Designation: F3106 – 14

Standard Guide for *in vitro* Osteoblast Differentiation Assays[1]

This standard is issued under the fixed designation F3106; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This document provides guidance on how to conduct *in vitro* osteoblast differentiation assays with progenitor stem cells including mesenchymal stromal cells.

1.2 This document describes the roles of various osteogenic supplements that are added to the cell culture medium of an osteoblast differentiation assay to encourage and support the differentiation of progenitor cells into matrix-producing osteoblasts.

1.3 This document provides recommendations for the concentrations of osteogenic supplements that may prevent the precipitation of artifactual mineral deposits that are not directly produced by osteoblasts, nor correlated with osteoblastic gene expression of the cells.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F2312 Terminology Relating to Tissue Engineered Medical Products

F2997 Practice for Quantification of Calcium Deposits in Osteogenic Culture of Progenitor Cells Using Fluorescent Image Analysis

3. Terminology

3.1 Unless provided otherwise in 3.2, terminology shall be in conformance with Terminology F2312.

3.2 *Definitions:*

3.2.1 *calcium deposits, n*—a calcium phosphate-containing substance synthesized in cell cultures during mineralization or osteoblast differentiation assays that may be directly produced by osteoblasts or precipitated out of the solution without cell participation.

3.2.2 *mineralized matrix, n*—a calcium phosphate-containing substance produced by cells typically in the osteoblast, odontoblast, and calcifying chondrocyte lineages, which is composed of crystals of calcium phosphate and contains collagen Type I and other non-collagenous proteins.

3.2.3 *osteoblasts, n*—secretory mononuclear cells that will initiate the formation of a matrix containing characteristic proteins, such as collagen, and non-collageneous proteins such as bone sialoprotein and osteocalcin, that will mineralize in the presence of a calcium and phosphate source.

4. Significance and Use

4.1 This guidance document describes the components and conditions used for *in vitro* osteoblast differentiation assays that can be used to screen for the osteogenic capability of progenitor stem cells from various human or animal sources, including mixed tissue-derived connective tissue progenitor populations, or cell populations that may be selectively isolated or manipulated through culture expansion, processing, transfection or genetic modification.

4.2 The osteoblast differentiation assay may be referred to as an osteogenesis assay or a mineralization assay.

4.3 It is important to carefully select the components and conditions used for *in vitro* osteoblast differentiation assays since high amounts of osteogenic medium components can lead to dystrophic, pathologic or artifactual calcium-based precipitates that do not indicate differentiation of the cells in culture to functional osteoblasts (**1**).[3] For example, when high concentrations of beta-glycerophosphate are used in the medium to function as a substrate for the enzyme alkaline phosphatase secreted by the cells, there is a marked increase in free phosphate, which then precipitates with Ca^{++} ions in the media to form calcium phosphate crystals independently of the differentiation status of the progenitor cell (**2**, **3**).

4.4 Alkaline phosphatase production is an early event associated with osteoblast differentiation but it can also be stimulated in other cell types by the addition of the osteogenic

[1] This test method is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.43 on Cells and Tissue Engineered Constructs for TEMPs.

Current edition approved Oct. 1, 2014. Published February 2015. DOI: 10.1520/F3106-14.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The boldface numbers in parentheses refer to the list of references at the end of this standard.

supplement dexamethasone to the medium. Alkaline phosphatase enhances the formation of calcified deposits prior to their natural occurrence in bone that typically coincides with bone sialoprotein and osteocalcin expression by mineralized matrix-producing osteoblasts. These kinds of calcified/mineral deposits are thus considered dystrophic, pathologic, or artifactual because they were not initiated by a mature osteoblast. A calcium measurement, such as that described in Practice F2997 for the Quantification of Calcium Deposits in Osteogenic Culture of Progenitor Cells Using Fluorescent Image Analysis, may thus result in a potentially false interpretation of the differentiation status of osteoprogenitor cells if used in isolation without gene or protein expression data.

4.5 In addition to screening for multipotentiality of undifferentiated stem cells, osteoblast differentiation assays are useful for assessing the osteoinductivity of cell culture substrates or biomaterial scaffolds or drugs or biomolecules; such as, cytokines or growth factors.

4.6 *In vitro* osteoblast differentiation assays are not predictive of *in vivo* bone formation, but are useful for comparison purposes to standardize performance between different types, sources or passages of progenitor cells, biomaterials, or types and concentrations of biomolecules.

