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ORIGINAL ARTICLE

Correlation between antizyme 1 and differentiation of vascular smooth muscle cells cultured in honeycomb-like type-I collagen matrix

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Abstract Vascular smooth muscle cells (SMC) are able to proliferate when cultured on plates, but become differentiated when maintained in three-dimensional type I collagen matrices (honeycombs). SMC grown in honeycombs contained a low level of polyamines due to the presence of antizyme 1 (AZ1), a negative regulator of ornithine decarboxylase (ODC) and of polyamine uptake. To clarify the role of AZ1 in differentiation of SMC in honeycombs, an ODC gene was stably transfected into SMC (ODC-SMC). Although proliferation of ODC-SMC on plates was accelerated together with an increase in phosphorylated focal adhesion kinase (FAK) and a decrease in α-actin and myosin, maker proteins of differentiation, growth of ODC-SMC ceased in honeycombs similarly to normal SMC with a low level of phosphorylated FAK and a high level of α-actin and myosin. AZ1 expression in ODC-SMC on plates was low, but that in honeycombs was high. Antizyme in ODC-SMC in honeycombs not only decreased the level of ODC but also inhibited polyamine uptake activity. These results taken together suggest that low levels of polyamines caused by AZ1 in SMC in honeycombs inhibit phosphorylation of FAK and enhance expression of α-actin and myosin, resulting in differentiation through inhibition of focal adhesions.

Keywords Polyamines · Antizyme 1 · Ornithine decarboxylase · Polyamine transport · Differentiation · Smooth muscle cells

Abbreviations

DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
ODC	Ornithine decarboxylase
ODC-SMC	ODC transfected SMC
PBS	Phosphate-buffered saline
SMC	Smooth muscle cells
Skp-2	S-phase kinase-associated protein-2
pTracer-AZΔT205	pTracer-CMV containing AZ1 without frameshift
pTracer-ODC	pTracer-CMV encoding the ODC gene

Introduction

Excessive proliferation of vascular smooth muscle cells (SMC) contributes to vascular diseases such as restenosis after percutaneous coronary intervention and the formation of atherosclerotic plaques. Thus, studies on inhibiting excessive proliferation of SMC and maintaining their differentiated state are useful for the treatment or prevention of vascular diseases (Consigny 1986; Faxon et al. 1987). Gizzard SMC derived from chick embryos are differentiated in the presence of insulin-like growth factors without serum when cultured on laminin-coated plates (Hayashi et al. 1998). However, the SMC can dedifferentiate in the

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presence of serum, even though the cells are maintained on laminin-coated plates. Previously, we reported a novel system for culturing differentiated SMC using type I collagen three-dimensional matrices, so-called "honeycombs" (Ishii et al. 2001). These matrices may represent an environment more akin to the normal vasculature than that represented by plastic or collagen-coated plates. When dedifferentiated SMC were cultured in honeycombs, cell proliferation ceased, together with a parallel decrease in the level of phosphorylated FAK. This was followed by gradual expression of marker proteins having characteristics of differentiated SMC. Because SMC cultured in honeycombs remain differentiated in the presence of serum, this system is more suitable to study the properties of SMC and the molecular events underlying differentiation than that reported using chick embryo SMC on laminin-coated plates (Hayashi et al. 1998).

Polyamines—putrescine, spermidine and spermine, abundant multivalent organic cations—are essential for cell growth (Igarashi and Kashiwagi 2006). Proliferation and transformation of cells induced by carcinogens, viruses and oncogenes are characterized by increases in cellular polyamine levels due to increased biosynthesis and uptake of polyamines (Pegg 1988). Ornithine decarboxylase (ODC) is the first and rate-limiting enzyme of polyamine biosynthesis. Thus, cells overexpressing ODC also show increased proliferation related to an increase in polyamine content (Kameji et al. 1993). On the other hand, overaccumulation of cellular polyamines accelerates the induction of antizyme 1 (AZ1) (Sakata et al. 1997), which inhibits the activity of ODC and increases its degradation by forming an AZ1–ODC complex (Matsufuji et al. 1995; Murakami et al. 1992). AZ1 can also decrease uptake of polyamines independent of its effects on ODC (Suzuki et al. 1994; Zhu et al. 1999).

In this study, we found that the level of AZ1 was high when SMC were cultured in honeycombs. Thus, we investigated the role of AZ1 in SMC differentiation in honeycombs using both normal and ODC transfected SMC (ODC-SMC).

Materials and methods

Honeycomb collagen tubes

Collagen sponges, called "honeycombs", consisting of type I collagen were obtained from Koken Co. The structure of the honeycomb is not only porous but consists of many tubes aligned side by side similar to a beehive (Ishii et al. 2001). The pore size of each tube is approximately 0.2 mm and each piece of honeycomb is $3 \times 3 \times 2$ mm^3.

Culture of SMC

SMC were prepared by the explant method from the medial layer of the thoracic aorta of male Japanese white rabbits weighing about 2 kg. SMC were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Ltd.) containing 100 units/ml streptomycin, 100 units/ml penicillin G and 10% fetal bovine serum (Gibco) (10% FBS/DMEM) at 37°C under an atmosphere of 5% CO_2 in air. Preparation of SMC by the explant method, subculture and culture methods in honeycombs were described in the previous report (Ishii et al. 2001).

