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Three-Dimensional "Honeycomb" Culture System that Helps to Maintain the Contractile Phenotype of Vascular Smooth Muscle Cells

Itsuko Ishii and Masashi Uchida

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Abstract

Vascular smooth muscle cells (VSMCs) in the normal aorta are described as having a contractile phenotype because they can contract and do not proliferate. VSMCs in pathological conditions such as atherosclerosis and restenosis can proliferate and migrate, but lose their ability to contract, which is referred to as a synthetic phenotype. VSMCs show plasticity by changing their phenotype according to the surrounding environment. When VSMCs are cultured on a plastic plate, which is a normal two-dimensional culture system, they display the synthetic phenotype because they proliferate and migrate without contraction. Recently, we successfully cultured VSMCs that display features similar to the contractile phenotype, using type I collagen three-dimensional matrices, "honeycombs," in the presence of abundant fetal bovine serum albumin. VSMCs cultured in honeycombs stop proliferating and can contract. The honeycomb culture system can maintain VSMCs in the contractile phenotype for a long period of time. In this chapter, we show the method of this new culture system and the characteristics of VSMCs in honeycombs. It is expected that the use of this culture system will generate new information on the characteristics of VSMCs.

Keywords: smooth muscle cell, three-dimensional culture, collagen, proliferation, contraction

1. Introduction

Vascular smooth muscle cells (VSMCs) are the major cell type in the vascular wall and their main role is contraction. VSMCs in the normal aorta are classified as having a contractile phenotype because they contract without proliferation. Conversely, VSMCs in vascular diseases, such as restenosis after percutaneous coronary intervention and the formation of atherosclerotic plaques, are described as having a synthetic phenotype because they can proliferate and migrate, but lose their ability to contract. The phenotypic modulation of VSMCs depends on the surrounding environment [1].

In order to conduct basic studies on the physiological function of VSMCs and to develop novel medical treatments, cultured VSMCs are used. When VSMCs are cultured on a plastic plate in the presence of 10% fetal bovine serum (FBS), which is the normal two-dimensional monolayer culture system, the cells can migrate and proliferate, but lose their contractile ability [2]. Cultured VSMCs are classified as having a synthetic phenotype and are used as a model of atherosclerotic lesion cells. As phenotypic modulation of VSMCs is responsible for restenosis and the progression of atherosclerosis, they could be the main target of medicinal treatment, and VSMCs cultured on plates are useful to evaluate the effects of medicines. However, there is no acceptable cell model that reflects the nature of VSMCs in the normal aorta of a living body. For this reason, information generated from synthetic VSMCs cannot be compared with that from contractile VSMCs in the normal aorta. In this chapter, we describe a new culture system for VSMCs that maintain their contractile phenotype.

2. Method of culture of VSMCs in type I collagen three-dimensional "honeycomb" matrices

We reported a method for culturing contractile VSMCs by using three-dimensional matrices, so-called "honeycombs." Honeycombs are type I collagen sponges that can be obtained from Koken Co., Ltd. (Japan). Honeycombs are prepared from 0.5% type I atelocollagen in an acid solution by neutralization with ammonia gas to separate the collagen fibrils and lyophilization [3]. The structure of the honeycomb is porous and consists of many tubes aligned side by side, similar to a beehive [4, 5]. The pore diameter of the honeycomb is controlled by altering the concentration of the collagen solution and ammonia gas. When a higher concentration of ammonia gas is used, smaller pores are produced, and vice versa [3]. The diameter of each pore of the tubes is 100–500 μm, and we usually use a pore size of 200–300 μm for rabbit and human VSMCs. The honeycombs are cut vertically into cubes of dimensions 5 × 5 × 2 or 3 × 3 × 2 mm for culturing VSMCs.

The initial culture of VSMCs in a honeycomb is as follows (Figure 1): rabbit or mouse VSMCs cultured on plates (synthetic phenotype) in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS are used. The honeycombs are pre-incubated with DMEM containing 10% FBS and the air in each pore of the honeycomb is removed by centrifugation. VSMCs cultured on plates are incubated with trypsin-EDTA, and the released cells are collected by centrifuga-

tion. The collected cells are suspended in 300–400 μL DMEM containing 10% FBS and are incubated with the honeycombs (approximately 2.0 – 3.0 × 10^6 cells per 30 honeycombs) on a dish (diameter, 6 cm) for 3 h at 37°C. Then, 3–5 mL culture medium is added. The medium is changed every 2–3 days. For human VSMCs, a commercial medium supplemented with epidermal growth factor, fibroblast growth factor-B, insulin, and 2% FBS is used.
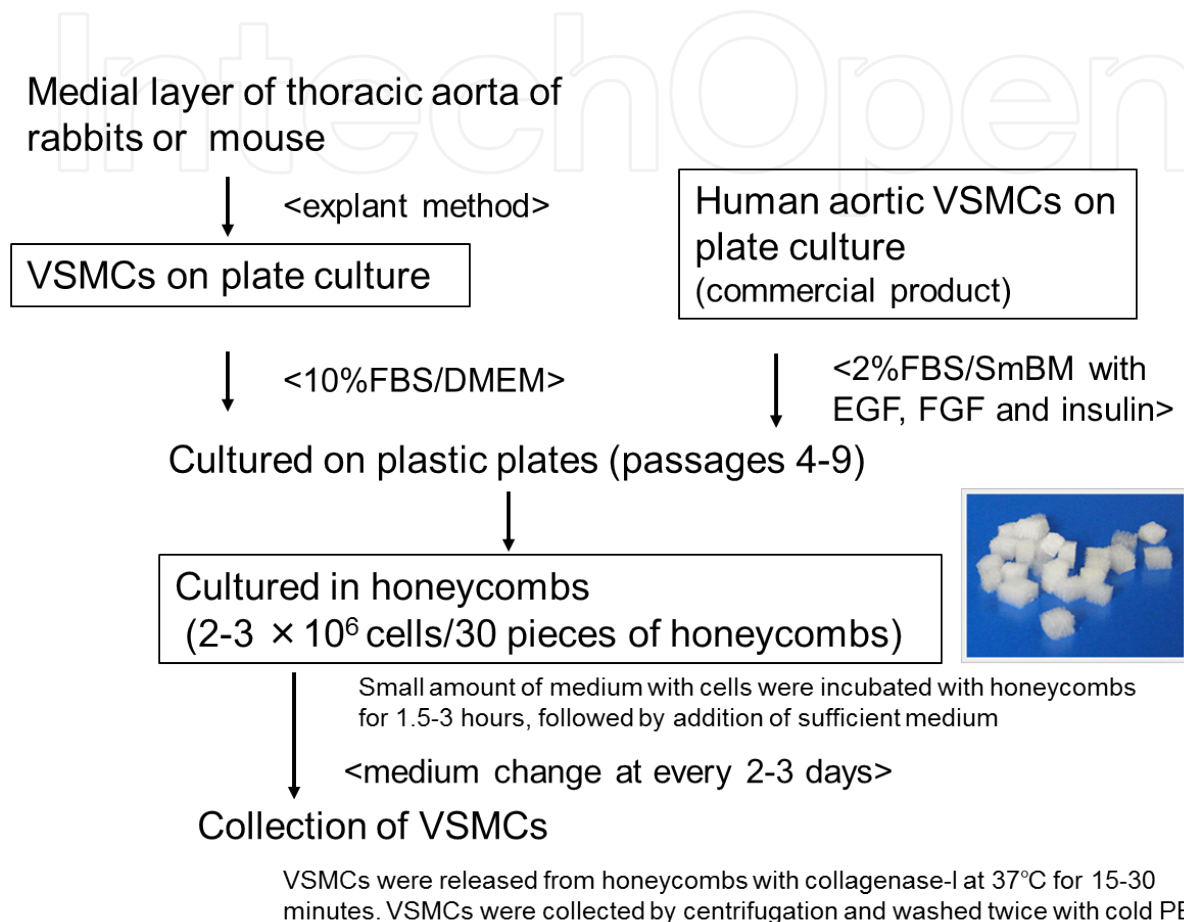

