Corning® PureCoat™ ECM Mimetic Cultureware Fibronectin Peptide: Novel Synthetic, Animal-free Surface for Culture of Human Bone Marrow-derived Mesenchymal Stem Cells

Kerry Thompson, Jeff Partridge, Paula Flaherty, Susan Qian, and Deepa Saxena

Corning Incorporated, Tewksbury, MA, USA

Application Note

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Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells with the ability to differentiate into bone cells (osteocytes), cartilage cells (chondrocytes) and fat cells (adipocytes)[1]. MSCs are an important tool in regenerative medicine and tissue engineering; the therapeutic potential of these cells is being evaluated for several disorders[2]. Ease of isolation and expansion, and suitability for allogenic transplant makes MSCs an attractive candidate for therapeutics. *Ex vivo* expansion of these cells requires either bovine serum containing media or coating of the culture vessel with human or animal-derived extracellular matrix (ECM) protein. Animal or human derived components can be a source of pathogens and may require additional screening to ensure safety. Growing concerns about introducing human and animal-derived pathogens into the culture necessitate the need for an animal free (xeno-free and human origin components-free) culture environment. Also, media components and coating matrices of biological origin may have batch to batch variability and can be undefined. Self-coating requires additional time, resulting in coated vessels with limited shelf-life.

Here, we report Corning PureCoat ECM Mimetic Cultureware Fibronectin Peptide for culture of cell types that require a Fibronectin coating. The Fibronectin mimetic surface is a pre-coated, synthetic, xeno-free, animal-free, and room temperature stable surface. Fibronectin peptide is covalently immobilized on the surface in a manner that presents a functionally active orientation to the cells. The peptide consists of the RGD amino acid sequence from the Fibronectin cell binding domain that facilitates cell attachment[3]. In this study human bone marrow derived mesenchymal stem cells were cultured on Corning PureCoat ECM Mimetic Cultureware Fibronectin Peptide for multiple passages in defined and xeno-free medium. MSC growth and morphology on the Fibronectin mimetic surface were comparable to cells grown on human-origin matrix-coated surface. Cells maintained their

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differentiation capability during the course of culture and were successfully differentiated into osteogenic and adipogenic lineages following multiple passages on the Fibronectin mimetic surface. Expression of MSC markers was also determined by flow cytometry; cells exhibited the characteristic marker profile established by International Society for Cell Therapy[4]. Corning PureCoat ECM Mimetic Cultureware Fibronectin peptide provides a ready to use alternative to Fibronectin coating for cell culture with comparable cell attachment and functionality.

Materials and Methods

Reagents

Human bone marrow-derived MSCs were purchased from Lonza (Cat. No. PT-2501). Cells were cultured in MesenCult™-XF medium (STEMCELL Technologies Cat. No. 05420 referred to here as, culture medium) on Corning PureCoat ECM Mimetic Cultureware Fibronectin Peptide 6-well plate (Corning Cat. No. 356240). Falcon® 6-well cell culture plates (Corning Cat. No. 353224) coated with MesenCult-XF Attachment Substrate (Cat. No. 05424) served as a control and is also referred to as human-origin matrix from the kit. Cells were passaged using MesenCult-ACF Enzymatic Dissociation Solution (STEMCELL Technologies, Cat. No. 05427); dissociation enzyme was neutralized by MesenCult-ACF Enzyme Inhibition Solution (STEMCELL Technologies, Cat. No. 05428). MSC immunophenotypic analysis was performed using a Human MSC Analysis Kit (BD Cat. No. 562245). HMSC Differentiation BulletKit™ – Adipogenic (Lonza Cat. No. PT-3004) was used for differentiation into Adipogenic lineage. Differentiation into Osteogenic lineage was performed using HMSC Differentiation BulletKit– Osteogenic (Lonza Cat. No. PT-3002). Mineralization by bone cells was quantified with OsteoImage™ Mineralization Assay kit (Lonza Cat. No. PA-1503). Cells differentiated to the osteogenic lineage were stained for Alkaline Phosphatase activity with Vector® Red Alkaline Phosphatase Substrate Kit I (Vector Lab Cat. No. SK-5100). Adipocytes were stained with Oil Red O from Mesenchymal Stem Cell Adipogenesis Kit (EMD Millipore Cat. No. SCR020). Paraformaldehyde 16% solution (EMS Cat. No. 15710) was diluted to 4% with 1 X Dulbecco Phosphate Buffer Saline (DPBS). Rat tail Collagen I was purchased (Corning Cat. No. 354236) and coated according to the manufacturer's instructions.

