Co-culture of osteocytes and neurons on a unique patterned surface

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(Received 25 August 2011; accepted 31 October 2011; published 22 December 2011)

Neural and skeletal communication is essential for the maintenance of bone mass and transmission of pain, yet the mechanism(s) of signal transduction between these tissues is unknown. The authors established a novel system to co-culture murine long bone osteocyte-like cells (MLO-Y4) and primary murine dorsal root ganglia (DRG) neurons. Assessment of morphology and maturation marker expression on perlecan domain IV peptide (PlnDIV) and collagen type-1 (Col1) demonstrated that PlnDIV was an optimal matrix for MLO-Y4 culture. A novel matrix-specificity competition assay was developed to expose these cells to several extracellular matrix proteins such as PlnDIV, Col1, and laminin (Ln). The competition assay showed that approximately 70% of MLO-Y4 cells preferred either PlnDIV or Col1 to Ln. To co-culture MLO-Y4 and DRG, we developed patterned surfaces using micro-contact printing to create $40\,\mu\text{m} \times 1\,\text{cm}$ alternating stripes of PlnDIV and Ln or PlnDIV and Col1. Co-culture on PlnDIV/Ln surfaces demonstrated that these matrix molecules provided unique cues for each cell type, with MLO-Y4 preferentially attaching to the PlnDIV lanes and DRG neurons to the Ln lanes. Approximately 80% of DRG were localized to Ln. Cellular processes from MLO-Y4 were closely associated with axonal extensions of DRG neurons. Approximately 57% of neuronal processes were in close proximity to nearby MLO-Y4 cells at the PlnDIV-Ln interface. The surfaces in this new assay provided a unique model system with which to study the communication between osteocyte-like cells and neurons in an *in vitro* environment. © *2011 American Vacuum Society*. [DOI: 10.1116/1.3664050]

I. INTRODUCTION

Osteocytes comprise approximately 90% of the cells found in bone. We propose that these cells are a likely component in neuronal communication due to their location throughout the entire bone, as well as their shared role in orchestrating the process of bone remodeling.

To study how osteocytes and neurons potentially could communicate with each other to regulate bone function *in vivo*, it was necessary to develop a comparative *in vitro* co-culture system with several key functions. These functions include support for the attachment and growth of both cell types in a matrix that provides cues for differentiation and/or cell survival and spatial guidance of cell growth to facilitate cell-cell communication.

Cell-extracellular matrix (ECM) interactions coordinate a variety of cellular responses including regulation of phenotype, gene expression, differentiation, cell growth and cytoskeletal structure.[1–3] A lack of appropriate signal(s) can inhibit cells from attaching to a specific matrix, resulting in apoptosis or anoikis.[4] Expression of specific cellular receptors and integrins is responsible for unique cellular responses to various ECM molecules; however, not all matrices equally support the attachment and growth of individual cell types. These differences in cell-matrix attachment allow for specific localization of various cell types in complex tissues, and regulates overall tissue function such as directional movement, secretion, conduction or transport.[5]

To prepare appropriate co-culture environments for *in vitro* studies of cell behavior, the original *in vivo* cellular matrix preference of the source cells must be considered. For example, a variety of different cellular matrices specify the differentiation of osteocytes from their osteoblast precursors. The cell bodies of osteocytes reside in small spaces, or lacunae, with dendrite-like processes extending from all sides of the cell through canals or passages in mineralized bone

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termed canaliculi. This canalicular system allows direct cell-cell contact between osteocytes and osteoblasts, but this lacunae-canalicular pore system itself must remain unmineralized so as not to compress the connecting processes.

Perlecan, or heparan sulfate proteoglycan 2, is a large, multidomain, multifunctional heparan sulfate proteoglycan with a core protein of approximately 450 kDa. Perlecan was recently shown to reside in the pericellular space around osteocytes and is specifically localized to the region surrounding osteocytic processes where it helps maintain the nonmineralized pericellular area.[6] Perlecan also has been shown to bind hydroxyapatite by interacting with the calcium phosphate mineral.[7,8] Due to its location in bone, perlecan is a promising candidate for the preparation of a DRG—osteocyte co-culture system.

Osteocytes begin expressing several molecules as they mature from their precursors, including E11/gp38,[9] dentin matrix protein 1 (DMP-1), and matrix extracellular phosphoglycoprotein (MEPE).[10] E11 is an osteocyte-specific molecule that is expressed at an early phase of maturation and is responsible for osteocyte process production and elongation.[9] DMP-1 and MEPE are secreted proteins, both of which are members of a larger group of proteins called small; integrin-binding LIgand, N-linked Glycoproteins (SIBLINGs).[11] DMP-1 is expressed exclusively by osteocytes in post-natal bone and is found along the canalicular wall and along the dendritic processes.[12] DMP-1 has been reported to be important in osteoblast differentiation into osteocytes, as well as in mineralization.[13] Matrix extracellular phosphoglycoprotein plays a major role in regulating bone metabolism as well as in inhibiting bone formation.[14] Nampei *et al*. demonstrated that MEPE is expressed specifically in osteocytes and is secreted along the cellular processes where it accumulates in the pericellular bone matrix around osteocytes.[15] These markers are used to identify differentiated osteocytes, and both DMP-1 and MEPE, like perlecan, may provide cell-matrix signals in the pericellular space around osteocytes.

Much like osteocytes, neurons also require specific ECM proteins for their regulation. Several matrix proteins, such as laminin, fibronectin, thrombospondin, various proteoglycans, collagens, and tenascin/entactin/J1, are known to be essential in nerve development and regeneration.[16] Thus, the ability of neurons to enter into bone must be regulated by their encounters with appropriate matrices for neurite outgrowth. Neuron growth has long been known to be regulated by ECM including Ln.[17] Immunohistochemical studies of bone in rat have shown that Ln primarily is localized to the blood vessel walls of the vasculature of bone.[18] Mach *et al*., have shown that nerves enter bone by tracking along the bone vasculature before entering into mineralized bone.[19] Ln has been identified as a key matrix component required for differentiation of canalicular cell processes in bone,[20] implying that Ln exists in the lacunae-canalicular system. Serre *et al*.[21] showed that glutamate immunoreactive fibers are in contact with osteocytes in mineralized bone, suggesting nerve fibers leave the bone vasculature and enter the lacunae-canalicular system. These data indicate that Ln is a key molecule for nerve movement and growth away from the vasculature and toward the mineralized bone and the cells that reside within it.