5. An Overview of the *in vitro* Osteoblast Differentiation Assay Procedure

5.1 Briefly, progenitor stem cells are seeded in monolayer on treated tissue culture plastic cell culture dishes or plates, or on biomaterial substrates, and allowed to proliferate in proliferation medium until they reach confluency with bi- or tri-weekly medium changes. After confluency is reached, cell culture medium is then changed from proliferation medium to differentiation medium that contains supplements to promote the osteogeneic differentiation of the progenitor cells and the formation of a mineralized matrix. The cells are cultured for up to 28 days in total with medium changes bi-weekly or every other day. Cells will undergo apoptosis during *in vitro* mineralization (**4**).

5.2 To assess the extent of differentiation of the progenitor cells after the osteoblast differentiation assay, the calcium deposits can be quantified using the non-destructive fluorescent image analysis as described in Practice F2997 or terminated and measured directly by conducting a total calcium content analysis using one of the many commercial colorimetric kits available for this purpose. Calcium deposition alone should never be used as a measurement of osteogeneic potential.

5.3 Quantifying the expression of osteogenic genes or proteins is another important measurement to use in conjunction with measurement of calcified deposits to confirm the presence of osteoblasts. Both gene and protein measurements should be performed at multiple time points, as many peak and then later decrease.

6. *In vitro* Osteoblast Differentiation Assay Components

6.1 *Cell Sources:*

6.1.1 Mixed tissue-derived connective tissue progenitors from various tissues (e.g. from marrow, bone, fat, synovium, periosteum, cartilage, muscle, vascular and perivascular cells, and cord blood).

6.1.2 Progenitor populations that have been selectively isolated or manipulated through culture expansion, processing, transfection or genetic modification.

6.1.3 Osteoprogenitors obtained from the pool of cells that grow out of bone chips during explant culture or from collagenase digestions of bone.

6.1.4 Embryonic stem cells, induced pluripotent stem cells or their progeny.

6.1.5 It should be noted that optimal conditions for osteogenesis vary between species, with human cells typically more resistant to differentiation, while rodent are more reproducible. Outcomes also differ between human and rodent cells, with rodent cells more likely to form discrete calcified nodules particularly when harvested from neonatal tissues by enzymatic digestion.

6.2 *Media for Osteoblast Differentiation Assays:*

6.2.1 *Proliferation Phase Cell Culture Medium*—Dulbecco's Modified Eagle Medium (DMEM), including low glucose DMEM, or DMEM F-12 supplemented with 10% Fetal Bovine Serum (FBS) is commonly used for the proliferation or expansion phase of the osteoblast differentiation assay. Some cells may have been propagated or expanded in alpha-MEM and should therefore remain in that medium for the proliferation phase, even although it contains ascorbate which can promote osteoblast differentiation.

6.2.2 *Differentiation Phase Cell Culture Medium*—For osteogenic differentiation cultures, α-MEM is commonly used during the differentiation phase, although, as mentioned, it can be used for proliferation as well. Low glucose DMEM with additional osteogenic supplements has also been used for human mesenchymal stem cell osteogenic differentiation.

6.3 *Fetal Bovine Serum (FBS):*

6.3.1 Fetal bovine serum (FBS), previously called fetal calf serum (FCS), is typically used at 10% of the medium volume during osteoblast differentiation assays in both the proliferation medium and the differentiation medium. Serum composition varies by lot and as such it is important to screen various lots to ensure osteogenic potential. Some lots of FBS are more efficient at promoting cell proliferation while others are better for promoting mineralization; thus, sera suitable for differentiation may not be the same as sera suitable for proliferation. Several companies offer serum qualified for human mesenchymal stem cells to support either their proliferation or differentiation. This testing can also be performed "in house" whereby serum batches can be compared to current stocks. Any serum testing should include information about the cell type used for screening, and serum source and lot number. Use of a standard cell line could be incorporated to compare results across serum lots. Heat inactivation of the serum is not necessary before use in an osteoblast differentiation assay, but may be required when growth factor supplements are being tested in this assay in order to avoid a high background.