Plasmids and transfection

pTracer-CMV (pTracer) encoding the ODC gene (pTracer-ODC) was constructed as follows. pODC188-*Xho*I (Shimogori et al. 1996) was digested with *Eco*RI and *Xba*I, and the fragment was inserted into the same restriction sites of pTracer-CMV (Invitrogen Corp.). Transformation of *Escherichia coli* DH5α with the plasmid and culture of *E. coli* were carried out according to the method of Maniatis *et al* (1989). The plasmid in *E. coli* was purified using the QIAGEN Plasmid Midi Kit (QIAGEN) according to the manufacturer's protocol. Confluent SMC (1×10^6 cells/dish, ϕ 10 cm) cultured on plates for 3 days were released with trypsin-EDTA and suspended in 0.8 ml of phosphate-buffered saline (PBS). Then, the plasmids pTracer and pTracer-ODC (2 μg/ml) were transfected into SMC by electroporation (Gene Pulser, Bio-Rad, Hercules) according to the methods of Kimura et al. (1994). Transfection of plasmid was confirmed by the fluorescence of GFP protein. The fluorescence of GFP was observed in all viable cells at for 24 h after transfection. SMC showing ODC overexpression (ODC-SMC) could be selected by 4–5 times passage because growth rate of ODC-SMC was 2–3-fold faster compared with that of N-SMC. ODC-SMC was used for 4–15 passages and the cells kept high ODC expression level during this period. Three kinds of pTracer-CMV containing AZ1 without frameshift were also used; pTracer-AZΔT205 containing natural sequence without T205 expresses 24.5 and 29 kDa AZ1, pTracer-AZΔT205-ΔAUG1 expresses 24.5 kDa AZ1 and pTracer-AZΔT205-ΔAUG2 expresses 29 kDa AZ1 (Hoshino et al. 2005). AZ1 encoded by pTracer-AZΔT205, pTracer-AZΔT205ΔAUG1 and pTracer-AZΔT205ΔAUG2 was transiently overexpressed using Lipofectamine^{TM} 2000 (Invitrogen). SMC (6×10^4 cells/well, 12-well plate) were plated and incubated for 24 h. Then, 3.2 μg/well of plasmids and 8.0 μl/well Lipofectamine^{TM} 2000 were added according to the manufacturer's protocol. SMC transfected with non-coding pTracer-CMV [pTracer-(−)] were used as control SMC.

Measurement of cell number and [^{3}H]thymidine incorporation

1×10^4 SMC on plastic plates (diameter, 3 cm) or 3–4 $\times$ 10^4 SMC in five pieces of honeycombs were cultured for the indicated time with 10% FBS/DMEM. Cells were then washed twice with PBS and released from plastic plates with trypsin–EDTA at 37°C for 1 min and from honeycombs with collagenase I at 37°C for 10 min. Cell number was determined with the Improved Neubauer Deep chamber hemocytometer. Cell viability was assessed by trypan blue exclusion. SMC on plates or in honeycombs were incubated with 10% FBS/DMEM containing 37 kBq of [^{3}H]thymidine (1.5 TBq/mmol, GH Healthcare)/well for 24 h, and [^{3}H]thymidine incorporation of SMC was measured as described previously (Ishii et al. 2001).

Measurement of ODC and AZ1 activities, and polyamine and protein contents

2×10^7 cells were suspended in 1 ml of ODC assay buffer [10 mM Hepes-KOH pH7.5, 0.2 mM pyridoxal phosphate, 2.5 mM dithiothreitol (DTT), 0.1 mM EDTA and 20 μM Fut-175] and were frozen and thawed four times. The lysate was centrifuged (12,000×g for 30 min at 4°C) and the supernatant was collected. Extracts were dialyzed twice against 800 ml of ODC assay buffer for over 3 h and used for assay. ODC activity was assayed by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine as described before (Mitchell and Chen 1990). The activity of AZ1 was determined as inhibitory percentage of ODC activity of the extract from ODC-overproducing FM3A (EXOD-1) cells (Kameji 1993). When 10 μg proteins of the extract were used, the specific activity of ODC was 2.2 nmol/min/mg protein. Polyamines in cells were measured using 6×10^6 cells as described previously (Igarashi 1986). Protein concentration was determined according to the method of Bradford (1976).

Visualization of cells

Fixation of SMC in honeycombs and electron microscopy were carried out as described previously (Ishii et al. 2001).

Western blotting

The level of AZ1, ODC, phosphorylated FAK, FAK, α-actin, myosin and β-actin, was measured using 5–20 μg protein as described previously (Ishii et al. 2001). The proteins were detected with the ECL Western blotting detection reagents (GE Healthcare) using rabbit polyclonal anti-AZ1 antibody (kindly supplied by Dr. Jänne at University of Helsinki) and rabbit anti-ODC polyclonal antibody (kindly supplied by Dr. S. Matsufuji and Dr. Y. Murakami, Jikei University School of Medicine), anti-phosphorylated tyrosine of FAK antibody (2A7, Upstate), anti-FAK (77, BD Transduction Laboratories), monoclonal anti-α-actin antibody (1A4, DAKO) and monoclonal anti-smooth myosin heavy chain antibody (hSM-V, Sigma) as primary antibodies. The membrane was incubated with the primary antibodies for 60 min at room temperature and with secondary antibodies (horse radish peroxidase-linked anti-mouse IgG or anti-rabbit IgG, GE Healthcare) for 30 min at room temperature. The intensity of protein band obtained by Western blotting was measured by a LAS-1000 plus luminescent image analyzer (Fuji Film) and exposing time was 10–60 s unless otherwise noted. Experiments were repeated three times to confirm the reproducibility of the results. The detected bands were quantified using the ImageJ1.43.

Spermine transport assay

SMC (1×10^6) in 1 ml of NaCl buffer (135 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 20 mM Hepes–Tris, pH 7.2) (Hoshino et al. 2005) was incubated at 30°C for 5 min, and then the uptake assay was started by the addition of [^{14}C] spermine at a final concentration of 10 μM (740 MBq/mmol). After incubation at 37°C for 30 min, cells (1×10^6) were washed three times with phosphate-buffered saline containing 0.2 mM spermine. Washed cells were lysed with 0.6 ml of Renilla luciferase assay lysis buffer (Promega), and a 0.5-ml aliquot was used for the measurement of radioactivity by a liquid scintillation counter. The uptake rate was linear during the incubation time.