Figure 1. Preparation of VSMCs into honeycombs. Rabbit, mouse, and human VSMCs can be cultured successfully in honeycombs with this chart. The required number of cells are cultured on a plate as synthetic VSMCs and transferred to the honeycombs. To collect VSMCs in honeycombs for analysis, the honeycombs are treated with collagenase-I.

By visualizing the binding process of VSMCs to honeycombs using a real-time cultured cell monitoring system [6], VSMCs are fixed at one attachment point and move little by little using other attachment points, resembling the movement of an inchworm. After 25 h incubation, the positions of the VSMCs are fixed, and they form cross-bridges in the honeycombs. VSMCs in honeycombs form 2–5 attachment points per cell (Figure 2). However, mouse VSMCs (length of long axis, 33–139 [80 ± 29] μm) do not make cross-bridges in honeycombs in which the diameter of the pore is about 500 μm. These results suggest that the relationship between pore size and the length of the long axis of VSMCs is important for the formation of cellular cross-bridges. The inner wall of honeycombs with a larger pore size may function as a "flat floor" for VSMCs, as if it were a plastic plate.
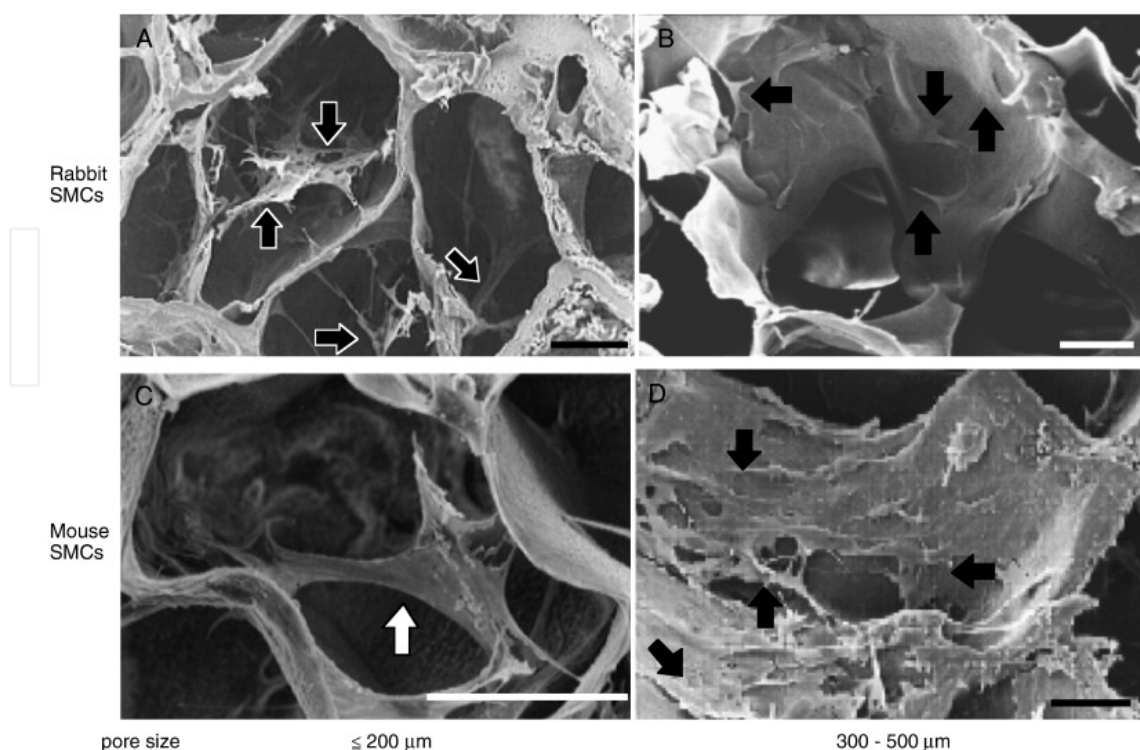
Figure 2. Cross-bridges of VSMCs on honeycombs. These pictures are electron microscopic observations of rabbit and mouse VSMCs cultured in honeycombs. (A) Rabbit VSMCs cultured in honeycombs of pore size ≤200 μm; (B) rabbit VSMCs cultured in honeycombs of pore size 300–500 μm; (C) mouse VSMCs cultured in honeycombs of pore size ≤200 μm; and (D) mouse VSMCs cultured in honeycombs of pore size 300–500 μm. Arrows show VSMCs. Scale bars, 50 μm. Data adapted from reference 6.

3. Proliferative inhibition of VSMCs cultured in honeycombs

Various kinds of cells, such as human fibroblasts, CHO-K1, BHK-21, and bovine endothelial cells [3], can be cultured successfully in honeycombs. These cells grow normally in honeycombs. However, the proliferation of VSMCs can be controlled easily by the pore size of the honeycombs.

