緩衝液を含むダルベッコ改変細胞培養液) とヘキスト 33342 色素を 5 μg/ml 濃度で加え、37℃で 30 分間反応させた。細胞洗浄後、上清を除去し、細胞ペレットに 100 μl の細胞洗浄液と 10 μl の PE (phycoerythrin) ラベルされた CD56 抗体を加え、氷中で 10 分間反応させた。細胞洗浄後、上清を除去し、1 ml の細胞洗浄液で細胞を懸濁後、40 μm のナイロン・メッシュに通してソーティング用チューブに回収した。

セルソーティング

得られた細胞懸濁液を定法に従ってセルソーター (JSAN) にて展開し、ヘキスト 33342 陽性の生細胞の中から CD56 陽性の細胞画分をソーティングした。

単離した CD56 陽性細胞が、筋幹細胞であることを確認するため、matrigel コートした 96 ウェルプレートに播種し、培養を行った。

細胞培養液としては、Primary Myocytes Growth Medium (pmGM, Wada et al., 2002) を用いた。また、気相は、「二酸化炭素：空気＝1：9」とし、培養温度は 36.6-36.8℃を保つように設定した。

（倫理面への配慮）

ヒト材料を用いた実験に関しては、国立長寿医療センター倫理委員会の承認を受けたうえで、説明と同意に関する所定の手続きを行い、注意深く実施した。

C. 研究結果

ソーティングによって得られたヘキスト 33342 陽性の生細胞で CD56 陽性の細胞画分 1,000 個を matrigel コートした 96 ウェルプレートに播種し、顕微鏡下で観察すると、単離直後は細胞質がほとんど認められないほどコンパクトで丸い形態をとっていた。培養 4 日後には、筋芽細胞と思われる紡錘形の単核細胞が観察され、培養 12 日後には、ウェル全体に増殖した紡錘形の細胞が認められた。これらの細胞は免疫染色により、MyoD 陽性の筋芽細胞であることが確認された。また、同時にソーティングしたヘキスト 33342 陽性かつ CD56 陰性の細胞画分を同様に培養したが、筋芽細胞は全く認められなかった。このことから、CD56 陽性の細胞画分に筋幹細胞が濃縮されていることがわかり、ヒト筋組織からセルソーターを用いて、筋幹細胞を単離できることが示された。

D. 考察

私たちはマウスの筋組織を用いた先行研究において、マウス筋幹細胞特異的抗体とセルソーターを用いて単離した筋幹細胞が静止期にあること、また、培養し、活性化した筋幹細胞と比べて、多数の遺伝子発現に差があることを見出した (Fukada et al., 2007)。さらに、マウス骨格筋への細胞移植実験により、筋組織から単離直後の筋幹細胞に比べて、培養下で増殖させた筋細胞は、移植後の筋再生能力が有意に低下することを明らかにした (Ikemoto et al., 2007)。しかし、患者から得られるヒト筋組織は少量であり、単離可能な筋幹細胞数は限られているため、自己筋細胞移植に必要な量の筋幹細胞を、培養を介さずに確保することはできない。したがって、筋細胞を高い増殖・分化能力を保ったまま、培養下で増幅させるための条件を確立することが大きな課題である。本年度、私たちは、CD56 を指標として高齢者筋組織からヒト筋幹細胞をセルソーターによって分離する条件を確立した。分離直後のヒト筋幹細胞を、培養した筋細胞と比較することによって、筋細胞の増殖・分化能を反映する分子マーカーの探索が可能となった。また、CD56 の発現自体が筋細胞の増殖・分化能を反映している可能性についても、検討が必要である。筋細

胞の増殖・分化能を反映する表面抗原を特定することができれば、迅速に移植用細胞の品質を検定することができるばかりではなく、培養筋細胞の中から高い増殖・分化能をもった細胞集団だけを選別することが可能になり、自己筋細胞移植の治療効果の増大および安全性の担保に大きく貢献することが期待できる。

E. 結論

高齢者から得られた少量の筋組織（100 mg程度）から、セルソーターを用いて再現性よくヒト筋幹細胞を分離することに成功した。この技術は、今後、移植用筋幹細胞の品質管理システム確立に大きく貢献することが期待できる。

F. 研究発表

1. 論文発表

1) Ikemoto M, Fukada S, Uezumi A, Masuda S, Miyoshi H, Yamamoto H, Wada MR, Masubuchi N, Miyagoe-Suzuki Y, Takeda S: Autologous transplantation of SM/C-2.6(+) satellite cells transduced with micro-dystrophin CS1 cDNA by lentiviral vector into mdx mice. Mol Ther. 15(12): 2178-2185, 2007
2) Fukada S, Uezumi A, Ikemoto M, Masuda S, Segawa M, Tanimura N, Yamamoto H, Miyagoe-Suzuki Y, Takeda S: Molecular signature of quiescent satellite cells in adult skeletal muscle. Stem Cells. 25(10): 2448-2459, 2007

2. 学会発表

なし

G. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（長寿科学総合研究事業）
分担研究報告書
高齢者腹圧性尿失禁に対する括約筋機能再生治療：
移植用筋細胞の安全性検定系の開発と筋増殖・肥大因子の有効性・安全性に関する検討

分担研究者　宋　時栄　徳島文理大学　香川校　神経科学研究所　教授

研究要旨

高齢者腹圧性尿失禁に対する有力な治療手段の１つとして、ヒト由来骨格筋細胞の移植が考えられる。しかしながら、人体に適応可能な筋幹細胞移植治療を実現するためには、移植細胞の品質（高い筋再生能力と安全性）を確保することが必須である。今年度の本研究では、マウスへの不死化ヒト筋前駆細胞の移植実験系を用いて、移植細胞の性状を病理組織学的に検討し、発癌性などの異常は検出されず、筋再生過程で正常な筋分化を遂げることを確認した。

A.　研究目的

治療手段として用い得る移植細胞の品質を確保するために、実験動物への移植実験系について病理組織学的解析を行い、細胞移植に伴う病理学的現象を検討することによって、筋細胞移植治療の安全性を見極める。

B.　研究方法

1. 実験動物として成熟した雌の NOD/Scid マウスを用い、その両側前脛骨筋に cardiotoxin を注射し、骨格筋再生を誘導。
2. 注射の翌日に、橋本らによって樹立された Venus（GFP の変異体）遺伝子導入不死化ヒト筋前駆細胞（E18V=E18）を 2 - 2.5x 10^6 個、上記マウスの前脛骨筋に移植。
3. 移植後 27 日に前脛骨筋を採取し、4% パラホルムアルデヒドで固定。
4. 固定された前脛骨筋を O.C.T. コンパウンドに包埋し、凍結。
5. 厚さ 10 μ の凍結切片を作成し、抗 GFP 抗体を用いた免疫組織化学的検索によって、Venus 遺伝子導入不死化ヒト筋前駆細胞に由来する蛍光を観察し、細胞の動態、筋線維への分化、腫瘍化などの悪性変化の有無を検索する。また、ヘマトキシリン・エオジン染色を行って、病理組織学的検索を行う。（1-4 の行程は国立長寿医療センター研究所　再生再建医学研究部において行われ、本研究では 5 の行程を分担研究として行った。）

（倫理面への配慮）

動物およびヒト材料を用いた実験に関しては、国立長寿医療センターの動物実験倫理委員会、倫理委員会の承認を得、規定にしたがって実施した。本研究ではこうした所定の倫理審査を経て得られた検体（不死化ヒト筋前駆細胞を移植されたマウス前脛骨筋）について検討した。

C.　研究結果

1. 不死化ヒト筋前駆細胞を移植したマウス前脛骨筋の凍結切片を抗GFP 抗体で免疫染色し、共焦点レーザー顕微鏡（オリンパス FV1000）で観察したところ、GFP 強陽性所見を示す筋線維が散在性に認めら

れる部分（図 1）。また GFP 弱陽性所見を示す筋線維が陰性筋線維とモザイク上に混在している部分（図 2）が認められた。こうした GFP 陽性所見を示す筋線維は、しばしば中心核の所見を示した（図 1, 2 矢印）。

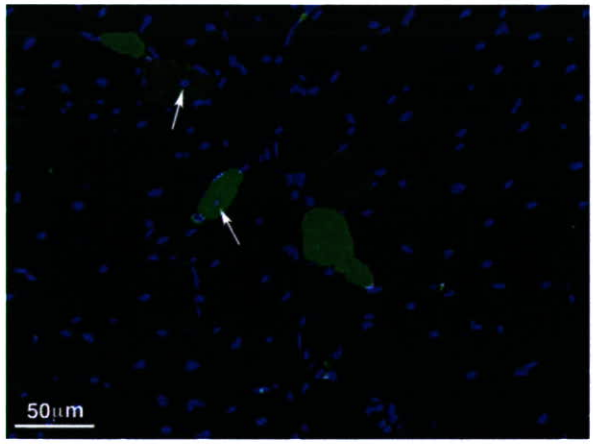

図 1

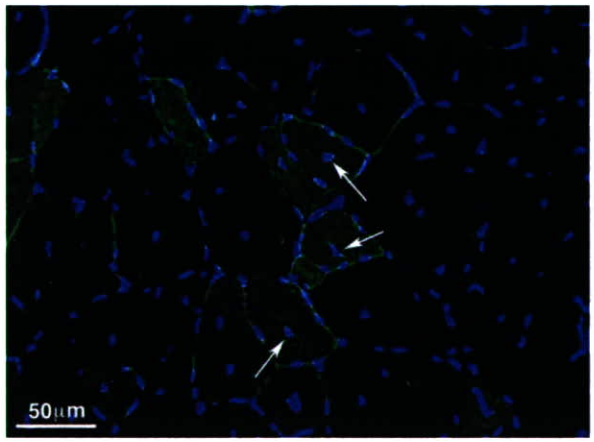

図 2

2. 同上の凍結切片をヘマトキシリン・エオジン染色し、病理組織学的検索を行った。上記 GFP 蛍光強陽性所見を示す筋線維の見られる部分には組織学的には目立った異常を認めない。またこの部位の筋線維は、蛍光観察でも確認されたように多数の中心核線維の所見を示す（図 3）。

