Experimental conditions and protein markers for redifferentiation of human coronary artery smooth muscle cells

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Abstract. A phenotype switch from contractile type to proliferative type of arterial smooth muscle cells is known as dedifferentiation, but to the best of our knowledge, little is known about redifferentiation of coronary artery smooth muscle cells. The purpose of the present study was to determine *in vitro* culture conditions for inducing redifferentiation of coronary artery smooth muscle cells. In addition, the present study aimed to determine protein markers for detection of redifferentiated arterial smooth muscle cells. Human coronary artery smooth muscle cells (HCASMCs) were cultured in the presence or absence of growth factors, including epidermal growth factor, fibroblast growth factor-B and insulin. Protein expression and migration activity of HCASMCs were evaluated using western blotting and migration assay, respectively. In HCASMCs 5 days after 100% confluency, expression levels of α-smooth muscle actin (α-SMA), calponin, caldesmon and SM22α were significantly increased, while expression levels of proliferation cell nuclear antigen (PCNA) and S100A4 and migration activity were significantly decreased, compared with the corresponding levels just after reaching 100% confluency, indicating that redifferentiation occurred. Redifferentiation was also induced in a low-density culture of HCASMCs in the medium without growth factors. When the culture medium for confluent cells was replaced daily with fresh medium, the expression levels of α-SMA, caldesmon, SM22α, PCNA and S100A4 and migration activity were not significantly different but the calponin expression was significantly increased compared with the levels in dedifferentiated cells just after reaching 100% confluency. Thus, redifferentiation was induced in HCASMCs by deprivation of growth factors from culture medium. The results suggested that α-SMA, caldesmon and SM22α, but not calponin, are markers of redifferentiation of HCASMCs.

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Introduction

Phenotype modulation of arterial smooth muscle cells (SMCs) is a key process during the development of atherosclerotic plaque (1,2). Local factors around the vascular wall, including growth factors, inflammatory mediators, mechanical stress, injury and lipoprotein, induce a phenotypic switch from a differentiated contractile state to a dedifferentiated proliferative state, which is characterized by decreased expression of contractile type SMC markers represented by α-smooth muscle actin (α-SMA) (3). When vascular SMCs are prepared for experiments, they are typically cultured in the presence of growth factors and thus exhibit a proliferative (dedifferentiated) phenotype (4).

To the best of our knowledge, there is limited information on redifferentiation of SMCs, which is reversion to the differentiated phenotype from the dedifferentiated phenotype (5). *In vitro* redifferentiation in human umbilical artery SMCs was induced by serum deprivation (6). Similar redifferentiation was observed in human uterine SMCs on the 6th day after reaching 100% confluency (7). There have been reports on molecules that are involved in redifferentiation of vascular SMCs (8-10): Hyperplasia suppressor gene has been shown to be involved in redifferentiation of rat aortic SMCs (RASMCs) (8). C-type natriuremic peptide has been reported to induce redifferentiation of RASMCs by stimulating the cGMP cascade (9). Exendin-4 has also been reported to promote redifferentiation of RASMCs via the AMPK/sirtuin 1/FOXO3a signaling pathway (10). However, it is unknown whether and how redifferentiation is induced *in vitro* in coronary artery SMCs. Moreover, it remains to be determined whether protein markers of differentiation also serve as markers of redifferentiation. Redifferentiation has been shown in SMCs of neointimal tissue following percutaneous transluminal coronary angioplasty (PTCA) in autopsied patients (11). Therefore, it is hypothesized to be clinically relevant to determine protein markers of redifferentiation of HCASMCs.

The aim of the present study was therefore to determine the culture conditions for inducing redifferentiation of human coronary artery SMCs (HCASMCs). Proliferation and migration, which are phenotypic characteristics of dedifferentiated

SMCs, were monitored, and expression levels of α-SMA, calponin, caldesmon and SM22α/tagln, known specific differentiation markers of vascular SMCs (12-14), were evaluated as potential protein markers of redifferentiated HCASMCs.