When synthetic rabbit VSMCs are used for culture in honeycombs (pore size, about 200 μm), they stop proliferating immediately when they form cross-bridges [4]. [^{3}H]Thymidine is incorporated at a low level into VSMCs cultured in honeycombs, and cell number does not change during culture. VSMCs can be cultured in honeycombs for approximately 3 months with medium change. However, when rabbit VSMCs are cultured in honeycombs with larger pore size (100–500 μm) (Figure 3), they proliferate for the first few days, but then stop proliferating and cell number does not change [6]. The reason why cell number increases only at the beginning of culture is as follows: in honeycombs with a smaller pore (≤200 μm), VSMCs form cross-bridges independently of each other, but a large number of connected cells form cross-bridges together at the wall of honeycombs with larger pores (100–500 μm) after the initial increase in cell number at the beginning of culture. These data suggest that the formation

of cross-bridges by VSMCs in honeycombs may be a significant step for the cessation of proliferation.
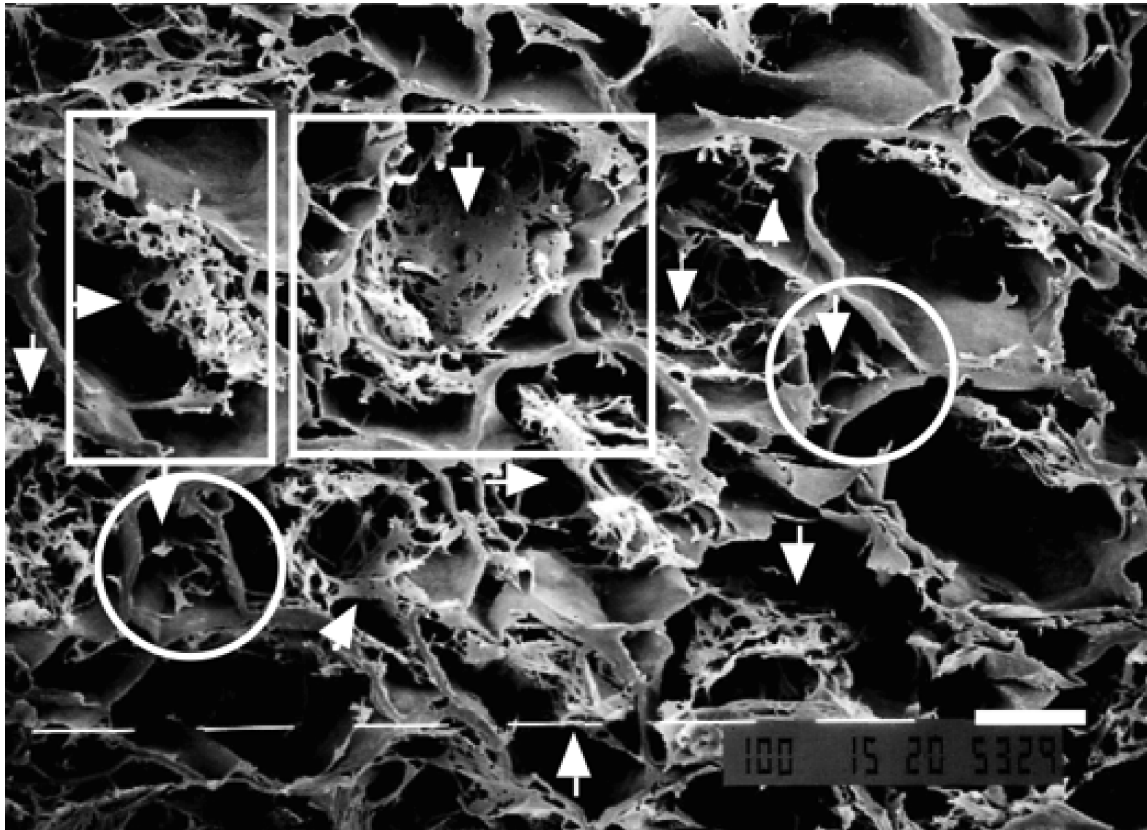

Figure 3. Cross-bridges of rabbit VSMCs cultured in honeycombs for 14 days. Electron microscopic observation of rabbit VSMCs cultured in honeycombs for 14 days. Rabbit VSMCs were cultured in honeycombs of pore size 100–500 μm. The pores enclosed by squares contain a large number of VSMCs, and the pores enclosed by circles contain a small number of VSMCs forming cross-bridges. Arrows show VSMCs. Scale bar, 100 μm. Data adapted from reference 6.

When VSMCs are cultured on collagen-coated plates, their proliferation rate increases. Conversely, when VSMCs are cultured on collagen gels, their proliferation stops via the cdk2 inhibitor p27^{kip1} [7]. However, proliferative inhibition persists for less than 1 week because VSMCs dissolve the collagen gel and start to proliferate again (unpublished data). Therefore, it is expected that both the higher-order structure of collagen and pore size of the honeycombs are considerable factors for the proliferative inhibition of VSMCs.

Although VSMCs in honeycombs stop proliferating immediately, p27^{kip1} expression increases after incubation for 2–3 days (unpublished data). From this observation, it is expected that p27^{kip1} may work to keep VSMCs at a resting state and not as the initiator of proliferative inhibition. Taken together with electron microscopic observations and data for growth in honeycombs, proliferative inhibition occurs in parallel with a decrease in the number of focal adhesions, including reduced levels of focal adhesion kinase (FAK). As a high level of phosphorylated FAK promotes proliferative activity [8], the low level of phosphorylated FAK is one of the reasons for proliferative inhibition of VSMCs in honeycombs.

An additional mechanism for the proliferative inhibition of VSMCs in this three-dimensional culture system is the expression of ornithine decarboxylase antizyme 1 (OAZ1), which is a key regulator of intracellular polyamines. Polyamines (putrescine, spermidine, and spermine), which are multivalent organic cations, are essential for cell growth [9]. The proliferation and transformation of cells induced by oncogenes, carcinogens, and viruses are characterized by increases in the levels of intracellular polyamines due to their increased biosynthesis and uptake [10]. Ornithine decarboxylase (ODC) is the rate-limiting enzyme of polyamine biosynthesis. OAZ1 inhibits the activity of ODC and increases its degradation by forming an OAZ1-ODC complex [11, 12]. OAZ1 also decreases the uptake of polyamines independent of its effects on ODC [13, 14].