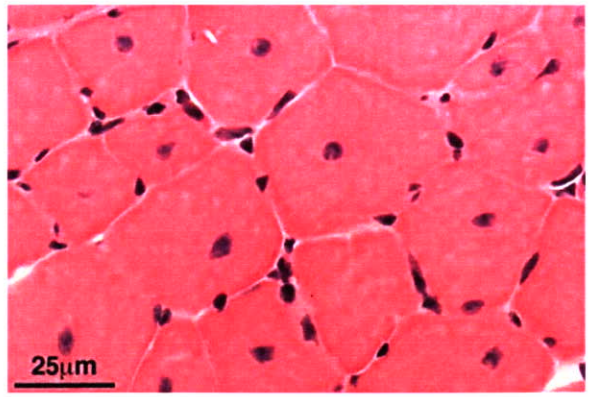

図 3

3. 同上ヘマトキシリン・エオジン染色には移植した細胞の腫瘍性増殖、炎症反応、肉芽形成などの所見は見いだされなかった。

D. 考察

1. 不死化ヒト筋前駆細胞を移植したマウス前脛骨筋の凍結切片で観察された GFP 強陽性および弱陽性筋線維は、移植された変異 GFP 遺伝子導入不死化ヒト筋前駆細胞に由来する筋線維であり、移植先の前脛骨筋における cardiotoxin 注射後の筋再生過程で、あるいは自発的な筋管形成を経て形成されたものと考えられる。強陽性線維は数が少なく、比較的多数の弱陽性線維と隣接している。一部では多数の弱陽性線維が陰性線維と混在してモザイク状の配列を取っている。これら弱陽性線維はしばしば中心核線維の所見を示すことから、明らかに cardiotoxin 注射後の筋再生過程で形成されたものと考えられる。強陽性線維も一部には中心核線維が認められ、周囲にやはり中心核線維の所見を示す弱陽性線維が認められることから、弱陽性線維と同様の機構で形成されたものと思われる。筋線維によって GFP 蛍光強度が異なる理由は明らかではないが、1 つの可能性としては、宿主 NOD/Scid マウス由来の GFP を持たない筋芽細胞と様々な比率で fusion した

ことであるが、筋管形成過程で異種細胞間のハイブリッド形成が起こるか否かは培養系で検証する必要がある。また、cardiotoxin注射後27日でもかなり多数の中心核線維が認められ、SCIDマウスでは筋再生が遅延しているものと考えられる。

2. 不死化ヒト筋前駆細胞を移植したマウス前脛骨筋の凍結切片では移植した細胞の腫瘍性増殖、炎症反応、肉芽形成などの所見は見いだされないが、炎症反応、肉芽形成が乏しい点はNOD/Scidマウスでは免疫反応が抑制されているためとも考えられる。これに対し、腫瘍性の細胞増殖に関しては、宿主NOD/Scidマウスからの抑制が強いとは考えられず、immune surveillanceと言う観点からはむしろ脆弱であると考えられる。この宿主環境下で目立った異常増殖の見られなかったことから、不死化ヒト筋前駆細胞の腫瘍性増殖に関しては、一定の安全性が確認されたと考えられる。

E. 結論

1. 変異GFP 遺伝子導入不死化ヒト筋前駆細胞をマウス前脛骨筋に移植した標本 9 例について病理組織学的検索を行った。
2. この標本にはGFP 蛍光陽性筋線維が散在性に認められ、移植した細胞は、移植先のマウス前脛骨筋へのcardiotoxin注射後の骨格筋再生過程で筋線維への分化を遂げたと判定され、移植先に於いて正常の分化能力を保持していることが示された。
3. ヘマトキシリン・エオジン染色標本の病理学的検索によって、移植細胞の腫瘍化、移植部での著しい炎症反応、肉芽形成などの移植に伴う病変は認められず、不死化ヒト筋前駆細胞の移植については一定の安全性が確認された。

F. 研究発表

1. 論文発表
   なし
2. 学会発表
   なし

G. 知的財産権の出願・登録状況

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

研究成果の刊行に関する一覧表

1. Mukai, A. and Hashimoto, N. Localized cyclic AMP-dependent protein kinase activity is required for myogenic cell fusion. Exp.Cell Res., 314: 387-397, 2008.

2. Hashimoto, N., Kiyono, T., Wada, M. R., Umeda, R., Goto, Y., Nonaka, I, Shimizu, S., Yasumoto, S., and Inagawa-Ogashiwa, M. Osteogenic Properties of Human Myogenic Progenitor Cells. Mech. Dev. 125：257-269, 2008.

3. Ikemoto M, Fukada S, Uezumi A, Masuda S, Miyoshi H, Yamamoto H, Wada MR, Masubuchi N, Miyagoe-Suzuki Y, Takeda S: Autologous transplantation of SM/C-2.6(+) satellite cells transduced with micro-dystrophin CS1 cDNA by lentiviral vector into mdx mice. Mol Ther. 15(12): 2178-2185, 2007.

4. Fukada S, Uezumi A, Ikemoto M, Masuda S, Segawa M, Tanimura N, Yamamoto H, Miyagoe-Suzuki Y, Takeda S: Molecular signature of quiescent satellite cells in adult skeletal muscle. Stem Cells. 25(10): 2448-2459, 2007.

EXPERIMENTAL CELL RESEARCH 314 (2008) 387-397

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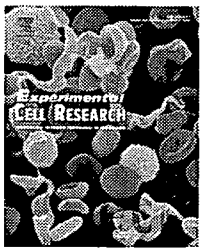

Research Article

Localized cyclic AMP-dependent protein kinase activity is required for myogenic cell fusion

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ARTICLE INFORMATION

Article Chronology:
Received 11 August 2007
Revised version received
7 October 2007
Accepted 10 October 2007
Available online 17 October 2007

Keywords:
Myogenesis
Cell fusion
Myotube
Myoblast
Muscle
Terminal differentiation
Lamellipodium

ABSTRACT

Multinucleated myotubes are formed by fusion of mononucleated myogenic progenitor cells (myoblasts) during terminal skeletal muscle differentiation. In addition, myoblasts fuse with myotubes, but terminally differentiated myotubes have not been shown to fuse with each other. We show here that an adenylate cyclase activator, forskolin, and other reagents that elevate intracellular cyclic AMP (cAMP) levels induced cell fusion between small bipolar myotubes in vitro. Then an extra-large myotube, designated a "myosheet," was produced by both primary and established mouse myogenic cells. Myotube-to-myotube fusion always occurred between the leading edge of lamellipodia at the polar end of one myotube and the lateral plasma membrane of the other. Forskolin enhanced the formation of lamellipodia where cAMP-dependent protein kinase (PKA) was accumulated. Blocking enzymatic activity or anchoring of PKA suppressed forskolin-enhanced lamellipodium formation and prevented fusion of multinucleated myotubes. Localized PKA activity was also required for fusion of mononucleated myoblasts. The present results suggest that localized PKA plays a pivotal role in the early steps of myogenic cell fusion, such as cell-to-cell contact/recognition through lamellipodium formation. Furthermore, the localized cAMP–PKA pathway might be involved in the specification of the fusion-competent areas of the plasma membrane in lamellipodia of myogenic cells.

Introduction

Skeletal muscle fibers are multinucleated, non-mitotic cells. This unique cell type is derived from multinucleated myotubes, which are formed by the fusion of mononucleated myogenic progenitor cells (myoblasts). Myoblasts fuse to each other or to existing myofibers during both development and repair of skeletal muscle. Myoblast fusion is cell-specific because myoblasts do not fuse with non-myogenic cells [1]. Myoblast fusion consists of a series of steps such as myoblast–myoblast contact, recognition, adhesion, and plasma membrane breakdown/union [2]. However, little is known about the molecular mechanisms that regulate cell fusion during muscle development and regeneration.

The genes essential to myotube formation have been identified in *Drosophila*. They encode proteins that mediate a particular step of myoblast fusion [3,4]. In contrast to the detailed knowledge of the molecules involved in muscle cell fusion in *Drosophila*, the molecular mechanisms that control each step of myogenic cell fusion in mammals remain to be resolved, although a number of molecules have been implicated in regulating muscle fiber formation. Extracellular matrix receptor integrins and adhesion molecules such as cadherins, NCAM, CD9, CD81, and ADAMs may contribute to the regulation of the

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doi:10.1016/j.yexcr.2007.10.006

steps of recognition and adhesion in myoblast fusion [5,6]. However, how they coordinate their functions in the recognition/adhesion steps prior to plasma membrane fusion remains puzzling. In addition, few molecules involved in the regulation of plasma membrane fusion have been identified, although many membrane molecules have been implicated in myoblast fusion. One of these is cholesterol. It is involved in maintaining membrane fluidity and the structure of lipid microdomains. Membrane fusion takes place within the cholesterol-free areas of the myoblast plasma membrane [7]. However, the role of cholesterol in muscle cell fusion is not well understood because inconsistent results have been reported [8,9]. Because an increase of membrane fluidity is assumed to be required for plasma membrane fusion, the concentration of membrane cholesterol in myoblasts should decrease prior to membrane fusion. Therefore, the molecular mechanisms that regulate the distribution of membrane cholesterol and other membrane molecules at the fusion-competent areas of the plasma membrane should be identified. Unfortunately, the fusion-competent area of a membrane prior to myoblast fusion has not been positively identified.

To identify the key molecules that render an area of plasma membrane fusion-competent, we determined the stimulus and culture conditions that enhance or suppress muscle cell fusion in vitro. In contrast to mononucleated myoblasts, it has been unclear whether myotubes fuse together in vitro and in vivo. However, we found here that several reagents, such as forskolin, which elevate intracellular cyclic AMP (cAMP) levels, induce fusion between myotubes. The results suggest that the localized cAMP-dependent protein kinase (PKA) activity plays a critical role in the specification of fusion-competent areas of the plasma membrane.

Materials and methods

Cell culture

The mouse myogenic cell clone Ric10 was established from multiclonal myogenic cells derived from muscle satellite cells in the normal gastrocnemius muscle of an adult female ICR mouse [10]. Ric10 cells were plated on dishes coated with type I collagen (Sumilon, Tokyo, Japan) and cultured at 37 °C under 10% CO_2 in primary cultured myocyte growth medium (pmGM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2% Ultroser G (Biosepra, Cedex-Saint-Christophe, France), and glucose (4.5 mg ml^{-1}). For induction of myogenic differentiation, 2×10^4 of the cells were replated on a 35-mm dish and cultured in primary cultured myocyte differentiation medium (pmDM) consisting of the chemically defined medium TIS [11,12] supplemented with 2% FBS.

COM3 cells were isolated from the mouse myoblastic cell line C2C12 and maintained in medium consisting of DMEM supplemented with 10% FBS and glucose (4.5 mg ml^{-1}) [13]. For induction of myogenic differentiation, 5×10^4 of COM3 cells were plated on a 35-mm dish, and then the medium was switched to TIS on the next day.

Primary cultured mouse myogenic progenitor cells were isolated from gastrocnemius muscles of adult C57BL/6J mice and cultured in pmGM as described previously [10,14].

Immunofluorescence and immunocytochemical analyses

Cells were grown on collagen-coated culture dishes for immunofluorescence and immunocytochemical analysis. The cultured cells were fixed with 4% paraformaldehyde for 10 min at room temperature or on ice, and then permeabilized in phosphate buffered saline (PBS) supplemented with 0.5% Triton X-100 and 1% donkey serum for 20 min. Then the cells were incubated for 12 to 36 h at 4 °C with a mouse monoclonal antibody to sarcomeric myosin heavy chain (undiluted culture supernatant; MyHC) [15] or a goat polyclonal antibody to cAMP-dependent protein kinase type II subunit (RII) (1:100 dilution; Upstate, Lake Placid, NY). Biotinylated donkey antibodies to mouse (1:1000 dilution; Jackson ImmunoReseach Laboratory, Bar Harbor, ME) or goat (1:1000 dilution; Zymed Laboratories, San Francisco, CA) immunoglobulin G were used as secondary antibodies. The biotinylated antibodies were detected with Alexa 488 (Molecular Probes, Eugene, OR) or horseradish peroxidase-conjugated streptavidin. The peroxidase reaction was performed with 3,3-diaminobenzidine (Sigma, St. Louis, MO). Cell nuclei were stained with 2, 4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI) (0.5 μg ml^{-1}; Sigma) or Mayer's hematoxylin (Wako Pure Chemicals, Osaka, Japan). Samples were observed under an inverted microscope (model IX71; Olympus, Tokyo, Japan). Images were taken by a CCD camera (DP70; Olympus) and post-processed using Adobe Photoshop (Adobe Systems, San Jose, CA).