MSC Culture

Corning PureCoat ECM Mimetic Cultureware Fibronectin Peptide is pre-coated and ready to use. Falcon 6-well cell culture plates were coated with human-origin matrix. MSCs were thawed from the frozen stock and spun at 200 x g for 5 minutes, resuspended in culture medium, counted using the Vi-CELL™ cell counter (Beckman Coulter) and seeded at a density of 4000 cells/cm^2 in 2 mL culture medium/well in 6-well plate. Cells were incubated in a humidified incubator at 37°C with 5% CO_2. When cells reached ~60-70% confluence as observed under the microscope, passaging was performed. For passaging, culture media was aspirated and cells were washed once with DPBS and 0.5 mL enzymatic dissociation solution was added to each well (6-well format), cells were examined under the microscope; when cells were detached from the surface, enzymatic solution was neutralized by adding 0.5 mL of enzyme inhibition solution. Cells were then transferred to a polystyrene Falcon tube (Corning Cat No. 352097). The remaining cells were recovered by rinsing each well with 1 mL culture medium. Cell suspension was centrifuged at 200 x g for 5 min, supernatant was removed, and the cells were resuspended in culture medium. Cells were counted, seeded in the culture vessels and incubated in a humidified incubator at 37°C with 5% CO_2.

MSC Differentiation

After 3 passages, cells were differentiated into osteogenic and adipogenic lineages. Differentiation kits are described in the reagent section.

Osteogenic Differentiation of Mesenchymal Stem Cells

MSCs were seeded at a density of 3.1x10^3 cells per cm^2 in 0.5 ml MSCGM™ medium in 24-well tissue culture plates coated with Collagen I. Cells were incubated for 24 hours in a humidified incubator at 37°C with 5% CO_2. Osteogenesis was induced by replacing MSCGM media with osteogenesis induction medium. Induced MSCs were fed every 3-4 days for 2-3 weeks by replacing spent medium with fresh induction medium. Uninduced controls were maintained in MSCGM. After differentiation, cells were stained for Alkaline Phosphatase activity, and mineral deposition was quantified by a fluorescence based detection method.

Alkaline Phosphatase Staining

Cells differentiated to the osteogenic lineage were fixed and stained for Alkaline Phosphatase activity. For staining, media was removed from the wells, cells were washed with DPBS and fixed in 4% paraformaldehyde (PFA) for 20 min. PFA was removed and cells were rinsed 3 times with water. Cells were then incubated with substrate solution for 20-30 minutes, washed once in DPBS and counterstained with the nuclear dye DAPI.

Quantification of Mineral Deposition

The mineral deposition assay is based on the specific binding of the fluorescent OsteoImage™ staining reagent to the hydroxyapatite portion of the bone-like nodules deposited by bone cells. After differentiation, medium was removed and cells were rinsed once with DPBS, and then fixed in 4% PFA. After fixing, the cells were rinsed twice with 1x wash buffer (supplied with the kit). To each well of the 24-well plates, 0.5 mL of staining reagent was added and plates were incubated in the dark at room temperature for 30 minutes. Following the incubation step, staining reagent was removed and plates were washed three times with 1 mL of wash buffer per well, allowing the wash buffer to remain in the plate for five minutes before proceeding to the next wash. Fresh wash buffer was added to all of the wells, and the plates were read in a Tecan® Safire²™ plate reader at 492 nm/520 nm.

Adipogenic Differentiation of Mesenchymal Stem Cells

MSCs were seeded at a density of $2.1x10^4$ cells per cm^2 in 0.5 mL MSCGM™ medium in Falcon® 24-well cell culture plates (Corning Cat. No. 353047). Cells were incubated in a humidified incubator at 37°C with 5% CO_2 and fed every 2-3 days by replacing the medium with fresh MSCGM until the cells reached confluence. Adipogenesis was induced by replacing MSCGM medium with adipogenesis induction medium for 3 days followed by culture in adipogenesis maintenance medium for 1-3 days. Uninduced controls were maintained in adipogenesis maintenance medium. After 3 cycles of induction/maintenance, the cells were fixed and stained with Oil Red O.

Oil Red O Staining of Adipocytes

After differentiation into Adipocytes, differentiation media was removed and cells were washed once with DPBS. Cells were fixed in 4% PFA for 30 minutes at room temperature and rinsed three times in DPBS, then rinsed with water two times. Water was removed from the wells and 0.5 ml of Oil Red O Solution was added to each well. Cells were stained for 50 minutes at room temperature, and then washed three times with water. Cells were examined under the microscope and images were captured.