Autophosphorylation of FAK in vitro

Truncated human FAK consisting of 376-1052 (end) amino acid residues (molecular weight, 103 kDa) was obtained from Carna Biosciences Inc. The reaction mixture (10 μl) consisting of 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.17 pmol FAK and 1 mM ATP with or without 0.5 or 1 mM $MgCl_2$ and/or spermine was incubated at 30°C for 1 h. After the incubation, the same volume of the 2× sample buffer of sodium dodecyl sulfate-polyacrylamide gel electrophoresis was added, and Western blot analysis was performed to detect phosphorylated FAK and FAK.

Results

Cell proliferation and polyamine content of ODC-SMC cultured on plastic plates

We first tried to confirm whether ODC enhances proliferation of SMC on plates as it does with other cells. In

ODC-SMC cultured on plates, the cell number and incorporation of [^{3}H]thymidine were higher than those in normal SMC (N-SMC) (Fig. 1a, b). The spermidine content of ODC-SMC was much higher than that in N-SMC until day 3 (Fig. 1c, d), after which the level of spermidine rapidly declined and polyamine levels in ODC-SMC were similar to those in N-SMC during days 5–14 (Fig. 1c, d).

Cell proliferation and polyamine content of ODC-SMC cultured in honeycombs

We next examined morphology of SMC, cell proliferation and polyamine content cultured in honeycombs using N-SMC and ODC-SMC. Morphologically, ODC-SMC appeared to be differentiated like N-SMC [4]—that is, they attached to the walls of honeycombs (Fig. 2a). When N-SMC were cultured in honeycombs, proliferation ceased (Fig. 2b), and [^{3}H]thymidine incorporation was very low (Fig. 2c). Polyamine content was maintained at a low level from day 1 to day 14 with a transient increase on day 3 (Fig. 2d). ODC-SMC, like N-SMC, did not proliferate either (Fig. 2b) and the incorporation of [^{3}H]thymidine in these cells was nearly equal to that of N-SMC (Fig. 2c). Although the levels of polyamines were slightly higher in ODC-SMC than in N-SMC on day 3 (Fig. 2e), they decreased gradually and remained at low levels during days 7–14. These data indicate that ODC-SMC stop their proliferation and are differentiated in honeycombs similarly to N-SMC.

Levels of AZ1, ODC, α-actin, myosin, and phosphorylated FAK in SMC

Since polyamine levels are regulated by AZ1 and two different AZ1s (24.5 kDa and 29 kDa) exist in cells (Hoshino et al. 2005), ODC inhibitory activity of the two AZ1s were measured using N-SMC on plates transfected with various types of AZ1 gene constructs which express 24.5 kDa AZ1 only, both 24.5 and 29 kDa AZ1, and 29 kDa AZ1 only, and ODC-overproducing cell lysate as the ODC enzyme source. Expression level of AZ1 and degree of inhibition of ODC activity were shown in

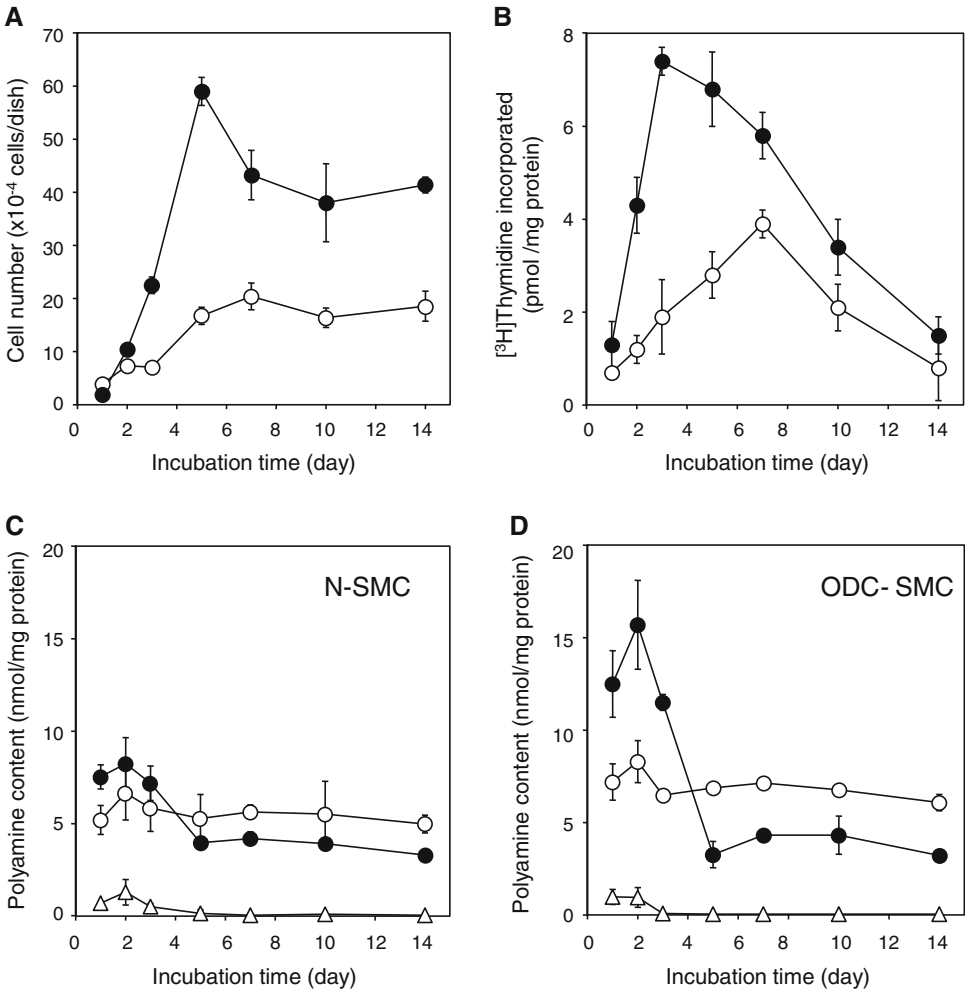


Fig. 1 Effect of ODC transfection on SMC cultured on plates. **a** Cell number of SMC cultured on plates. Cell number was counted with a chamber hemocytometer. *Open circle* Normal SMC (N-SMC), *filled circle* ODC-SMC. **b** [^{3}H]Thymidine incorporation to N-SMC (*open circle*) and ODC-SMC (*filled circle*) on plates. **c**, **d** Polyamine content in N-SMC and ODC- SMC cultured on plates. *Open triangle* putrescine, *filled circle* spermidine, *open circle* spermine. Values are mean ± SD of triplicate determinations