Immunoblotting

Sample preparation and immunoblot analysis were performed as described [11,13]. Immune complexes were detected by colorimetry with a BCIP/NBT detection kit (Nacalai, Kyoto, Japan).

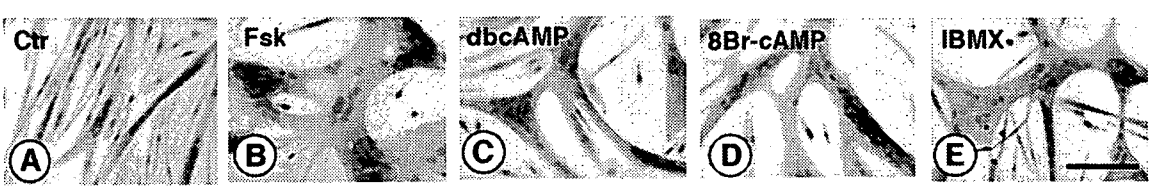

Fig. 1 – Intracellular cAMP-elevating reagents induce muscle cell hypertrophy. (A–E) Ric10 cells (2×10^4 cells per 35-mm dish) were cultured for 36 h in pmDM (A), or in pmDM supplemented with 24 μM forskolin (B), 10 μM dbcAMP (C), 10 μM 8Br-cAMP (D), or 10 μM IBMX (E) to elevate intracellular cAMP levels. Then the cells were subjected to immunostaining for MyHC (brown). Nuclei were counterstained with Mayer's hematoxylin (blue). Images were obtained by bright field microscopy. Scale bar: 100 μm.

Time-lapse recording

Cells were placed in a humid chamber (Olympus, Tokyo, Japan) maintained at 37 °C, and observed under a phase-contrast microscope (IX71, Olympus) with a 10× Plan Apo Fluor objective lens. Time-lapse images were taken with a CCD camera (DP70, Olympus).

Quantification of muscle cell hypertrophy, fusion, and myogenic differentiation

To establish the potentials for cell hypertrophy, fusion, and differentiation, myogenic cells were immunostained with anti-MyHC antibody, and the nucleus was stained with hematoxylin; then at least 1500 nuclei were counted from three randomly chosen areas of three independent cultures. To assess cell hypertrophy, the myotube size was estimated by counting the number of nuclei per cell. The distribution of myogenic cell sizes was determined by calculating the percentage of nuclei in myogenic cells with different numbers of nuclei in the total number of nuclei (myoblasts plus myotubes). The percentage of differentiated cells was measured by calculating the percentage of nuclei in MyHC-positive cells in the total number of nuclei (myoblasts plus myotubes). The incidence of cell fusion, also called the fusion index, was determined by calculating the percentage of nuclei

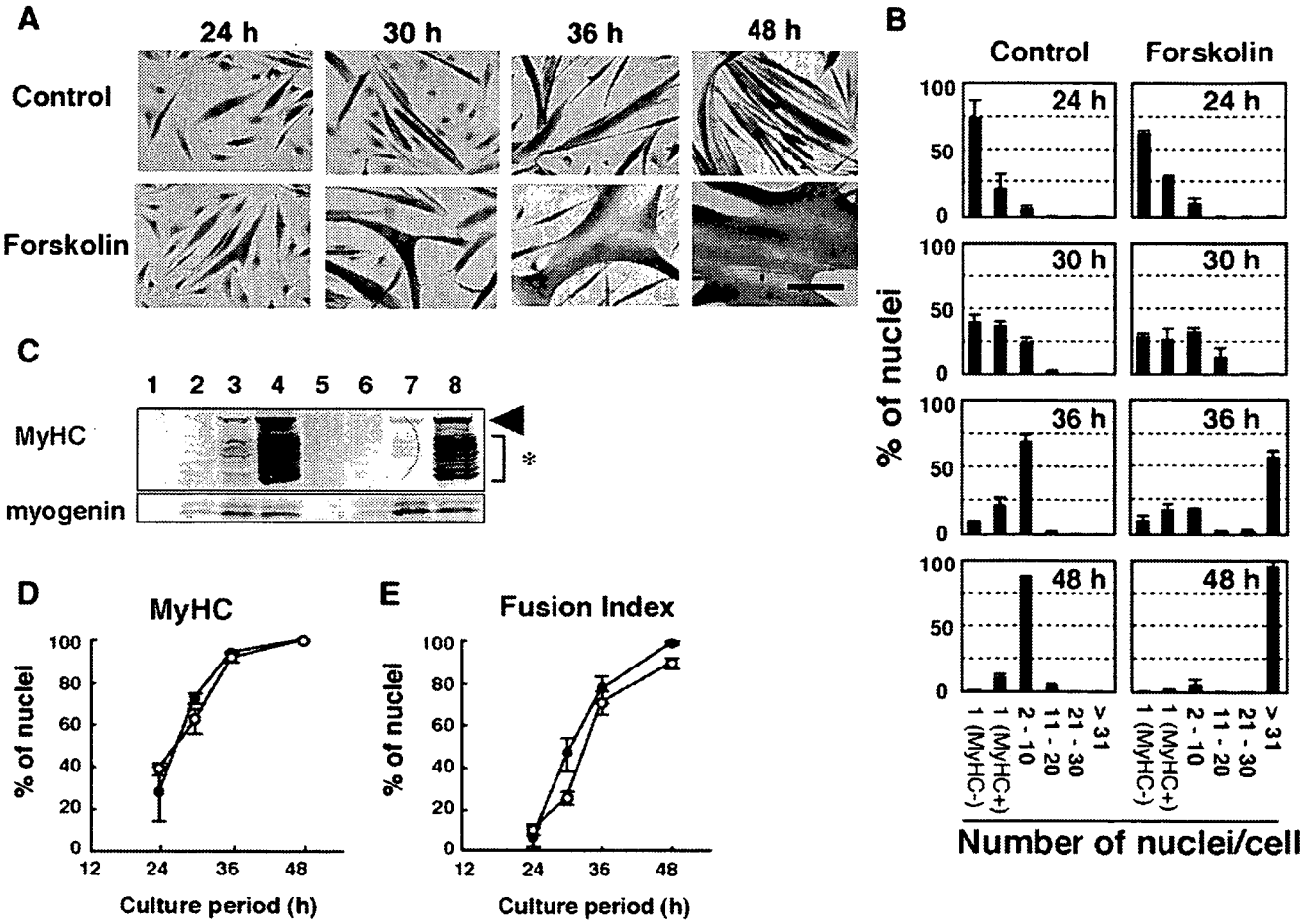

Fig. 2 – Forskolin induces muscle cell hypertrophy but does not enhance cell proliferation and differentiation. Ric10 cells (2×10^4 cells per 35-mm dish) were cultured in pmDM (control) or in pmDM supplemented with 24 μM forskolin for up to 48 h. (A) The cells were fixed after the indicated periods of culture and subjected to immunostaining for MyHC (brown). Nuclei were counterstained with Mayer's hematoxylin (blue). Images were obtained by bright field microscopy. Scale bar: 100 μm. (B) Histograms represent the distribution of myogenic cells with different numbers of nuclei in unstimulated (left panels) and forskolin-stimulated cultures (right panels) after the indicated periods of culture. Mononucleated cells were classified to two subpopulations: one expressed MyHC (MyHC+) and the other did not (MyHC–). (C) Total lysates (20 μg of proteins) were prepared from cells that were cultured in the absence (lanes 1–4) or presence of forskolin (lanes 5–8) for 0 h (lanes 1 and 5), 12 h (lanes 2 and 6), 24 h (lanes 3 and 7) or 36 h (lanes 4 and 8), and then subjected to immunoblot analysis for MyHC and myogenin. MyHC is sometimes degraded in 1% SDS-containing lysis buffer by unknown reasons. Arrowhead and asterisk represent full-length and degraded MyHC species, respectively. (D) Differentiated cells were detected by immunostaining with anti-MyHC antibody in unstimulated (open circles) and forskolin-stimulated cultures (filled circles). (E) Fusion indexes were calculated as percentages of nucleus numbers in multinucleated cells of unstimulated (open circles) and forskolin-stimulated (filled circles) cultures. Averages and standard deviations of three independent cultures are shown in panels B, D and E.

in myotubes in the total number of nuclei (myoblasts plus myotubes).

Reagents and peptides

Dibutyryl-cyclic AMP (dbcAMP), 8-bromo-cyclic AMP (8Br-cAMP), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma. Stearated Ht31 peptide (StHt31S), which is capable of inhibiting PKA anchoring, and its control peptide, StHt31P, were purchased from Promega (Madison, WI). Forskolin (Sigma) was dissolved in ethanol at 24 mM. H89 (Biomol, Devon, UK) was dissolved in dimethylsulfoxide (DMSO) at 10 mM. The other reagents and peptides were dissolved in PBS. Each reagent was diluted with medium immediately before use. For control cultures, the medium was supplemented with the same concentration of vehicle as that used in experimental cultures.

Results

Elevated intracellular cAMP levels induce muscle cell hypertrophy in vitro

We have established a unique primary culture system of mouse myogenic cells derived from muscle satellite cells in adult skeletal muscle [10]. Ric10 is a single cell-derived clone isolated from multiclonal mouse primary cultured myogenic cells, which retains a high capacity for myogenic differentiation. To determine whether cAMP is involved in the regulation of myogenesis, we determined the effects of reagents that elevate intracellular cAMP levels on terminal skeletal muscle differentiation of Ric10 cells in vitro. Ric10 cells were cultured for 36 h in pmDM supplemented with an activator of adenylate cyclase, forskolin, a phosphodiesterase inhibitor, IBMX, or the cAMP analogues 8Br-cAMP and dbcAMP. Ric10 cells gave rise to small bipolar myotubes under differentiation-inducing conditions (Fig. 1A). In contrast, Ric10 treated with the reagents formed large sheet-like multinucleated cells, which we designated "myosheets" (Figs. 1B–E). The results imply that the elevation of intracellular cAMP levels induced muscle cell hypertrophy in vitro.