A vital component for the study of cell-cell communication *in vitro* is an environment that can spatially control the growth of the two cell types toward each other. A variety of approaches have been used to spatially control cell growth on a surface including soft lithography (micro-contact printing), photo-patterning, and laser-scanning lithography.[22] Specifically, different types of micro-patterning have been used to create co-cultures that include a variety of cell types, such as co-culture of hepatocytes and endothelial cells on thermally responsive grafted polymer surfaces[23] or co-culture of hepatocytes and fibroblasts using hydrogel microstructures.[24] Directed co-culture of osteocytes and neurons has not been accomplished previously. Micro-contact printing techniques allow for various biological molecules to be placed on the same surface in defined patterns at controllable coverages, allowing for cell-specific attachment cues at known regions on the surface. The design of the patterns also provides an interface that supports cell-cell contact.

The purpose of this study was to prepare and characterize a reproducible and reliable *in vitro* system for co-culture of osteocytes and neurons. The aim of this study was to prepare a two-dimensional (2-D) environment that would facilitate co-culture of osteocytes and neurons and that would provide regions of specific spatial cellular location. Spatial control of the growth of these cells provides predictable regions of contact that can be probed in future studies of cell-cell communication.

For this study, we utilized a surface patterning chemistry developed by Zhang *et al*. to prepare patterned surfaces with alternating 40 μm $\times$ 1 cm lanes of Ln and 40 μm $\times$ 1 cm lanes of a novel 18-amino-acid peptide derived from domain IV of perlecan, PlnDIV.[25] We previously identified functional peptides of domain IV and domain V of perlecan,[26] and showed that PlnDIV peptide induces rapid cell adhesion, spreading, and focal adhesion kinase activation in some, but not all, cell types including osteoblastic cells. We hypothesize that PlnDIV would promote proper attachment, cell morphology and maturation of cells from the osteocyte-like cell line, MLO-Y4. We also propose that preparation of dual-patterned PlnDIV/Ln surfaces will allow for specific attachment of mouse DRG neurons to Ln regions and MLO-Y4 cells to PlnDIV regions. This degree of spatial control will provide well defined areas of cell-cell contact that can be utilized to study cell-cell communication between osteocytes and neurons.

II. EXPERIMENT

A. MLO-Y4 cell culture

MLO-Y4 cells (kindly provided by Dr. Lynda F. Bonewald, Department of Oral Biology, University of Missouri at Kansas City School of Dentistry, Kansas City, MO) were cultured on either Col1 (BD Biosciences, San Jose, CA) or PlnDIV.[26] Culture plates were prepared by coating with Col1 (15 μg/cm^2 in 0.02 N acetic acid) or PlnDIV (0.5 μg/cm^2)[26] in

phosphate buffered saline (PBS; Mediatech, Inc., Manassas, VA) for 1 h, followed by drying for 1 h. MLO-Y4 cells were cultured on prepared plates at 37 °C in a humidified atmosphere of 5% CO_2 in alpha minimal essential medium (αMEM; Mediatech) supplemented with fetal bovine serum (2.5% [v/v]) (FBS; Hyclone, Logan, UT) and penicillin/streptomycin (P/S, 1% [w/v], Invitrogen, Carlsbad, CA).

B. Dorsal root ganglia primary cultures

Adult male friend leukemia virus B strain N mice were sacrificed according to a University of Delaware IACUC-approved protocol and accepted practices with CO_2 and cervical dislocation. Dorsal root ganglia were excised from L3-L5 vertebrae, rinsed in P/S (1%) in DMEM/F12 (Mediatech), then placed in culture media containing FBS (10%), P/S (1%) and N1(1%[w/v]; N1 is a nutrient supplement that contains 0.5 mg/mL insulin from bovine pancreas, 0.5 mg/mL human transferrin, 0.5 μg/mL sodium selenite, 1.6 mg/mL putrescine, and 0.73 μg/mL progesterone) (Sigma-Aldrich, St. Louis, MO). Dorsal root ganglia were chopped into several large pieces and digested with 1000 U/mL collagenase (Sigma-Aldrich) at 37 °C for 25 min. Dorsal root ganglia then were triturated 15 – 20 times with a flame-polished pipette and returned to 37 °C for an additional five min. After collagenase treatment, cells were washed in DMEMB (DMEM/F12 with 1% [w/v] BSA) and centrifuged at 100 × *g* for five min. Cells then were triturated 15 – 20 times and incubated in trypsin/EDTA (0.25% [w/v], Mediatech) at 37 °C for five min. Culture media with FBS was added to inactivate trypsin, followed by triturating 15 – 20 times and centrifugation at 100 × *g* for five min. Cells were washed two more times in DMEMB and cultured on protein surfaces (either patterned or unpatterned) in DMEM/F12 with FBS (5%), P/S (1%) and N1 (1%). Cells were treated the following day with 10 nM of the mitotic inhibitor arabinosylcytosine hydrochloride (ARAC; Sigma-Aldrich) to reduce the number of Schwann cells in culture.

C. Dependence of MLO-Y4 cells on ECM

To assess the effect of extracellular matrix on morphology, MLO-Y4 cells were cultured on PlnDIV (0.5 μg/cm^2) or Col1 (15 μg/cm^2) coated polystyrene plates (plates were coated as described above) and grown to 80 – 90% confluence. Cells were washed three-times in tris-buffered saline (TBS) and fixed in paraformaldehyde (4% in TBS; Electron Microscopy Sciences, Hatfield, PA) for 45 min. Samples were washed again in TBS three-times, and then placed in blocking media containing TBS, BSA (3%, Sigma-Aldrich), goat serum (10%, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and Triton-X 100 (0.25% [v/v], Thermo Fisher Scientific, Pittsburg, PA) for 2 h. After blocking, samples were reacted with Syrian hamster monoclonal anti-E11 (antibody 8.1.1 at 1:250 dilution; a generous gift from Dr. Lynda Bonewald) prepared in TBS, BSA (3%) and goat serum (10%) and reacted with samples at 4 °C for 12 h. Samples then were washed six-times for ten min each in TBS, BSA (3%), goat serum (10%), followed by treatment with Alexa Fluor® 488 goat anti-hamster (1:200, Invitrogen) for 1 h at room temperature. Samples were subsequently washed two-times for five min each and two-times for ten min each in TBS. Cells were imaged using a Nikon TE2000 inverted microscope with a CoolSNAP-Pro$_{cf}$ CCD camera (Roper Scientific Photometrics, Trenton, NJ). Images were processed using Image Pro® Plus 4.5 software (Media Cybernetics, Bethesda, MD).

