) or ascorbic acid (to stimulate matrix formation), results in the deposition of mineral.[117]

Primary cultured ameloblasts are reported to keep differentiated phenotype in vitro, including the expressions of ameloblast specific genes and the potential to form calcified nodules, but have restricted proliferation potential.[118−120]

3.1.3. Cell Lines

Cell lines offer the advantage of reproducing the same phenotype every time they are grown under the same conditions; thus, they are very useful for probing the effects of different genetic or chemical modifications on mineral deposition. However, because they are cell lines, they do not recapitulate the situation in vivo as closely as organ cultures or even primary cultures. These cell lines are generally produced from tumor cells or by immortalization by a virus and hence provide a more homogeneous population, although they are not regulated in the same way as primary cells. Techniques for generation cell lines are reviewed elsewhere.[121,122]

There are numerous mesenchymal cell lines that can be differentiated into osteoblasts and chondrocytes in different media. These include ATDC5,[123] C3H10T1/2,[124] HEPM (human embryonic palatal mesenchyme),[125] HFOB1.1.9 (human fetal osteoblasts),[126] MC3T3-E1,[127] 2T3,[128] Oct-1,[129] ROS 17/2.8,[130] SAOS-2,[131−133] TE-85,[134] and UMR 106.[135] Since mineralization has only been reported in some of these, this discussion will be restricted to those cell lines. The MCT3T-E1 cells isolated and cloned from newborn mouse calvarial cells[127,136] express all the bone phenotypic markers and represent mature osteoblasts but require additives (osteogenic media or BMP2) to reproducibly form hydroxyapatite mineral.[137,138] BGP, ascorbic acid, and dexamethasone are not mandatory additives to get mineralization in these cultures.[139] The OCT-1 cells, well-differentiated secretory osteoblast-like cells isolated from rat calvaria, when cultured on a scaffold, deposit hydroxyapatite mineral.[140] The rat osteosarcoma cell line (ROS 17/2.8) differentiates in the presence of mineral ions,[141] but there are no reports of the analysis of the mineral formed in this system, although these cells form osteocalcin (which usually is a sign of mineral deposition[142]) when treated with TGF-beta1[143] and accumulate calcium in the presence of BGP.[144] When implanted in diffusion chambers in vivo, the ROS 17/2.8 cells form nodules identified as mineral by electron microscopy.[145] SAOS-2 (human) and UMR-106 (rat) cells are both derived from osteosarcomas, and both deposit mineral in the presence of either 4 mM inorganic phosphate or 5−10 mM BGP, as demonstrated by calcein labeling,[146] in response to 17-beta estradiol (SAOS-2), as evidenced by electron microscopy[147] or for the UMR106 cells by X-ray diffraction, TEM, and SEM.[148]

In an interesting paper,[108,149] Cornellissen compared primary osteoblasts from both adult fetal rat calvaria and long bones and UMR 106 cells. While all cultures contained mineral based on histochemistry and infrared spectroscopy, alkaline phosphatase activity was reported to be essentially absent in the cells from the fetal calvaria. Calvarial cell also maintained lower alkaline phosphatase activities than the UMR106 cells, but collagen fibril formation (and mineralization of these fibrils) was not detected in the UMR106 cells and in the long bone derived cultures. The poor mineralization of the UMR106 cells most likely was due to the impaired collagen production. These findings are very important to note because they indicate that many of these cell lines, and even primary cultures from long bones, may not produce physiologic mineral; thus, care must be taken in evaluating papers that report the presence of alkaline phosphatase as evidence of physiologic mineralization.

The osteocyte cell line MLO-A5 was established by Bonewald's group[150,151] by cloning from cultures of long bone cells of transgenic mice expressing the SV40 Large T antigen oncogene under the control of an osteocalcin promoter. Osteocalcin mRNA is expressed just prior to mineralization, and the protein is expressed during mineralization.[142] These cells express long processes comparable to dendrites, have low levels of alkaline phosphatase but high levels of the osteocyte phenotypic markers DMP-1, E11, and connexin 43,[21] and mineralize spontaneously in culture.[152] The mineral properties in these cultures were established by FTIR.[152]

There are fewer detailed studies of mineralization in odontoblast cell lines. Magne et al.[76] characterized mineralization in the M2H4 rat odontoblast cell line by FTIR microspectroscopy. The cells, cultured in α-MEM with additional 3 mM inorganic phosphate at pH 7.3, in the presence of 10 ng/mL TGF-beta1 and 100 ng/mL BMP-4, formed a mineralized matrix, as shown by FTIR to be equivalent to that in rat dentin.