Materials and methods

Cell culture. HCASMCs were purchased from Kurabo Co., Ltd. (cat. no. KS-4209) and cultured in medium (HuMedia-SB2; Kurabo Co. Ltd) supplemented with 5% fetal bovine serum, human epidermal growth factor (hEGF) at 0.5 ng/ml, human fibroblast growth factor B (hFGF-B) at 2 μg/ml, insulin at 5 μg/ml, gentamicin at 50 μg/ml and amphotericin B at 50 ng/ml (all obtained from Kurabo Co. Ltd) under 95% air-5% CO_2 at 37˚C. For cell observation and protein extraction or cell migration, cells were seeded at 90% confluency (day 0) and reached 100% confluency after 48 h incubation (day 2). Cells were seeded at 90% confluency ($3.5x10^5$ cells in a 35-mm dish for cell observation and western blotting and $7.5x10^5$ cells in a 60-mm dish for the migration experiment) (day 0). In experiments for testing the effects of removal of growth factors on expression of phenotype marker proteins, cells were seeded with a lower density of 10% confluency ($2.0x10^5$) in a 100-mm dish in the presence or absence of the aforementioned growth factors. Phase-contrast and fluorescence images were captured by microscopy (TE300, Nikon Corporation) with x10 magnification every day from day 0 to day 6 after seeding the cells and representative images were obtained. Fluorescence images were captured after staining with Calcein-AM (sc-203865, Santa Cruz Biotechnology) for 1 h under 95% air-5% CO_2 at 37˚C and analyzed using a software (Lumina Version, MITANI Corporation, Ver.3.3.6.1). Other chemicals were purchased from Sigma-Aldrich (Merck KGaA) unless otherwise stated.

Western blotting. Western blotting was performed as described previously (15). Percentages of the SDS-PAGE used were 6% for caldesmon-1, 8% for calponin, α-SMA, PCNA and GAPDH, and 15% for S100A4 and SM22α/tagln. Proteins were extracted from HCASMCs at day 1 to day 6 after seeding the cells. The proteins transferred onto the nitrocellulose membrane following separation with SDS-PAGE were incubated with primary antibodies at 4˚C overnight. Antibodies to detect α-smooth muscle actin (α-SMA; 1:1,000, cat. no. 19245S), proliferating cell nuclear antigen (PCNA; 1:2,000, cat. no. 2586S), caldesmon-1 (1:250, cat. no. 2980S), SM22α/tagln (1:1,000, cat. no. 40471S), S100A4 (1:250, cat. no. 13018S), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:10,000, cat. no. #2118) were purchased from Cell Signaling Technology, Inc. A calponin antibody (1:1,000, cat. no. 13938-1-AP) was obtained from Proteintech Group, Inc. The proteins bound to the primary antibodies were incubated with secondary antibodies at room temperature for 1 h. The secondary antibodies used were anti-mouse IgG, HRP-linked antibody (1:2,000, Cell Signaling, cat. no. #7076) for PCNA and anti-rabbit IgG, HRP-linked antibody (1:2,000, Cell Signaling, cat. no. #7074) for α-SMA, calponin, GAPDH, caldesmon-1, S100A4 and SM22α/tagln. Immunoreactive protein bands were visualized using an ECL plus detection system (Pierce™ ECL Plus Western Blotting Substrate 32132, Thermo Fisher Scientific, Inc.) with LAS4000mini (Cytiva). The protein band intensity was quantified by ImageQuant TL (ver.8.2, Cytiva) and the relative changes of band intensities were calculated compared with the control at the earliest time point (designated as 1.0). The values were obtained from at least three independent experiments.

Evaluation of migration. Migration activity of HCASMCs with 100% confluency was evaluated in the presence of 5% fetal bovine serum by migration assay, as previously described (16,17). At 0 and 24 h after the culture from day 2 and day 5, the culture was scratched with a sterile sharp knife and the medium (HuMedia-SB2) was replaced with fresh medium. Following 23 h incubation at 37˚C, cells were pre-stained using calcein-acetoxymethyl ester (calcein-AM) at 1 μg/ml for 1 h at 37˚C and images were taken. Migrating cells were counted manually under a light microscopy (TE300, Nikon Corporation) with x10 magnification and a fluorescent microscopy as mentioned above.

Statistical analysis. All data are presented as the mean ± SEM of at least three independent experiments. Differences in mean values between groups for multiple comparisons were subjected to one-way ANOVA and Dunnett or Tukey's post hoc test and comparisons between two groups were performed using Student's unpaired t test. Statistical analysis was performed using JMP Pro 16 (SAS Institute Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Changes in protein expression levels of α-SMA, calponin and PCNA in HCASMCs after reaching 100% confluency. Fig. 1A shows representative phase-contrast images of HCASMCs on days 1, 2 and 6. HCASMCs were seeded at 90% confluency on day 0 (the day when culture was started). The confluency was similar on days 0 (data not shown) and 1. This may be due to the fact that HCASMCs did not start proliferating immediately after being seeded. The cells reached 100% confluency on day 2, which was maintained without cell death until day 6; no cells came off from the bottom face of the dish, which indicated there were no dying cells. Thus, cell viability was not changed post-confluence. Representative protein expression of α-SMA and calponin, typical markers of contractile-type SMCs, and PCNA, a marker protein of proliferating cells (12-14), in HCASMCs before and after reaching 100% confluency is shown in Fig. 1B. The levels of α-SMA increased in a time-dependent manner and were significantly higher on days 5 and 6 compared with day 2 (Fig. 1B and C). The calponin expression also increased in a time-dependent manner and was significantly higher on days 4-6 than on day 2 (Fig. 1B and D). By contrast, PCNA expression decreased in a time-dependent manner and was significantly lower on day 6 than on day 2 (Fig. 1B and E).