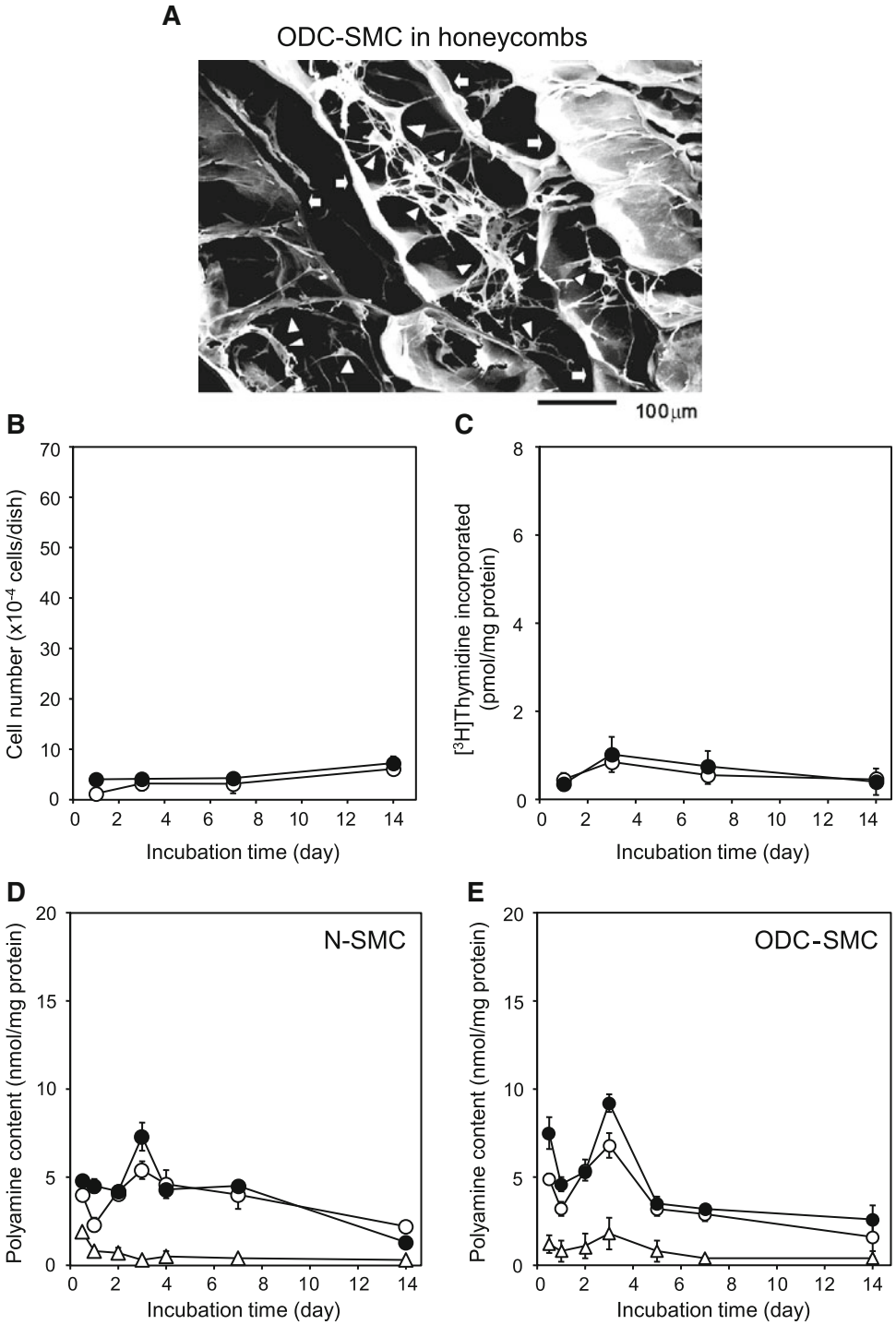


Fig. 2 Effect of ODC transfection on SMC cultured in honeycombs. **a** Morphology by electronmicroscopy of ODC-SMC cultured in honeycombs for 14 days. *Arrows* show honeycombs and *arrowheads* show ODC-SMC. **b** Cell number of SMC in honeycombs. Cell number was counted with a chamber hemocytometer. *Open circle* N-SMC, *filled circle* ODC-SMC. **c** [^{3}H]Thymidine incorporation to N-SMC (*open circle*) and ODC-SMC (*filled circle*) in honeycombs. **d**, **e** Polyamine content in N-SMC and ODC-SMC cultured in honeycombs. *Open triangle* putrescine, *filled circle* spermidine, *open circle* spermine. Values are mean ± SD of triplicate determinations

Fig. 3A (a) and (b). The degree of inhibition was parallel with the expression level of AZ1, i.e. N-SMC expressing 24.5 kDa AZ1 alone showed the strongest ODC inhibitory activity. These results suggest that both 24.5 and 29 kDa AZ1s function similarly. Then, the AZ1 level in N-SMC on plates and in honeycombs was compared. As shown in Fig. 3B, the degree of inhibition of ODC activity was higher in cell lysate prepared from N-SMC cultured in honeycombs than that cultured on plates, indicating that AZ1 level is higher in SMC in honeycombs than in SMC on plates.

Then, levels of AZ1, ODC, phosphorylated FAK, α-actin, myosin, and β-actin were measured in normal and ODC-SMC cultured on plates (Fig. 3C). ODC expression in ODC-SMC on plates was greater than that in N-SMC. ODC activities in normal and ODC-SMC on day 3 were 2.5 and 7.6 pmol/min/mg protein, respectively. AZ1 was not detectable in N-SMC but 24.5 kDa AZ1 was weakly

expressed in ODC-SMC on plates. Although the level of α-actin and myosin was decreased greatly, that of phosphorylated FAK was increased in ODC-SMC on plates. Because α-actin and myosin are marker proteins of differentiation of SMC (Gabbiani et al. 1981; Sobue et al. 1999) and phosphorylated FAK levels link to proliferation activity (Hauck et al. 2000), ODC-SMC was thought to be more de-differentiated.