D. Osteocyte-specific marker expression/production

Total RNA was extracted from the cells using TRIzol® (Invitrogen) per the manufacturer's instructions. Ribonucleic acid extracts then were treated with DNA-*free*™ DNase Treatment and Removal Kit (Ambion, Inc., Austin, TX) to remove any contaminating DNA according to the manufacturer's instructions. Complementary DNA was synthesized using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA), using a 1 μM Oligo-d(T)$_{16}$ primer (Applied Biosystems, Foster City, CA) 1 μg template RNA, and 10 U rRNasin RNase inhibitor (Promega, Madison, WI) according to manufacturer's instructions. Amplification by PCR of DMP-1, MEPE and β-actin cDNAs were performed using PTC-100™ Programmable Thermal Controller (MJ Research, Inc, Watertown, MA) under the following conditions: cDNA was initially denatured at 95 °C for 15 min followed by 40 cycles during which DNA was denatured at 94 °C for 30 s, primers were annealed at 56 °C for 30 s, and primer extension proceeded at 72 °C for 1 min. After the 40 cycles, final elongation was performed at 72 °C for ten min, and the resultant product was held at 4 °C. All conditions were the same for E11, except that the primers were annealed at 58 °C. The primer sequences used for PCR amplification were designed based on cDNA sequences of mRNA for murine E11 (GenBank XM_992534, 1064–1146 nt), murine DMP-1 (GenBank NM_016779, 1222–1498 nt), murine MEPE (GenBank NM_053172, 1016–1322 nt), and murine β-actin (GenBank NM_007393, 305–877 nt). Primers were designed utilizing San Diego Supercomputer Center 3.2 and assessed using Integrated DNA Technologies OligoAnalyzer tool. The primer sequences used were 5′-ATAATGCAGGGGATGAAACG-3′ and 5′-ATGGCTAACAAGACGCCAAC-3′ for murine E11, generating an 83-bp fragment; 5′-AGTGAGGAGGACAGCCTGAA-3′ and 5′-TCCCTGTGGAGTTGCTGTGT-3′ for murine DMP-1, generating a 277-bp fragment; 5′-CCCAAGAGCAGCAAAGGTAG-3′ and 5′-AACTCCCACTGGATGACGAC-3′ for murine MEPE, generating a 307-bp fragment; 5′-TGTTACCAACTGGGACGACA-3′ and 5′-AAGGAAGGCTGGAAAAGAGC-3′ for murine β-actin, generating a 573-bp fragment. Primers were synthesized by Sigma-Aldrich. Amplified products were visualized on an agarose gel (1.5% [w/v]) stained with ethidium bromide.

E. Validation of neuron isolation

Primary neurons were assessed for their ability to express synaptophysin, a protein found on the cell membrane and in synaptic vesicles of healthy neurons. Neurons were cultured

on Ln-coated cell culture dishes (5 μg/cm^2) and fixed as previously described for MLO-Y4 cells. Ln was physically adsorbed from a 50 μg/mL solution for 12 h at 4 °C, followed by three rinses with PBS. Fixed samples were incubated with rabbit monoclonal anti-synaptophysin (1:250; Abcam, Cambridge, MA) at room temperature for 1 h. Samples were washed three-times for ten min each in blocking solution [TBS, BSA (3%), goat serum (10%)] and then reacted with goat anti-rabbit Alexa Fluor® 555 (1:200; Invitrogen) and Draq-5 (1:1000, Biostatus Limited, Leicestershire, UK) at room temperature for 1 h. Samples then were washed three-times for ten min each in blocking solution and imaged using confocal microscopy as described above.

F. Surface modification

For all assays involving the determination of marker expression levels (described above), substrates were prepared using the incubation of multiwell plates in solutions of PlnDIV, Col1 or Ln at the concentrations and times specified above. In all assays involving patterned lanes of proteins or peptides, covalent surface-attachment chemistry was used as described here. Functionalized surfaces were utilized for co-culture of MLO-Y4 cells and dorsal root ganglia neurons. Glass coverslips (18 mm, Thermo Fisher Scientific) were immersed in a "Piranha" cleaning solution (7:3 (v/v) H_2SO_4:H_2O_2) for 30 min. The surfaces were rinsed with distilled water three to five times, and then underwent five ten-min ultrasonic cleanings in distilled water. The coverslips then were dried at 140 °C for 1 h. Following cleaning, glass coverslips were placed in a glove bag under an inert dry nitrogen atmosphere and then placed into a solution of 3-mercaptopropyltrimethoxysilane (2% [w/v]) (MTS, Sigma-Aldrich) in dry toluene for 2 h. Coverslips then were removed from the glove bag, rinsed in toluene three-times, and allowed to air dry. N-γ-maleimidobutyryloxy succinimide ester ("GMBS"; Sigma-Aldrich) was dissolved in a minimal volume of dimethylformamide (DMF; Sigma-Aldrich), and diluted to 2 mM in absolute ethanol. Coverslips were placed in 2 mM GMBS for 1 h, followed by rinsing in absolute ethanol three-times. GMBS-functionalized surfaces are reactive with lysine residues in proteins/peptides.[25] Such coverslips were placed in a clean vacuum oven ($<10^{-3}$ mBar) and allowed to dry overnight at room temperature. Protein-functionalized coverslips were created with either Col1 (30 μg/cm^2) in 0.02 N acetic acid or PlnDIV (1 μg/cm^2) in PBS for 2 h at room temperature. Following incubation, coverslips were rinsed three-times in distilled water and allowed to dry briefly. Protein-modified surfaces then were analyzed using x-ray photoelectron spectroscopy (XPS) or used for micro-contact printing followed by cell culture.

G. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) analysis was performed on an ESCALAB 250i-XL electron spectrometer (Thermo Scientific, East Grinstead, UK) with a monochromatic Al $K\alpha$ (1486.7 eV) x-ray source. Survey spectra and high-resolution spectra were acquired at pass energies of 100 eV and 20 eV, and data spacing of 1 eV and 0.1 eV, respectively. Sample charging was compensated using a low-energy electron flood gun operated at 3.6 Amps and 6 eV and ~0.05 mA emission. All peaks were linearly shifted by the amount required for the methylene component of the C1*s* peak to be centered at 284.6 eV, typically ~6 eV. Subsequent spectra were analyzed with a Shirley-type background removed. Peak fitting and quantification were performed with CasaXPS v2.2.24 software (Casa Software Ltd., UK).