To elucidate the process of myosheet formation induced by elevated intracellular cAMP levels, Ric10 cells were cultured in pmDM in the presence or absence of forskolin for up to 48 h. No difference in myotube formation was found between unstimulated and forskolin-stimulated Ric10 cells during 24 h of differentiation culture. After 24 h, the size of the myotubes in forskolin-stimulated Ric10 culture increased in proportion to the length of the culture period (Fig. 2A). In control cultures, most myotubes contained 2 to 10 nuclei, and the maximum number of nuclei in myotubes was no more than 20 throughout the culture period (Fig. 2B, left panels). In

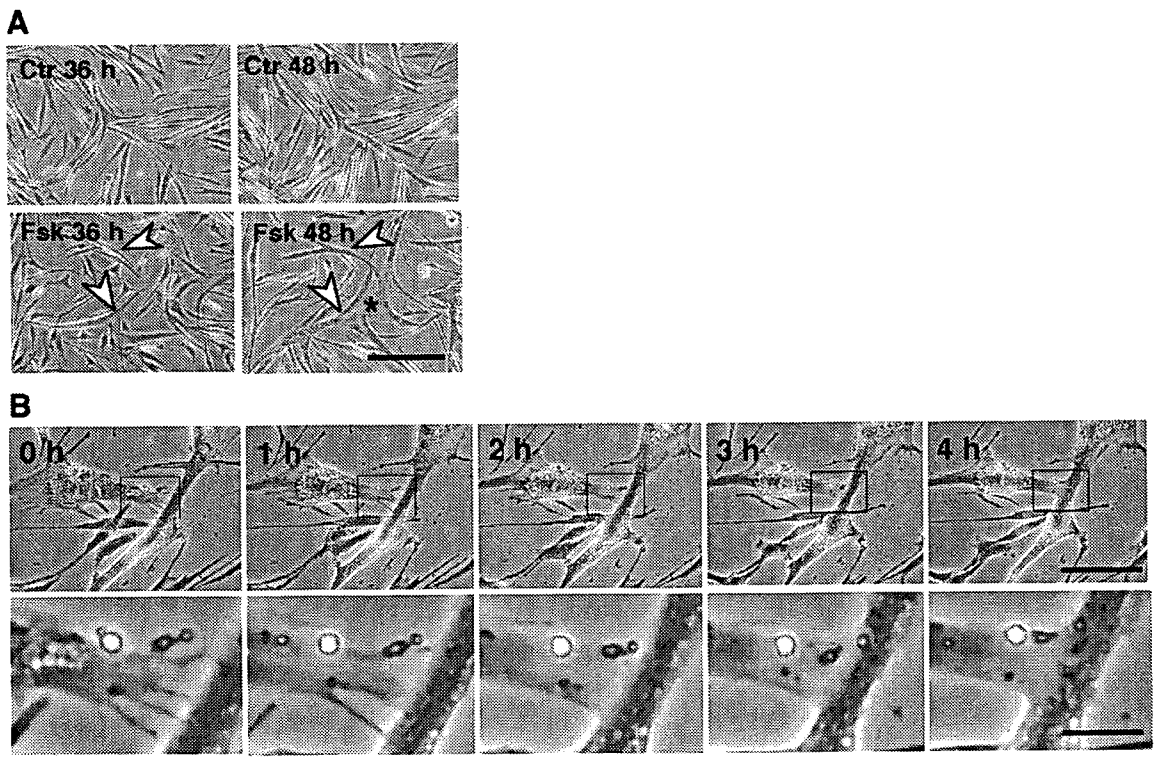

Fig. 3 – Forskolin induces cell fusion between myotubes. Ric10 cells (1×10^4 cells per 35-mm dish) were cultured in pmDM (upper panels in A), or in pmDM supplemented with 24 μM forskolin (lower panels in A and panels in B) for up to 48 h. (A) The same areas of cultures were photographed after 36 and 48 h of differentiation culture. Arrowheads represent myotubes fused with each other to form a large syncytium (asterisk). Scale bar: 100 μm. (B) The process of myotube fusion in Ric10 culture stimulated with forskolin was recorded by phase-contrast, time-lapse microscopy. The lamellipodia of one myotube fused to the lateral plasma membrane of another myotube. Images were obtained at the indicated time points. Squares in the top panels indicate the areas magnified in the bottom panels. Scale bars: 100 μm in top panels; 25 μm in bottom panels.

contrast, forskolin-stimulated Ric10 cells formed extra-large myotubes containing more than 31 nuclei (Fig. 2B, right panels). In forskolin-stimulated culture, more than 90% of nuclei were incorporated into only a few syncytia (Fig. 2A, bottom right panel and Fig. 2B, bottom right panel).

In the next experiments, the effects of forskolin on myogenic differentiation of Ric10 cells were analyzed. Expression patterns of the muscle-specific transcription factor myogenin and the muscle differentiation marker sarcomeric MyHC were similar in unstimulated and forskolin-stimulated Ric10 cells (Fig. 2C). The differentiation index, which represents the ratio of the number of nuclei in MyHC-expressing cells to the total number of nuclei, reached 100% during 48 h of differentiation culture, even in the presence of forskolin (Fig. 2D). In addition, the ratio of the number of nuclei in myotubes to the total number of nuclei, which is called the "fusion index", reached more than 80% after 48 h of culture in the presence or absence of forskolin (Fig. 2E). Therefore, it is unlikely that forskolin affects the myogenic differentiation potential of Ric10 cells. The numbers of nuclei in unstimulated and forskolin-stimulated cultures were 302±64 and 313±11 per mm^2 after 36 h of differentiation culture, respectively, when 2×10^4 cells were plated in a 35-mm dish. The total amounts of cellular protein in unstimulated and forskolin-stimulated cells were 138±32 μg and 125±17 μg after 36 h of differentiation culture when 1×10^5 cells were plated. The results imply that myotube hypertrophy is induced by forskolin independently of stimulation of protein synthesis, cell differentiation, or proliferation. It is noteworthy, however, that fusion of mononucleated progenitor cells was slightly but statistically significantly enhanced in forskolin-stimulated Ric10 cells after 30 h of differentiation culture (Fig. 2E).

Forskolin induces cell fusion between myotubes

Ric10 cells were seeded at low density (1×10^4 cells per 35-mm dish) to slow down the speed of myosheet formation. Then, serial observation of Ric10 differentiation cultures revealed that myosheets were formed through cell fusion between small bipolar myotubes that were stimulated with forskolin (Fig. 3A, lower panels). Although myotube fusion also occurred in control cultures, the incidence was relatively quite low (Fig. 3A, upper panels). The small bipolar myotubes that appeared during the earlier period of differentiation culture formed lamellipodia at their polar ends. Time-lapse recording revealed that myotube-to-myotube fusion in forskolin-stimulated Ric10 cultures always occurred between the leading edge of the lamellipodia of one myotube and the lateral plasma membrane of the other (Fig. 3B and Supplementary data). The results suggest that forskolin enhances cell fusion between myotubes and induces muscle cell hypertrophy, and that lamellipodia of myotubes may retain the capacity for plasma membrane fusion.

Forskolin enhances lamellipodium formation in myotubes prior to cell fusion

A previous study proposed that PKA may be involved in lamellipodium formation of fibroblastic cells [16]. Therefore, the effects of forskolin on the formation of lamellipodia in small bipolar myotubes were determined. Ric10 cells gave rise to small bipolar myotubes containing 2–10 nuclei during 30 h of differentiation culture in pmDM (Fig. 2B). To determine involvement of PKA in forskolin-enhanced lamellipodium formation in myotubes, the small myotubes were cultured for further 6 h with forskolin alone or forskolin plus H89. Forskolin treatment doubled the frequency of lamellipodium formation of unstimulated Ric10 myotubes (Fig. 4A, middle

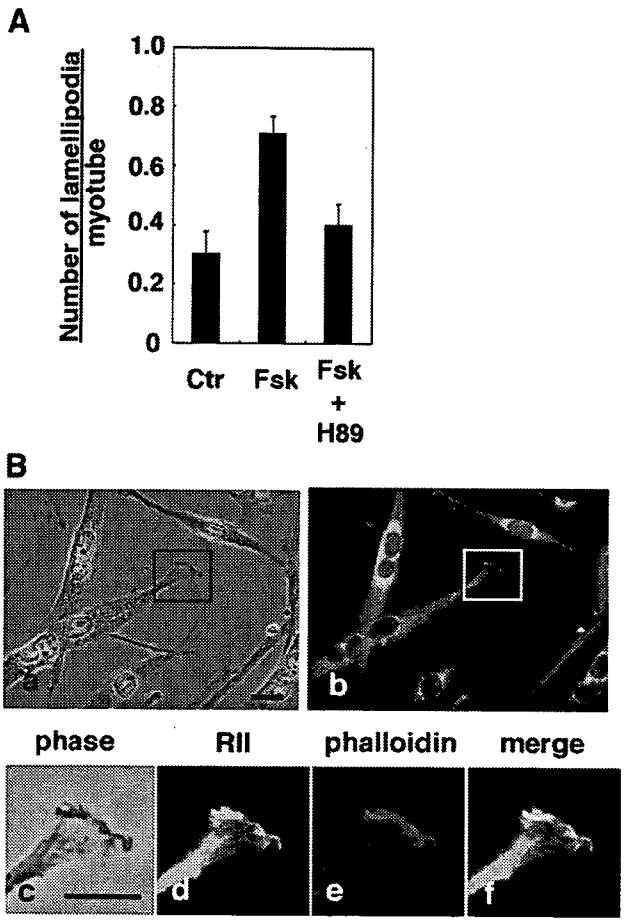

Fig. 4 – Forskolin enhances lamellipodium formation where PKA is located. (A) Ric10 cells (1×10^4 cell per 35-mm dish) were cultured for 36 h in pmDM (Ctr), pmDM supplemented with 24 μM forskolin alone (Fsk), or forskolin plus 10 μM H89 (Fsk+H89). Actin filaments were stained with Alexa Fluor 546-labelled phalloidin. The number of lamellipodia in at least 100 myotubes per dish was counted under a phase-contrast and epifluorescence microscope. Each myotube formed 0, 1, or 2 lamellipodia at their polar ends. Averages and standard deviations of numbers of lamellipodia per myotube from three independent cultures are shown. (B) After 36 h of culture in the medium containing forskolin, Ric10 cells were subjected to immunostaining with anti-PKA RII antibody (RII) (b and d). Actin filaments were stained with Alexa Fluor 546-labelled phalloidin (e). Images in (a) and (c) were obtained by phase-contrast microscopy, and those in (b), (d)–(f) were obtained by epifluorescence microscopy. Cell nuclei stained with DAPI are superimposed in the left panel. (b) Shows the same area in (a). A square in the upper panels represents the area that is magnified in the lower four panels. Scale bars: 20 μm.

column). H89 significantly suppressed the forskolin-enhanced lamellipodium formation (Fig. 4A, right column). Furthermore, immunostaining analysis showed that the type II regulatory subunit of PKA was enriched in the leading edge of lamellipodia where F-actin was accumulated (Fig. 4B), as previously shown in fibroblasts [16]. Taken together with the results here, PKA is likely to be involved in forskolin-enhanced lamellipodium formation.