When N-SMC and ODC-SMC were cultured in honeycombs, a low level of 29 kDa AZ1 was expressed in N-SMC, and of both 24.5 and 29 kDa AZ1 in ODC-SMC (Fig. 3C). Expression level of total AZ1 was greater in ODC-SMC than in N-SMC. ODC activity in both N-SMC and ODC-SMC cultured in honeycombs was less than 1 pmol/min/mg protein. Phosphorylated FAK in ODC-SMC was a little more abundant than that in N-SMC in honeycombs on day 1 and 3, but it became negligible in both SMC in honeycombs on days 7 and 14. α-Actin level in ODC-SMC increased in a time-dependent manner similarly to N-SMC in honeycombs, and its expression level in ODC-SMC after day 7 was almost the same with in N-SMC. However, the level of myosin in ODC-SMC in honeycombs was higher than that in ODC-SMC on plates. The results confirmed that even ODC-SMC was differentiated in honeycombs (see Fig. 2a). β-Actin level was kept at the same level in all samples.

Degradation of AZ1 in ODC-SMC after treatment with cycloheximide was examined because the expression level of AZ1 was greater in ODC-SMC in honeycombs than on plates. As shown in Fig. 3D, AZ1 of ODC-SMC on plates was degraded till 3 h after treatment with cycloheximide. However, degradation of AZ1 in honeycombs was limited during 12 h incubation. The difference in the stability may be the major reason for the existence of a high level of AZ1 in SMC in honeycombs.

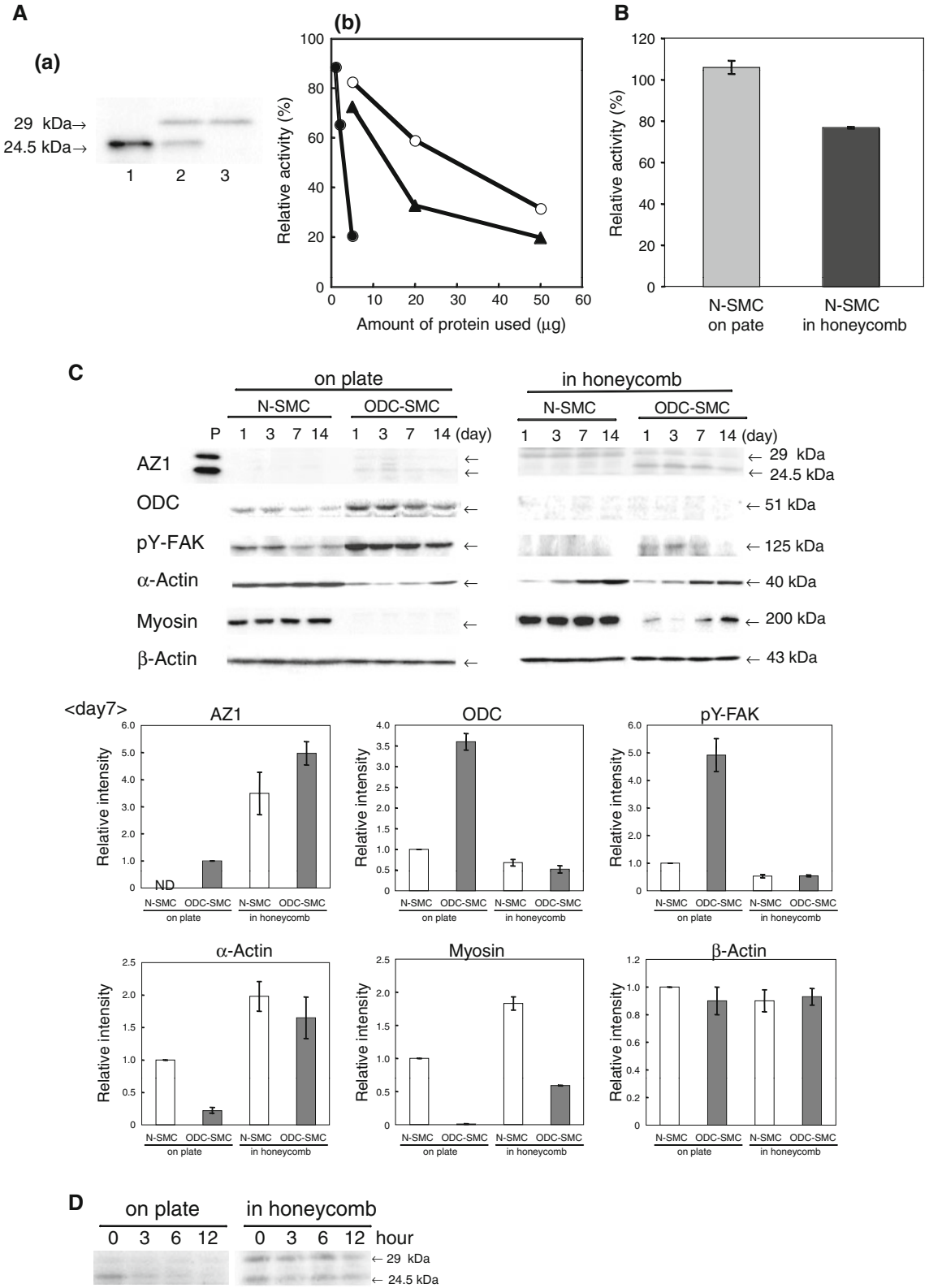
Fig. 3 AZ1 in N-SMC and ODC-SMC, and levels of various proteins expressed in SMC cultured on plates and in honeycombs. **a** *a* N-SMC were transfected with pTracer-AZΔT205ΔAUG1 (*lane 1*), pTracer-AZΔT205 (*lane 2*) and pTracer-AZΔT205ΔAUG2 (*lane 3*). After transfection, SMC was collected at 24 h and cell extract was prepared as described in "Materials and methods". Western blotting was performed using 5 μg protein after dialysis for ODC assay buffer. *b* Degree of inhibition of ODC by AZ1 was measured as described in "Materials and methods". *Filled circle* the extract of N-SMC expressing 24.5 kDa AZ1, *filled triangle* the extract of N-SMC expressing 29 and 24.5 kDa AZ1, *open circle* the extract of N-SMC expressing 29 kDa AZ1. The specific activity of ODC activity in EXOD-1 cells was 2.2 nmol/min/mg protein, and the relative activity of three extracts was calculated. Values are means of duplicate determinations. **b** Inhibition of ODC activity using the extract of N-SMC cultured on plates and in honeycombs. The extract containing 2 mg protein was used in the assay. Values are means ± SD of triplicate determinations. **c** Protein levels involved in cell proliferation and differentiation. AZ1, ODC, tyrosine-phosphorylated FAK (pY-FAK), α-actin, myosin, and β-actin of N-SMC and ODC-SMC cultured on plates and in honeycombs were detected by Western blotting using 20, 20, 15, 5, 5 and 5 μg proteins, respectively. *P* EXOD-1 cells as positive control [7]. Relative intensity on day 7 was quantified as described in "Materials and methods". Intensity of AZ1 was quantified as the sum of 29 and 24.5 kDa bands, *ND* not detectable. Values are mean ± SD of triplicate determinations. **d** Degradation of AZ1. ODC-SMC cultured on plates and in honeycombs for 3 days was treated with 20 μg/ml cycloheximide (Sigma) for indicated time. AZ1 was detected by Western blotting using 40 μg protein