6.4 *Osteogenic Supplements:*

6.4.1 *Ascorbic Acid*—In osteogenic cell cultures, ascorbic acid is necessary to promote extracellular matrix protein production. It has been shown to act as a co-factor in the hydroxylation of proline and lysine residues in collagen (**5**). There are two forms of this supplement that are typically used in the differentiation medium. The first is ascorbic acid which is typically used at a concentration of 50 µg/mL. Due to its fast degradation, it is necessary to add this supplement to the cultures daily. The other form of ascorbic acid is the more stable ascorbic acid-2-phosphate (AA2P) which is added to the culture media typically at a final concentration of 50 µM (**6**). If this form is chosen, daily addition is not necessary and can be replenished at the same time as media change (every 2-4 days). A range of AA2P can be used; however, when used at 500 µM, there may be cellular damage that reduces proliferation of human mesenchymal stem cells (**7**). AA2P at 1 mM or higher causes cell death of human mesenchymal stem cells (**6**).

6.4.2 *Beta-glycerolphosphate*—β-glycerolphosphate (β-GP) is a phosphate source required for *in vitro* mineral deposition and is added to the differentiation medium at a range of 2-10 mM during osteoblast differentiation assays. There are concerns that high concentrations of β-GP, such as 10 mM or higher, lead to dystrophic mineralization because of the ability of alkaline phosphatase produced by osteogenic cells to cleave phosphate groups from β-GP and cause calcium phosphate deposits not deposited directly by a mature osteoblast (**8**). This confounds the accuracy of calcium measurements to assess osteoblast differentiation status. To avoid dystrophic or non-osteoblast mediated mineralization concentrations of 2.5-4 mM are recommended for this supplement. Dystrophic calcium phosphate deposition within the cultures appears to promote osteogenic differentiation of the cells and thus 10 mM β-GP continues to be commonly used. When β-GP is used at 10 mM, calcium content measurements alone may not adequate to distinguish if osteogenic differentiation has occurred.

6.4.3 *Dexamethasone*—Dexamethasone is a glucocorticoid which can act in both a stimulatory and inhibitory manner on osteogenic differentiation depending on dose, duration, stage of cell differentiation, and species of responding cell (**6**). Glucocorticoids have been shown to be involved in the bone formation/remodeling axis (**9**). Some studies have provided evidence for the necessity of dexamethasone in *in vitro* mineral formation, particularly in rat marrow-stromal derived cells (**10**), and human mesenchymal stem cells (**6**). In general, dexamethasone is used at a concentration range from 1-100 nM. 100 nM can be used during the first three weeks, but if used longer inhibits mineralization, therefore 10 nM dexamethasone is preferred throughout the culture time period (**11**). The use of dexamethasone with human cells is a matter of debate with regards to osteocalcin expression, which has been shown to decrease in osteoblastic cells after dexamethasone exposure (**12**).

6.4.4 *Vitamin D3*—1,25-dihydroxyvitamin D3 (vD3) is known to accentuate osteoblast gene expression when added to the differentiation media if cells have already begun to differentiate to the pre-osteoblast phase (**13**) in particular osteocalcin. It may be added to later stage cultures, for example after 2 weeks, at a dose of 10 nM. It will also stimulate mineral deposition.

6.4.5 *Bone Morphogenetic Protein-2 (BMP-2)*—BMP-2 may be used to stimulate *in vitro* osteogenic differentiation of MSCs when the combination of ascorbic acid, β-GP, and dexamethasone are not sufficient (**14**).

6.4.6 *Non-Essential Amino Acids and Glutamine*—Non-essential amino acids (NEAA) and L-glutamine are two supplements commonly used in proliferation medium to support progenitor cell expansion. They are not known to influence osteoblast mineralization.

6.4.7 *Antibiotics or Fungicides*—The addition of antibiotics or fungicides to prevent bacterial and fungi growth does not affect the mineralization or osteogenic potential of cultures. The mostly commonly used antibiotics are penicillin and streptomycin.

6.5 *Seeding Density:*

6.5.1 Cell seeding density has been shown to affect *in vitro* mineralization (**5, 15**). Low seeding densities that do not lead to confluent cell cultures may generate poorly mineralized cultures. Cell seeding at higher densities such as 20,000 cells/cm^2 can accelerate the differentiation and mineralization process. Human mesenchymal stem cells are typically seeded between 3,000-25,000 cells/cm^2 and more commonly at 10,000 cells/cm^2.