Odontoblast cell lines have also been established by a number of investigators. One of the first was derived by Panagakos[153] by transfecting primary cultures of human pulp cells with an SV40-adenovirus construct; the development of the transformed pulp cell (HPC-T) was followed by the development of the mouse MO6-G3 line produced by MacDougall for evaluation of gene expression.[154] MacDougall also produced the M2H4 cell line[155] that mineralizes in the presence of 3 mM inorganic phosphate.[76,156] George's group developed a cell line immortalized by telomerase with a high proliferation potential and the ability to make a mineralized matrix (based on von Kossa staining) in vitro.[157] Immortalized odontoblasts were more recently established from porcine pulp.[158] This cell line makes mineralized nodules and expresses the dentin specific markers dentin sialoprotein (DSP) and dentin matrix protein 1 (DMP1).

The pluripotent C3H10T1/2 cells can differentiate into osteoblasts,[159] chondrocytes,[160] adipocytes,[161] and odontoblasts.[162] The requirements for inducing the expression of each of these cell types are quite variable. Where mineralization has been noted in these cultures, for osteoblasts, both exogenous BMP2 and adenovirus expression of Runx2 or BMP2 have been used[159,163] for chondrocytes, BMP2,[160,164] and for odontoblasts Wnt10.[165] There is a long list of chondrocyte cell lines, reviewed elsewhere,[166] but only a few of these, ATDC5,[167] RCJ3.IC.18,[168] and N1511[169] formed mineral deposits in the presence of dexamethasone and 10 mM BGP, as evidenced by histochemical stains and electron microscopy. The ADTC5 cells mineralize, as shown by FTIR, in long-term insulin-treated culture (5 weeks) in both the presence and absence of BGP.[167]

Cementoblast cell lines,[170] for example OCCM-30,[171] a tumor derived cell line,[172] and BCPb8,[173] all have been shown to differentiate into cementoblasts and produce a mineralized matrix, as identified by histochemical stains. The mineralization in the tumor cell line was shown to be apatitic by electron diffraction.[172]

There are also several ameloblast cell lines that have been used to study mineralization. There are reports on immortalization of ameloblast-lineage cells using T-antigen of SV40 or polyoma viruses;[118,174] however, while these cultures expressed phenotypic markers, their ability to support mineralization was not established. In contrast, Nakata et al.[175] used low Ca (0.2 mM) media to develop a spontaneously immortalized cell line that expressed amelogenin, tuftelin, and enamelin and deposited mineral when 2.5 mM calcium was added to the culture in the absence of BGP.

3.2. Culture Conditions

3.2.1. Confluent Cultures

There are a variety of ways in which single cells can be cultured resulting in a matrix that will mineralize. Most cultures are developed to confluence (i.e., 100% of the culture dish is covered). Cells generally have been shown to stop proliferating when matrix production begins. Some are plated in high density or left in suspension, sometimes within carriers, while others are placed upon a feeder culture. Each of these, under the proper conditions, can produce a mineralized matrix.

Some cultures require confluency to mineralize in a monolayer. This is true for both osteoblast cultures as well as chondrogenic cultures such as the ATDC5 cells.[167] The cultured cells often form matrix vesicles (membrane bound bodies which bleb out from the cell bodies and provide a protected environment for initial nucleation) as well as an extracellular matrix, and the cultures generally mineralize over a 3−5 week period depending on cell type.

3.2.2. Suspension Cultures

In suspension cultures, it has been shown that chondrocytes will form aggregates that can act more "tissue-like". These chondrocytes will produce a matrix and in turn mineralize that matrix. Embryonic stem cells have also been grown in suspension cultures which will then form embryonic bodies that can go on to differentiate into an ostegenic lineage and form mineral.[176−179] In some instances, groups have allowed embryonic bodies to remain in culture and then disrupted them in order to drive them down the ostegenic line.[177] These have been shown to have mineralizing potential when they differentiate into hypertrophic chondrocytes or osteoblast-like cells; however, this appears to require an anchorage dependent step.[180,181]

3.2.3. High Density Cultures

The most common type of high density culture are micromass cultures.[182] In this system, a high density of cells is plated in a low volume of media and allowed to attach to the dish and to each other for a few hours before media is added. This method has been used extensively for primary chick 3-D cultures and for some mouse cultures.[183,184] Each have been shown to mineralize[71,185,186] in the presence of inorganic phosphate or 2.5 mM BGP using X-ray diffraction, TEM, and FTIR.

