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근육유래 줄기세포의 평활근으로 분화유도를 위한 VEGF/Laminin 탑재 맴브레인

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VEGF/Laminin Co-Immobilized Membrane for Enhanced Differentiation of Muscle-derived Stem Cells into Smooth Muscle Cells

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초록: 본 연구에서는 생리활성인자[vascular endothelial growth factor(VEGF) 및 laminin]가 탑재된, 비대칭구조를 가지는 다공성 polycaporlactone(PCL) 기반의 맴브레인을 제조하였으며, 맴브레인에 탑재된 생리활성인자에 의한 근육유래 줄기세포(muscle-derived stem cells, MDSCs)의 평활근세포(smooth muscle cells, SMCs)로의 분화거동을 연구하였다. PCL 기반 맴브레인에 탑재된 VEGF 및 laminin은 맴브레인으로부터 각각 28일과 14일 동안 지속적으로 방출되었다. 각 생리활성인자가 단독 혹은 공동으로 탑재된 PCL 기반 맴브레인에서 MDSCs의 SMCs로의 분화거동을 DNA 정량화, RT-PCR 및 면역염색을 통해 분석하였으며, 두 생리활성인자가 공동으로 탑재된 PCL 기반 맴브레인에서 가장 우수한 평활근으로의 분화거동을 보임을 확인하였다. 이러한 결과로부터, VEGF/laminin이 탑재된, 비대칭구조를 가지는 다공성 PCL 기반의 맴브레인은 평활근 재생을 위한 재생막으로 사용이 가능할 것이라 판단되었다.

Abstract: It is important to control the stem cell differentiation into target cell types for clinical use of the stem cells. In this study, asymmetrically porous polycaprolactone (PCL)/Pluronic F127 membranes immobilized with bioactive molecules [vascular endothelial growth factor (VEGF) and/or laminin] were prepared to investigate the effect of muscle-derived stem cells (MDSCs) on smooth muscle cell (SMC) differentiation. The VEGF and laminin immobilized on the PCL/F127 membrane surface were released with a sustained manner for 28 and 14 days from the membrane, respectively. The SMC differentiation behavior of MDSCs on the membranes immobilized with single or dual bioactive molecules was compared by DNA quantification, RT-PCR, and immunohistochemical analyses. The dual VEGF/laminin-immobilized membrane group showed higher cell growth and more effective SMC differentiation than the single VEGF- or laminin-immobilized group. From our findings, we suggest that the dual VEGF/laminin-immobilized membrane may be applicable to use as a guided smooth muscle regeneration membrane.

Keywords: porous membrane, vascular endothelial growth factor, laminin, stem cell, differentiation.

Introduction

Stem cells are widely used in tissue engineering for the regeneration of various tissues or organs.[1,2] It is well known that the stem cells are differentiated into various cell types, such as bone marrow stem cells into osteogenic, chondrogenic, myogenic, vascular, and neurogenic cells;[3,4] adipose stem cells into osteogenic, myogenic, and chondrogenic cells;[5,6] skin stem cells into neurons and smooth muscle cells;[6] and muscle-derived stem cells (MDSCs) into smooth muscle cells (SMCs).[7-9] Various growth factors and cytokines are commonly adapted to achieve the differentiation of stem cells into specific target cells. It is important to precisely control the stem cell differentiation into specific cell types for the clinical use of

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stem cells. Particularly, the MDSCs capable of differentiating into SMCs have been widely used for the smooth muscle tissue regeneration in urethra,[10] anus,[11,12] and bladder.[13] In our previous studies,[14,15] dual growth factors [vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)]-immobilized polycaprolactone (PCL)/Pluronic F127 beads were prepared as an injectable bioactive urethral bulking agent. Both bFGF and VEGF are well known as the growth factors which induce smooth muscle differentiation[9,16] as well as angiogenesis. It was observed that the growth factors (bFGF and VEGF)-immobilized porous beads stimulate SMC differentiation of MDSCs and thus defect tissue regeneration around urethra (mainly smooth muscle) to improve the sphincter function.