Characterization of Markers by Flow Cytometry

Cells were grown for 3 passages and expression of markers was analyzed by flow cytometry using the Human MSC Analysis Kit. Cells were dissociated using BD Accutase™ Cell Detachment Solution (BD Cat. No. 561527), washed with PBS and resuspended in PBS with 10% fetal bovine serum at a concentration of $5x10^6$ cells/mL. For staining, 0.01 mL cell suspension was transferred to each tube and antibodies were added. Tubes were incubated in the dark for 30 minutes on ice. Cells were washed twice with PBS containing 10% fetal bovine serum, and then resuspend in 0.3-0.5 mL of the same buffer. Cells were analyzed using a BD FACSCalibur™ flow cytometer, and data were analyzed with BD CellQuest™ 3.0 software (BD).

Results and Discussion

MSC Culture and Population Doubling

MSCs from the frozen stock were seeded on the human-origin matrix-coated surface and on the Fibronectin mimetic surface. Cells were cultured in serum-free and xeno-free culture medium. No pre-adaptation was required on the Fibronectin mimetic surface; cell attachment on the Fibronectin mimetic surface was comparable to the human-origin matrix control. MSCs cultured on the Fibronectin mimetic and human-origin matrix surfaces exhibited similar morphology (Figure 1). Cell growth was determined by population doubling at each passage. After MSCs reached 60-70% confluence, cells were collected by enzymatic dissociation and counted. Population doubling was determined for each passage and growth on the two surfaces was compared by cumulative population doubling over the course of the experiment. As shown in Figure 2, population doubling on the Fibronectin mimetic and human-origin matrix surfaces was comparable, indicating similar growth properties on both surfaces. Thus, Fibronectin mimetic surface supported cell attachment and growth for multiple passages demonstrating suitability for MSC culture in xeno-free medium.

Figure 1. Phase contrast images of human bone marrow-derived MSCs at different passages in a defined and xeno-free medium. Top row represents images from the Corning PureCoat ECM Mimetic Cultureware Fibronectin Peptide surface, and the bottom row shows MSCs cultured on human-origin matrix-coated plates. Images captured using 10X objective. Note comparable morphology on both surfaces.

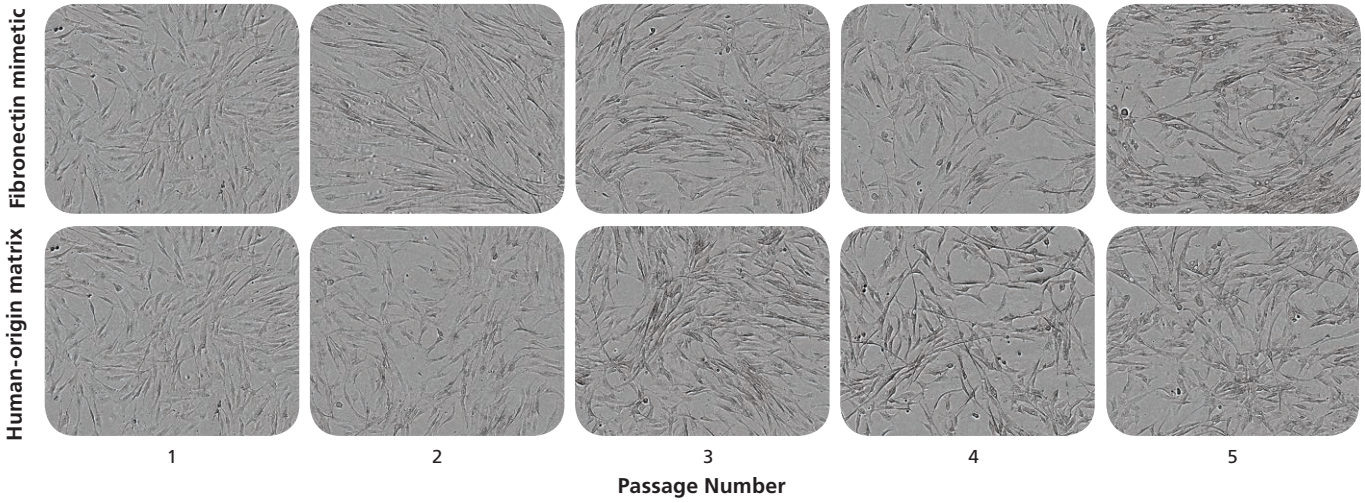
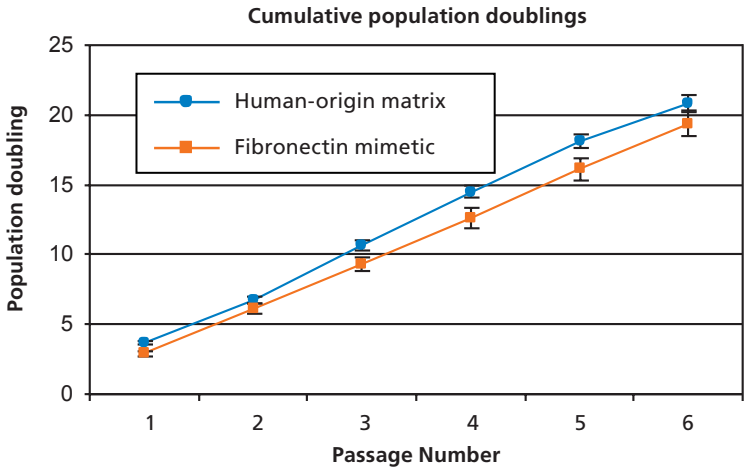