3.2.4. Encapsulation Cultures

In many culture systems, it has been shown that the morphology of the cell is important to gene expression and matrix production.[187] Both to provide mechanical support and to deliver cells to an in vivo defect site, it can be beneficial to use "encapsulation" techniques. In these methods, cells are included in beads made from agarose, alginate, or other biocompatible hydrogels. Beads are often small enough to be injectable, while maintaining the ability to support the growth and differentiation of cells and matrix. With these methods, a large number of cells can be delivered or grown at once.

To promote a differentiated phenotype, alginate[188] and collagen−agarose beads have been used.[35,189] These systems are based on the concept that cells maintained with a 3-D matrix will differentiate into mature chondrocytes or osteoblasts. It has been shown that this is a valuable way to encourage differentiation while preventing dedifferentiation of these cells into fibroblasts.[187] It is also useful for driving them down an ostegenic lineage. In one recent study, murine embryonic stem cells encapsulated in alginate beads over one month in a rotating bioreactor were shown to mineralize in the presence of 10 mM BGP based on histochemistry, microCT, and FTIR.[190]

3.3. Matrices for Cell Culture

Matrices are primarily used to lend mechanical support to in vitro culture systems. They can be used to provide a three-dimensional shape to a construct, aid in delivery of growth factors and other additives, as well as promote growth and differention of cells and matrix depending on the chemical and physical properties of the scaffold. Scaffolds are also very important because morphology and adhesion and the organization of cytoskeletal elements are very important to gene expression and matrix mineralization.

3.3.1. Plastics

Many studies of mineralization are carried out in dishes made from polystyrene (tissue culture plastic). This is very common when cells are grown in monolayer cultures for mineralization. There are commercially available coated surfaces which can aid in attachment and mineralization of cells. Such coatings include laminin, type I collagen, fibronectin, and matrigel, a basement membrane matrix containing growth factors. The proteins in these coatings have domains with cell binding (RGD) sequences that facilitate cell adherence. There are several examples where using tissue culture plastic coated with type I collagen,[191−193] fibronectin,[194,195] and matrigel[165] promoted mineralization relative to uncoated dishes.

3.3.2. Polymers

There are various polymer scaffolds that have been used as scaffolds for in vitro growth and differentiation of cells. Many of these are resorbable scaffolds that are biocompatible and support cell growth and matrix accumulation. Poly(lactic acid) (PLA) or poly-L-lactic acid and PLGA [poly(lactic glycolic acid)] are commonly used polymers in tissue engineering techniques. In bone tissue engineering, PLA and PLGA scaffolds have been shown to support adhesion and proliferation of osteogenic cells.

3.3.3. Surface Topography

There have been many studies that show that the topography of a substrate can affect mineralization. It has been shown that grooved polystyrene influences collagen alignment by osteoblast-like cells,[196] and a number of studies show increases in mineralization with micropatterning or roughened growth surfaces.[197−200] The reports vary with the types of micro- or nanopatterning used; groove size can vary from millimeter all the way to nanometer scales, resulting in differences in matrix alignment and bone nodule formation depending on the size and type of grooves.[199] Boyan's group has shown that MG63 cells have a more differentiated osteoblast phenotype with greater alkaline phosphatase activity and osteocalcin production on roughened titanium surfaces than on smooth surfaces.[201] Boyan et al. also correlated the mineral:matrix ratio with FTIR imaging of titanium surfaces with microtopographies (grit blasted/acid etched or plasma sprayed) seeded with fetal rat calvarial cells and showed increased bonelike apatite deposition on these surfaces.[75] In another study that looked at surface topography, they took into account the hydrophobicity and hydrophilicity of pyramid-like structured polydimethylsilozane, showing surface-dependent differences in mineral production by osteoblasts.[198] Because surface topography may influence differentiation, gene expression,[197,202] and matrix formation, it may be important in bone tissue engineering techniques as well as in osteointegration studies. Because there is not a great amount of uniformity in the studies, it is still unclear as to exactly what type of patterning or grooving is preferred or necessary for mineralization.