In this study, we prepared dual bioactive molecules [VEGF (growth factor) and laminin (protein)]-immobilized PCL/Pluronic F127 asymmetrically porous membrane to use as a guided tissue (smooth muscle) regeneration membrane. Laminin is the major protein of the basal lamina of various tissues, and is known to be a key protein responsible for cell migration and differentiation.[17,18] In particular, it is known that the laminin plays important roles in SMC differentiation and maintenance of the SMC phenotype.[19] We tested whether using cell differentiation-stimulating growth factor (VEGF) and protein (laminin) together provides a synergistic effect on SMC differentiation of MDSCs on the membrane surface. VEGF, which is one of heparin-binding growth factor families, was loaded on the surface of porous PCL/Pluronic F127 membrane via specific interactions between heparin and Pluronic F127 (hydrogen bonding) and subsequent interactions between growth factor and heparin (ionic interaction), which preserve the biological activity of growth factor without denaturation. Laminin was immobilized on the membrane pore surface by physical adsorption. The VEGF and laminin immobilized on the membrane can be released in a sustained manner for continuous stimulation of the cells attached on the membrane surface. Figure 1 illustrates the schematic diagrams of the membrane with the immobilization of single VEGF, single laminin, and dual VEGF and laminin [successive binding of VEGF and laminin]. The SMC differentiation behavior of MDSCs on the membranes immobilized with single or dual bioactive molecules was compared by DNA quantification, RT-PCR, and immunocytochemical analyses.

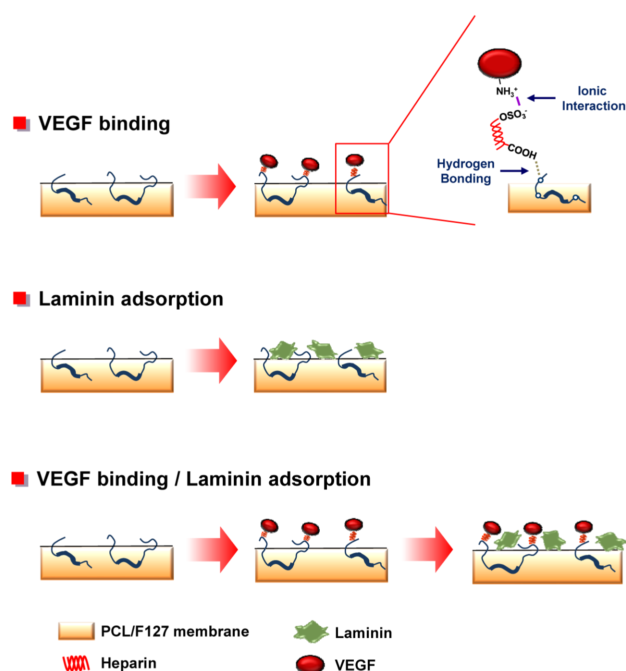

Figure 1. Schematic diagrams of the PCL/Pluronic F127 membrane with the immobilization of single VEGF, single laminin, and dual VEGF and laminin [successive binding of VEGF and laminin (VEGF/laminin)].

Experimental

Materials. PCL (Mw 70~90 kDa; Sigma, USA) and Pluronic F127 (triblock copolymer of ethylene glycol and propylene glycol; BASF, USA) were used to fabricate Pluronic F127-incorporated PCL porous membranes. Tetraglycol (glycofurol; Sigma) was used as a nontoxic co-solvent to dissolve PCL and Pluronic F127. VEGF and laminin were purchased from R&D Systems (USA). All other chemicals (analytical grade) were used without further purification.