Overexpression of AZ1 in ODC-SMC cultured on plates and in honeycombs

In order to clarify that AZ1 present in ODC-SMC cultured in honeycombs is enough for differentiation of SMC, 24.5 kDa AZ1 (see Fig. 3A (a)) was transiently overexpressed in ODC-SMC. Figure 4a shows the timecourse of transient expression of AZ1 in ODC-SMC cultured on plates and in honeycombs. Transient expression of AZ1 on day 1–3 decreased the cell number of ODC-SMC cultured on plates on day 3, but did not change the cell number of ODC-SMC in honeycombs (Fig. 4b). Transient expression of AZ1 only influenced the levels of myosin in ODC-SMC cultured on plates and in honeycombs and the levels of α-actin cultured in honeycombs, but did not influence the levels of ODC and phosphorylated FAK significantly in ODC-SMC cultured on plates and in honeycombs (Fig.4). These results suggest that AZ1 presents in ODC-SMC cultured on plates and in honeycombs is enough to regulate the level of ODC, and that transient expression of AZ1 does not strongly influence ODC-SMC differentiation in honeycombs. The level of AZ1 in honeycombs on day 7 was approximately two-times higher compared with that on plates on day 7, confirming that AZ1 in ODC-SMC in honeycombs is more stable than on plates.

Relationship between AZ1 expression and polyamine uptake in ODC-SMC

AZ1 not only stimulates degradation of ODC but also inhibits polyamines uptake (Suzuki et al. 1994; Zhu et al. 1999). If the level of AZ1 is high in ODC-SMC cultured in honeycombs, polyamine uptake should be low in these cells. When 10 μM [^{14}C]spermine was added to SMC culture medium (Fig. 5A), [^{14}C]spermine incorporated into ODC-SMC on plates was much higher than that into ODC-SMC in honeycombs. Similarly, accumulated spermine in ODC-SMC on plates was much higher than that in ODC-SMC in honeycombs, when ODC-SMC were cultured for 12 h in the presence of 30 μM spermine (Fig. 5b). Instead, the level of spermidine greatly decreased in ODC-SMC cultured on plates in the presence of 30 μM spermine (data not shown). Essentially the same results were obtained with



N-SMC cultured on plates and in honeycombs (data not shown). The results indicate that high level of AZ1 in ODC-SMC cultured in honeycombs not only enhances degradation of ODC but also inhibits polyamines uptake.

Effect of spermine on phosphorylation of FAK

It is known that SMC proliferation is up-regulated via increase in stability of S-phase kinase-associated protein-2