Migration activity of confluent and post-confluent HCASMCs. Fig. 2A shows representative phase-contrast and fluorescence images of migration assay using HCASMCs. When culture from day 2 was scratched, cells migrated from the baseline following 24 h incubation, while only a small number of cells

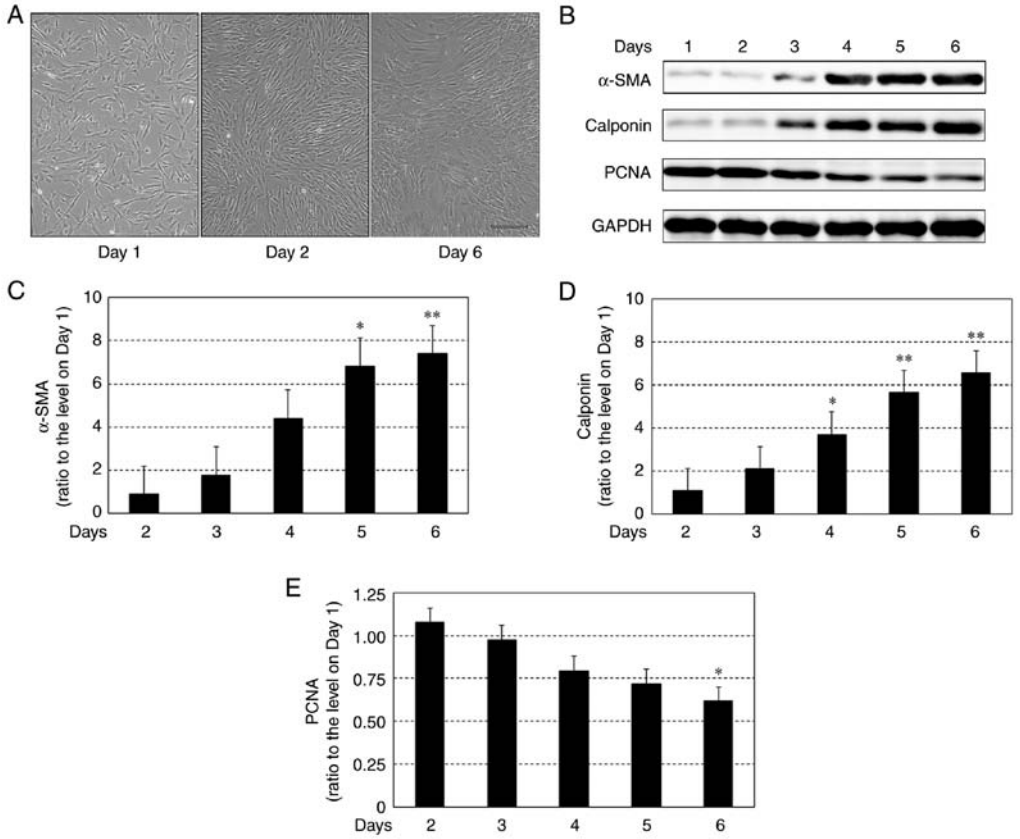

Figure 1. Time course of changes in protein expression of α-SMA, calponin and PCNA in HCASMCs before and after reaching 100% confluency. (A) Representative phase-contrast images (x10 magnification) of HCASMCs on days 1, 2 and 6 after seeding at 90% confluency. Scale bar, 200 μm. (B) Representative western blots for α-SMA, calponin, PCNA and GAPDH on each day. Densitometrical analysis of expression of (C) α-SMA, (D) calponin and (E) PCNA. Data are expressed as the ratio to GAPDH-corrected levels of each protein on day 1 and are shown as the mean ± SEM of three independent experiments. *P<0.05, **P<0.01 vs. day 2. SMA, smooth muscle actin; PCNA, proliferating cell nuclear antigen; HCASMC, human coronary artery smooth muscle cell.