Fabrication of PCL/Pluronic F127 Membrane. PCL/Pluronic F127 membrane was prepared by an immersion precipitation method.[20] Briefly, PCL pellets were dissolved in tetraglycol (12 wt%) at 90 °C and then Pluronic F127 powders were added to the PCL solution (5 wt%, PCL base). The PCL/Pluronic F127 mixture solution was casted in a mold (50 mm ×50 mm×0.6 mm) and immersed in excess water for 1 hr at room temperature. Upper part of the PCL/F127 mixture solution was precipitated by water contact (water, non-solvent for PCL) and the sub-layer was gradually solidified by the diffusion of water into the solution (solvent/non-solvent exchange). The precipitated PCL/Pluronic F127 membrane was rinsed by excess water to remove the residual solvent. The PCL/Pluronic F127 membrane was obtained after vacuum drying (Figure 2). Surface and cross-section morphologies of the

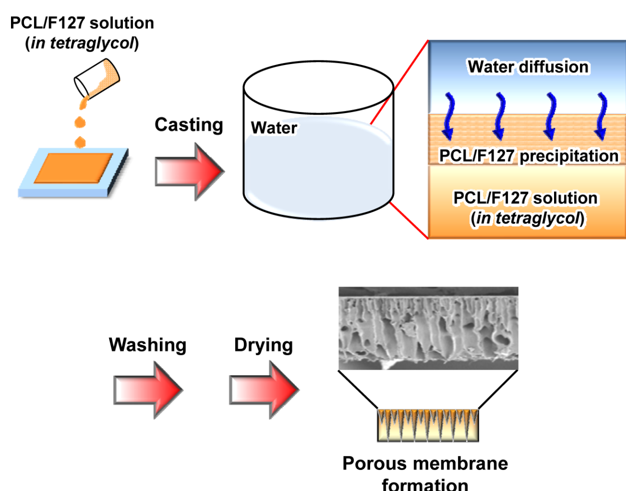

Figure 2. Schematic diagram showing the fabrication steps of PCL/Pluronic F127 membrane.

PCL/Pluronic F127 membrane were investigated by a scanning electron microscopy (SEM) (Model S-3000N, Hitachi, Japan).

Bioactive Molecules Immobilization and Release Test. VEGF was immobilized on the pore surfaces of PCL/Pluronic F127 membrane (10 mm×10 mm×0.6 mm) *via* heparin binding. To immobilize heparin, the membrane was soaked in a heparin solution [3 mg/mL (in 2 wt% NaCl solution)] for 3 hrs at 4 °C. The heparin-immobilized membrane was washed with 2 wt% NaCl solution and then water to remove the unbound or weakly bound heparin. The amount of heparin incorporated on the membrane was estimated using a Toluidine blue assay.[21] In order to immobilize VEGF onto the heparin-bound membrane, the membrane was soaked in VEGF solution (200 ng/mL) for 6 hrs at room temperature. The growth factor-immobilized membrane was rinsed by phosphate buffered saline (PBS; pH ~7.4) three times to eliminate the free growth factor. The amount of VEGF immobilized on the membrane was measured using an enzyme-linked immunosorbent assay kit (ELISA kit; R&D systems).[22] Laminin was immobilized on the heparin-bound PCL/Pluronic F127 membrane by physical adsorption. For this, the membrane was soaked in a laminin solution (0.1 mg/mL in PBS; 37 °C) for 1 hr and the following rinse with PBS twice to remove unadsorbed or weakly adsorbed laminin.[23] The amount of adsorbed laminin on the membrane was quantified by a directed ELISA technique, according to the standard procedure of the kit (Abcam, USA). Some of the VEGF-immobilized membranes were also treated with laminin for successive binding of VEGF and laminin (VEGF/laminin) (refer to Figure 1).

The bioactive molecules (VEGF and/or laminin)-immobilized membranes were incubated in 1 mL of PBS supplement with 0.1% bovine serum albumin (Sigma) at 37 °C under mild shaking (~50 rpm). At pre-set time intervals (up to 35 days), the whole medium were sampled and replaced with fresh one. The amount of released VEGF and laminin in the collected solution was analyzed using the corresponding ELISA kits, respectively.