3.3.4. Demineralized Bone and Ceramics

Although both demineralized bone and ceramics have been used as scaffolds for in vitro cell growth techniques, it is very difficult to determine the extent of mineralization on these scaffolds, in part because the ceramics already consist of mineral or silicate glasses doped with mineral; thus, it is difficult to distinguish new from pre-existing mineral. One example in which new mineral deposition (as opposed to the existing mineral in the ceramic) was characterized used SEM and EDAX to study rat osteoblast cells cultured with 10 mM BGP, ascorbate, and fetal calf serum. An electron dense layer formed on the surface of the ceramic consisted of a layer of collagen and then a layer of mineral crystals.[203] Other studies use alkaline phosphatase activity and osteocalcin expression as a measure of mineralization, but these measures, especially in cultures with BGP, do not show physiologic mineral has formed. On the other hand, a phosphate-free ceramic was used to culture human osteoblasts without any additives (ascorbate, BGP, dexamethasone) and type I collagen was deposited prior to the formation of bone nodules.[204] Since ascorbate is required for collagen hydroxylation, one wonders whether the inclusion of ascorbic acid might have enhanced the bone formation in these cultures. Unfortunately, the detailed properties of the mineral were not described.

3.3.5. Cultures with Feeder Layers

One of the ways to maintain cells before development is to maintain them on a feeder layer. This has been done with embryonic marrow stromal cells which differentiate into osteoblasts[205] and with epithelial cells that differentiate into ameloblasts,[206] or cells that produce ameloblast markers,[207] and become mineralized.

3.4. Media for Cell Cultures

The chemical composition of the media used to induce and monitor mineralization of these different cell types is variable. Classically, the liquid substance used to nurture the cells and to study mineralization was BJGb media with and without the Fitton−Jackson modification, or Dulbecco's modified eagle medium (DMEM) and variations thereof (Table 3). Low phosphate media is often used for chondrocyte culture and "osteogenic media" for osteoblast and odontoblast cultures. Several different such osteogenic media have been described. The first, called "osteogenic media", contained 10% fetal calf serum (FCS) and 10 nM vitamin D along with DMEM.[208] In this media, there was no added phosphate source, yet mesenchymal cells expressed osteoblast markers. Similarly, chondrocyte cultures will mineralize without BGP present.[209] In later studies, "osteogenic media" consisted of 1−100 nM dexamethasone or retinoic acid, 10 mM BGP, and 50 μM ascorbate added to one of the basal media. Most recently, osteogenic media is defined as 100 nM dexamethasone, 10 mM BGP, and 50 μM ascorbate. The dexamethasone accelerates cell proliferation and expression of "osteogenic genes".[210]

The variation in the Ca x P products and other major ingredients of this media determine whether mineralization will occur spontaneously without cells or matrix (i.e., it is not physiologic). As is apparent from Table 3, the major variables in these media are the Ca x P mM product and the Ca/Mg ratio. Since, at pH 7.4, solutions in which the Ca x P product exceeds 5.5 mM^2 will precipitate unless mineralization inhibitors are present,[211] it is obvious that many of the media used in cell culture are supersaturated with respect to hydroxyapatite. The addition of serum (which increases calcium concentration but provides both mineralization inhibitors and promoters[91,212] as well as beneficial growth factors) can be avoided using a defined medium, such as ITS[76] (insulin, transferrin, selenium) or other serum supplements. Many investigators add calcium or inorganic phosphate (or phosphate sources) to the media in addition to what is in the basal media. To ensure that these media are not supersaturated with respect to hydroxyapatite, and that any mineral deposition observed is not artifact, control cultures without cells should be shown not to form mineral. However, BGP supplements can deposit mineral if the enzyme alkaline phosphatase is present, even if cells are not.[213]