H. Micro-contact printing

A poly(dimethyl siloxane) (PDMS) stamp presenting 40 μm × 1 cm raised features was cleaned in a 8:2 (v/v) ethanol: water solution, blown dry with air, and coated with 25 μg/cm^2 Oregon Green® (Invitrogen)-conjugated Ln (BD Biosciences) or unconjugated Ln. Ln was incubated on stamps at 4 °C for 2 h in a closed humidified environment to avoid evaporation to dryness. Following stamp incubation, excess Ln solution was removed from stamps by pipette and blown dry with clean, dry N_2 gas. Stamps then immediately were brought into conformal contact with Col1- or PlnDIV-functionalized glass coverslip (prepared, as described above). A weight (200 g) was placed on top of each stamp to regularize pressure and enhance protein transfer. Samples were contact incubated at 37 °C for 30 min. Following contact incubation, stamps were removed from glass coverslips, and the resulting patterned coverslips were rinsed three-times with PBS, followed by incubation in antibiotic/antimycotic solution (Mediatech, Inc.) at 4 °C for 24 h, followed by rinsing in PBS three-times. Surfaces patterned with Oregon Green-conjugated Ln were imaged using a Nikon TE2000 inverted microscope with a CoolSNAP-Pro$_{cf}$ CCD camera. Images were processed using Image Pro® Plus 4.5 software.

I. Assessment of MLO-Y4 cell protein selection on patterned surfaces

MLO-Y4 cells were cultured at 5 000 cells/cm^2 on either PlnDIV/Oregon Green®-Ln or Col1/Oregon Green-Ln dual-patterned surfaces for 24 h in MEMα with FBS (2.5%) and P/S (1%). Cells were washed three-times in TBS and fixed in paraformaldehyde (4%) for 45 min. Samples were washed again in TBS three-times, and then imaged on a Nikon TE2000 inverted microscope with a CoolSNAP-Pro$_{cf}$ CCD camera. Images were processed using Image Pro® Plus 4.5 software. The number of cells attached to lanes of either ECM protein were visually counted.

J. DRG neuron-MLO-Y4 cell co-cultures

Following 48 h of culture, patterned coverslips with DRG neurons were washed three-times with PBS to remove ARAC and MLO-Y4 cells were added at a plating density of 1×10^5 cells per coverslip. Dorsal root ganglia neurons and MLO-Y4 cells were co-cultured for an additional 24 h in DMEM/F12 with FBS (5%), P/S (1%) and N1 (1%).

K. Co-culture Immunostaining

After culture, cells were washed three-times in TBS and fixed in paraformaldehye (4%) for 45 min. Samples were washed again in TBS three-times, and then placed in blocking media containing TBS, BSA (3%), goat serum (10%), and Triton-X 100 (0.25%) for 2 h. After blocking, coverslips were reacted with rat monoclonal anti-Ln (AL-4; 1:100; Abcam), rabbit monoclonal anti-synaptophysin and Syrian hamster monoclonal anti-E11 (antibody 8.1.1 at 1:250 dilution). All primary antibodies were prepared in TBS, BSA (3%) and goat serum (10%) and reacted with samples at 4 °C for 12 h. Samples then were washed six times for ten min in TBS, BSA (3%), goat serum (10%), followed by treatment with secondary antibody: Alexa Fluor® 555 goat anti-rabbit, Alexa Fluor® 488 goat anti-hamster, and Alexa Fluor® 633 donkey anti-rat (all 1:200, Invitrogen) for 1 h at room temperature. Nuclei were stained with Draq-5 at 1:1000. Samples were subsequently washed two-times for five min each and two times for ten min each in TBS and visualized via confocal microscopy as described above. Images were processed using Zeiss LSM Image Examiner (Version 4.2.0.121), and Ln lanes were enhanced using the intensify tool in Adobe Photoshop® CS2 (Version 9.0) to define clear boundaries between protein/peptide lanes. This was done post-acquisition by increasing the contrast in one of the image channels prior to a multichannel image overlay/merge. This enhancement was performed to allow a clear visual distinction to be drawn between the different lanes and no quantitative results were dependent upon such contrast enhancement. The number of DRG neurons located in specific protein/peptide lanes were counted visually. Contacts between DRG neurons and MLO-Y4 cells were also visually counted. A contact was defined to be any cell-cell interaction for which it was not possible to see a gap between the cells when magnified. Given the resolution and size of the images taken (resolution of 1024×1024; image size of $460.7\ \mu m \times 460.7\ \mu m$), the average distance between pixels is $0.45\ \mu m$. Thus, a contact determined through this imaging and processing procedure implies a distance of $\leq 0.45\ \mu m$.

L. Statistical analysis

Protein specificity on patterned surfaces data are presented as percent of total ± SEM. Data was analyzed using a two-tailed exact binomial test at $p < 0.05$ for statistical significance.

III. RESULTS AND DISCUSSION

The purpose of this study was to define, develop and test both a suitable ECM environment to support cell growth and maturation marker expression of MLO-Y4 osteocyte cells, and to support co-culture of MLO-Y4 cells with primary DRG neurons in order to study cell-cell interactions between these cell types.

A. MLO-Y4 cell morphology is more osteocyte-like when cells are cultured on PlnDIV

We have previously shown that MLO-Y4 cells produce and secrete perlecan. We have also shown that perlecan is localized to the lacunae-canalicular system in bone where it functions to maintain the pericellular space.[6] However, the role of perlecan in establishing osteocyte morphology and maturation has not been studied. To determine if Col1 or perlecan produced a cellular morphology and phenotype similar to that seen in osteocytes *in vivo*, MLO-Y4 cells were cultured on either PlnDIV or Col1. Figure 1 shows fluorescence photomicrographs of MLO-Y4 cells stained for E11 after they had been cultured to ~80 – 90% confluency. E11 was expressed diffusely in the majority of cells cultured on Col1, with some cells displaying punctate staining along the cell processes [Fig. 1(b)]. MLO-Y4 cells cultured on PlnDIV showed a similar pattern of E11 expression [Fig. 1(d)], however, there appeared to be a greater amount of punctate staining on cells that displayed longer, thinner processes than for cells cultured on Col1(see insets). The majority of MLO-Y4 cells cultured on PlnDIV exhibited a distinct morphology that is characteristic of osteocytes [Figs. 1(c) and 1(d)]. As E11 is a type-1 transmembrane glycoprotein, punctate staining is consistent with expression at the membrane. Expression of E11 has been shown to be required for cell process elongation.[9] When cultured on Col1 [Figs. 1(a) and 1(b)], MLO-Y4 cells displayed a more flattened morphology. These observations suggest that MLO-Y4 cells may attach to PlnDIV where they exhibit a more osteocytic phenotype than do cells cultured on Col1.