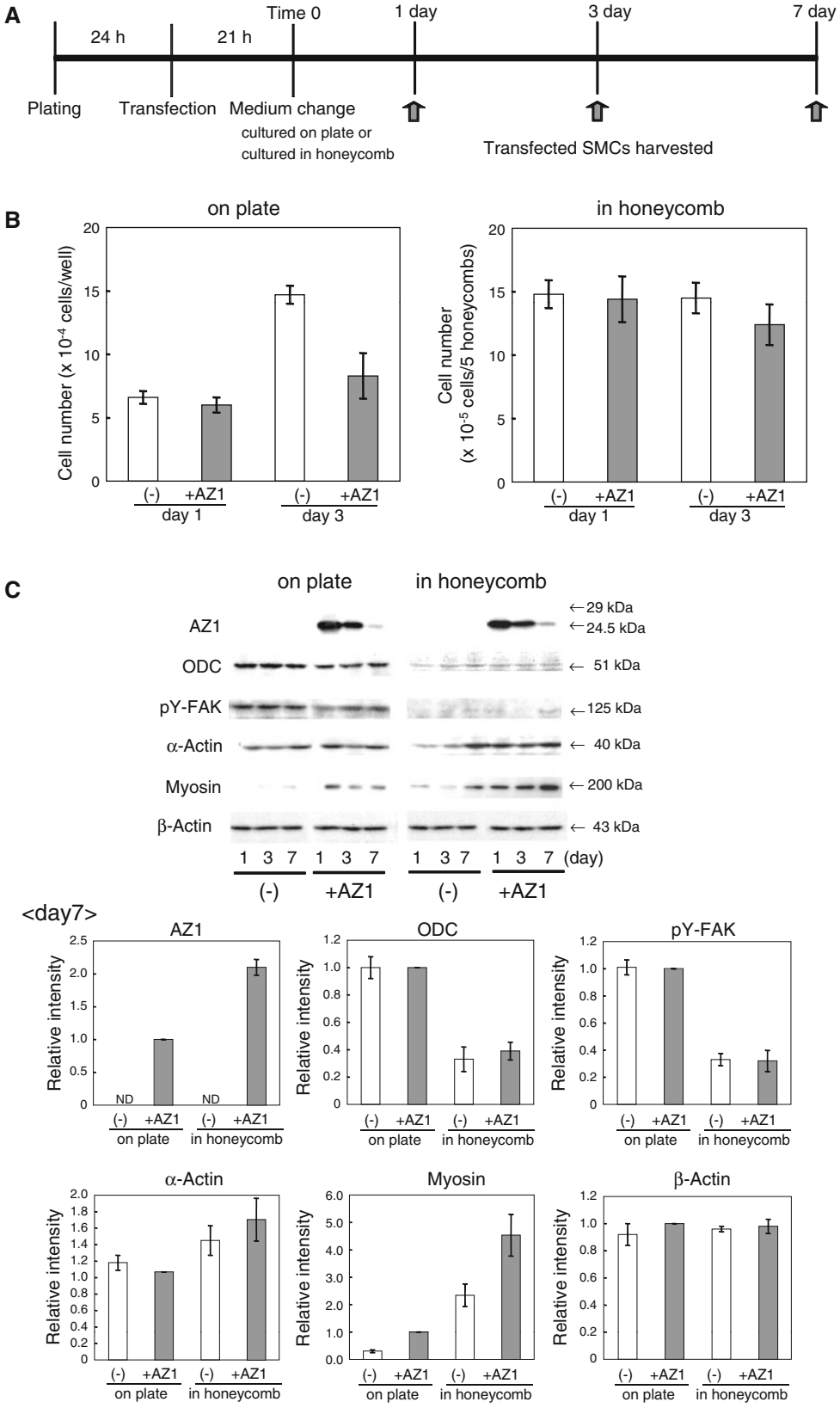


Fig. 4 Effect of transient expression of AZ1 in ODC-SMC cultured on plates and in honeycombs. After ODC-SMC was transfected with pTracer-AZΔT205ΔAUG1 (24.5 kDa AZ1), the cells were cultured as shown in (**a**). Transfected ODC-SMC were then cultured either on plates or in honeycombs for 1, 3 and 7 days. **b** Cell number was counted on day 1 and 3 as described in "Materials and methods". **c** Levels of AZ1, ODC, pY-FAK, α-actin, myosin, and β-actin were measured by Western blotting using 5, 20, 20, 15, 5 and 5 μg proteins, respectively. Relative intensity of proteins on day 7 was quantified as described in "Materials and methods". Values are mean ± SD of triplicate determinations

(Skp-2) by autophosphorylation of FAK-Tyr397 (Odenlund et al. 2009). Phosphorylated FAK disappeared in SMC cultured in honeycombs (see Figs. 3, 4). To investigate the role of spermine on autophosphorylation of FAK, we measured autophosphorylation of FAK in vitro. Fig. 6 shows the level of phosphorylated tyrosine in FAK and the

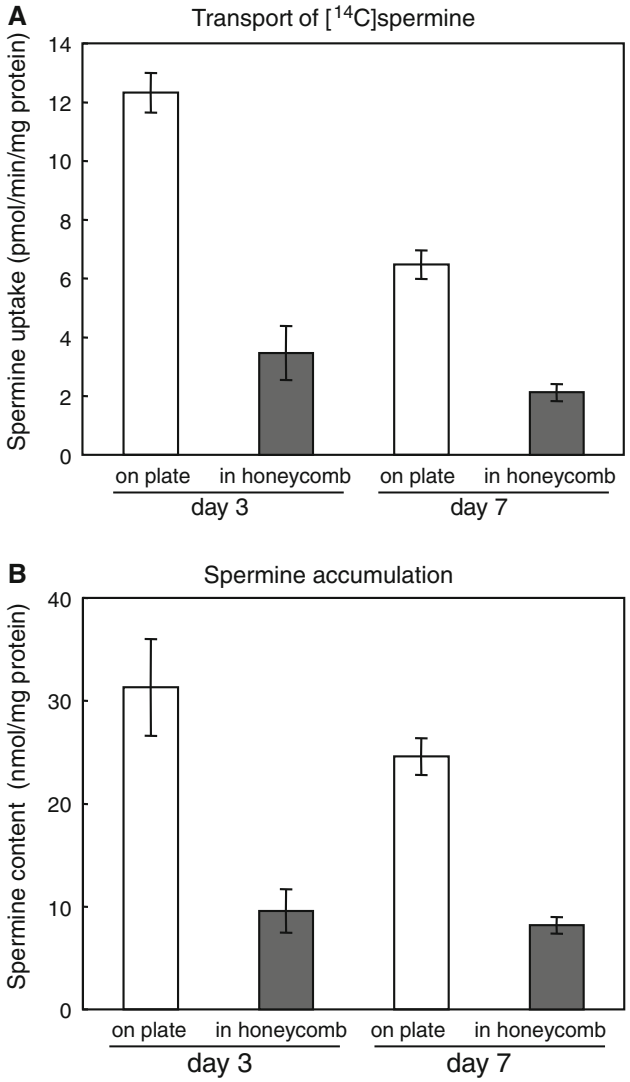


Fig. 5 Spermine transport and accumulation in ODC-SMC cultured on plates and in honeycombs. ODC-SMC was cultured on plates and in honeycombs for 3 and 7 days. **a** [^{14}C]Spermine transport into SMCs. Values are mean ± SD of triplicate determinations. **b** Spermine accumulation in SMC. Spermine content of SMC was measured after incubation for 12 h with 30 μM spermine in the medium containing 1 mM aminoguanidine, an inhibitor of serum amine oxidase. Values are the mean ± SD of triplicate determinations

ratio of phosphorylated FAK/FAK. When ATP was absent, FAK was decreased during incubation. Surprisingly, FAK was autophosphorylated without Mg^{2+} and spermine, and even in the presence of 0.5 mM EDTA to eliminate the contaminating Mg^{2+}. Phosphorylation of FAK was slightly inhibited by Mg^{2+}, but was stimulated by spermine. Furthermore, inhibition of phosphorylation by Mg^{2+} was recovered by spermine. These results suggest that spermine plays an important role on autophosphorylation of FAK-Tyr397.