Cell Culture. Before *in vitro* cell culture, the PCL/F127 membranes were sterilized with ethylene oxide and underwent VEGF and/or laminin immobilization procedures. The membranes were placed on 12-well polystyrene (PS) plates (Corning, USA) with a micro-pore side, up-position. To investigate the potential of SMC differentiation by the bioactive molecules (VEGF and/or laminin)-immobilized membranes, MDSCs were isolated from the hindlimb muscle of a rat and purified using the preplate technique.[24] The MDSCs, after four passages [in Dulbecco's modified eagle's medium/nutrient mixture F-12 (DMEM/F-12) containing 10% fetal bovine serum (FBS), 5% horse serum, and 1% antibiotics (Gibco, USA)], were seeded on the microporous membrane surface with a density of 1×10^4 cells/cm^2, and the membrane was maintained for 2 hrs at 37 °C in an incubator (humidified 5% CO_2 atmosphere) for cell adhesion. The cell-adhered membranes were cultured for 7, 14, and 28 days with mild shaking (~50 rpm) for sufficient medium exchange. The culture medium in each plate well (3 mL/well) was freshly changed every third day.

DNA Quantification. Cell adhesion and proliferation behavior on the bioactive molecules-immobilized membranes were determined by the measurement of DNA content. MDSCs attached on the membranes were digested overnight using a papain buffer at 60 °C after cell culture [2 hrs (for adhesion); 7, 14, and 28 days (for proliferation)]. DNA contents were determined using a Hoechst 33258 (DNA-binding fluorochrome, Sigma-Aldrich). A 10 μL sample of each lysate was shifted to a 96-well PS plate, and 90 μL of TNE buffer [tris (10 mM), NaCl at pH 7.4 (2 M) and EDTA (1 mM)] was added. 100 μL of Hoechst 33258 solution (1 mg/mL) was added in the wells, and then the plates were read using a Fluostar Optima fluorescence plate reader (BMG Labtech, USA; emission wavelength, 460 nm and excitation wavelength, 355 nm). A standard curve to estimate the DNA content was prepared by serial dilution of calf thymus DNA. All experiments to evaluate the cell adhesion and proliferation were

performed in triplicate for each sample.

Real-time Polymerase Chain Reaction (RT-PCR) Analysis. After cell culture on the bioactive molecules-immobilized membranes, RNA from the cells adhered on membrane was extracted using Trizol (Invitrogen, USA). After DNAse I treatment, the obtained RNA was reverse-transcribed using Multiscribe reverse transcriptase oligo (dT) primer (Invitrogen) in a 40 μL reaction volume. All the PCR reactions were conducted by a LightCycler 480 System (Roche Diagnostics, USA) in standard 10 reactions. The primers used for RT-PCR were α-smooth muscle actin (α-SMA), calponin, caldesmon, and smooth muscle-myosin heavy chain (SM-MHC) for smooth muscle cell differentiation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was adapted as an internal standard. The relative normalization ratio of PCR resultants derived from the target genes was determined using LightCycler System software (Roche Diagnostics). The lowest value in each target gene analysis was used as a control (relative expression level, 1).

Immunocytochemical Analysis. To visualize the SMC differentiation of the MDSCs on the bioactive molecules-immobilized membranes, immunocytochemical staining of the membranes was also conducted after 28 days of cell culture. The membranes were blocked for 2 hrs in a blocking solution (0.3% Triton X-100 in PBS, 1% bovine serum albumin, and 10% normal goat serum). For SMC expression, the membranes were incubated with primary antibody (1:500 dilution; -SMA) for 2 hrs. The appropriate fluorescence-tagged secondary antibody (R&D Systems) was used for visualization. All procedures were performed under room temperature. The nuclei of the cells attached the membranes were stained using a 4'6-diamidino-2-phenylinodole (DAPI; Vector Laboratories, USA), and visualized using a fluorescence microscope (Leica, DMI 6000B, Germany).

Statistical Analysis. The data obtained from the experiments were expressed as the mean ± standard deviation. The significance of differences between membranes was assessed by one-way analysis of variance with Tukey's multiple comparison test. The differences were considered to be statistically significant at $p<0.05$.