B. MLO-Y4 cells express osteocyte-specific markers when cultured on PlnDIV

To determine the effect of matrix on osteocyte maturation marker expression, RT-PCR was performed to study the matrix-specific expression of the osteocyte markers E11, DMP-1, and MEPE in MLO-Y4 cells compared to expression in total mouse embryo (TME) positive controls (Fig. 2).

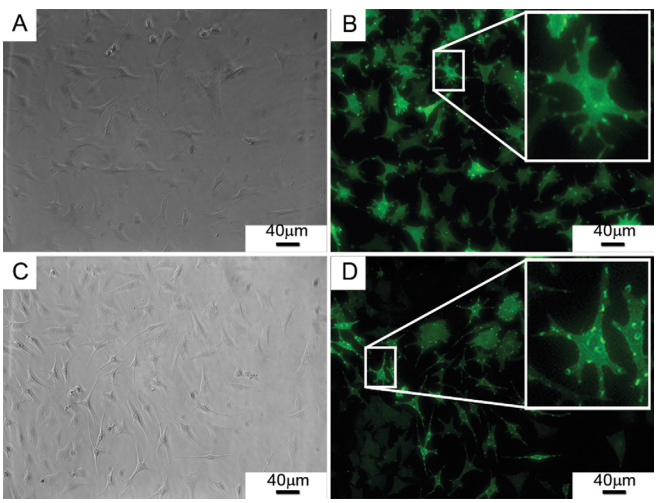

FIG. 1. (Color online) MLO-Y4 cells cultured on PlnDIV or Col1. MLO-Y4 osteocyte-like cells display a more mature morphology with smaller cell bodies having dendrite-like processes when cultured on PlnDIV (c) in comparison to Col1 (a). E11 expression by MLO-Y4 cells is localized to the cell processes and more punctate when cultured on PnDIV (d) than on Col1 (b). Insets in (d) and (b) show a representative cell cultured on the specified molecules.

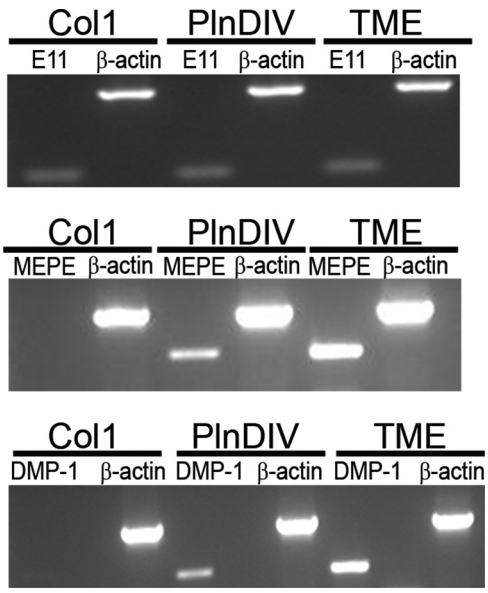

FIG. 2. MLO-Y4 osteocyte-like cells express RNA for the osteocyte-specific markers E11, DMP-1 and MEPE differently when cultured on Col1 (columns 1 and 2) and PlnDIV (columns 3 and 4). Ribonucleic acid expression of E11 was similar when cells were cultured on either substrate, but RNA expression of DMP-1 and MEPE was more evident when cells were cultured on PlnDIV in comparison to cells cultured on Col1. Total mouse embryo (TME) positive control experiments (lanes 5 and 6) indicate the upper limits of RNA available in this assay.

MLO-Y4 cells expressed similar quantities of E11 transcripts when cultured on either surface. However, MLO-Y4 cells that were cultured on PlnDIV showed greater mRNA expression levels of DMP-1 than did those cultured on Col1. Matrix extracellular phosphoglycoprotein was only expressed by MLO-Y4 cells that were cultured on PlnDIV. This data indicates that MLO-Y4 cells cultured on PlnDIV express osteocyte-specific markers to a greater degree than they do when cultured on Col1. Coupled with morphological data, we concluded that the use of PlnDIV in our culture system was a suitable choice to produce cells with a more osteocyte-like phenotype.

C. Heterobifunctional cross-linker provides successful attachment of proteins to surface

Dual-protein patterned surfaces for future co-culture experiments were prepared using a heterobifunctional cross-linker chemistry [Fig. 3(a)] to covalently attach Ln and PlnDIV to glass substrates. Attachment of molecules was assessed using x-ray photoelectron spectroscopy (XPS). The series of XPS data panels [Figs. 3(b)–3(e)] for the C1*s* region showed a progression of complexity, first as the MTS (a), then GMBS cross-linking reagents were added (b). In panel (c) (Col1) and (d) (PlnDIV), the increase of the amide component relative to the other carbon types was consistent with the presence of protein.[25] With addition of protein to the covalent linking components, MTS and GMBS, the C1s spectra indicates presence of proteins by increases in the carboxyl and amino functionalities in comparison to the C-C, and C-H functionalities.

D. Dual-patterned surfaces created by micro-contact printing exhibit lane resolution and image contrast in confocal microscopy

Following initial protein attachment, surfaces were modified further by micro-contact printing of Oregon green-labeled Ln onto the PlnDIV or Col1 monolayers (Fig. 4). Using micro-contact printing, surfaces displayed two distinct regions observed as vertical stripes. These regions include dark stripes that consists of unlabeled protein (either PlnDIV or Col1), and gray regions that consist of Oregon green-labeled Ln. This particular image shows Ln regions that are 40 μm wide x 1 cm long opposed to Col1 regions that are 40 μm wide $\times$ 1 cm long. Using this technique, we produced surfaces with 250 alternating lanes of Ln and PlnDIV or Ln and Col1, and thus, a large number of regions where osteocytes and neurons can be cultured and potentially interact in a covalent, modular and reproducible 2-D platform. These surfaces provide a unique tool for co-culture of MLO-Y4 cells and DRG neurons.