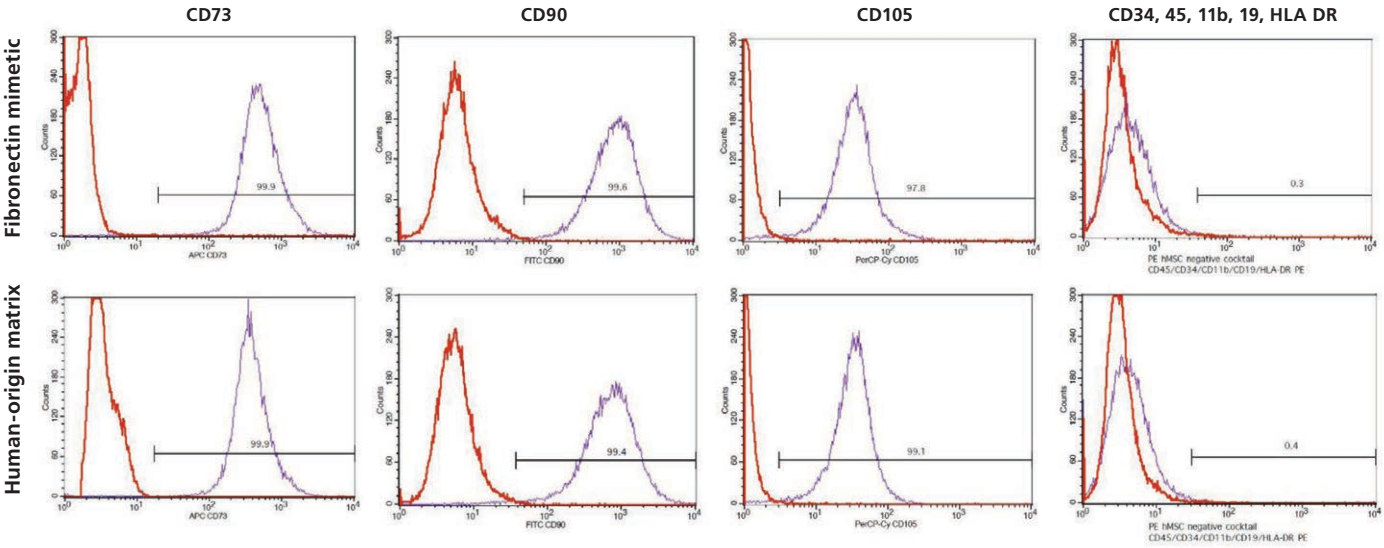
Figure 2. Cumulative population doubling of MSC for 6 passages on Fibronectin mimetic and human-origin matrix coated surface.



Analysis of MSC Markers by Flow Cytometry

After MSCs were cultured on Fibronectin mimetic surface for 3 passages, cells were collected by enzymatic dissociation, stained for established MSC markers using the respective antibodies, and then analyzed by flow cytometry. Cells were stained with antibodies CD90, CD105, CD73, CD34, CD11b, CD19, CD45 and HLA-DR using the BD™ Human MSC Analysis Kit. Immunophenotyping analysis captured in Figure 3 demonstrated that the MSC population was positive for CD90, CD105, and CD73 markers, and negative for CD34, CD11b, CD19, CD45 and HLA-DR. A similar marker profile was exhibited by cells cultured on the human-origin matrix-coated surface. Thus, MSCs cultured on Fibronectin mimetic surface exhibited the established marker profile.