It is important to comment on the use of BGP as a phosphate source, especially in terms of its concentration. Assuming 80% hydrolysis (higher proportions have been measured[109]), if alkaline phosphatase is made by the cells, the solution will contain higher than 8 mM phosphate concentrations, and most basal media have more than 1 mM calcium, making the deposition of hydroxyapatite inevitable and nonphysiologic if the deposition does not occur on a proper matrix. Inorganic phosphate and other phosphate sources, ATP, phosphoethanolamine, etc., have all been used in lower concentrations to elevate phosphate concentrations, but care must be taken that the media alone does not cause apatite precipitation. BGP has been used as a phosphate source at 2−10 mM concentrations. The Canadian group found increased mineral deposition with 10 mM BGP, and this soon became the classic "osteogenic media". It was known early on, however, that if alkaline phosphatase was added to this media, without cells or matrix present, mineral deposition would occur.[213] Thus, many of the studies of osteogenic media provide proof that alkaline phosphatase activity is present in the cultures, not that physiologic mineral is being deposited, a feature that must be identified by one of the physicochemical approaches described previously. An alternate control would be media with phosphatase inhibitors, which would prevent hydrolysis of BGP.[109,110]

The observation that increasing BGP concentration from 1 to 3 mM[62] in marrow stromal cell cultures did not alter mineral properties suggests that where BGP levels are low (and where the Ca x P mM product is below supersaturation), BGP may be an acceptable source of inorganic phosphate for nucleation and growth of hydroxyapatite crystals. However, high concentrations of BGP are problematic.

Cultures of ameloblasts and odontoblasts also often use "osteogenic media", yet the reason for this is not known. Interestingly, in all these cases, there is mineral deposition, suggesting that there is some alkaline phosphatase activity. Den Besten has established media for growth of preameloblasts and ameloblasts. LHC-9 media, which is selective for epithelial cells, maintains them in a confluent state and exhibits strong expression of secreted amelogenin and ameloblastin proteins.[119] More recently for cultures of epithelial cells released from embryonic tooth buds, she has used KGM-2 media with or without serum supplemented with 0.05 mM calcium and has observed production of all markers of amelogenesis and no evidence of mesenchymal cell phenotypic markers.[214] It is of interest to note that alkaline phosphatase is expressed during enamel/ameloblast maturation,[215] indicating why users of BGP have observed mineralization in their cultures.[216] However, the initial studies of ameloblast mediated mineralization found that mineral deposition occurred in α-MEM without supplemented phosphate.[119]

3.5. Other Additives

One of the advantages of studying mineralizing in culture is the ability to modify the environment in which mineralization is occurring to test hypotheses about the factors affecting mineral deposition. Culture media is usually supplemented with serum (to provide needed growth factors) or cocktails containing specific growth factors. Some of these, along with proteins in the serum, can affect the mineralization process, and it is important to verify that observed effects are not simply due to the interaction of the additive with mineral crystals. More importantly, in culture, one can vary oxygen tension or pH or block the expression of specific genes hypothesized to be crucial in inducing the formation of mineral or regulating the proliferation of mineral crystals.

Table 4. Growth Factors and Cytokines—Exogenous Agents that Affect Mineralization in Culture[a]

factor	system	effect on mineralization	ref
TGFbeta	primary osteoblast	decrease	297
FGF	primary osteoblast	decrease	297
FGF-2	tooth buds	decrease	298
1,25D3 and 24,25D3	primary osteoblast	increase	297, 299
BMP2	MC3T3; primary osteoblast	increase	81, 137, 300
BMP6	chondrocyte	increase	72
BMP7	chondrocyte	decrease	301
dexamethasone	osteoblasts, odontoblasts, chondrocyte cell lines, MSCs	increase or decrease	302
		(depends on cell maturity)	265
estrogen	bone organ culture	increase	303
FGF	primary osteoblast	decrease	297
FGF-2	tooth buds	decrease	298
IL-1 b	periodontal ligament cells	decrease	304
IL-6	osteoblasts	decrease	253, 305
LMP	mesenchymal stem cells	increase	306
leptin	stem cells	increase	307
osteoprotegrin	tooth buds	decrease	308
IL-1 b	periodontal ligament cells	decrease	304
IL-6	osteoblasts	decrease	253, 305
dexamethasone	Oosteoblasts, odontoblasts	increase	302
PTH	MCT3TE1 cells	increase	309, 310
	primary chondrocytes	increase	311
	chondrocytes	retard	
PGE2	osteoblasts, cementoblast cell line	increase	312
retinoic acid	osteoblasts, periodontal ligament cells	decrease	313, 314
	chondrocytes, osteoblast cell line	increase	117, 315
		decrease	316
Runx2	osteoblasts	increase	264
	fibroblasts	no effect	
1,25-dihydroxy Vitamin D3 & 24,25-dihydroxy Vitamin D3	primary osteoblast	increase	297, 299

[a] See review by Declercq[84] for concentrations of many of these additives that are used in osteoblast cultures.