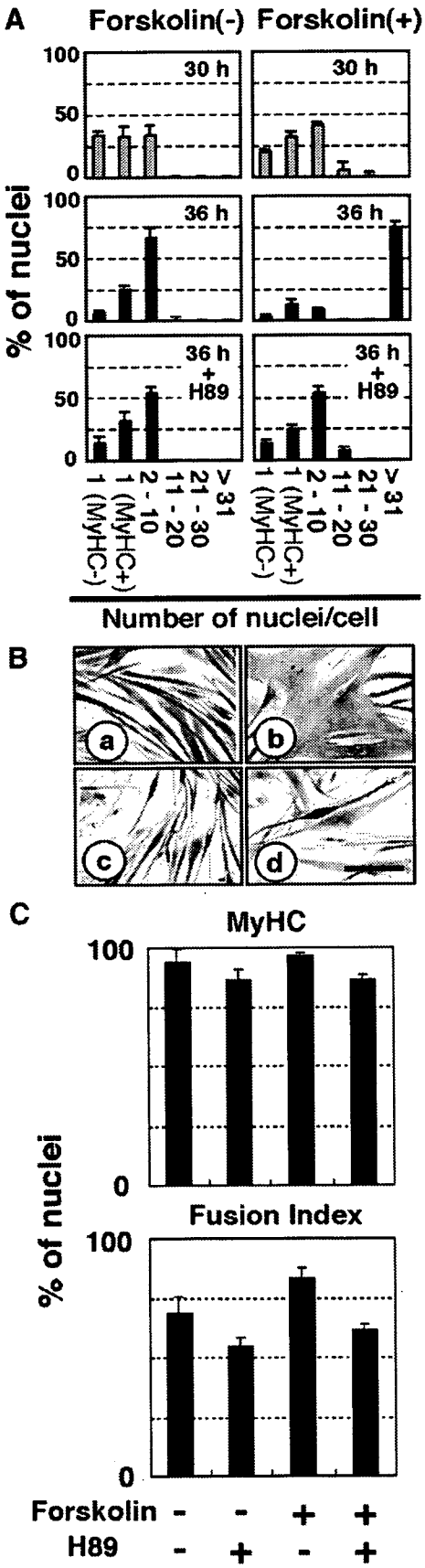

Fig. 5 – Enzymatic activity of PKA is required for induction of myotube fusion by forskolin. (A and B) Ric10 cells (2×10^4 cells per 35-mm dish) were cultured for 30 h in pmDM (upper left panel in A) or in pmDM supplemented with 24 μM forskolin (upper right panel in A). Then the medium was switched to pmDM supplemented with 0.1% DMSO (middle left panel in A and Ba), forskolin plus DMSO (middle right panel in A and Bb), 10 μM H89 (bottom left panel in A and Bc), or forskolin plus H89 (bottom right panel in A and Bd). After 6 h of further culture, the cells were subjected to immunostaining with anti-MyHC antibody. Nuclei were counterstained with Mayer's hematoxylin. Panels show the distribution of myogenic cells with different numbers of nuclei. Mononucleated cells were classified to two subpopulations: one expressed MyHC (MyHC+) and the other did not (MyHC–). Averages and standard deviations of three independent cultures were shown. Scale bar in panel B: 100 μm. (C) Ric10 cells were treated as described in panel A, and then the potential for differentiation and fusion was analyzed as described in Materials and methods. Averages and standard deviations of three independent cultures are shown.

Localized PKA activity is required for forskolin-induced muscle cell hypertrophy

Localization of PKA in lamellipodia of myotubes suggests that blocking PKA activity or anchoring it on these structures will affect forskolin-induced muscle cell hypertrophy. Ric10 cells produced small bipolar myotubes containing 2–10 nuclei during 30 h of differentiation culture in the presence or absence of forskolin (Fig. 5A, upper panels). Then the small myotubes were cultured for further 6 h with forskolin alone or forskolin plus H89 (Fig. 5A, middle and lower right panels, and B). At the end of culture, H89 had prevented myosheet formation in the forskolin-stimulated Ric10 myotube cultures, whereas small and medium-sized myotubes containing 2–20 nuclei had increased in number (Fig. 5A, bottom right panel). The results suggest that the administration of H89 during the last 6 h of culture inhibits mainly forskolin-enhanced cell fusion between small myotubes, resulting in suppression of muscle cell hypertrophy. Both the differentiation and fusion indexes of forskolin-stimulated cultures declined slightly in the presence of H89 (Fig. 5C). However, the reduction of the fusion index or the differentiation index was too small to explain the prevention of myosheet formation in cultures treated with forskolin plus H89 (Fig. 5A, middle and lower right panels). Fusion index was also slightly reduced in the culture treated with H89 alone since H89 inhibited fusion of remnant mononucleated cells during the last 6 h of differentiation culture (Fig. 5C).

In the next series of experiments, stearated Ht31 peptide (StHt31S) was used to inhibit PKA anchoring because it contains a peptide from A kinase anchoring protein (AKAP) and disrupts PKA–AKAP interaction [17]. StHt31S perfectly prevented the muscle cell hypertrophy induced by forskolin,

whereas the control peptide StHt31P, which is incapable of disrupting PKA–AKAP interaction, did not inhibit it (Figs. 6A and B). The forskolin-enhanced lamellipodium formation of small myotubes was suppressed by StHt31S in a similar manner (data not shown).

The decline of the fusion index in StHt31S-treated cells (75.1±7.1% in StHt31P-treated cells versus 51.4±4.8% in StHt31S-treated cells) suggests that StHt31S inhibits cell fusion of mononucleated cells as well as myotubes stimulated with forskolin (Fig. 6C, lower panel). In contrast to the disappearance of myosheets, StHt31S markedly increased the incidence of nuclei in small myotubes in Ric10 cultures treated with forskolin (Fig. 6B). Therefore, the partial inhibition of fusion or differentiation (Fig. 6C, upper panel) of mononucleated cells by StHt31S is unlikely to prevent myosheet formation. We concluded that StHt31S prevents the muscle cell hypertrophy induced by forskolin through suppression of fusion between myotubes. These results suggest that localized PKA activity is required for lamellipodium formation and subsequent cell fusion between myotubes.

In the absence of forskolin, StHt31S also suppressed cell fusion of mononucleated Ric10 cells (64.2±5.6% in StHt31P-treated cultures versus 21.9±5.0% in StHt31S-treated cultures) without significant effects on the expression of MyHC (Fig. 6C) and myogenin (data not shown).

Forskolin induces muscle cell hypertrophy in established and primary cultured myogenic cells

To assess whether myosheet formation enhanced by forskolin is dependent on the type of muscle cells, we determined the effects of forskolin on the myogenic differentiation of a C2C12-derived subclone, COM3 [13], and primary cultured mouse myogenic cells derived from muscle satellite cells [10,14]. COM3 cells differentiated into cylindrical myotubes under the differentiation-inducing condition (Fig. 7A). In contrast, they gave rise to large myotubes when stimulated with forskolin (Fig. 7B). For the assay using primary cultured mouse myogenic cells, colonies derived from single-muscle satellite cells were formed on a 35-mm dish, and then the medium was changed to pmDM. Primary

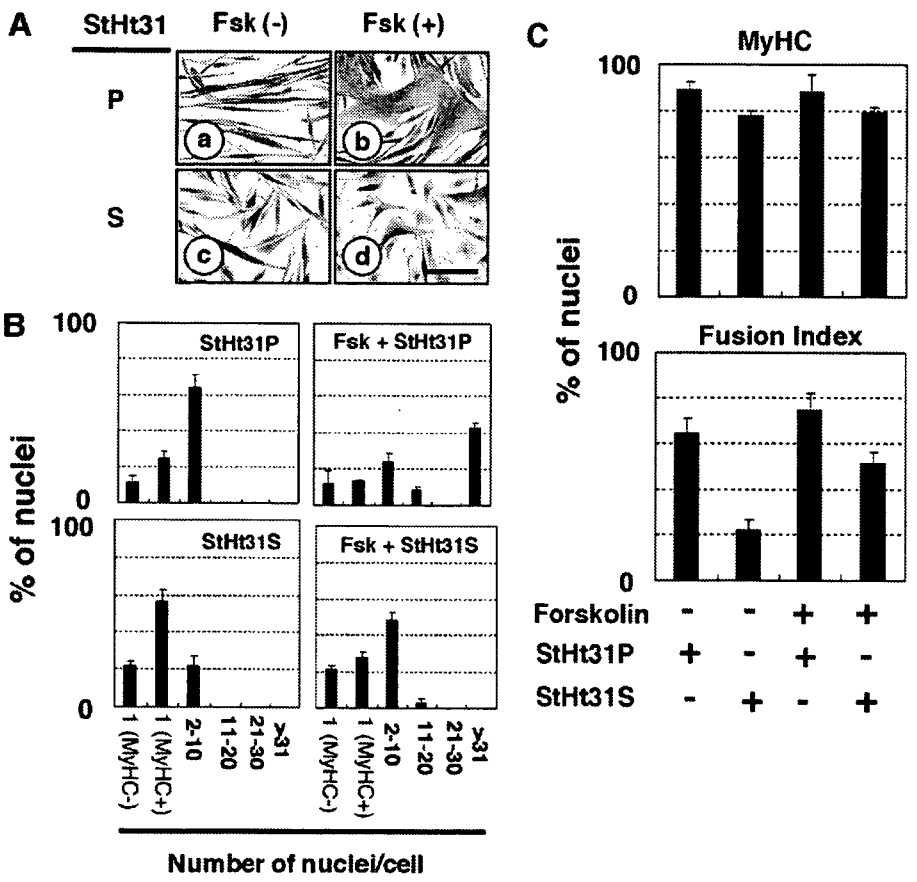

Fig. 6 – Localized PKA activity is required for induction of myotube fusion by forskolin. (A) Ric10 cells (2×10^4 cells per 35-mm dish) were cultured in pmDM (a and c) or pmDM containing 24 μM forskolin (b and d) for 18 h. Then the cells were cultured for further 18 h in medium containing 50 μM StHt31P (a) or StHt31S (c) alone, or forskolin plus 50 μM StHt31P (b) or StHt31S (d). MyHC was immunostained with horseradish peroxidase. Nuclei were counterstained with Mayer's hematoxylin. Images were obtained by bright field microscopy. Scale bar: 100 μm. (B) Panels show the distribution of myogenic cells with different numbers of nuclei in cultures treated as described in panel A. Averages and standard deviations of three independent cultures are shown. (C) Differentiation and fusion potential in cultures treated as described in panel A were analyzed according to Materials and methods. Averages and standard deviations of three independent cultures are shown.