Results and Discussion

Characterization of PCL/Pluronic F127 Membrane. Asymmetrically porous PCL/Pluronic F127 membrane was fabricated by the immersion precipitation method (Figure 2). Pluronic F127 was used as an intermediator to incorporate heparin and VEGF on the membrane surface as well as a hydrophilic additive to prepare hydrophilized PCL membrane. The Pluronic F127 chains can be stably immobilized on the PCL membrane by hydrophobic interaction between the polypropylene glycol in the Pluronic F127 and the PCL as well as the entrapment of the chains on the PCL matrix.[25] The membrane had approximately 400 μm thickness with nano-pores (~100 nm) on the top surface and micro-pores (~100 μm) on the bottom surface, and both surfaces were connected by a column-shaped pore as observed by SEM. The formation of an asymmetrically porous structure of the membrane can be explained by gradual phase separation between the polymer solution (in tetraglycol) and the non-solvent (water).[26] The water contact side of the PCL/F127 mixture solution forms smaller pores (nano-pores) by precipitation of the polymer with a higher initial polymer concentration, whereas the sublayer creates lager ones (micro-pores) by the precipitation of the polymer having a lower polymer concentration compared to the water contact side.

Bioactive Molecules Immobilization and Release Behavior. VEGF and laminin were selected as promoting growth factor and protein for smooth muscle differentiation, respectively.[9,18] To incorporate VEGF on the PCL/Pluronic F127 membrane, heparin was immobilized on the membrane via hydrogen bonding between the ether oxygen in Pluronic F127 on the pore surfaces of membrane and the carboxylic acid group of heparin (Refer to Figure 1). Figure 3 shows the loading amount of heparin on the surfaces of the PCL and PCL/F127 membranes. It was observed that the PCL/F127 membrane allowed much larger heparin immobilization (7.41

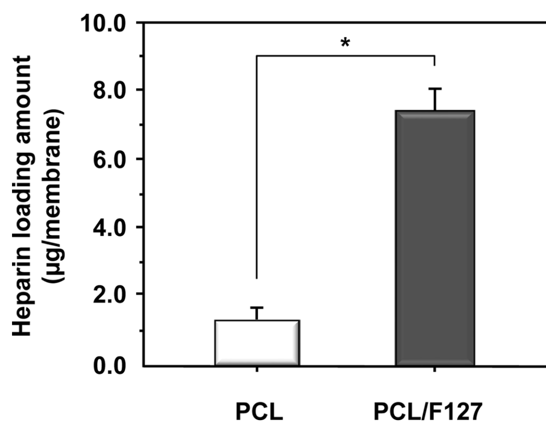

Figure 3. Loading amount of heparin on the PCL/Pluronic F127 membrane with and without heparin binding (n=3, *$p<0.05$).

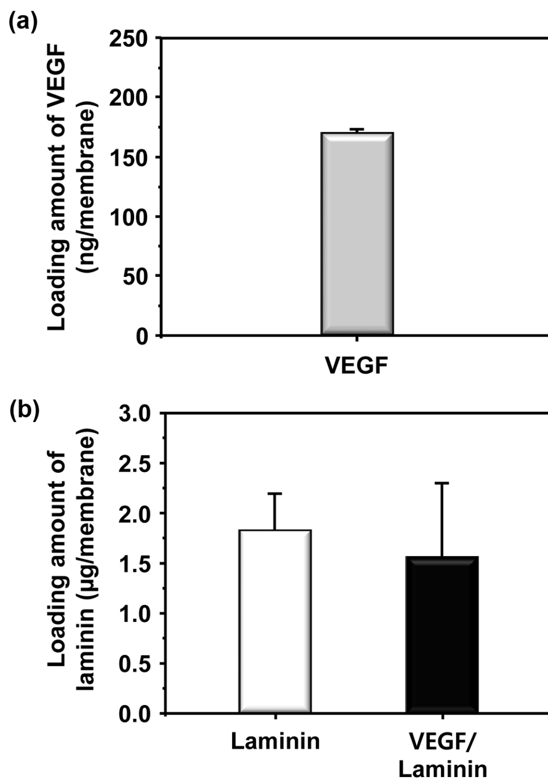

Figure 4. Loading amount of (a) VEGF on the heparin-bound PCL/Pluronic F127 membrane; (b) laminin on the heparin-bound PCL/Pluronic F127 membrane with and without VEGF pre-immobilization (n=3).