6.6 *Cell Passage Number:*

6.6.1 The number of times that progenitor cells have been passaged prior to initiating a mineralization experiment can greatly affect the differentiation capability of the cells. Typically, the greatest ability to mineralize occurs in early passage cells (within 4 passages), or primary cultures in which the cells are taken directly from tissue or marrow and plated directly. The passage number will also affect the mineralized matrix appearance, with primary cultures showing clear distinct nodules, while multi-passaged cells will result in a wide-spread, small particulates of mineral.

7. Methods of Characterizing the Outcomes of an Osteogenesis Assay

7.1 *Characterization of the Mineralized Matrix:*

7.1.1 *Calcium Content*—Typically measured by a commercially available colorimetric assay.

7.1.2 *Mineral Staining*—For visualization and quantification of mineralized matrix. There are a variety of stains that can be used for this purpose including von Kossa, alizarin red, alizarin complexone, and the non-toxic dyes xylenol orange and calcein. Alizarin red chelates calcium and is often solubilized and quantified spectrophotometrically. von Kossa is a silver stain that causes silver phosphate to precipitate and oxidize leaving a black precipitate that can be visualized. Fluorescent dyes such as xylenol orange can also be used to illustrate and quantify the distribution of calcium in the culture matrix without affecting cell viability as described in Practice F2997.

7.1.3 *Total Area, Number of Mineralized Nodules*—The total area of mineralized matrix and the number of mineralized

nodules (if they are discrete) are two commonly used parameters to quantify the extent of mineralization.

7.1.4 *Structure and Composition of Mineral*—The *in vitro* mineralization process is not the same as *in vivo* bone formation and may include mineralized deposits that are not directly associated with the cells or the collagen component of bone mineral, or other phases of calcium phosphate not found in bone. Images should be taken at a magnification sufficient to determine individual cells so that it can be determined if the mineralization is associated with the cells. Rapid cell death can also lead to dystrophic calcification, which is easily detected at a higher magnification where the deposits are circular in nature and the same size as the individual cells. Transmission electron microscopy may be used to obtain very high magnification images that can confirm the co-localization of the inorganic mineral within the fibrous organic collagen component.

7.2 *Characterization of the Differentiation Status of the Cells:*

7.2.1 *Alkaline Phosphatase (ALP) Staining*—Alkaline phosphatase is an early pre-osteoblast protein that is produced by osteoblasts prior to and also in conjunction with mineralized matrix deposition. Human embryonic stem cells (hESCs) have a high level of ALP expression in their undifferentiated state and will lead to mineral precipitation without being differentiated to an osteoblast.

7.2.2 The expression of genes associated with osteoblast differentiation may be measured to assess differentiation status. The typical panel of osteogenic genes includes alkaline phosphatase, Type I collagen, and osteocalcin (ALP, Col1α1, OCN). A useful transcription factor marker of osteogenesis is osterix, which is upstream of Runx2. In human mesenchymal stromal cells the expression of Runx2 is often not predictive of osteogenic potential. Bone sialoprotein expression is a late stage marker occurring prior to osteocalcin that may also be included to determine osteoblast differentiation. It is important to conduct the gene expression analysis at multiple time points to capture peak expression levels since expression level varies over time.

7.2.3 Protein analysis of osteoblast associated matrix proteins can be conducted to assess the extent of osteoblast differentiation. In addition to the early protein alkaline phosphatase, bone sialoprotein and osteocalcin are late stage mineralized matrix proteins that are also commonly analyzed to confirm osteoblast differentiation. Osteocalcin expression at high levels is not typically observed in differentiated human cultures that have been passaged many times (e.g. more than 4-6 times) before initiating the assay.

7.2.4 Morphology is another way to assess the differentiation status of progenitor cells. The initial elongated, spindle-shaped progenitor cells typically become cuboidal in shape as they differentiate to osteoblasts.

7.2.5 Quantitative imaging modalities as described in ASTM Standard Method F2944-12 the area fraction of mineralization, nodules, expression of alkaline phosphatase, bone associated matrix proteins and gene expression can be uses as a metric to quantitatively measure features of osteogenic potential and to define differences in osteogenic potential between different cell populations.

8. Keywords

8.1 connective tissue progenitor stem cells; mesenchymal stem cells; mineralization assay; osteoblastic progenitor cells; osteoblasts

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