When VSMCs proliferate on plates, the intracellular content of polyamines increases. Conversely, polyamine content is maintained at a low level in VSMCs cultured in honeycombs [15]. VSMCs stably transfected with the ODC gene (ODC-VSMCs) and cultured on plates increase their rate of proliferation, which is accompanied by an increase of polyamine content (especially spermidine) and phosphorylated FAK and a decrease in marker proteins of differentiation, α-actin, and myosin heavy chain in comparison to VSMCs cultured on plates (Figure 4). As ODC is an oncogene and induces an excess of polyamines [16], it is assumed that ODC may promote the proliferative activity of VSMCs cultured in honeycombs. However, the proliferation of ODC-VSMCs also ceases in honeycombs, similar to normal VSMCs with low levels of spermidine and phosphorylated FAK (Figure 4). Finally, culture of ODC-VSMCs in honeycombs increases the expression of α-actin and myosin heavy chain.

As shown in Figure 4, OAZ1 levels are higher in both VSMCs and ODC-VSMCs cultured in honeycombs than in both VSMCs and ODC-VSMCs cultured on plates. OAZ1 is degraded in ODC-VSMCs cultured on plates for up to 3 h after treatment with cycloheximide, but OAZ1 degradation in ODC-VSMCs cultured in honeycombs is limited over a 12 h incubation period (Figure 4). This difference in OAZ1 stability may be the major reason for the high levels of OAZ1 observed in VSMCs cultured in honeycombs. These results suggest that OAZ1 in VSMCs cultured in honeycombs might enhance the degradation of ODC and inhibit polyamine uptake. In fact, our data show that polyamine uptake is significantly lower in ODC-VSMCs cultured in honeycombs than in ODC-VSMCs cultured on plates [15]. This could be one of the reasons for the lower polyamine levels in VSMCs cultured in honeycombs than in VSMCs cultured on plates.

When OAZ1 is transiently overexpressed in ODC-VSMCs (Figure 5), OAZ1 decreases the number of ODC-VSMCs cultured on plates at day 3, whereas OAZ1 overexpression does not change the number of ODC-VSMCs cultured in honeycombs. OAZ1 overexpression slightly decreases the expression of ODC and phosphorylated FAK levels in ODC-VSMCs cultured on plates. Surprisingly, OAZ1 overexpression influences the levels of myosin heavy chain in ODC-VSMCs cultured on plates and in honeycombs, and influences the levels of α-actin in ODC-VSMCs cultured in honeycombs. These results suggest that OAZ1 in VSMCs might regulate intracellular polyamine levels and cellular proliferation. Moreover, OAZ1 levels may influence the expression of α-actin and myosin heavy chain, which are the main components of the contractile apparatus.
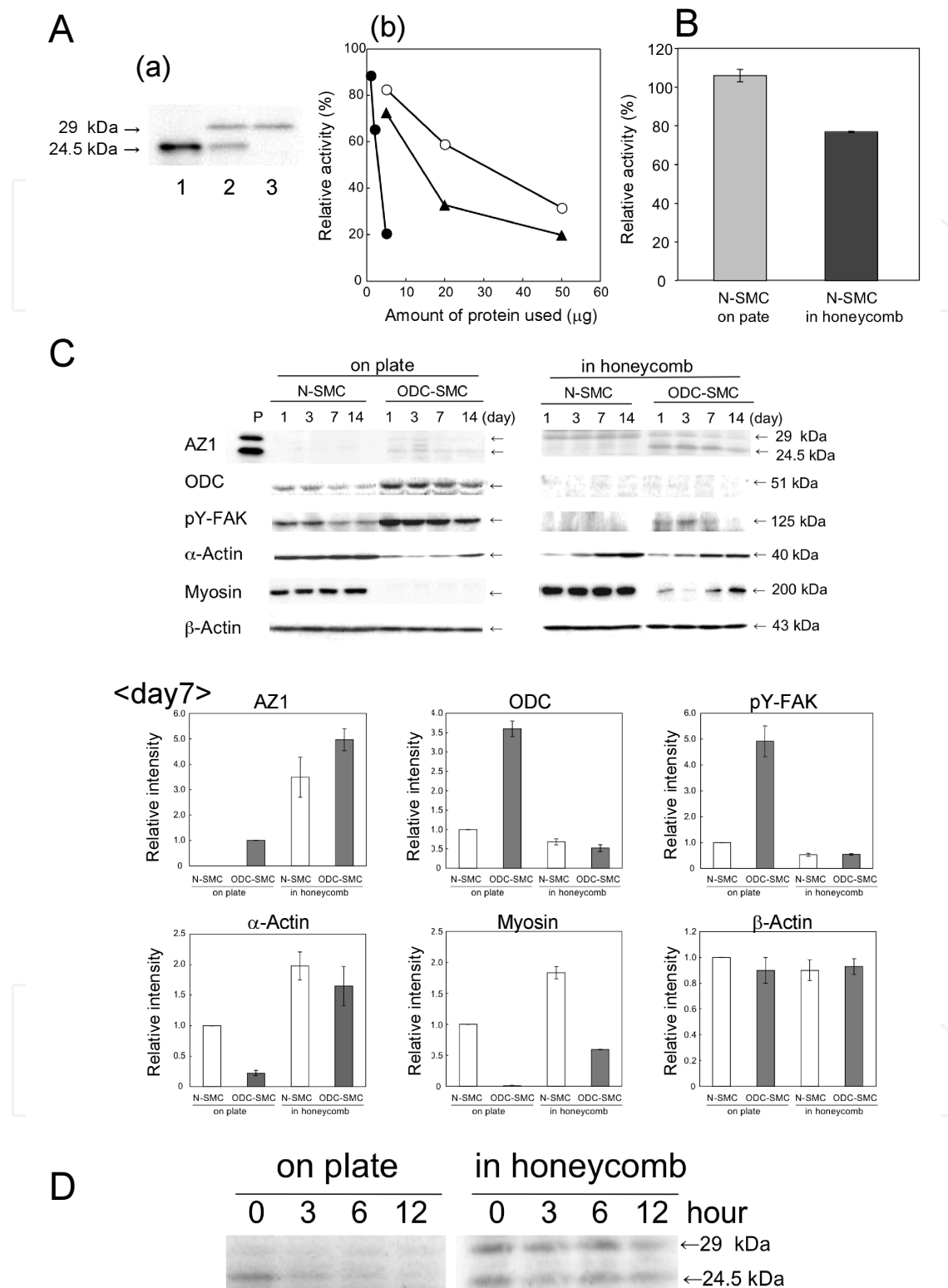
Figure 4. OAZ1 in normal VSMCs and ODC-VSMCs, and the levels of various proteins expressed in VSMCs cultured on plates and in honeycombs. (A) (a) VSMCs were transfected with three kinds of pTracer-CMV containing OAZ1 without T205, which do not need a frameshift for their expression of each type of OAZ1. Lane 1, pTracer-OAZΔT205ΔAUG1, which expresses 24.5-kDa OAZ1; lane 2, pTracer-OAZΔT205, which expresses 24.5- and 29-kDa