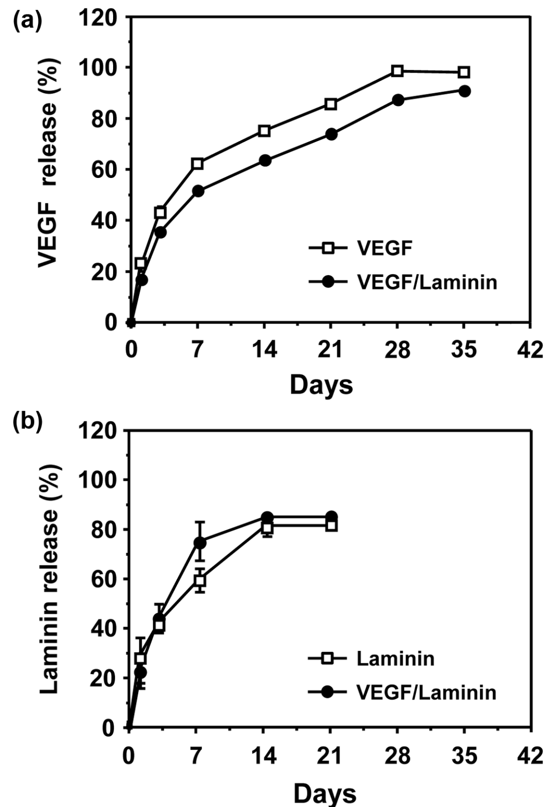

Figure 5. Cumulative released amount of (a) VEGF on the PCL/Pluronic F127 membrane; (b) laminin on the PCL/Pluronic F127 membrane with and without VEGF pre-immobilization (n=3).

±1.06 μg/membrane) compared with the PCL membrane (1.23 ±0.49 μg/membrane). The heparin loading on the PCL membrane without Pluronic F127 may be caused by the physical adsorption (non-specific binding) of the heparin on the pore surfaces. VEGF was immobilized onto the heparin-bound PCL/F127 membrane through ionic interactions between the N-sulfate and O-sulfate groups of the heparin molecule and lysine and arginine groups in the growth factor (loading amount, 169.46±2.39 ng/membrane) (Figure 4(a)). Laminin was immobilized on the heparin-bound PCL/F127 membrane with and without VEGF pre-immobilization by physical adsorption. The loading amount of laminin was much higher than that of VEGF on the PCL/F127 membrane, probably owing to nonspecific physical adsorption on the membrane surface. It was not significantly different on the membranes with and without VEGF pre-immobilization (1.83±0.37 μg/membrane for laminin and 1.56±0.74 μg/membrane for VEGF/laminin) (Figure 4(b)).

Figure 5 shows the release profiles of VEGF and laminin from the membranes. The VEGF was continuously released from the VEGF- and VEGF/laminin-immobilized membrane surfaces for more than 28 days (Figure 5(a)). The sustained release of the VEGF from the membrane may be explained by the gradual dissociation of ionic bonds of the VEGF molecules on the membrane surface in PBS. The laminin was released shorter time than VEGF release (for about 14 days) from both membranes with and without VEGF pre-immobilization (Figure 5(b)), probably owing to desorption of the laminin physically adsorbed on the membranes. The VEGF pre-immobilization did not significantly affect the laminin release behavior.

Proliferation and Differentiation of MDSCs on Bioactive Molecules-immobilized Membranes. To investigate the *in vitro* proliferation and differentiation behavior of MDSCs into SMCs by continually releasing bioactive molecules from the membrane, MDSCs were seeded onto the PCL/F127 membranes with bioactive molecules immobilization (single VEGF, single laminin, and dual VEGF/laminin) as well as the membrane without the bioactive molecule immobilization (Control). The cells were seeded and attached on the micro-pore side of the membranes. The cell proliferation on the mem-