3.5.1. Serum

Most culture systems include 1−20% of bovine or fetal calf serum, while the majority of studies use 10% fetal bovine serum. The reason for including serum is to enhance exposure of the cell to cytokines and growth factors that will aid in growth and differentiation. The caveat is the tremendous variability in the composition of fetal calf serum[217] and that serum contains detectable amounts of calcium, making it essential to measure the total Ca x P of the "full media" after addition of all materials to ensure that the solution is not supersaturated.

3.5.2. Antibiotics

To prevent bacterial and fungi growth, antibiotics (penicillin and streptomycin) and antimycotics (fungizone) are often added to culture media. These reagents do not generally affect mineral ion concentrations in the media and will not be further discussed. Some metabolites such as glutamine (low in most media) or glucose are added to mineralizing cultures to maximize metabolism; these are reviewed in detail elsewhere.[218]

3.5.3. Other Additives

To address questions about effects of growth factors, cell signaling, or specific proteins on mineralization, such factors may be added exogenously, antagonists to their receptors added to alter signaling, silencing RNA or viral transcripts included to alter expression, or blocking antibodies added to test the effect of specific proteins. While these generally do not alter the supersaturation of the solution, they can and do alter the rate of mineral deposition and the physicochemical properties of the matrix. Despite the fact that these additives may not generally alter supersaturation, it is urged that control, nonmineralizing cultures, always be included. Table 4 provides examples of factors that have been included in mineralizing cultures, resulting in alteration of the mineral formed in culture.

3.6. Physical Factors

3.6.1. Oxygen Tension and pH

While most culture studies are performed in incubators with 5% CO_2 and media at pH 7.4, there have been some studies where the oxygen tension is reduced to mimic the conditions thought to exist in calcifying cartilage[219] or the hypoxia hypothesized to exist in osteocytes.[220] Hypoxia in cultures of an osteoblast cell line (MC3T3) in the presence of 10 mM BGP resulted in increased accumulation of alizarin red staining (for mineral) and expression of osteocalcin, MEPE, connexin 43, DMP1, and FGF23, all osteocyte markers, suggesting that hypoxia accelerated the formation of osteocytes.[220] In contrast, hypoxia in cultures of chondrocyte cell lines[221] increases matrix synthesis but retards transformation of cells to hypertrophic chondrocytes and thereby retards mineralization. In certain cell lines such as the ATDC5 cells, which have been shown to undergo chondrogenic differentiation, 3% CO_2 is regularly used to induce mineralization along with other additives to the media.[167]

Table 5. In Vitro Effects of Modifying Proteins Linked to Mineralization

protein	culture system	evaluation method	observed effect	ref
alkaline phosphatase	osteoblasts	KO	no mineralization	240, 317
	MC3T3-E1 cells	antisense	decreased mineral	185
	chondrocytes	levamisole	no effect	
α-2HS glycoprotein	osteoblasts	addition	inhibition	318
amelogenin	organ culture	antisense	inhibition of growth	239
BAG-75	UMR-106 cells	laser capture	decreased mineral	319, 320
	osteoblasts	proteolytic degradation	decreased	
bone sialoprotein	chondrocytes	immunoblocking	decreased	191, 252, 321
	osteoblasts	RNAi	decreased	321
	MC3T3-E1 cells	overexpression	increased	
collagen I	chondrocyte	immunoblocking	inhibition	191
DSPP	stromal cells	overexpression	enhanced	274
matrix gla-protein	chondrocyte	KO	accelerated mineralization	322
osteocalcin	tooth buds	study of KO; blockade of carboxylation with warfarin	none	237
osteopontin	osteoblast	overexpression	decreased	323, 324
	vascular smooth muscle cells	immunoblocking	increased	
phosphophoryn	fibroblasts	overexpression	increased	
proteoglycans	chondrocytes	degradation	increased	186
thrombospondin 1	MC3T3E1 cells	antisense to TSP1	increased	241
		overexpression	decreased	
		exogenous addition	decreased	