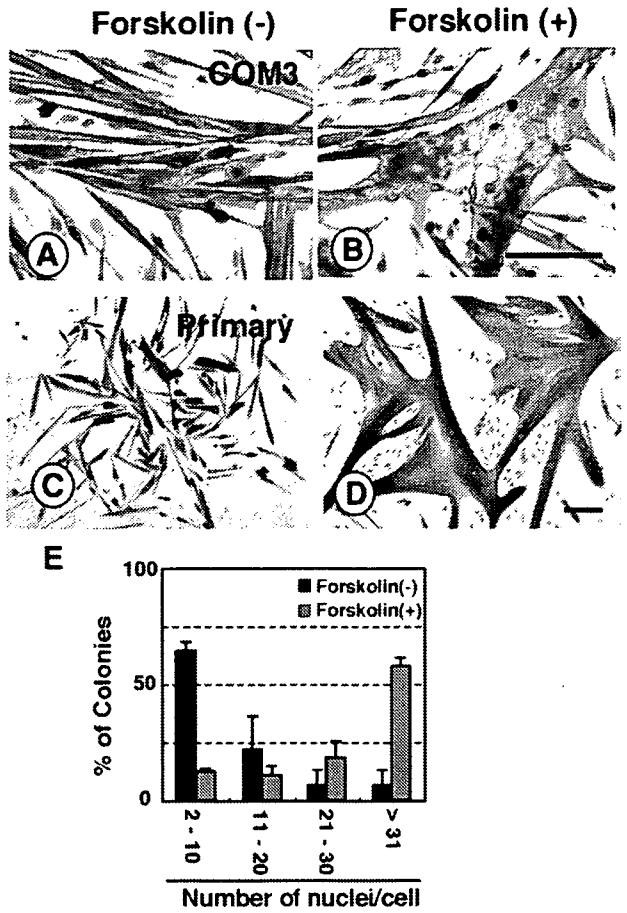

Fig. 7 – Forskolin induces muscle cell hypertrophy in established and primary cultured mouse myogenic cells. COM3, a subline of C2C12 (A and B) and primary cultured mouse myogenic cells (C and D) were cultured in pmDM with (B and D) or without (A and C) stimulation of forskolin (24 μM). MyHC was immunostained with a horseradish peroxidase. Nuclei were counterstained with Mayer's hematoxylin. Images in A–D were obtained by bright field microscopy. (E) Colonies of primary cultured mouse myogenic cells were classified by the size of largest myotube within each colony. Forty-five colonies from three independent dishes were examined in unstimulated (Forskolin (–)) and forskolin-stimulated (Forskolin (+)) cultures, respectively. Histogram represents percentages of colonies that contain the largest myotube with the indicated number of nuclei in the total number of colonies. Averages and standard deviations of three independent cultures are shown.

cultured myogenic cells produced small myotubes during 4 days of differentiation culture (Figs. 7C and E). Forskolin induced myosheet formation in 60.0±6.7% of myogenic cell colonies (Figs. 7D and E). Therefore, the results indicate that the elevation of intracellular cAMP levels by forskolin induced muscle cell hypertrophy in both an established myogenic cell line and primary cultured myogenic cell clones.

Localized PKA activity is also required for cell fusion of mononucleated myogenic progenitor cells

To examine whether localized PKA also plays a pivotal role in fusion of mononucleated myogenic progenitor cells, Ric10 cells were treated with H89 alone. Differentiating mononucleated Ric10 cells were cultured in pmDM for 18 h to avoid the cell detachment caused by the prolonged exposure to H89, and then they were treated with H89 for a further 18 h. H89 markedly inhibited cell fusion of Ric10 cells (69.5±5.6% in control cultures versus 28.5±6.6% in H89-treated cultures) (Fig. 8A, left panel) without significant effects on the

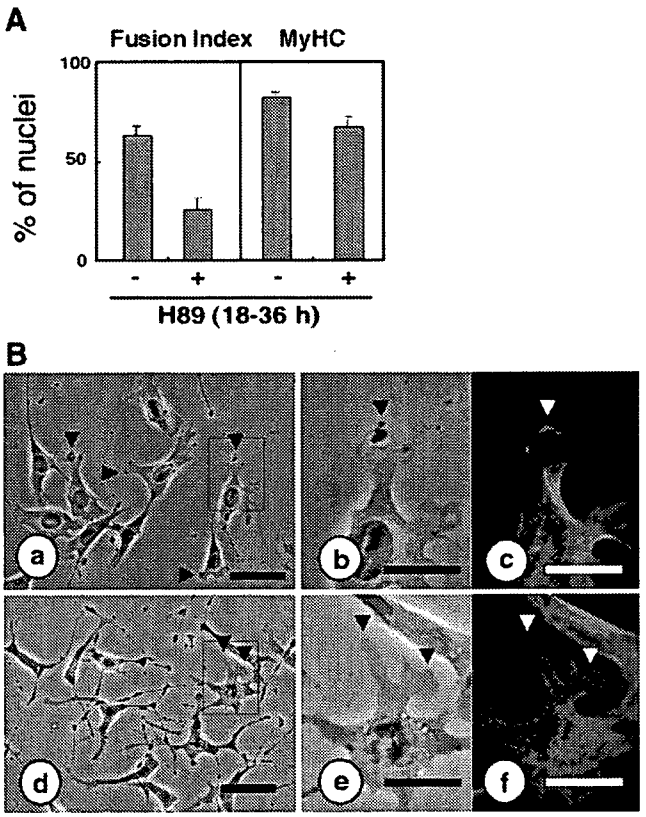

Fig. 8 – Localized PKA activity is required for mononucleated myoblast fusion. (A) Ric10 cells were cultured for 18 h in pmDM. Then the cells were cultured for further 18 h in pmDM supplemented with 0.1% DMSO (–) or 10 μM H89 (+). Fusion index and differentiation potential (MyHC) were analyzed as described in Materials and methods. (B) Ric10 cells were cultured in pmDM supplemented with 0.1% DMSO (a–c) or 10 μM H89 (d–f) for 6 h. Lamellipodium formation (arrowheads in a, b, and c) was severely inhibited by H89 (d). Accumulation of F-actin (b and c) on the leading edge of lamellipodia was not observed in lamellipodia that were formed in H89-treated cells (arrowheads in d, e, and f). Squares in (a) and (d) represent the areas that were magnified in (b) and (c), and (e) and (f), respectively. Images in (a), (b), (d) and (e) were obtained by phase-contrast microscopy, and those in (c) and (f) were obtained by epifluorescence microscopy. Scale bars: 50 μm in a and d; 25 μm in b, c, e, and f.

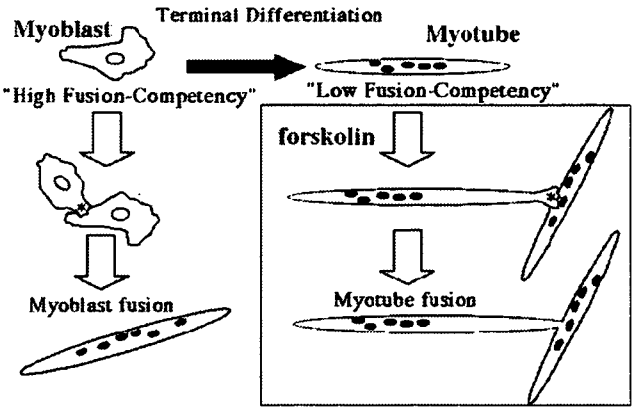

Fig. 9 – Role of localized PKA on myogenic cell fusion. Differentiating myoblasts have the capacity of cell fusion whereas myotubes tend to lose it during terminal muscle differentiation. Myoblast fusion requires the PKA activity localized in lamellipodia. Ability of cell fusion is induced by forskolin through elevating activity of PKA localized in lamellipodia of myotubes. Asterisks represent PKA-localized lamellipodia. A square indicates a process of forskolin-induced myotube–myotube fusion.

expression of MyHC (Fig. 8A, right panel) and myogenin (data not shown).

To determine whether the PKA pathway is involved in lamellipodium formation by mononucleated progenitor cells, Ric10 cells were cultured for 6 h in pmDM supplemented with DMSO (vehicle) or pmDM supplemented with H89. Ric10 cells frequently protruded lamellipodia in control cultures (Figs. 8Ba, b, and c). In contrast, H89 prevented lamellipodium formation, resulting in marked morphological changes in Ric10 cells (Fig. 8Bd). F-actin was accumulated in the leading edge of lamellipodia in mononucleated progenitor cells (Figs. 8Bb and c), as shown in forskolin-stimulated myotubes (Fig. 4B). However, F-actin was not accumulated on the leading edge of lamellipodia in H89-treated Ric10 cells even if they were formed (Figs. 8Be and f). These data suggest that localized PKA activity is required for the lamellipodium formation that is prerequisite for cell fusion of both mononucleated myoblasts and small myotubes (Fig. 9).

Discussion

In the present study, we describe the cell fusion of terminally differentiated myotubes, which has not been shown previously either in vivo or in vitro. Multinucleated myotube fusion results in large, branched myotubes, which we designated "myosheet". Time-lapse recording strongly suggests that branched myotubes are formed by cell fusion of a myotube with another myotube but not with a myoblast. Branched myotubes are usually found in cultured myogenic cells under differentiation-inducing conditions. In addition, branched myofibers are rare in healthy muscle but can be found in regenerated muscle. These observations indicate that terminally differentiated multinucleated myotubes still have the capacity to fuse with each other under appropriate conditions both in vitro and in vivo. The myotube–myotube fusion shown here was not an unusual event and did not depend on a specific cell line or the culture conditions used in our experiments.

We show here that the cAMP–PKA pathway localized in lamellipodia plays a pivotal role in myotube fusion, which is a multistep process. The enhancement of lamellipodium formation by localized PKA activity demonstrates that PKA regulates the early steps of myotube fusion, such as myotube–myotube contact and recognition. Furthermore, the localized PKA might also be involved in the specification of fusion-competent areas of the plasma membrane because only the leading edge of lamellipodia seems to acquire fusion competency.

The role of cAMP in myogenesis has not been established because inconsistent results were reported in previous papers. Membrane-permeable cAMP analogs or compounds that stimulate adenylate cyclase inhibited the expression of muscle-specific genes in the cell line BC3H1 [18,19]. BC3H1 cells are non-fusing cells derived from a mouse intracranial tumor [20], and their expression of muscle-specific genes is rapidly reversible by altering the level of serum or growth factors in the culture medium [21]. BC3H1 cells have been used as a unique model system to reveal the mechanisms by which the expression of muscle-specific genes is regulated. However, they are not true myogenic cells that have the ability to differentiate irreversibly into myofibers. Therefore, we should note that the effects of cAMP in BC3H1 cells are not always the same as in true myogenic cells derived from muscle satellite cells.

Dibutyryl cAMP inhibits the expression of the muscle-specific transcription factor myogenin [22] and terminal muscle differentiation in the mouse myoblastic cell line C2C12 and rat myoblastic cell line L6 [23]. However, the effective concentration of dbcAMP was extremely high (1–3 mM) in those studies. Thus, these results might represent a spurious effect of dbcAMP rather than a true effect of intracellular cAMP because butyrate inhibits myogenesis by interfering with the transcriptional activity of MyoD and myogenin [24]. Cyclic AMP also inhibits the myogenesis induced by vasopressin or insulin-like growth factor I (IGF-I) in L6-C5, a rat myoblastic cell clone selected for its ability to undergo myogenesis when stimulated by these peptides [25–27]. Thus, the inhibition seems to be a specific event in the vasopressin- or IGF-I-induced myogenesis of certain myogenic cell lines. In contrast, the present study demonstrates that intracellular cAMP does not inhibit the expression of muscle-specific genes and cell fusion in both established and primary cultured mouse myogenic cells derived from muscle satellite cells. Similar results have been shown in C2C12 cells [28] and primary cultured chicken myoblasts [29]. Therefore, we should note that an appropriate dose of a cAMP-elevating reagent does not inhibit the expression of muscle-specific genes in primary cultured myogenic cells that are derived from muscle satellite cells.