OAZ1; lane 3, pTracer-OAZΔT205ΔAUG2, which expresses 29-kDa OAZ1. After transfection, VSMCs were collected at 24 h, a cell extract was prepared, and Western blot analysis was performed. (b) Degree of inhibition of ODC by OAZ1 was measured. The activity of OAZ1 was determined as an inhibitory percentage of ODC activity of the extract from ODC-overproducing FM3A (EXOD-1) cells. ●, extract from VSMCs expressing 24.5-kDa OAZ1; ▲, extract from VSMCs expressing 29- and 24.5-kDa OAZ1; and ○, extract from VSMCs expressing 29-kDa OAZ1. Values are means of duplicate determinations. (B) Inhibition of ODC activity using extracts from VSMCs cultured on plates and in honeycombs. OAZ1 activity was determined as an inhibitory percentage of ODC activity of the extract from EXOD-1 cells. Values are means ± standard deviation (SD) of triplicate determinations. (C) Western blot analysis of OAZ1, ODC, tyrosine-phosphorylated FAK (pY-FAK), α-actin, myosin heavy chain, and β-actin of VSMCs and ODC-VSMCs cultured on plates and in honeycombs. P, EXOD-1 cells as a positive control. Relative intensity on day 7 was quantified. The intensity of OAZ1 was quantified as the sum of the 29- and 24.5-kDa bands. ND, not detectable. Values are means ± SD of triplicate determinations. (D) OAZ1 degradation. ODC-VSMCs cultured on plates and in honeycombs for 3 days were treated with 20 μg/mL cycloheximide for the indicated time. Data adapted from reference 15.

VSMC proliferation is reportedly up-regulated via an increase in the stability of S-phase kinase-associated protein-2, E3 ubiquitin protein ligase (SKP2) by autophosphorylation of FAK-Tyr397 [17]. In ODC-VSMCs cultured on plates, the increased rate of proliferation is accompanied by an increase of phosphorylated FAK. Interestingly, when the effect of spermine on FAK autophosphorylation *in vitro* was investigated, FAK phosphorylation was stimulated by spermine [15]. The low levels of intracellular polyamines in VSMCs cultured in honeycombs could inhibit the autophosphorylation of FAK, and this may contribute to the proliferative inhibition of VSMCs.

4. Differentiation of VSMCs cultured in honeycombs

Caldesmon is an actin-linked regulatory protein, and caldesmon heavy chain (h-CaD) is expressed abundantly and specifically in contractile VSMCs. h-CaD localizes to the actomyosin contractile structure in VSMCs as an integral component of smooth muscle thin filaments [18]. Caldesmon also works as a potent repressor of cancer cell invasion because its ectopic expression reduces the number of podosomes/invadopodia and decreases extracellular matrix degradation. The depletion of caldesmon facilitates the formation of podosomes/invadopodia and cell invasion [19]. As VSMCs cultured in honeycombs for 14 days express h-CaD [4, 6], these cells could be classified to the contractile phenotype.

Some other cells also differentiate in honeycombs. By usage of honeycomb scaffolds, rat axonal regeneration [20], differentiation of pluripotent embryonic stem (ES) cell-derived embryoid bodies (EBs) into hepatocyte-like cells [21], and differentiation of mesenchymal stem cells into osteoblasts [22] have been reported. For example, EBs formed from ES cells become liver-specific gene-positive and albumin-positive cells, and they form cord-like structures in honeycombs that are not present in two-dimensional monolayer culture systems [21]. When a honeycomb scaffold including EB-derived hepatocyte-like cells is transplanted into the median lobes of partially hepatectomized nude mice, cells that are positive for both albumin and cytokeratin 18 appear in the transplant and form clustered aggregates after 7 and 14 days. These data suggest that a honeycomb is a useful scaffold culture system for regulation of cellular differentiation and for advanced tissue engineering.
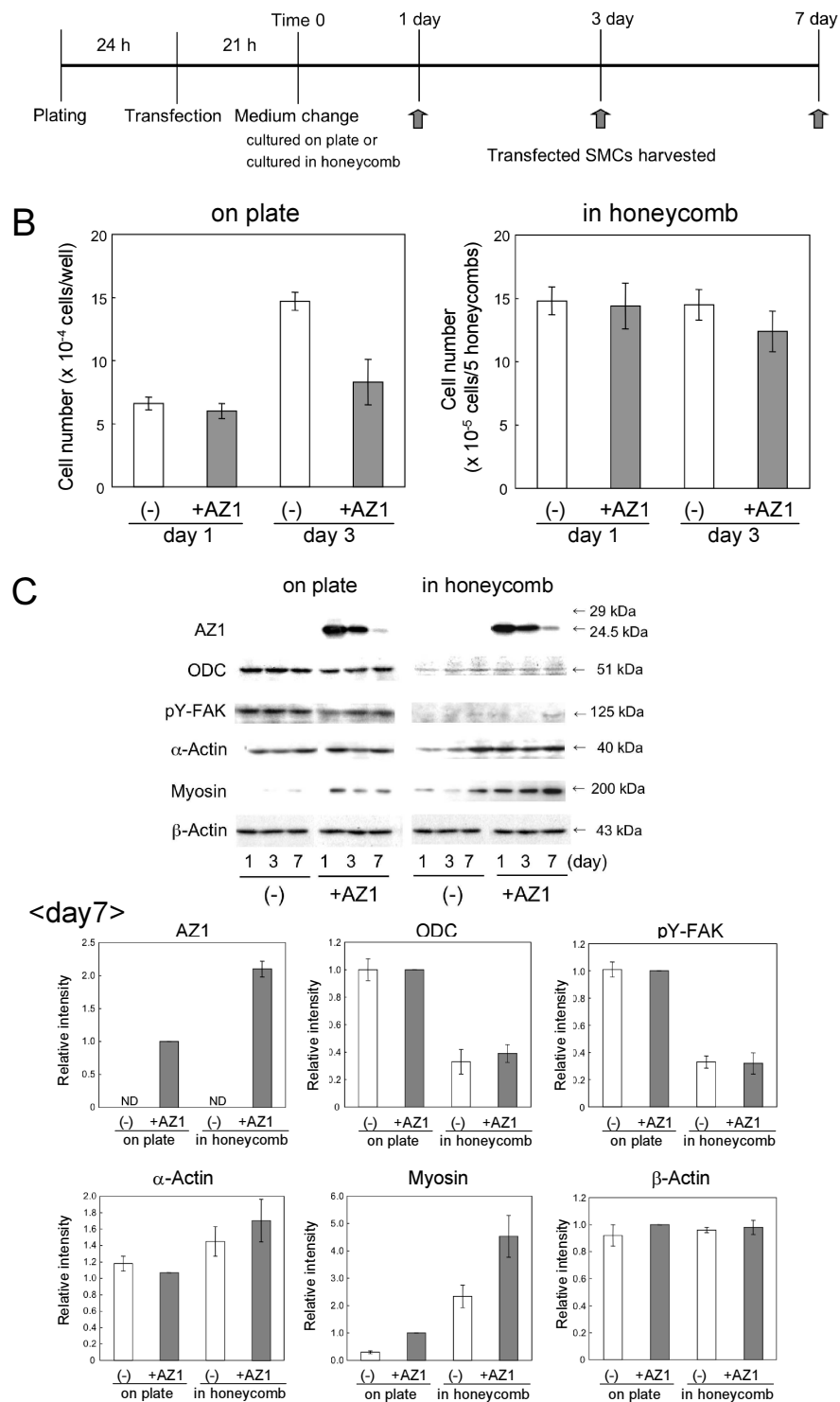
Figure 5. Effect of the transient expression of OAZ1 in ODC-VSMCs cultured on plates and in honeycombs. After ODC-VSMCs were transfected with pTracer-OAZΔT205ΔAUG1 (24.5-kDa OAZ1), the cells were cultured as shown in (A). Transfected ODC-VSMCs were then cultured either on plates or in honeycombs for 1, 3, and 7 days. (B) Cell number on days 1 and 3. (C) Western blot analysis of OAZ1, ODC, pY-FAK, α-actin, myosin heavy chain, and β-actin. Relative intensity of proteins on day 7 was quantified. Values are means ± SD of triplicate determinations. Data adapted from reference 15.