The supersaturation of the media is very dependent on pH. However, to mimic the pH in body fluids, the media pH may be increased to be closer to the 7.6 values found at mineralizing sites.[222] Reducing the pH to mimic acidotic conditions reduces mineral accumulation in osteoblast cultures.[223]

3.6.2. Pressure, Loading, and Perfusion

Pressure increases mineralization during in vitro loading of cartilage organ cultures, as it mimics in vivo loading models.[224] These methods are not only useful in looking at the mechanisms and factors affecting mineralization in vitro but also in creating tissue engineered bone constructs and understanding therapies for fracture healing. In tissue engineering techniques, one group has looked at both the effects of hydrodynamic compression as well as cell stretching on osteoblasts grown on titanium coated scaffolds. The effect of compression was dependent on cell type, and the effects of stretching were dependent on intermittent versus static loading techniques.[225−227] These compression methods may be modulated to create different properties of in vitro grown tissues.

There have been a few systems that use low pressure and/or perfusion to seed cells into scaffolds.[228,229] Such systems do not necessarily mimic in vivo loading but enhance cell seeding or nutrient delivery to the scaffolds. These methods have also been shown to increase mineralization.[230] In more traditional tissue engineering techniques, cyclic mechanical compression has been shown to increase mineralization in polymer seeded scaffolds.[231,232]

3.6.3. Hypogravity

During space flight, life on the International Space Station, and travel on the space shuttle, there is a reduction in gravity. This hypogravity has been shown to decrease mineralization and increase calcium release in mouse long bone organ cultures[233] and in chick osteoblasts.[234] Mineral resorption is also increased in hypogravity.[235] It was not clear, however, from these studies if the harsh launch conditions, rather than the exposure to hypogravity caused the reduction in cell proliferation, matrix production, and mineralization. Culture studies planned for the International Space Station should address these issues; however, to date this information is not available.

4. What Sorts of Questions Can Successfully Be Addressed by Culture Studies

The major limitations of cell culture studies are that, unlike in the body, dead cells are not removed, and other cells with which there are interactions in situ are not generally present. While organ culture avoids the second limitation, it too is subject to the first problem. The other drawback is that the selections of culture conditions determine the final observations. There are questions that have been answered in vitro that are difficult or almost impossible to answer in animals. For example, where knockout (KO) or transgenic animals with proposed mineral defects are nonviable, cultures of fetal cells have been useful in providing insight into the effects of such proteins (Table 5).

Similarly, use of antisense RNA, siRNA, and antibody blocking can be used to prevent the expression of specific proteins or exposure of those proteins to the environment. But transfection efficiency varies among cell types, and often, with siRNA and even when methodologies are optimized, these techniques may not give complete inhibition.[236] Addition of reagents that might have affects on other organ system can be used to demonstrate the importance of certain enzymes, matrix proteins, and cellular organelles in the mineralization process. Such studies avoid the need to sacrifice numerous animals, enable exogenous factors to be examined, and provide greater reproducibility because of the innate heterogeneity of even inbred animals.

Some of the earliest studies on the effect of matrix proteins in culture were performed by Bronckers et al.,[237] who used organ cultures of hamster tooth germs to demonstrate that exogenous osteocalcin had no effect on dentinogenesis or dentin mineralization. Later studies, summarized in Table 5, used genetic manipulation (overexpression, gene ablation, or chemical treatment) to modify the amount and structure of different proteins.

There are a variety of techniques for blocking expression of specific proteins, either by binding an antisense chain to the proliferating RNA chain or knocking down its expression with siRNA, shRNA, and iRNA.[238] Antisense techniques for blocking RNA expression of a specific gene with small RNA segments (18−24 mers) were useful for showing that amelogenin could regulate the hydroxyapatite crystal morphology[239] or for demonstrating that blocking alkaline phosphatase expression[240] and thrombospondin 1 expression,[241] respectively, in MC3T3-E1 cultures resulted in decreases and increases in mineral accretion. Interference with RNA expression by small-interfering (si)RNA knockdown[238,242] has been used to demonstrate the importance of the Runx proteins in chondrocyte maturation and mineralization[184] and of annexin V for matrix-vesicle induced mineralization by chondrocytes,[243] while short interfering RNA (siRNA) was used to demonstrate that the calcium binding protein S100A4 when knocked down caused increased expression of osteoblastic markers, suggesting that a function of S100A4 may be to inhibit mineralization by blocking expression of osteoblastic genes.[244] Small hairpin RNA (shRNA) blocking the formation of the transcription factor Lef1 in osteoblasts accelerated matrix mineralization,[245] while knockdown of bone sialoprotein in osteoblast cultures with shRNA inhibited matrix mineralization. In all cases, knockdown with short or hairpin RNA segments is rarely 100% effective, thus mandating confirmation by other techniques. More importantly, sustained knockdown during long-term cultures is difficult to achieve and may be confounded by “off target” knockdown (or knockdown of genes other than that designated for ablation), again requiring use of other validation techniques.