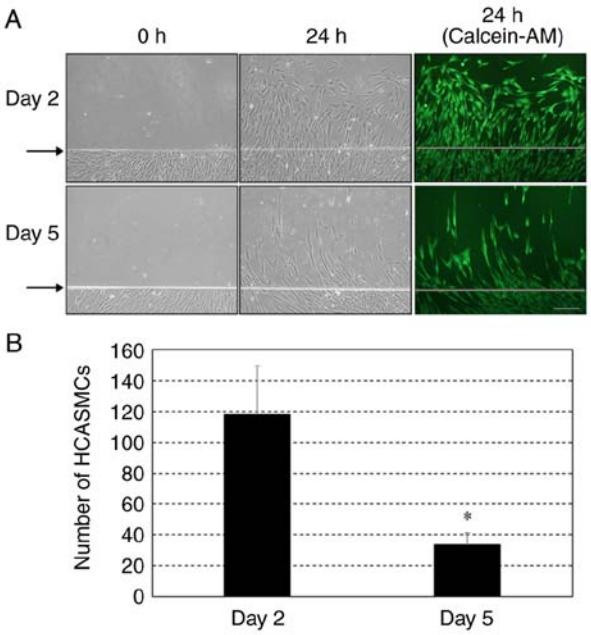

Figure 2. Migration activity of HCASMCs after reaching confluency. (A) Representative phase-contrast and fluorescence images (x10 magnification) of HCASMCs on days 2 and 5 after seeding at 90% confluency without (0 h) and with injury (24 h). Scale bar, 200 μm. (B) Number of cells that had migrated at 24 h after injury on days 2 and 5. Data are shown as the mean ± SEM of three independent experiments. *P<0.05 vs. day 2. HCASMC, human coronary artery smooth muscle cell; AM, acetoxymethyl ester.

migrated at 24 h following injury when the culture from day 5 was scratched. The number of migrating cells from day 5 was significantly smaller than that from day 2 (Fig. 2B).

Effects of growth factors on protein expression levels of α-SMA, calponin and PCNA in HCASMCs before reaching 100% confluency. When culture of HCASMCs was started at 10% confluency, the cells did not reach 100% confluency on day 4 in the presence or absence of the growth factors (Fig. 3A). The number of cells was larger in the presence of growth factors than in the absence but this was not significant (Fig. 3B). Fig. 3C shows representative protein expression of α-SMA, calponin and PCNA in cells cultured in the presence or absence of growth factors. Expression levels of α-SMA (Fig. 3D) and calponin (Fig. 3E) on day 4 were significantly higher and those of PCNA (Fig. 3F) tended to be lower (but not significantly) in the absence of the growth factors.

Effect of growth factors on protein expression levels of α-SMA, calponin and PCNA in HCASMCs after reaching 100% confluency. Cells seeded at 90% confluency were cultured and maintained for five days with or without replacement of fresh medium including growth factors every day (Fig. 4A). In the cells cultured in the medium that was replaced every day with fresh medium, the protein expression