E. MLO-Y4 cells prefer PlnDIV or Col1 lanes in comparison to Ln

To assess the degree of cell-surface-specific attachment, MLO-Y4 cells were cultured on dual-patterned surfaces and the numbers of cells that bound to each surface were determined (Fig. 5). The majority of MLO-Y4 cells (approximately 70%) were retained within either the PlnDIV (Fig. 5(a); 81 out of 117 cells; $n = 2$ substrates) or the Col1 (Fig. 5(b); 44 out of 64 cells; $n = 6$ substrates) lanes in comparison to the Ln lanes. This demonstrates that either PlnDIV/Ln or Col1/Ln can be appropriate for co-culture of MLO-Y4 cells and DRG neurons, because each combination can provide multiple replicates of unique interfaces on a single substrate. However, because morphological and maturation marker data has already shown that PlnDIV is a better substrate than Col1 for culturing mature MLO-Y4 cells, all subsequent studies were performed using PlnDIV/Ln-modified surfaces.

F. MLO-Y4 cells and DRG neurons are cultured together on dual-protein patterned surfaces with alternating lanes of Ln and PlnDIV

To determine if surfaces would support co-culture of both cell types, cells were co-cultured on dual-patterned surfaces and assessed by confocal microscopy. Confocal microscopy showed that both MLO-Y4 cells (green) and DRG neurons (red) could be cultured on the same surfaces [Fig. 6(a)], and that DRG neurons preferred to grow on the Ln lanes (blue antibody staining) in comparison to the PlnDIV lanes (unlabeled) [Fig. 6(b)]. The majority of DRG neurons (approximately 83%) were retained within the Ln lanes (10 out of 12 cells; $n = 2$ substrates) in comparison to the PlnDIV lanes. These surfaces also allowed DRG neurons and MLO-Y4 cells to come into close contact with one another at or close to the protein lane interfaces. A contact was defined as instances where there was no gap between the red pixels of a DRG neuron process, specifically at the growth cone, and the green pixels from any part of an MLO-Y4 cell. With the

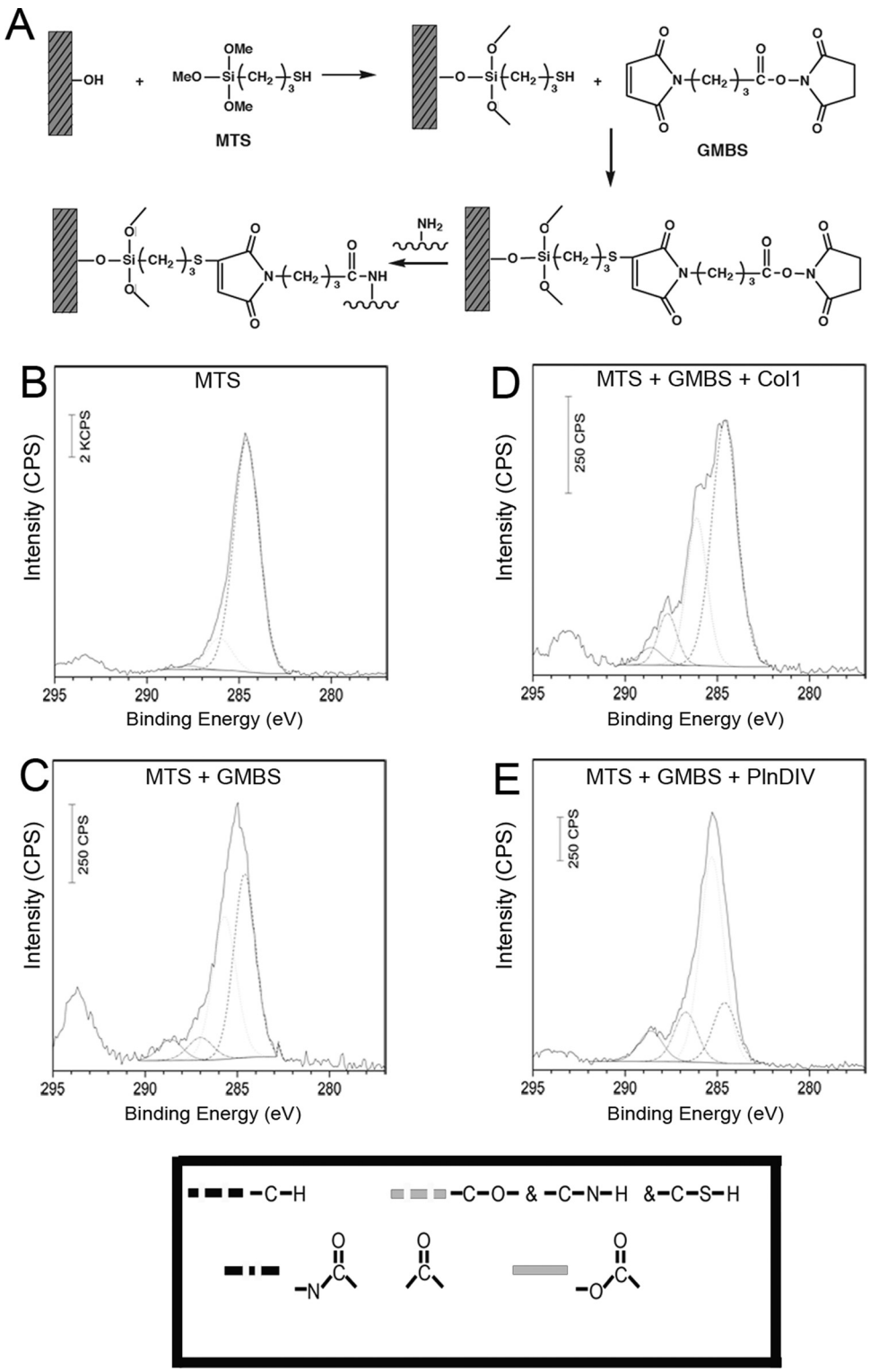

FIG. 3. X-ray photoelectron spectroscopy spectra and chemical reaction scheme for covalent protein attachment. Heterobifunctional cross-linker (GMBS) allows for covalent attachment of various proteins and peptides as assessed by XPS (a). X-ray photoelectron spectroscopy analysis was performed after surface modification with MTS (b) and GMBS (c), followed by attachment of either Col1 (d) or PlnDIV (e). Increase in carboxylic acid and amide functionalities indicate successful protein attachment.

resolution of the instrumentation and images taken, this distance is $\leq 0.45\ \mu$m. Approximately 57% the DRG neurons co-cultured with MLO-Y4 cells came into contact with at least one MLO-Y4 cell. These contacts generally occurred at points where the cells began to crossover into the neighboring lanes. However, without the spatial control of the patterned region, there is little predictability of where osteocytes and neurons would interact on a surface.