Transfection with viral or nonviral agents (such as calcium phosphate granules[246] and related strategies [247]) is frequently used to overexpress regulatory factors in culture and, as reviewed elsewhere, has been used in tissue engineering to overexpress certain growth factors in preparation for implantation into ectopic sites.[248] These implanted constructs may not be mineralized, but it is hoped that they may be used in the regeneration of bone and other mineralized tissues. The caution is that some cells, such as hematopoietic cells, have low transfectability[249] and other cells, such as mesenchymal stem cells, may require unique methods to allow expression of specific gene products at physiologic levels.[247] It is imperative that the level of expression not exceed physiologic values, as the effects of exposing cells to supraphysiologic levels of growth factors have not been well documented, although it has been shown that exogenous growth factor delivery alters the expression of other cytokines and their receptors and the cellular phenotype in 3D (alginate) cultures.[250]

Antibody blocking has been used to study cell-based factors[251] and extracellular matrix proteins.[252] For example, immunoblocking of type I collagen in differentiating mesenchymal cell micromass cultures retarded mineralization,[191] suggesting a role for type I collagen in cartilage calcification; blocking leukemia inhibitor factor (LIF) in osteoblast cultures increased the number of calcifying nodules.[253] Other examples are summarized in Table 5. There are limits to these methods also: first because the antibody itself might interact with the mineral and second because the antibody might not react with those epitopes of the protein that are involved in the mineralization process. Thus, a negative result might be refuted by an independent method, and finally, antibodies are not always available and even when available may not penetrate the matrix.

5. Conclusions: Advice for Cell Culture Studies of Calcification

As stated throughout this paper, the authors strongly believe that the mineralized tissue formed in culture should compare closely with that which exists in nature. This means that for bone, dentin, and cementum, hydroxyapatite mineral crystals should be found oriented with respect to the axes of the collagen fibrils. This in turn mandates that the cultures be examined in some way that will reveal this orientation (by scanning or transmission electron microscopy or AFM). Second, the hydroxyapatite mineral that is formed should be poorly crystalline, carbonate containing, with substitutions in its lattice. This can be demonstrated by a combination of a variety of techniques, including but not limited to chemical analysis of Ca/P ratio, X-ray diffraction, and/or vibrational spectroscopy.

In the case of enamel, the mineralized matrix should contain few proteins when the cell mediated process is complete, and the hydroxyapatite crystals should be approximately 10 times as long as those found in bone and dentin. Cell processes and membranes should not be included in the final matrix.

Finally, and perhaps most important, it is crucial that the process of mineralization being studied is cell-mediated. Thus, control cultures without cells should not form mineral precipitates (as is the case in cell-free cultures with BGP and exogenous alkaline phosphatase[213] or cell-free cultures on collagen sponges that have not been appropriately washed[254]). Cultures should not form mineral if matrix proteins are inactive, which may be demonstrated by blocking RNA and protein synthesis with agents such as actinomycin and cycloheximide, respectively.

This brings us to the final point of the review. BGP, in the authors’ opinion, is not a necessary component of “osteogenic media”. BGP can be replaced with physiologic levels of inorganic phosphate or other phosphate esters (ATP, pyridoxal phosphate, phosphoserine, etc.[255]), and mineralization will occur in the absence of BGP.[191,204] In addition, expression of some key genes relevant to mineralization has been shown to be comparable in mineralizing cultures with BGP or inorganic phosphate.[72,185,256] Thus, authors and readers alike should remember that the presence of hydroxyapatite in cultures with BGP simply indicates the presence of alkaline phosphatase or other phosphatase activity, especially since mineralization does not occur in these systems when the phosphatases are inhibited.

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