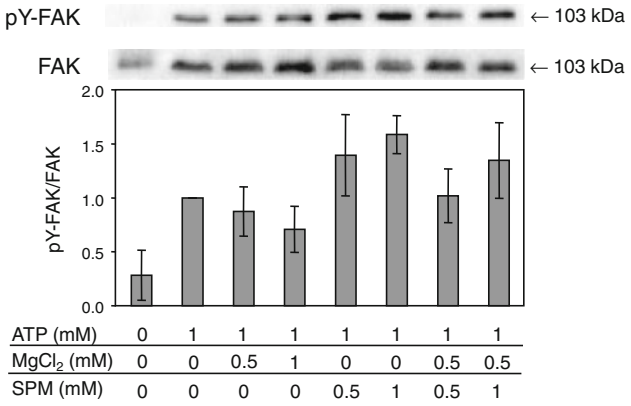


Fig. 6 Effect of Mg^{2+} and spermine on autophosphorylation of FAK in vitro. After incubation of FAK with ATP in the presence or absence of Mg^{2+} and/or spermine as indicated in the figure, the levels of pY-FAK and FAK were measured by Western blotting as described in "Materials and methods". Then, the relative ratio of pY-FAK/FAK was calculated assuming the ratio obtained in the absence of Mg^{2+} and spermine as 1

Discussion

Our results show that SMC cultured in honeycombs exhibited lower proliferation rates through a decrease in polyamines and phosphorylated FAK due to the existence of a high level of AZ1, subsequently the cells increased the levels of α-actin and myosin for differentiation.

Primary culture of SMC on plates also contained low level of polyamines and showed slow proliferation rate (doubling time was almost 2 days) compared to other cell lines. When ODC was overproduced in NIH3T3, AZ1 level increased together with the increase in polyamines (Auvinen et al. 1997). Since synthesis of AZ1 is regulated by a +1 frameshift of its mRNA, increase in polyamines was necessary for +1 frameshift (Matsufuji et al. 1995). Thus, ODC-overproducing SMC was used to study the role of AZ1 on differentiation of SMC, because the level of AZ1 was higher in ODC-SMC than in N-SMC (Auvinen et al. 1997). We found the following using ODC-SMC: (1) AZ1 in SMC was maintained at a relatively high level in honeycombs due to the decrease in its degradation; (2) through the decrease in polyamine content by AZ1 in SMC in honeycombs, autophosphorylation of FAK-Tyr397 was inhibited; and (3) then, the level of α-actin and myosin, marker proteins of differentiation, increased.

Therefore, it is important to clarify why AZ1 is stable in SMC in honeycombs. Since it has been reported that AZ1 is rapidly degraded by the 26S proteasome (Gandre et al. 2002), activity of the 26S proteasome may be prohibited in SMC in honeycombs. So, it is expected that the level of AZ1 originally existing in SMC is slowly degraded in honeycombs.

It has been reported that decreased polyamines in IEC-6 cells delayed phosphorylation of FAK and greatly reduced focal adhesion complex formation (Ray et al. 2002). It is also well known that phosphorylation of FAK is the initial step of proliferation of SMC (Hauck et al. 2000). Our results indicate that spermine increased autophosphorylation of FAK by ATP in the absence or presence of Mg^{2+} in vitro (see Fig. 6). It has been also reported that aminoacyl-tRNA formation was stimulated by polyamines in the absence of Mg^{2+} (Igarashi et al. 1971). In this case, ATP without Mg^{2+} could be recognized by aminoacyl-tRNA synthase in the presence of tRNA and polyamines. This was explained by the concerted mechanism in which aminoacyl-tRNA is formed without intermediate complex, i.e., aminoacyl-AMP-enzyme complex, instead of two step mechanism in the presence of Mg^{2+} (Igarashi et al. 1972). A similar situation may occur in case of autophosphorylation of FAK by ATP. FAK may be able to recognize several conformations of ATP in the presence and absence of Mg^{2+} and/or spermine. It is an important phenomenon that spermine activates autophosphorylation of FAK in the presence or absence of Mg^{2+} without influencing the structure of ATP.

Polyamine contents of SMC in honeycombs were low and persisted at the same level after day 3 even though the ODC gene was transfected. One of the reasons is a stable AZ1 expression in honeycombs. Total protein syntheses of SMC in honeycombs greatly decreased compared with those on plates [4]. However, α-actin and myosin were maintained at a relatively high level. These proteins are markers of SMC differentiation. One of the reasons why α-actin and myosin were expressed at the relatively high level in SMC in honeycombs may be explained by the fact that the mRNAs encoding α-actin and myosin have a short 5′-untranslated regions (5′-UTR), i.e., 5′-UTR of α-actin and myosin mRNAs are short, 116 and 94 nucleotides, respectively (Spruill and McDermott 2009). We have previously reported that polyamine stimulation of ODC synthesis is dependent on the size of 5′-UTR of ODC mRNA in a cell-free system (Ito et al. 1990). If the size of 5′-UTR is longer, the degree of polyamine stimulation of ODC synthesis became greater. Thus, α-actin and myosin may be synthesized at the relatively high level in SMC in honeycombs, in which polyamine content is low.

It is of interest to know whether a relatively high level of AZ1 exists in most kinds of differentiated cells. Further studies are necessary to clarify the role of AZ1 in differentiation of SMC and other differentiated cells.

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