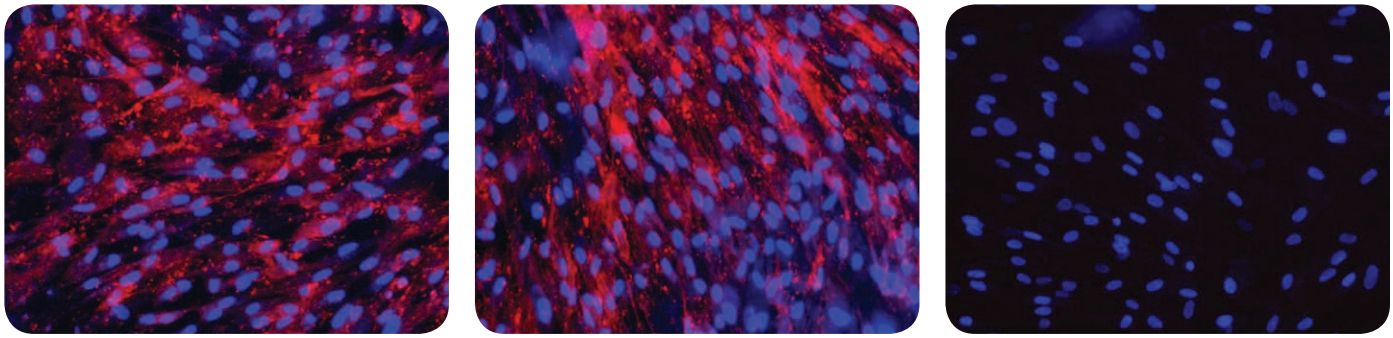
Figure 3. Immunophenotyping of MSCs after 3 passages on human-origin matrix-coated or Fibronectin mimetic surfaces. Cells expressed CD73, CD90, and CD105, and were negative for CD34, CD45, CD11b, CD19, and HLA DR.



Differentiation to Osteogenic Lineage

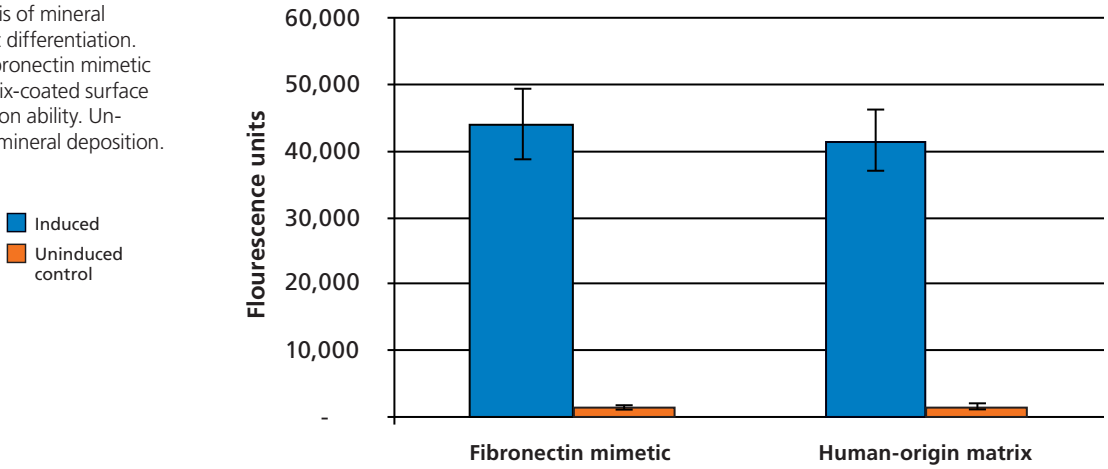
After 3 passages, MSCs were differentiated into osteogenic lineage. Post-differentiation, cells were fixed and then analyzed for Alkaline Phosphatase activity using fluorescence detection of an enzyme-substrate reaction product (red staining). Nuclei were stained with DAPI and images were captured. Bright red regions in Figure 4a demonstrated that differentiated cells exhibited Alkaline Phosphatase activity. Alkaline phosphatase activity associated with bone cells has been previously described[5]. No enzymatic activity was detected in uninduced controls. Mineralization by osteocytes was also quantified by a fluorescence-based staining method that detects the hydroxyapatite portion of the mineralized bone matrix. As shown in Figure 4b, cells differentiated after culture on the Fibronectin mimetic surface exhibited mineral deposits comparable to cultures on human-origin matrix. No bone forming activity was detected in uninduced controls. This experiment has demonstrated that MSCs remained multipotent and successfully differentiated into osteogenic lineage when cultured on the Fibronectin mimetic surface.