5. Contraction of VSMCs cultured in honeycombs

VSMCs cultured on plates express the main components of the contractile apparatus and cytoskeleton (including β-non-muscle actin filaments), but VSMCs cultured on plates do not contract under normal culture conditions [23]. The size of honeycombs containing VSMCs is significantly reduced over time [5], which could be due to the contraction of the VSMCs. A key protein for the contraction of VSMCs cultured in honeycombs is filamin. Filamin A has a molecular weight of 280 kDa and a molecular length of approximately 160 nm [24]. Filamin dimers link actin filaments into orthogonal networks or parallel bundles, and an *in vitro* study showed that the manner in which actin filaments are organized depends on the ratio of filamin to actin [25]. Filamin mainly co-localizes with the β-non-muscle actin filaments in gizzard SMCs [26, 27]. When ODC-VSMCs are used, the function of filamin in VSMCs becomes clear. Filamin expression is dramatically decreased in ODC-VSMCs compared to VSMCs (Figure 6). β-Non-muscle actin filaments in ODC-VSMCs cultured on plates are thinner than those of VSMCs, although β-actin expression, as determined by Western blot analysis, is almost the same in ODC-VSMCs as in VSMCs (Figure 6). These results suggest that the 280-kDa form of filamin is responsible for the bundling of β-non-muscle actin filaments in VSMCs.

It is well known that filamin is degraded by μ- and m-calpain [28, 29]. When VSMCs are cultured in honeycombs, 280-kDa filamin is degraded to 180-kDa filamin by calpains [5]. It is expected that degraded filamin cannot induce the bundling of actin filaments, resulting in the relative structural weakness of β-non-muscle actin filaments in VSMCs cultured in honeycombs. Observation of the intracellular distribution of filamin and β-actin by immunofluorescence microscopy shows that filamin is situated along fibers and co-localizes with β-actin in subconfluent VSMCs cultured on plates (Figure 7A and B). In honeycombs, filamin staining is detected evenly in the cytoplasm and is not stained as fibers in most cells (Figure 7C and D). It can be assumed that degraded filamin, which does not bind to β-non-muscle actin filaments, is present in the cytoplasm of VSMCs cultured in honeycombs.

The expression levels of proteins involved in both the contractile apparatus (α-actin, myosin heavy chain, and tropomyosin) and cytoskeleton (β-actin and α-actinin) in VSMCs cultured in honeycombs are almost identical to those in VSMCs cultured on plates [5]. However, VSMCs on plates express the 280-kDa form of filamin, whereas VSMCs cultured in honeycombs express both the 280- and 180-kDa forms of filamin. α-Actin and β-actin co-localize in subconfluent VSMCs cultured on plates and in VSMCs cultured in honeycombs, suggesting that the contractile apparatus is aligned with β-non-muscle actin filaments [5].