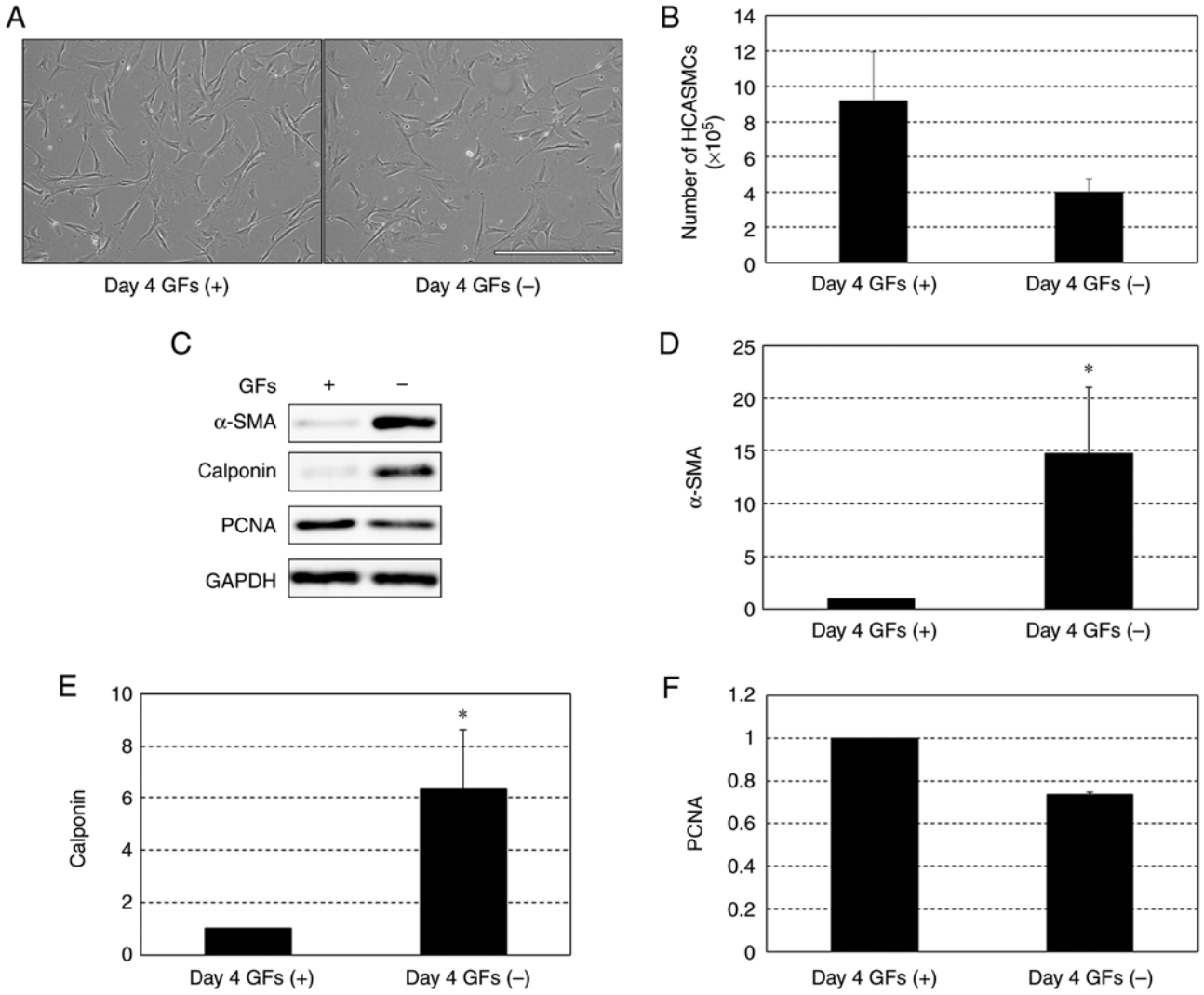

Figure 3. Protein expression levels of α-SMA, calponin and PCNA in HCASMCs in the presence and absence of GFs, including epidermal GF, fibroblast GF-B and insulin, on day 4 after seeding at 10% confluency. (A) Representative phase-contrast images (x10 magnification) on day 4 of HCASMCs cultured in the presence and absence of GFs. Scale bar, 200 μm. (B) Numbers of HCASMCs on day 4 in the presence and absence of the GFs. (C) Representative western blots for α-SMA, calponin, PCNA and GAPDH expressed in HCASMCs on day 4 in the presence and absence of GFs. Densitometrical analysis of expression levels of (D) α-SMA, (E) calponin and (F) PCNA. Data are expressed as the ratio to GAPDH-corrected levels of each protein in the presence of GFs and are shown as the mean ± SEM of three independent experiments. *P<0.05 vs. GFs (+). SMA, smooth muscle actin; PCNA, proliferating cell nuclear antigen; HCASMC, human coronary artery smooth muscle cell; GF, growth factor.

of α-SMA and PCNA on day 5 was similar to that on day 2 (Fig. 4B, C and E). On the other hand, in the cells with daily replacement with fresh medium, levels of calponin on day 5 were significantly higher than those on day 2 (Fig. 4D). This change in calponin expression levels was similar to the change under a culture condition without daily medium replacement: expression levels of calponin in the cells cultured without daily medium replacement were significantly higher on day 5 than those on day 2.

Effects of growth factors on protein expression levels of caldesmon, SM22α/tagln and S100A4 in HCASMCs after reaching 100% confluency. Protein expression levels of caldesmon, SM22 α/tagln and S100A4 were compared on days 2 and 5 with and without daily medium replacement (Fig. 5). The expression levels of caldesmon and SM22α in cells without medium replacement were significantly higher on day 5 than on day 2. The levels of caldesmon and SM22α on day 5 in the cells with daily medium replacement were significantly lower than in the cells without medium replacement (Fig. 5A-C). The expression levels of S100A4 on day 5 in cells without medium replacement tended to be lower than the expression levels on day 2 but this was not significant. The expression levels of S100A4 on day 5 in the cells with daily medium replacement tended to be higher than in cells without medium replacement and were comparable to those on day 2 (Fig. 5A and D); however, these results were not significant.

Effects of growth factors on migration of HCASMCs after reaching 100% confluency. Cell migration activity on day 5 without medium replacement was significantly decreased compared with that on day 2. Cell migration on day 5 with daily medium replacement was significantly higher than that without medium replacement and was comparable to that on day 2 (Fig. 6A and B).