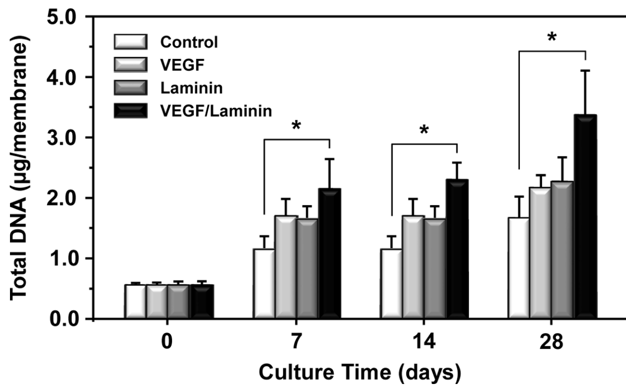

Figure 6. DNA content with time after MDSC culture on the PCL/Pluronic F127 membranes with and without bioactive molecules immobilization (n=3, *p<0.05).

branes was assessed by the measurement of their DNA contents at pre-determined time intervals (0, 7, 14, and 28 days) after cell culture. The cells were proliferated with time, but their growth was not significant after 7 days (Figure 6). Higher cell growth was observed on the membranes with the bioactive molecule immobilization than the membrane without the bioactive molecule immobilization. The VEGF/laminin group showed higher cell growth on the membrane compared to the single VEGF or laminin group, especially at 28 days of culture.

In order to further investigate the effect of MDSC differentiation into SMCs on the bioactive molecules-immobilized membrane, RT-PCR analysis of several genes was conducted for up to 28 days of culture. SMC differentiation was evaluated using smooth muscle specific markers, such as α-SMA, calponin, caldesmon, and SM-MHC. The expressions of all SMC markers evaluated by the RT-PCR increased over time, indicating the continuous differentiation of MDSCs into SMCs (Figure 7). For single bioactive molecule-immobilized groups, the expression of all the SMC markers was greater on the laminin group than the VEGF group, indication better differentiation of MDSCs into SMCs. The expression of the SMC markers were also greater on the dual VEGF/laminin group than the single VEGF or laminin group, suggesting that the membrane immobilized with both VEGF and laminin provided a better environment for the SMC differentiation. As discussed earlier, the loading amount of laminin was much higher than that of VEGF on the membrane surface, while its release time was shorter than VEGF release (about 14 days for laminin and about 28 days for VEGF). It seems that the fast release