These data from VSMCs cultured in honeycombs could explain why contraction, which is a result of shortening of the contractile apparatus, is not observed in VSMCs cultured on plates. VSMCs attach to rigid plastic plates via focal adhesions, and the resulting cytoskeletal tension, which is maintained by β-non-muscle actin filaments, inhibits shortening of the contractile apparatus. In honeycombs, VSMCs can contract because filamin degradation reduces cytoskeletal tension and allows shortening of the contractile apparatus [5].
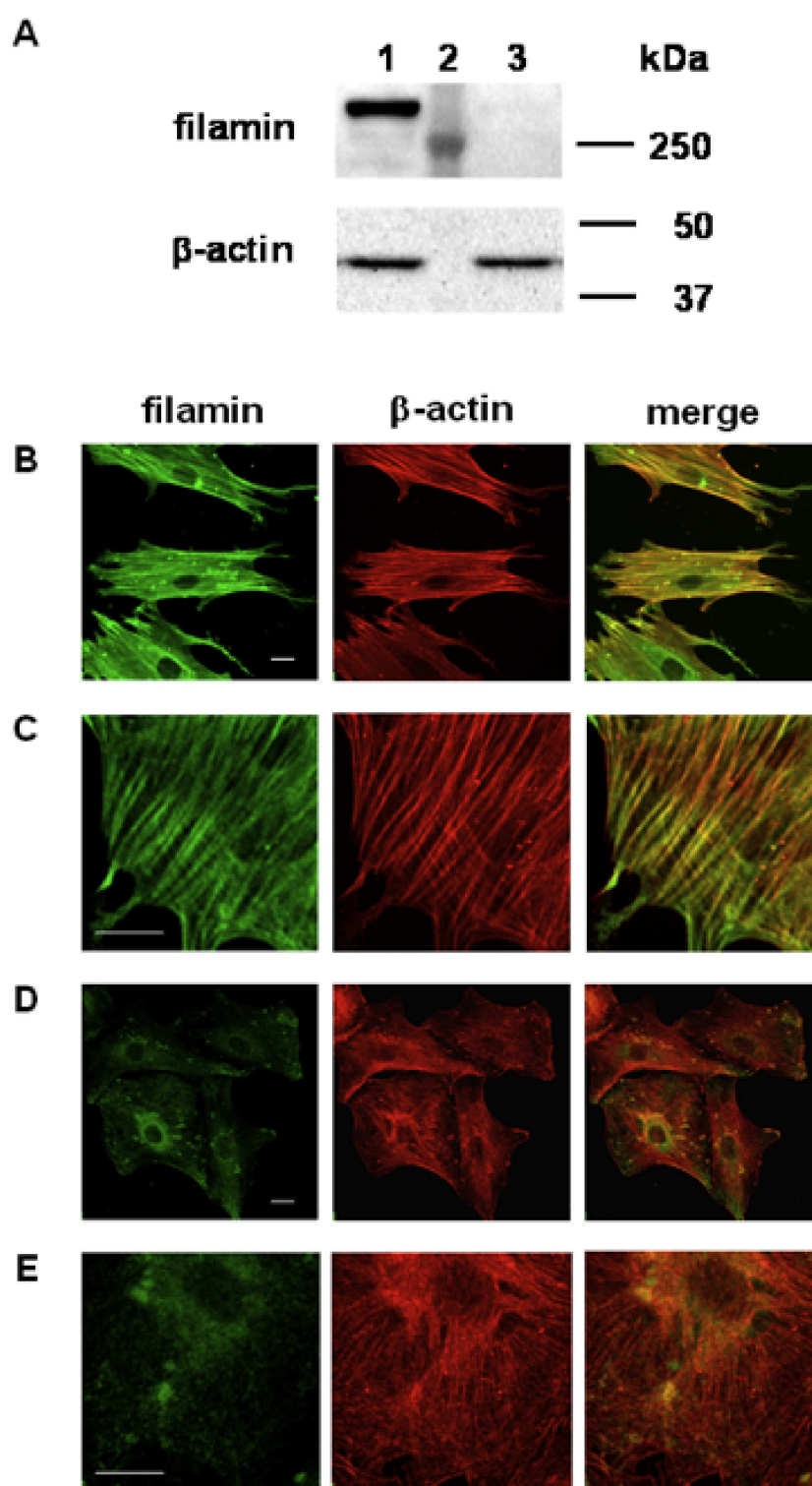
Figure 6. Expression and localization of filamin and β-actin in ODC-VSMCs. (A) Western blot analysis of filamin and β-actin in VSMCs and ODC-VSMCs. VSMCs and ODC-VSMCs were cultured on plates to subconfluence. Lane 1, VSMCs; lane 2, molecular marker; and lane 3, ODC-VSMCs. Localization of filamin and β-actin in VSMCs (B, C) and ODC-VSMCs (D, E). VSMCs and ODC-VSMCs were cultured on plates to subconfluence. Green indicates filamin, and red indicates β-actin. Scale bars, 20 μm (B, D); 5 μm (C, E). Data adapted from reference 5.
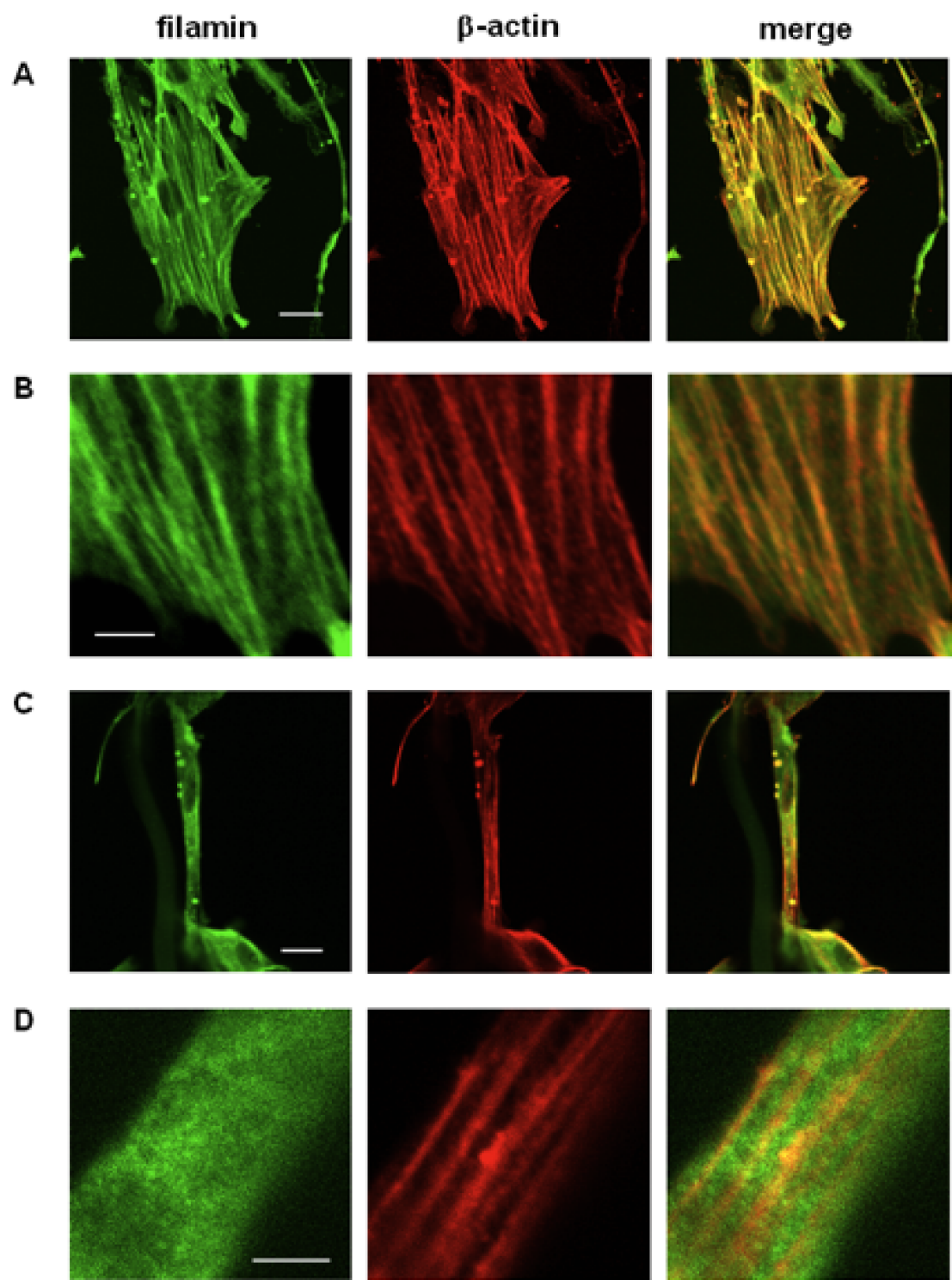
Figure 7. Localization of filamin and β-actin in VSMCs. (A, B) VSMCs were cultured to subconfluence on a plate. (C, D) VSMCs were cultured in honeycombs for 3 days. Green indicates filamin, and red indicates β-actin. Scale bars, 20 μm (A, C); 5 μm (B, D). Data adapted from reference 5.