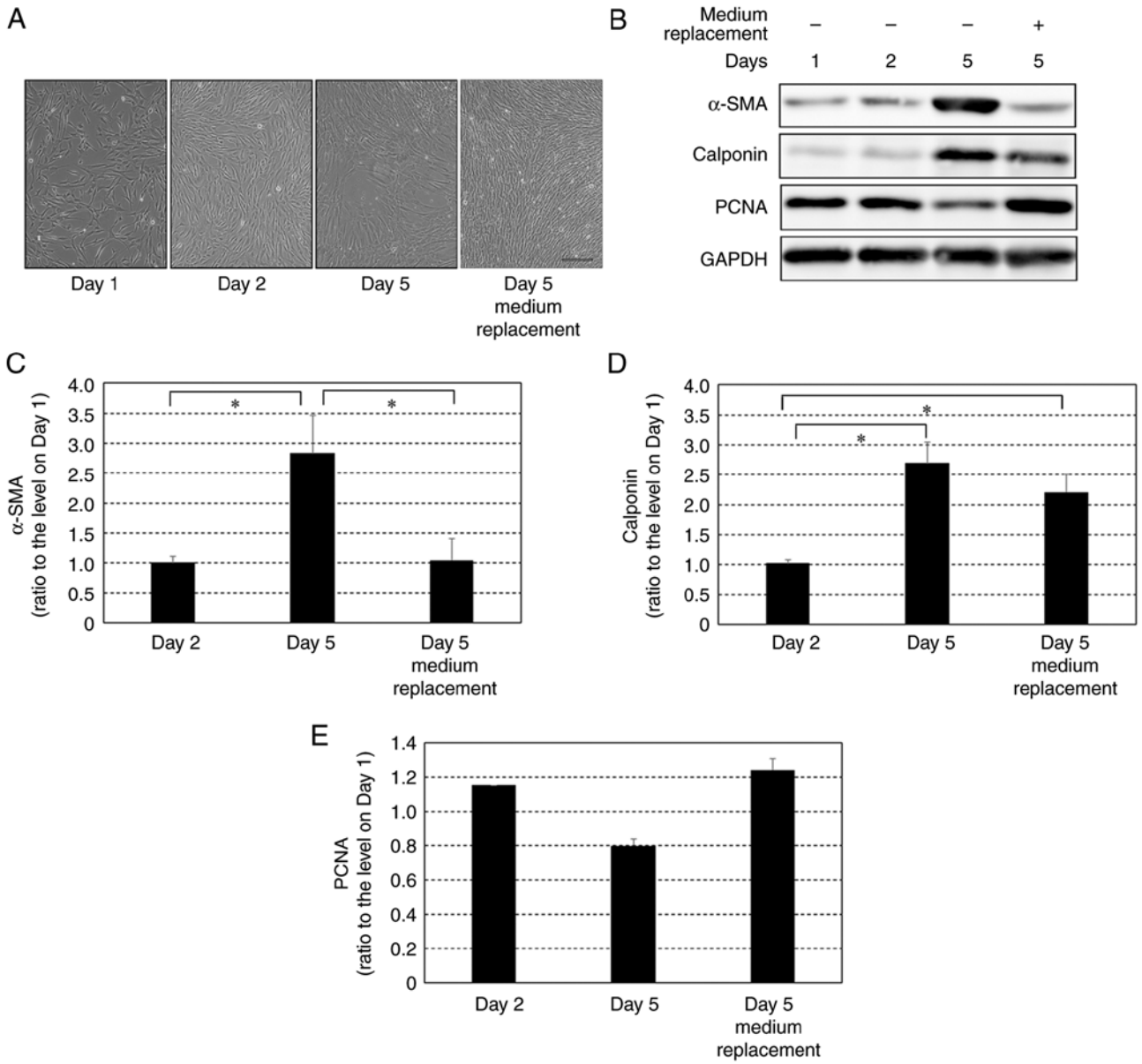

Figure 4. Effects of medium replacement on protein expression of α-SMA, calponin and PCNA in HCASMCs after reaching 100% confluency. (A) Representative phase-contrast images (x10 magnification) of HCASMCs on days 1, 2 and 5 (with and without medium replacement) after seeding at 90% confluency. Scale bar, 200 μm. (B) Representative western blots for α-SMA, calponin, PCNA and GAPDH. Densitometrical analysis of expression of (C) α-SMA, (D) calponin and (E) PCNA. Data are expressed as the ratio to GAPDH-corrected levels of each protein on day 1 and are shown as the mean ± SEM of three independent experiments. *P<0.05. SMA, smooth muscle actin; PCNA, proliferating cell nuclear antigen; HCASMC, human coronary artery smooth muscle cell.

Discussion

In the present study, HCASMCs cultured post-100% confluence without fresh medium replacement showed increased expression levels of contractile phenotype markers and decreased expression levels of proliferative phenotype markers. These results suggested that redifferentiation was induced in post-confluent HCASMCs. Similar changes in protein expression levels were observed in HCASMCs cultured in medium without growth factors before reaching 100% confluency. In the presence of a fresh medium including growth factors, the aforementioned changes in expression of phenotype marker proteins, except for calponin, were not observed in post-confluent HCASMCs. Therefore, deprivation of growth factors was required for induction of redifferentiation of HCASMCs. This is consistent with the results of a previous study showing that serum deprivation induces redifferentiation of umbilical artery SMCs (6) since serum contains growth factors. The present study investigated changes in migration, which is a representative functional characteristic of proliferative phenotype vascular SMCs, of HCASMCs. Post-confluent HCASMCs showed lower migration activity than that cells at 100% confluency; this decreased migration activity was not found in the post-confluent cells when medium was exchanged with a fresh medium every day. Accordingly, the results of both protein expression and migration activity indicated that deprivation of growth factors induces redifferentiation of HCASMCs. Similar changes in expression of contractile phenotype markes such as α-SMA and SM22α