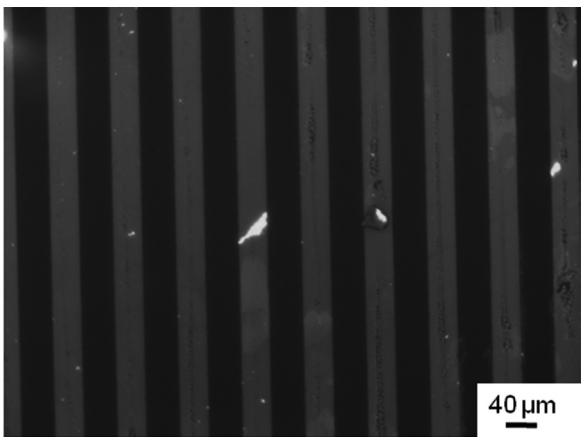

FIG. 4. Confocal microscopy of dual-patterned protein surfaces. Preparation of micro-patterned dual-protein surfaces produces clearly defined 40 μm × 1 cm lanes of alternating proteins. Uniformly covered surfaces first were produced with either unlabeled Col1 or PlnDIV (black lanes above). These surfaces were then micro-contact printed with Oregon green-labeled Ln1 (gray lanes above) using PDMS stamps with 40 μm wide lanes. The occasional dark features in the centers of the gray (Ln) lanes result from defects in the stamp's surface. The occasional bright features in the centers of gray (Ln) lanes result from residual protein aggregates after gentle rinsing with PBS.

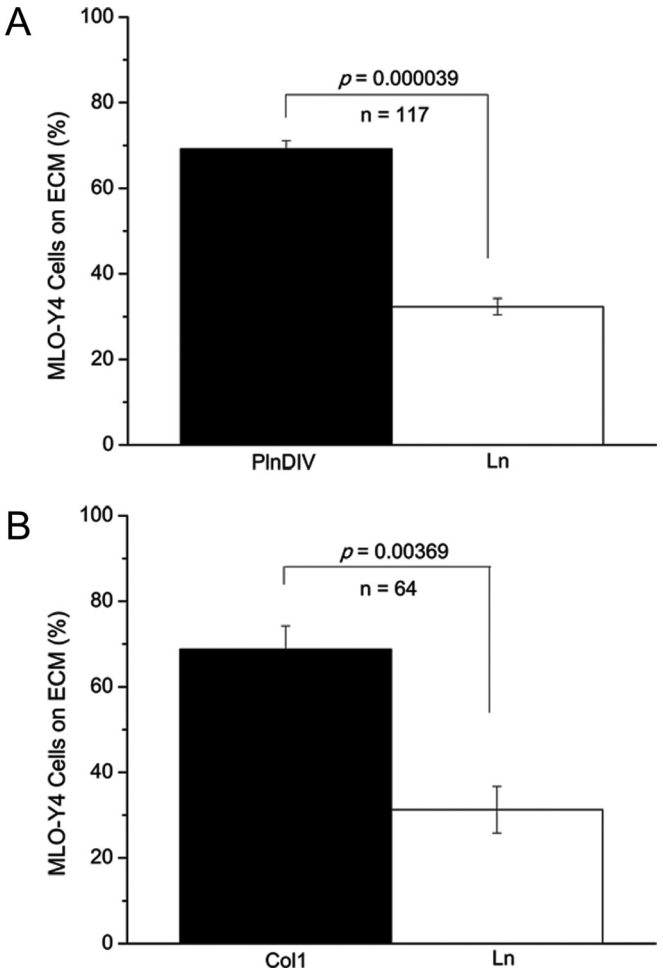

FIG. 5. Osteocyte-like MLO-Y4 cells cultured on dual-patterned PlnDIV/Ln (a) and Col1/Ln (b) substrates. Culture of MLO-Y4 cells on dual-patterned surfaces showed a preference for PlnDIV or Col1 regions over Ln regions. Approximately 70% of MLO-Y4 cells, when cultured on dual-patterned surfaces, preferred to attach to PlnDIV ($p = 0.000039$; $n = 117$) or Col1 ($p = 0.00369$; $n = 64$) lanes in comparison to Ln lanes. Two-tailed binomial test, $p < 0.05$.

IV. DISCUSSION

We have previously suggested[27] that bone cells behave much like a neuronal network due to the morphology of osteocytes and their use of the neurotransmitter, glutamate, in cell-cell communication. Based on numerous studies suggesting neural regulation of bone mass and afferent transmission of bone pain,[28] we propose that osteocytes and neurons can communicate to both regulate bone cell function and to transmit signals to the central nervous system. Thus, the need to develop a surface in which neural cells and bone cells could differentiate and communicate is apparent. Here, we demonstrate a convenient, modular 2-D ECM-modified surface culture environment to facilitate co-culture of MLO-Y4 cells and DRG neurons. This patterned surface produced osteocytes and neurons that expressed differentiation markers and resulted in these cells coming into close proximity to each other that could facilitate studies in cell-cell communication.

To define the best environment for co-culture, we first examined the effect of individual ECM molecules on MLO-Y4 cell morphology and RNA levels of expression markers. Col1 and PlnDIV were used because Col1 is the most abundant protein found in bone and because MLO-Y4 cells express and secrete perlecan.[6] We show that both cell morphology and RNA levels of marker expression mimicked that of a mature osteocyte when cells were cultured on PlnDIV. We have recently demonstrated that perlecan is secreted into the pericellular space of the osteocyte and lines the lacuna-canalicular system (LCS).[6] Further, we have shown that perlecan/*Hspg2* deficient mice have a reduced pericellular space in the LCS, suggesting that perlecan can regulate osteocyte function and that the binding of osteocytes to perlecan may be responsible for providing a signal for morphological maturation and gene expression of the osteocyte-specific markers E11, MEPE and DMP-1. Conventionally, MLO-Y4 cells are cultured on Col1. However, our data suggest that perlecan, or its derivative 18-amino-acid analog, PlnDIV, may be a better substrate for consistently maintaining mature osteocytes.