We and others have demonstrated that cAMP enhances myogenic cell fusion [29–31]. Intracellular cAMP levels rise upon the onset of cell fusion and then decline after fusion in myogenic cell culture [30,32,33]. The nuclear cAMP-PKA pathway was previously assumed to be involved in the regulation of transcriptional activity of the MyoD family

[22,23] or the expression of cAMP phosphodiesterase during myogenesis [34]. In addition, the present study suggests that the localized activity of PKA in lamellipodia is an important facet of a cell-to-cell contact/recognition step of muscle cell fusion.

Compared with the enhancement of myotube fusion, the effect of forskolin on myoblast fusion was not so clear. However, H89 or StHt31S inhibited myoblast fusion severely. Therefore, while localized PKA activity is also required for myoblast fusion, intracellular cAMP levels should not limit the PKA activity because cAMP is markedly produced in myoblasts upon the onset of fusion. In contrast, intracellular cAMP levels might limit PKA activity in myotubes because cAMP levels decline after myoblast fusion [30,32,33]. Here we propose that intracellular cAMP modulates the competence for cell fusion in myogenic cells. The present study reveals a new aspect of the cAMP–PKA pathway in terminal skeletal muscle differentiation.

Acknowledgments

We thank T. Kurisaki for useful discussion. This work was supported by a grant to N.H. from the Ministry of Health, Labor, and Welfare of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2007.10.006.

REFERENCES

[1] M.J. Wakelam, The fusion of myoblasts, Biochem. J. 228 (1985) 1–12.
[2] K.A. Knudsen, L. Smith, S. McElwee, Involvement of cell surface phosphatidylinositol-anchored glycoproteins in cell–cell adhesion of chick embryo myoblasts, J. Cell Biol. 109 (1989) 1779–1786.
[3] E.H. Chen, E.N. Olson, Towards a molecular pathway for myoblast fusion in *Drosophila*, Trends Cell Biol. 14 (2004) 452–460.
[4] H.A. Dworak, H. Sink, Myoblast fusion in *Drosophila*, BioEssays 24 (2002) 591–601.
[5] V. Horsley, G.K. Pavlath, Forming a multinucleated cell: molecules that regulate myoblast fusion, Cells Tissues Organs 176 (2004) 67–78.
[6] R.S. Krauss, F. Cole, U. Gaio, G. Takaesu, W. Zhang, J.S. Kang, Close encounters: regulation of vertebrate skeletal myogenesis by cell–cell contact, J. Cell Sci. 118 (2005) 2355–2362.
[7] T. Sekiya, T. Takenawa, Y. Nozawa, Reorganization of membrane cholesterol during membrane fusion in myogenesis in vitro: a study using the filipin–cholesterol complex, Cell Struct. Funct. 9 (1984) 143–155.
[8] R.B. Cornell, S.M. Nissley, A.F. Horwitz, Cholesterol availability modulates myoblast fusion, J. Cell Biol. 86 (1980) 820–824.
[9] C.S. Mermelstein, D.M. Portilho, R.B. Medeiros, A.R. Matos, M. Einicker-Lamas, G.G. Tortelote, A. Vieyra, M.L. Costa, Cholesterol depletion by methyl-beta-cyclodextrin enhances myoblast fusion and induces the formation of myotubes with disorganized nuclei, Cell Tissue Res. 319 (2005) 289–297.
[10] M.R. Wada, M. Inagawa-Ogashiwa, S. Shimizu, S. Yasumoto, N. Hashimoto, Generation of different fates from multipotent muscle stem cells, Development 129 (2002) 2987–2995.
[11] N. Hashimoto, M. Ogashiwa, S. Iwashita, Role of tyrosine kinase in the regulation of myogenin expression, Eur. J. Biochem. 227 (1995) 379–387.
[12] N. Hashimoto, M. Ogashiwa, E. Okumura, T. Endo, S. Iwashita, T. Kishimoto, Phosphorylation of a proline-directed kinase motif is responsible for structural changes in myogenin, FEBS Lett. 352 (1994) 236–242.
[13] N. Hashimoto, M. Ogashiwa, Isolation of a differentiation-defective myoblastic cell line, INC-2, expressing muscle LIM protein under differentiation-inducing conditions, Dev. Growth Differ. 39 (1997) 363–372.
[14] N. Hashimoto, T. Murase, S. Kondo, A. Okuda, M. Inagawa-Ogashiwa, Muscle reconstitution by muscle satellite cell descendants with stem cell-like properties, Development 131 (2004) 5481–5490.
[15] D. Bader, T. Masaki, D.A. Fischman, Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro, J. Cell Biol. 95 (1982) 763–770.
[16] A.K. Howe, L.C. Baldor, B.P. Hogan, Spatial regulation of the cAMP-dependent protein kinase during chemotactic cell migration, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 14320–14325.
[17] S. Vijayaraghavan, S.A. Goueli, M.P. Davey, D.W. Carr, Protein kinase A-anchoring inhibitor peptides arrest mammalian sperm motility, J. Biol. Chem. 272 (1997) 4747–4752.
[18] D.J. Kelvin, G. Simard, A. Sue-A-Quan, J.A. Connolly, Growth factors, signaling pathways, and the regulation of proliferation and differentiation in BC3H1 muscle cells: II. Two signaling pathways distinguished by pertussis toxin and a potential role for the *ras* oncogene, J. Cell Biol. 108 (1989) 169–176.
[19] J.S. Hu, E.N. Olson, Regulation of differentiation of the BC3H1 muscle cell line through cAMP-dependent and -independent pathways, J. Biol. Chem. 263 (1988) 19670–19677.
[20] D. Schubert, A.J. Harris, C.E. Devine, S. Heinemann, Characterization of a unique cell line, J. Cell Biol. 61 (1974) 398–413.
[21] R. Munson Jr., K.L. Caldwell, L. Glaser, Multiple controls for the synthesis of muscle-specific proteins in BC3H1 cells, J. Cell Biol. 92 (1982) 350–356.
[22] A. Salminen, T. Braun, A. Buchberger, S. Jurs, B. Winter, H.H. Arnold, Transcription of the muscle regulatory gene *Myf4* is regulated by serum components, peptide growth factors and signaling pathways involving G proteins, J. Cell Biol. 115 (1991) 905–917.
[23] B. Winter, T. Braun, H.H. Arnold, cAMP-dependent protein kinase represses myogenic differentiation and the activity of the muscle-specific helix–loop–helix transcription factors Myf-5 and MyoD, J. Biol. Chem. 268 (1993) 9869–9878.
[24] L.A. Johnston, S.J. Tapscott, H. Eisen, Sodium butyrate inhibits myogenesis by interfering with the transcriptional activation function of MyoD and myogenin, Mol. Cell. Biol. 12 (1992) 5123–5130.
[25] F. Naro, V. De Arcangelis, C. Sette, C. Ambrosio, H. Komati, M. Molinaro, S. Adamo, G. Nemoz, A bimodal modulation of the cAMP pathway is involved in the control of myogenic differentiation in L6 cells, J. Biol. Chem. 278 (2003) 49308–49315.
[26] F. Naro, C. Sette, E. Vicini, V. De Arcangelis, M. Grange, M. Conti, M. Lagarde, M. Molinaro, S. Adamo, G. Nemoz, Involvement of type 4 cAMP-phosphodiesterase in the myogenic differentiation of L6 cells, Mol. Biol. Cell 10 (1999) 4355–4367.
[27] V. De Arcangelis, D. Coletti, M. Conti, M. Lagarde, M. Molinaro, S. Adamo, G. Nemoz, F. Naro, IGF-I-induced differentiation of L6 myogenic cells requires the activity of cAMP-phosphodiesterase, Mol. Biol. Cell 14 (2003) 1392–1404.

[28] C.C. Tsai, J.E. Saffitz, J.J. Billadello, Expression of the G_s protein alpha-subunit disrupts the normal program of differentiation in cultured murine myogenic cells, J. Clin. Invest. 99 (1997) 67–76.
[29] E.J. Aw, P.G. Holt, P.J. Simons, Myogenesis in vitro. Enhancement by dibutyryl cAMP, Exp. Cell Res. 83 (1974) 436–438.
[30] R.J. Zalin, W. Montague, Changes in adenylate cyclase, cyclic AMP, and protein kinase levels in chick myoblasts, and their relationship to differentiation, Cell 2 (1974) 103–108.
[31] K. Stygall, R. Mirsky, The effect of cholera toxin on myogenesis in rat skeletal muscle cultures, Dev. Biol. 78 (1980) 14–24.
[32] U.B. Schutzle, M.J. Wakelam, D. Pette, Prostaglandins and cyclic AMP stimulate creatine kinase synthesis but not fusion in cultured embryonic chick muscle cells, Biochim. Biophys. Acta 805 (1984) 204–210.
[33] N.L. Siow, R.C. Choi, A.W. Cheng, J.X. Jiang, D.C. Wan, S.Q. Zhu, K.W. Tsim, A cyclic AMP-dependent pathway regulates the expression of acetylcholinesterase during myogenic differentiation of C2C12 cells, J. Biol. Chem. 277 (2002) 36129–36136.
[34] T. Kovala, I.A. Lorimer, A.M. Brickenden, E.H. Ball, B.D. Sanwal, Protein kinase A regulation of cAMP phosphodiesterase expression in rat skeletal myoblasts, J. Biol. Chem. 269 (1994) 8680–8685.

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Osteogenic properties of human myogenic progenitor cells

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ARTICLE INFO

Article history:
Received 6 August 2007
Received in revised form
11 November 2007
Accepted 14 November 2007
Available online 22 November 2007

Keywords:
Muscle satellite cell
Muscle regeneration
Osteogenesis
Myogenesis
Stem cell
Multipotentiality

ABSTRACT

Here, we identified human myogenic progenitor cells coexpressing Pax7, a marker of muscle satellite cells and bone-specific alkaline phosphatase, a marker of osteoblasts, in regenerating muscle. To determine whether human myogenic progenitor cells are able to act as osteoprogenitor cells, we cultured both primary and immortalized progenitor cells derived from the healthy muscle of a nondystrophic woman. The undifferentiated myogenic progenitors spontaneously expressed two osteoblast-specific proteins, bone-specific alkaline phosphatase and Runx2, and were able to undergo terminal osteogenic differentiation without exposure to an exogenous inductive agent such as bone morphogenetic proteins. They also expressed the muscle lineage-specific proteins Pax7 and MyoD, and lost their osteogenic characteristics in association with terminal muscle differentiation. Both myoblastic and osteoblastic properties are thus simultaneously expressed in the human myogenic cell lineage prior to commitment to muscle differentiation. In addition, C3 transferase, a specific inhibitor of Rho GTPase, blocked myogenic but not osteogenic differentiation of human myogenic progenitor cells. These data suggest that human myogenic progenitor cells retain the capacity to act as osteoprogenitor cells that form ectopic bone spontaneously, and that Rho signaling is involved in a critical switch between myogenesis and osteogenesis in the human myogenic cell lineage.