When myocardial cells derived from newborn rats are cultured in honeycombs, the cells attach to the honeycombs and start beating from the first day. The rate of beating increases gradually,

and reaches the highest frequency of 162 beats per minute at day 8 [30]. However, the number of myocardial cells increases significantly. From these data, it is shown that the honeycomb scaffold may induce the functional aspects of cells even though it is unclear whether or not cells cultured in honeycombs proliferate.

6. Reversibility of VSMCs between the contractile and synthetic phenotypes

When VSMCs cultured in honeycombs are treated with collagenase-I and the released cells are seeded in plastic plates, they start to proliferate again [5]. This result shows the remarkable plasticity of VSMCs. The relationship between the culture system and phenotypic modulation is shown in Figure 8.

Filamin A mRNA expression is almost identical in VSMCs cultured in honeycombs and on plates, and VSMCs moved to plates mainly express the 280-kDa form of filamin after 12 h [5]. These observations indicate that filamin might contribute to the phenotypic modulation seen in VSMCs.
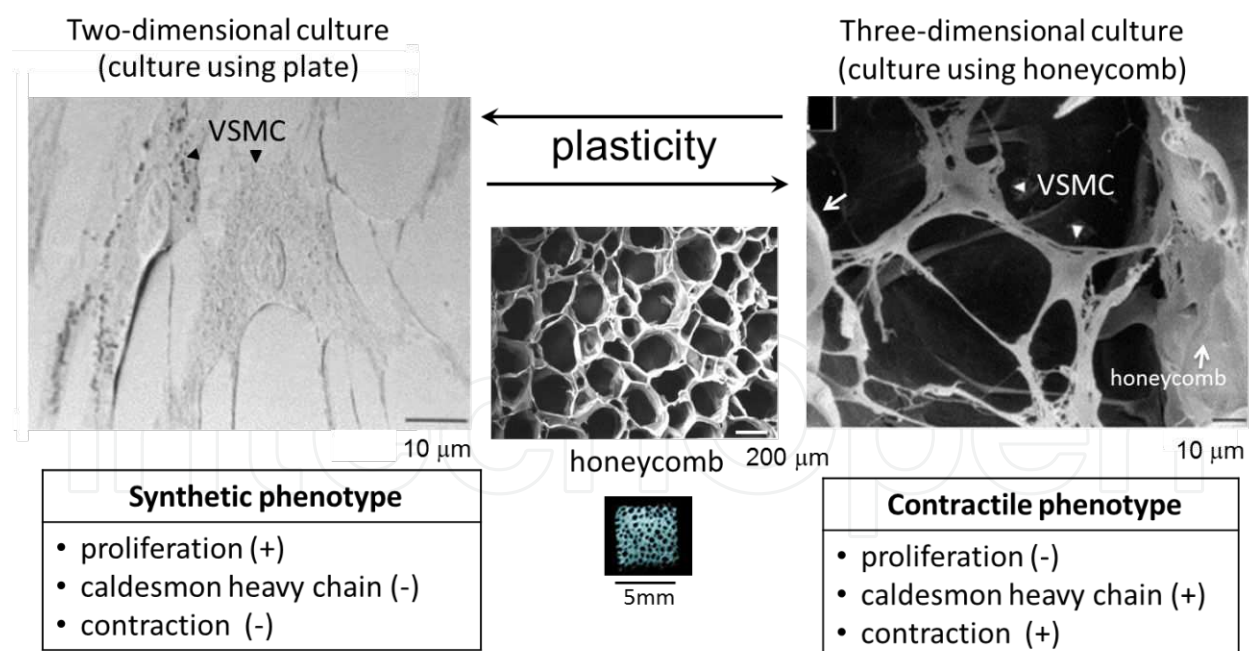

Figure 8. Summary of VSMC phenotypic modulation in culture. These images are scanning electron microscopic observations. Arrowheads show VSMCs and arrows show honeycombs. VSMCs cultured on plates express the synthetic phenotype. This type of VSMC is de-differentiated and can proliferate. VSMCs cultured in honeycombs display similar features to the contractile phenotype. These two phenotypes are reversible depending on the type of culture used. These photographs were taken from reference 4 to make it easy to understand the culture system.

7. Vision and agenda

The honeycomb culture system has been used for tissue engineering. For example, when honeycombs filled with grown human adipose-derived stem cells were transplanted into nude mice, bone formation was observed *in vivo* [31]. Another report showed that new bone induction by KUSA/A1cells was promoted by using honeycomb culture [32]. Furthermore, when human embryonic stem cells (hESCs) were co-cultured with Swiss 3T3 cells in honeycombs, type I collagen synthesis by Swiss 3T3 cells contributed to the hepatic maturation of hESCs [33]. From these data, it can be seen that the honeycomb culture system has the potential to help the regeneration of organs and to facilitate the analysis of intercellular interactions. In the near future, in order to clarify the mechanism of the phenotypic modulation of VSMCs involved in atherosclerosis, we intend to co-culture VSMCs and macrophages. In addition, the reason for the proliferative inhibition of VSMCs cultured in honeycombs is still unclear. This is our agenda that remains to be solved.

8. Conclusion

A summary of VSMC phenotypic modulation in culture is shown in Figure 8. The contractile phenotype of VSMCs can be cultured stably using type I collagen three-dimensional matrices, i.e., honeycombs, in the presence of 10% FBS. VSMCs in honeycombs stop proliferating and can contract. The expression of OAZ1 and reduction of phosphorylated FAK levels contribute partially to the proliferative inhibition of VSMCs in honeycombs. Filamin degradation reduces cytoskeletal tension and allows shortening of the contractile apparatus. However, the detailed mechanism by which VSMCs display features similar to those of aortic VSMCs when cultured in honeycombs is still unclear. In order to clarify the regulation of phenotypic modulation, further studies are needed.

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