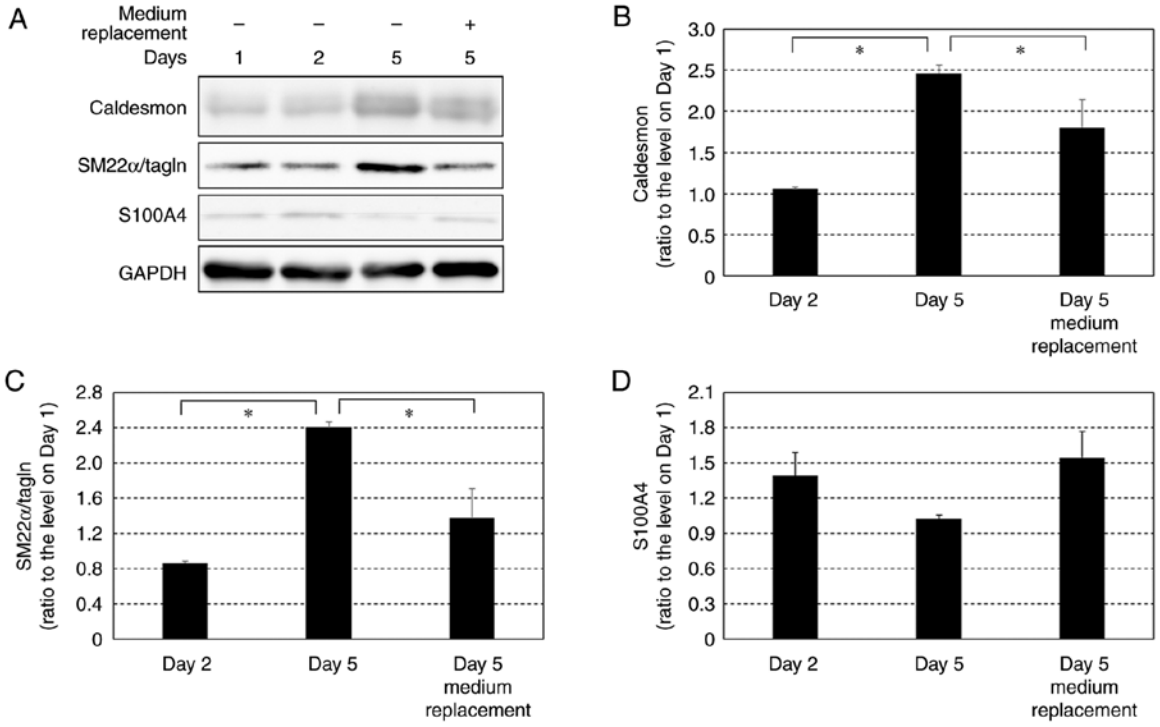

Figure 5. Effect of medium replacement on protein expression of caldesmon, SM22α and S100A4 in HCASMCs after reaching 100% confluency. (A) Representative western blots for caldesmon, SM22α, S100A4 and GAPDH. Densitometrical analysis of expression of (B) caldesmon, (C) SM22α and (D) S100A4. Data are expressed as the ratio to GAPDH-corrected levels of each protein on day 1 and are shown as the mean ± SEM of three independent experiments. *P<0.05. HCASMC, human coronary artery smooth muscle cell.