In this study, we produced a micro-patterned dual-protein cell culture surface. X-ray photoelectron spectroscopy analysis of chemically modified surfaces showed that both Col1 and PlnDIV could be covalently linked to surfaces using heterobifunctional cross-linking chemistry that remains robust in cell culture. To pattern the surface, micro-contact printing was employed to produce a dual-protein patterned substrate with alternating lanes of Col1 and Ln or PlnDIV and Ln. On any given 1-cm by 1-cm test substrate there are 250 total lanes, each 40-μm wide. On a dual-protein substrate, 125 PlnDIV lanes contact 125 Ln lanes on each side. All cells cultured on this substrate have been exposed to the exact same culture conditions. Our data indicate that micro-contact printing of these molecules on protein-modified surfaces is an effective way to produce predictable 40-μm × 1-cm protein regions. Such

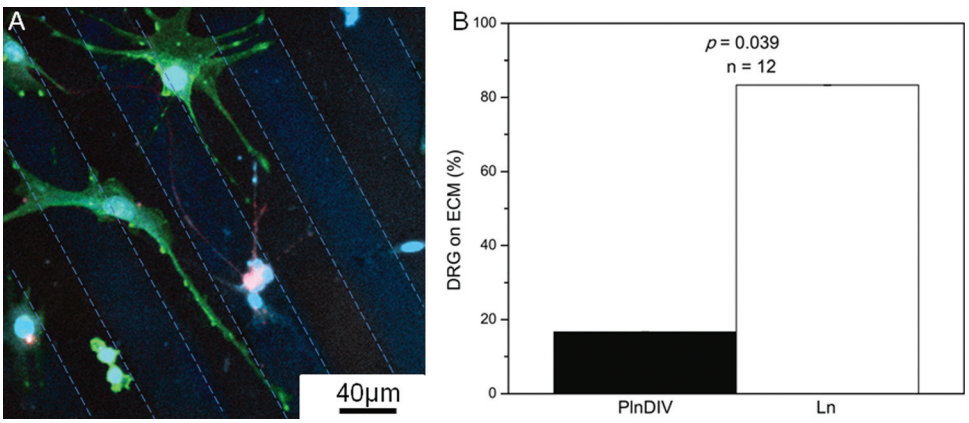

FIG. 6. (Color) Confocal microscopy of DRG neurons and MLO-Y4 cells co-cultured on Ln (anti-laminin: blue), PlnDIV (unlabeled) 40 μm x 1 cm micro-patterned lanes (a). MLO-Y4 cells, labeled with anti-E11 (green) localize primarily to PlnDIV lanes (black). Dorsal root ganglia neurons labeled with anti-synaptophysin (red) are localized primarily to Ln lanes (blue). Using dual-patterned surface, cells were co-cultured on their preferred respective proteins, and allowed to form contacts with one another at protein-protein interfaces. Dotted blue lines were drawn in to better view edges of patterned lanes. Culture of DRG neurons on dual-patterned surfaces showed a preference for Ln lanes in comparison to PlnDIV lanes. Approximately 80% of DRG neurons cultured on dual-patterned surfaces preferred to attach to Ln ($p = 0.039$ $n = 12$). Two-tailed binomial test, $p < 0.05$.

surfaces provide spatial control while allowing multiple regions of contact for cells in co-culture, affording appropriate statistical sampling of cell-cell behavior. Importantly, we have previously shown that stamping laminin onto surfaces in the described manner does not alter the biological activity of the molecule as determined by its ability to bind antibody.[29]

These micro-patterned surfaces also provide cell-matrix specificity. This characteristic presents unique and identifiable regions to support cell growth and provides specific and appropriate cues to various cell types. By exploiting these specificities, we created a cell-patterned surface that allows defined locations of DRG neurons and MLO-Y4 cells on their preferred matrices. This spatial localization allows for control of possible contacts between two different cell types.

We were able to use dual-protein PlnDIV/Ln surfaces for co-culture of DRG neurons and MLO-Y4 cells. This co-culture system provided an environment that facilitated cell-cell contacts between the two different cell types on a 2-D surface. Points of contact are predictable because they occurred along or close to the protein-protein interfaces. Experiments were performed to study cell-cell contacts on mixed matrices of PlnDIV/Ln. However, cell health was compromised on these substrates, leading to large vesicle formation in the cells indicative of apoptosis (data not shown). The advantage of this system is that cell-cell interactions can be easily studied as cells are grown in the same plane. Co-culture in the same plane provides the ability to study numerous cell interactions such as cell-cell communication on these co-cultures using electrophysiological and microscopic techniques. Many studies that examine cell-cell interactions use well inserts in tissue culture plates that allow cells to be cultured in two different planes, matrices and in different culturing media. While quite useful for certain types of experiments, the major disadvantage of this approach is that if one wanted to study direct cell-cell communication between two cell types, only one cell type could be studied at a time. Using planar micro-patterned dual-protein surfaces, the response of each cell type can be determined simultaneously. Additionally, this co-culture system allows the manipulation or stimulation of one cell type while observing the other cell type without a barrier inhibiting the researcher's ability to make measurements on the cells. Thus, these dual-protein micro-patterned surfaces will be ideal for determining if cells from different tissues can communicate, such as osteocytes initiating action potentials in local neurons or neurons controlling osteocyte function.

V. SUMMARY AND CONCLUSIONS

In this study, we describe the production and characterization of a unique dual-protein micro-patterned surface for co-culture of osteocyte-like MLO-Y4 cells and DRG neurons. The advantage of this modular system is that it utilizes biological matrix proteins that are similar to those found in the body to mimic the *in vivo* environment. We show that MLO-Y4 cells prefer to attach to PlnDIV in comparison to Ln and that this attachment produces an osteocyte-like phenotype. Attachment to PlnDIV also results in a more osteocyte-like mRNA marker expression in MLO-Y4 cells. Unlike MLO-Y4 cells, DRG neurons prefer to grow on Ln coated substrates. We have exploited this matrix preference to develop a DRG neuron—MLO-Y4 cell co-culture system that allows cells to elaborate in close proximity and come into contact with each other in a 2-D platform. These surfaces are ideal for future cell-cell communication studies between MLO-Y4 cells and DRG neurons.

ACKNOWLEDGMENTS

The authors wish to thank Deni Galileo, Ulhas Naik, Jeff Twiss, Kirk Czymmek, Zhanping Zhang, Shawn Sullivan, Holt Bui, Bill Theilacker, and Ben Rohe for helpful discussions and assistance with some experiments. This work was partially supported by the NIH (Grant Nos. R01-N543928 and R01-EB00463 (TPB) and R01-AR051901 (RLD), and CA098192 (MCFC). The UD Surface Analysis Facility was supported by the NSF (Grant No. DMR-9724307 and CHE-9814477).

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