Figure 4a. Qualitative analysis of Alkaline Phosphatase activity to demonstrate the osteogenic differentiation of MSCs following the culture on Fibronectin mimetic or human-origin matrix coated surfaces. Red staining indicates alkaline phosphatase activity. Uninduced cells did not exhibit enzymatic activity.



Fibronectin mimetic **Human-origin matrix** **Uninduced control**

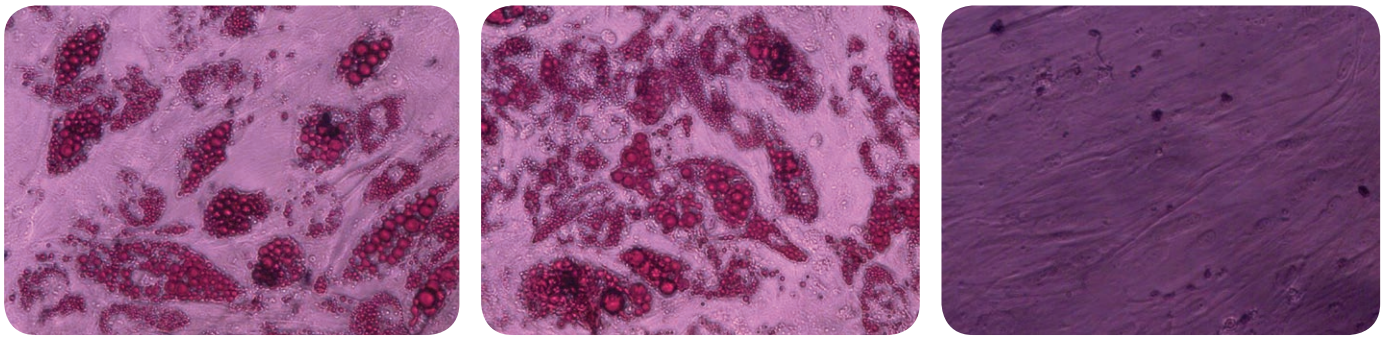
Figure 4b. Quantitative analysis of mineral deposition to assess osteogenic differentiation. Cells differentiated from the Fibronectin mimetic surface and human-origin matrix-coated surface exhibit comparable mineralization ability. Uninduced controls did not show mineral deposition.



Differentiation to Adipogenic Lineage

Adipogenesis was induced after 3 passages as described in Materials and Methods. Post-differentiation, cells were fixed and stained with Oil Red O. MSCs differentiated into adipocytes and deposited lipid vacuoles, which exhibit red staining with Oil Red O (Figure 5). Uninduced cells did not develop lipid vacuoles. Thus, multipotent MSCs were successfully differentiated into adipogenic lineage after multiple passages on the Fibronectin mimetic surface.

Figure 5. Qualitative analysis of adipogenesis by Oil-Red O staining to assess adipogenic differentiation of MSCs. Cells were cultured on Fibronectin mimetic and human-origin matrix-coated surfaces and differentiated to adipogenic lineage. Adipocytes exhibited staining of lipid vacuoles. Uninduced controls did not show staining.



Fibronectin mimetic **Human-origin matrix** **Uninduced control**

Conclusions

- Corning PureCoat ECM Mimetic Cultureware Fibronectin Peptide supported MSC attachment and growth for multiple passages in xeno-free and serum-free medium.
- MSCs retained their characteristic marker profile after culture on the Fibronectin mimetic surface.
- MSCs remained multipotent and successfully differentiated into adipogenic and osteogenic lineages when cultured on the Fibronectin mimetic surface.
- Corning PureCoat ECM Mimetic Cultureware Fibronectin Peptide can be used for the culture of MSCs where a defined environment is desirable as well as in basic and applied research.

References

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Corning Incorporated
Life Sciences
836 North St.
Building 300, Suite 3401
Tewksbury, MA 01876
t 800.492.1110
t 978.442.2200
f 978.442.2476
www.corning.com/lifesciences