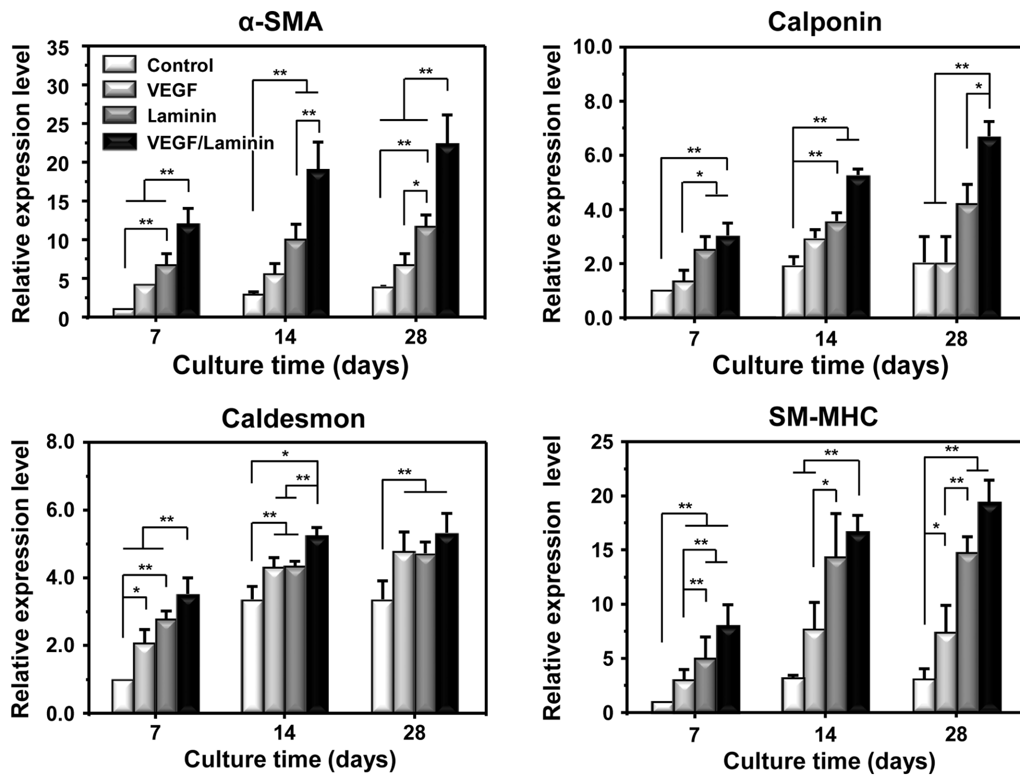

Figure 7. RT-PCR analysis with time after MDSC culture on the PCL/Pluronic F127 membranes with and without bioactive molecules immobilization (n=3, *p<0.05, **p<0.01).

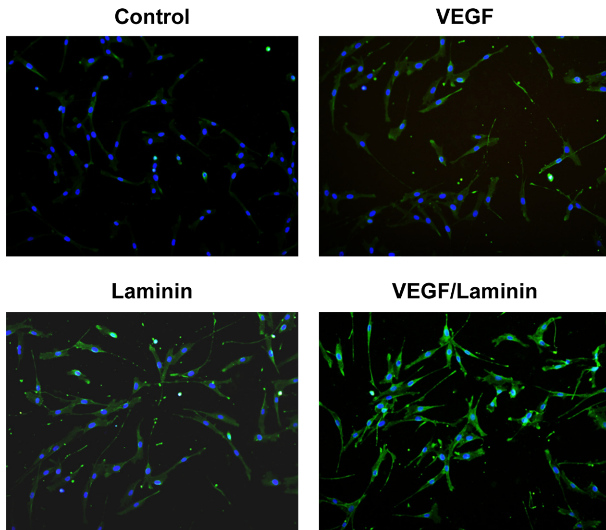

Figure 8. Immunocytochemical observations at 28 days after MDSC culture on the PCL/Pluronic F127 membranes with and without bioactive molecules immobilization [blue, cell nucleus (DAPI); green, α-SMA].

of large amount of laminin and the following continuous VEGF release from the dual VEGF/laminin group may be effective for the differentiation of MDSCs into SMCs in our system. From the immonocytochemical observations of MDSCs cultured on the bioactive molecules-immobilized membranes, it was observed that the cells (blue color, cell nucleus) were uniformly distributed on the membranes (Figure 8). The α-SMA expression (green color) was almost not detected on the control membrane group without bioactive molecule immobilization. However, all the bioactive molecule-immobilized membrane groups showed α-SMA expression. The VEGF/laminin group showed greater levels of α-SMA expression than the single VEGF or laminin group, indicating the greater differentiation of MDSCs into SMCs. This observation was agreed with the result of RT-PCR analysis (refer to Figure 7), and was also similar to our previous result[14] using dual growth factors (VEGF and bFGF)-immobilized PCL/Pluronic F127 beads. Based on these results, we can expect that using cell differentiation-stimulating growth factor (VEGF) and protein (laminin) together provides a synergistic effect on SMC differentiation of MDSCs on the membrane surface.

Conclusions

In the present study, PCL/F127 membranes immobilized with bioactive molecules (single VEGF, single laminin, and dual VEGF/laminin) were used to investigate the effect of MDSCs on SMC differentiation. The VEGF was immobilized on the PCL/F127 membrane surface via heparin binding. The VEGF immobilized on the heparin-bound membrane was released with a sustained manner for more than 28 days. The laminin was easily immobilized on the PCL/F127 membrane surfaces with or without VEGF pre-immobilization by physical adsorption. The laminin was released shorter time than VEGF release (for about 14 days) from both membranes with and without VEGF pre-immobilization. The dual VEGF/laminin-immobilized membrane group demonstrated more effective SMC differentiation than single VEGF- or laminin-immobilized membrane groups, and can be a potential to be used as a guided tissue (smooth muscle) regeneration membrane.

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