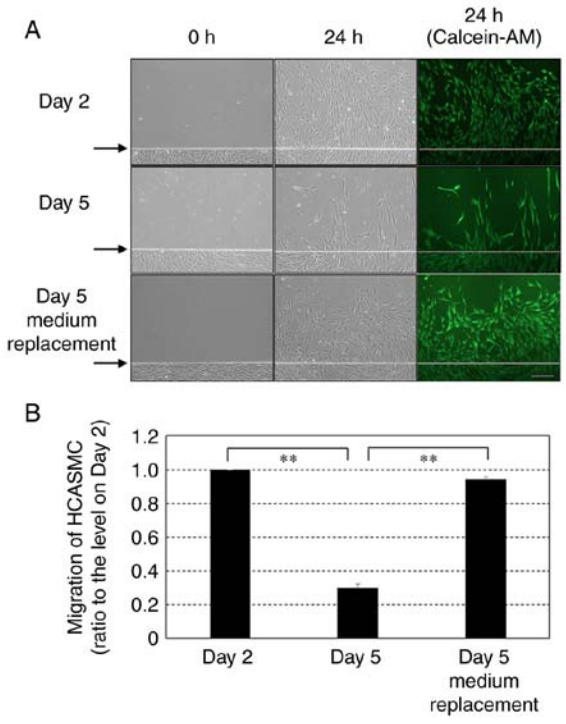

Figure 6. Effect of medium replacement on migration activity of HCASMCs after reaching 100% confluency. (A) Representative phase-contrast and fluorescence images (x10 magnification) of the migration assay. HCASMCs from days 2 and 5 (with and without medium replacement) after seeding at 90% confluency were compared before (0 h) and 24 h after injury. Scale bar, 200 μm. (B) Numbers of cells that migrated at 24 h after injury from days 2 and 5 with and without medium replacement. Data are corrected as the ratio to the number of migrating cells from day 2 and are shown as the mean ± SEM of three independent experiments. **P<0.01. HCASMC, human coronary artery smooth muscle cell; AM, acetoxymethyl ester.

and migration activity were shown in RASMCs in which redifferentiation was induced by serum deprivation (8). To the best of our knowledge, the present study is the first to determine experimental conditions for inducing redifferentiation of HCASMCs *in vitro*.

The present study demonstrated that deprivation of growth factors, including EGF, FGF-B and insulin, induced redifferentiation of HCASMCs. Redifferentiation of SMCs has been demonstrated in post-PTCA neointimal tissues in a human specimen (11). Blockade of signals stimulated by growth factors may facilitate redifferentiation of SMCs at loci of PTCA and in atherosclerotic plaque (11). This indicates potential therapeutic application of gene suppression of growth factors and their receptors in HCASMCs at atherosclerotic lesions.

A novel finding of this study was the difference in marker proteins for differentiation and redifferentiation. Calponin and α-SMA are protein markers of differentiated (contractile phenotype) SMCs (12-14). However, the present study obtained different results regarding calponin and α-SMA in cells with and without daily replacement with a fresh medium: Compared with expression level of calponin in confluent HCASMCs, the calponin expression was increased in post-confluent dedifferentiated HCASMCs in the presence of fresh medium, although calponin is a phenotype marker of differentiated arterial SMCs. On the other hand, α-SMA expression level was not different in confluent and post-confluent dedifferentiated HCASMCs in the presence of growth factors. In addition, the present study investigated expression levels of caldesmon and SM22α, other protein markers of differentiated-phenotype SMCs. Similar to

the results of α-SMA, levels of caldesmon and SM22α in post-confluent cells with daily medium replacement were not significantly different from those in confluent cells. These results suggested that α-SMA, caldesmon and SM22α, but not calponin, may serve as markers of redifferentiation of HCASMCs. Thus, medium replacement maintained HCASMC dedifferentiation after 100% confluency, while expression of calponin, a marker of differentiated arterial smooth muscle cells, was increased in post-confluent cells in the presence of growth factors. This means that calponin expression is more prone to be upregulated in post-confluent HCASMCs and is less affected by stimulation with growth factors compared with other contractile-phenotype markers such as α-SMA, caldesmon and SM22α. However, the reason for these findings remains unknown and future studies are needed. Levels of calponin, α-SMA, caldesmon and SM22α were all significantly higher in post-confluent cells without medium replacement than in confluent cells. Therefore, one potential explanation for the aforementioned difference in results is a difference in signal transduction of growth factor-induced inhibition of gene transcription of calponin and other contractile-phenotype marker proteins. Myocardin, a transcriptional coactivator of serum response factor, serves key roles in differentiation of SMCs (18,19) and expression levels of α-SMA, SM22 and calponin have been shown to be similarly affected by modulating myocardin activity in human aortic SMCs (20). Therefore, it is a possibility that a myocardin-independent pathway is involved in the expression of contractile-phenotype proteins in redifferentiated HCASMCs. Expression levels of α-SMA in post-confluent HCASMCs with daily medium replacement were comparable to those in the confluent cells, while expression levels of caldesmon and SM22α were slightly but not significantly higher in post-confluent cells with daily medium replacement than in confluent cells. Thus, α-SMA may be a more sensitive marker of growth factor-dependent redifferentiation of HCASMCs than caldesmon and SM22α. Although it would be ideal to confirm α-actin as a marker for redifferentiation of HCASMCs functionally by using RNA interference-α-SMA *in vitro*, the present study did not perform such an experiment due to hypothesized damage to cells by loss of α-SMA, a fundamental cytoskeletal element (21).

In conclusion, deprivation of growth factors, including EGF, FGF-B and insulin, from culture medium induced redifferentiation of HCASMCs. α-SMA, caldesmon and SM22α, but not calponin, may serve as markers for redifferentiation of HCASMCs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RS, RE and IW designed the study. RS performed the experiments and data analysis. RE and IW contributed to data interpretation. RS, RE and IW confirm the authenticity of all the raw data. IW drafted the manuscript. RS and RE reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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