1. Introduction

Ectopic ossification in skeletal muscle is characteristic of certain muscle diseases in humans (Kocyigit et al., 2001; Mahboubi et al., 2001). Bone marrow stromal cells are thought to be the primary source of osteoprogenitor cells in the body. Although intravenously transplanted mouse bone marrow cells settle in skeletal muscles (Brazelton et al., 2003; Ferrari

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doi:10.1016/j.mod.2007.11.004

et al., 1998; LaBarge and Blau, 2002), these cells have not been shown to migrate to muscle during ectopic ossification. Previous studies have suggested that osteoprogenitor cells reside in skeletal muscle, although their source has remained unknown (Bosch et al., 2000; Levy et al., 2001). Recent studies, however, suggest that vascular smooth muscle cells are candidates for osteoprogenitors in human skeletal muscle (Hegyi et al., 2003; Levy et al., 2001).

Muscle satellite cells are skeletal muscle stem cells that are activated to proliferate and then fuse with each other to form myofibers during muscle regeneration. Histopathologic and molecular biological studies indicate that progenitor cells derived from muscle satellite cells differentiate exclusively into myotubes and myofibers in vivo. However, our clonal analysis demonstrated that mouse muscle satellite cells actually preserve the ability to undergo osteogenic terminal differentiation in vitro in a bone morphogenetic protein (BMP) stimulation-dependent manner (Wada et al., 2002). Our previous results also suggest the possibility that failure of the restriction of phenotypic plasticity triggers ectopic ossification in muscle satellite cells (Wada et al., 2002).

A previous study referred to bone marrow stromal cells, which form bone spontaneously, as "determined osteoprogenitor cells" and to osteoprogenitor cells from extraskeletal sources, which require inductive agents to express osteogenic phenotypes, as "inducible osteoprogenitor cells" (Friedenstein and Kuralesova, 1971). In mice, descendants of muscle satellite cell undergo osteogenic differentiation only when exposed to BMPs in vitro (Asakura et al., 2001; Hashimoto et al., 2004; Wada et al., 2002). In the absence of BMPs, these cells preferentially undergo myogenic terminal differentiation in vitro. However, it remains to be determined whether osteogenic differentiation of human myogenic cells depends on BMP stimulation, although our previous study demonstrated that these cells are able to undergo osteogenic terminal differentiation in vitro (Wada et al., 2002). In addition, the involvement of BMPs in ectopic bone formation in skeletal muscle has not been shown in human disorders. Therefore, it is likely that ectopic bone formation in human skeletal muscle can be triggered in a BMP-independent manner, although "determined osteoprogenitor cells" have not been identified in human skeletal muscle.

We here show that muscle progenitor cells spontaneously expressed both a marker of muscle satellite cells and a marker of osteoblasts in regenerating human muscle. We then cultured both primary and immortalized human myogenic progenitor cells to determine whether they can undergo osteogenic differentiation without exogenous BMPs. We found that exogenous BMP stimulation is not necessary to induce ossification in both primary and immortalized human myogenic progenitor cells. Therefore, we propose the hypothesis that human myogenic progenitor cells preserve the ability to act as "determined osteoprogenitor cells" and undergo osteogenic terminal differentiation when myogenic differentiation is impaired in human muscle diseases. In addition, the immortalized human myogenic progenitor cell lines used have a multipotentiality that enables us to analyze the nature of human myogenic progenitor cells in detail, as previously done in mouse and rat myoblastic cell lines. They will also provide a model system for genetic modification and transplantation of human myogenic cells.

2. Results

2.1. Osteogenic properties of a human myogenic cell lineage in vivo

The transcription factor Pax7 is expressed exclusively in muscle satellite cells in mouse skeletal muscle (Seale et al., 2000). To determine whether Pax7 is a specific marker for human muscle satellite cells, we probed cryosections prepared from normal back muscle with antibodies to this protein. Pax7-positive nuclei were detected exclusively between the basement membrane and the sarcolemma, which were recognized by antibodies to laminin and dystrophin, respectively (Fig. 1A), suggesting that Pax7 is indeed specifically expressed in human muscle satellite cells (Reimann et al., 2004). Quiescent muscle satellite cells expressing Pax7 did not express bone-specific alkaline phosphatase (ALP), an early marker of osteogenic differentiation. In contrast, ALP was detected histochemically in the small regenerating myofibers present in humans with skeletal muscle diseases including Duchenne muscular dystrophy (DMD) (Fig. 1B–D) (Nonaka et al., 1981). To determine whether human myogenic progenitor cells express osteogenic markers during muscle regeneration, we examined the expression of ALP in muscle of individuals with DMD. In DMD muscles, muscle regeneration is spontaneously triggered and myogenic progenitor cells are present, although ectopic bone has not been found. Immunofluorescence analysis revealed that the ALP activity in small myofibers of a 5-month-old boy with DMD was attributable to bone-specific ALP located at the plasma membrane (Fig. 1C and D). Probing cryosections with antibodies to Pax7 and bone ALP also revealed several mononucleated cells coexpressing Pax7 and ALP in the regenerating muscle of a 4-month-old boy with DMD (Fig. 1B). One or two double-positive cells were found in each cryosection (~20 mm^2) of muscles from these two boys with DMD. These results thus indicate that myogenic progenitor cells with both myoblastic and osteoblastic properties are present in regenerating human skeletal muscles in vivo. Pax7 expression suggests that these cells might be descendants of muscle satellite cells. The results suggest that human myogenic progenitors are probable candidates for osteoprogenitor cells.

2.2. Coexpression of myoblast- and osteoblast-specific proteins in primary human myogenic progenitor cells in vitro

We previously isolated a primary human myogenic progenitor cell clone, Hu5, from the healthy subcutaneous muscle of a nondystrophic woman (Fig. 2A). To determine whether human muscle progenitor cells are able to act as osteoprogenitor cells, we characterized Hu5 cells. Expression of Pax7 was detected in the majority of Hu5 cells (more than 80%) in early passages (less than 6 passages), but not during successive passages (Fig. 2B). Further, Hu5 cells differentiated spontaneously into prominent myotubes in vitro on achieving confluence (Fig. 2C). They also underwent terminal osteogenic

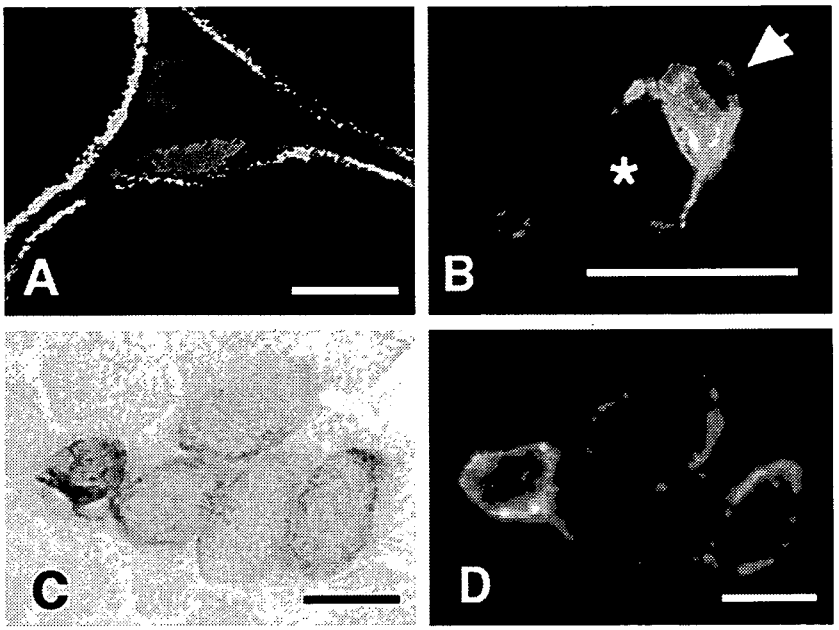

Fig. 1 – Coexpression of myogenic- and osteogenic-specific markers in cells present in skeletal muscle of individuals with DMD. (A) Confocal immunofluorescence analysis of a skeletal muscle section from a nondystrophic 62-year-old woman using antibodies to Pax7, laminin, and dystrophin. A muscle satellite cell expressing Pax7 (red) was detected between the basement membrane containing laminin (blue) and the sarcolemma containing dystrophin (green). (B) Cryosections of the biceps brachii muscle of a 4-month-old boy with DMD were subjected to immunofluorescence analysis with antibodies to Pax7 (red) and to bone-specific ALP (green). The arrow indicates a cell expressing both Pax7 and ALP that was located adjacent to a small, newly regenerated myofiber expressing ALP (asterisk). (C) Cryosections of the biceps brachii muscle of a 5-month-old boy with DMD were subjected to staining of ALP activity with Fast Blue RR. ALP activity was restricted to small, newly regenerated myofibers. (D) Immunofluorescence analysis revealed the presence of bone-specific ALP at the plasma membrane of the same myofibers shown in (C). Scale bars: 10 μm (A) and 20 μm (B–D).

differentiation accompanied by calcification in the presence of both BMP2 and βGP, whereas myogenic differentiation is prevented (Hashimoto et al., 2006). In addition, a phosphate donor, βGP, alone induced calcification in Hu5 cells without BMP2 stimulation (Fig. 2D). Unexpectedly, ALP was detected histochemically in all unstimulated Hu5 cells (Fig. 2E) whereas primary cultured mouse myogenic cells express bone ALP only when stimulated with BMP2 (Wada et al., 2002). The observation is consistent with result that Hu5 cells underwent osteogenic terminal differentiation without BMP2 stimulation. Double staining revealed that more than 80% of undifferentiated Hu5 cells coexpressed Pax7 and bone ALP in the absence of BMP2 (Fig. 2F) in a similar manner to mononucleated myogenic cells in vivo (Fig. 1E). In addition, Hu5 cells also express determination genes, including those for MyoD and Runx2, which are specific for myogenic and osteogenic differentiation, respectively (Table 2). The Hu5 cell, which is a myogenic progenitor cell clone derived from the healthy muscle, thus exhibits both myoblast- and osteoblast-specific properties. The expression of both myogenic- and osteogenic-specific proteins was also demonstrated in six independent clones of myogenic progenitor cells isolated from the subcutaneous muscle of another woman (see Fig. S1 online). We found no clones that retain only myoblast- or osteoblast-specific properties under our culture conditions. The results suggest that human myogenic progenitor cells coexpress myoblast- and osteoblast-specific proteins during in vitro culture.

2.3. *Preservation of dual lineage-specific properties in immortalized human myogenic progenitor cells*

In contrast to primary cultured mouse myogenic cells, the primary myogenic progenitor cell clone Hu5 ceased proliferation and underwent replicative senescence after 10–12 passages. Hu5 cells also lost differentiation potential along with cellular senescence, making it difficult to characterize Hu5 cells undergoing replicative senescence. Therefore, to facilitate analysis of the myogenic and osteogenic properties of Hu5 cells at the molecular level, we used the cells that were immortalized in vitro by introduction of the reverse transcriptase component of human telomerase (hTert) and human papillomavirus (HPV)-16 E7 (Hashimoto et al., 2006).

One of the Hu5-derived clones retaining myogenic differentiation potential, E18, was subjected to further analyses, although similar results were obtained with the other eight independent clones derived from individual Hu5 cells. The muscle lineage markers desmin, nestin, and a master gene for myogenesis, *MyoD*, were expressed in both parent Hu5 and immortalized E18 cells (Table 2).

In addition, Runx2 (Cbfa1), an essential transcription factor for osteogenesis (Komori et al., 1997; Otto et al., 1997),