C．研究結果

1．術後再発は全体224例中68例に認められ、再発率は30.4％であった。単変量解析では、年齢、不妊症、子宮筋腫、子宮腺筋症の有無、子宮内膜症性卵巣嚢胞の手術既往の有無、嚢胞が単数か複数か、片側か両側か、術後薬物治療の有無はいずれも、再発に有意な影響を与えなかった。子宮内膜症に対する薬物療法既往のある症例、最大嚢胞径の大きい症例、rASRMが高得点の症例では、再発を起こしやすい傾向があり、一方術後妊娠した症例では再発を起こしにくい傾向があった。ロジスティック回帰分析では、他の因子と独立して統計学的に有意に再発率を上げる因子として、子宮内膜症に対する薬物既往、大きい最大嚢胞径の2因子があげられた。rASRMスコア得点は再発率に統計学的に有意な影響は与えなかった。一方術後妊娠は、他の因子と独立して統計学的に再発率を低下させた。

2．子宮腺筋症と子宮内膜症の有無により分類して比較した。hMG総投与量はD群に比べA群で有意に多く、hCG投与時estradiol値はB群、D群に比べA群で有意に少なく、採卵数はB群、D群に比べA群、C群で有意に少なく、受精卵数はB群、D群に比べA群で有意に少なかったが、着床率、臨床妊娠率、流産率は4群間に有意差を認めなかった。

3．Metforminの添加は，ESC培養上清中のIL-8産生量を対照に比べ濃度依存性に67％まで低下させた。また，aromataseの遺伝子発現および活性を濃度依存性に最大58％と66％に抑制した。さらに，metforminの添加は，細胞増殖能を濃度依存性に最大22％に抑制したが，細胞毒性は認められなかった。

D．考察

　子宮内膜症合併不妊の治療は腹腔鏡手術を行った後に、保存的療法ならびに体外受精をおこなうのが妥当と考えられるが、今回の研究結果が示すように子宮内膜症性卵巣嚢胞の摘出後に約3割が再発することを考慮しなくてはならない。すなわち、現在不妊治療中である場合にはできるだけ早期の妊娠を目指して短期間に治療をステップアップする必要があると考えられる。一方で、妊娠を先に延ばしたい場合などは再発を予防する方法が必要となってくる。一般に、低用量ピルが子宮内膜症の発症を低下させると言われている。今後、術後に低用量ピルを使用した場合に実際に再発率が低下するか否かのエビデンスを構築し、もし、そうであれば低用量ピルの使用をガイドラインなどに盛り込む必要がでてくるかも知れない。一方、低用量ピルでは不十分な結果が得られたならば、さらに一歩進んだ薬物療法が必要となるであろう。この意味で、今回の研究で子宮内

膜症に対しメトフォルミンが治療薬となる可能性が示唆されたため、メトフォルミンの更なる検討が必要かと思われる。メトフォルミンは副作用が少なく比較的長期間使える薬物である。今回はin vitroの研究のみであったが、今後はin vivoの研究も含めてメトフォルミンが子宮内膜症の治療薬となり得るかを一層研究する必要がある。

　子宮腺筋症は社会状況の変化によりますます不妊との関係で重要な疾患となると考えられる。今回の研究では子宮腺筋症が明かに体外受精の成績を低下させるというデータは得られなかった。しかしながら、子宮筋腫がそうであるように、子宮腺筋症のなかのあるサブグループが妊孕性を低下させる可能性が残っており、今後より詳細な検討が必要である。他方、今回のデータをポジティブにとらえるなら、子宮腺筋症を合併する不妊症に対しては体外受精が治療のよい選択肢になるともいえる。また、子宮腺筋症は痛みなども伴い子宮摘出を選択する場合も少なくないが、何らかの方法で子宮腺筋症を早期に発見し、発育を抑制しておけば妊娠が可能であるともいえるので、早期発見し薬物療法などで進展を予防するといった長期戦略を各個人に合わせて考えることができよう。

E．結論

　子宮内膜症による不妊治療に手術療法と体外受精は有効であるが、いまだ効果は十分でなく更なる妊娠率向上のためには手術療法の限界まで踏まえた管理が必要である。子宮内膜症卵巣嚢胞の保存手術では約3割の再発があり、再発率をあげる要因として嚢胞の大きさなどがある。今後は再発まで考えた長期管理の指針を検討していく必要がある。子宮腺筋症は体外受精の成績を低下させなかったため、子宮腺筋症の適切な管理と体外受精を組み合わせることにより妊孕性の向上が期待できると考えられた。メトフォルミンは子宮内膜症に対し治療効果をもつ可能性が示唆されたので、より研究をすすめる必要性が認められた。来年度以降の研究では、子宮内膜症・子宮腺筋症の妊娠率向上にむけての基礎的・臨床的研究を一層発展させるとともに子宮筋腫にや多嚢胞性卵巣症候群に対する生殖補助医療の成績向上を目指した研究にも取り組みたい。

F．健康危険情報

　特記すべき事なし

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H．知的財産権の出願・登録状況
　　特記すべき事なし

卵子の質の低下を引き起こす原因究明に関する基礎的研究

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研究要旨：哺乳動物の卵子は母体内で受精から着床、さらに個体への発生が起こるため、受精および胎児の発育に影響を与える母体因子の研究は進んでいない。本研究では、母体へのストレスにより、排卵後の卵子が影響を受け、10 分以内に死滅、または、受精能力が極度に低下することを見出した。今後、このような卵子の質の低下を引き起こす母体因子を同定し、母体のストレス応答と卵子の質の低下との関連を明らかにする。

A．研究目的

卵子や精子といった生殖細胞は体細胞とは異なり、老化による質の低下を受けない唯一の細胞であると従来は考えられてきた。しかし、ヒトの不妊症に関わる臨床研究から、生殖細胞も例外ではなく老化することがわかってきた。その結果として、女性の妊娠率は20歳代前半でピークを向かえ、30歳代では急激に低下する傾向があることが報告されている。しかし、生殖細胞の老化には個人差があり、個体の老化との相関関係はいまだに不明な点も多い。また、生殖細胞の老化がこの10年程度で急速に進むようになったというのも生物学的には理解しがたいことである。本研究では、卵子の質の低下を引き起こす原因の候補として“母体のストレス応答”に注目して検討をおこなった。ヒト不妊症の原因究明と対処法の確立は出生率低下による急激な人口の減少を食い止めるために必要である。

B．研究方法

マウスをモデル動物として用いることにより、母体へのストレスと卵子の質の低下との関連について検討をおこなった。マウスへのストレスとしては母体の温度を急速に低下させた。具体的には、母体を安楽死させたのち、約23度に設定された金属トレー上で一定時間保温したのち、採卵をおこなった。また、用いたマウスの週齢は、加齢との関連も加えて検討を行うために、8週齢（性成熟個体）と3週齢（未成熟個体）の2種類のグループのマウスについて検討を行った。さらにマウスの系統による差を検討するため、C57BL/6、ICR、BDF1の3系統を用いた。コントロールとしては、頚椎脱臼後すみやかに卵子を取り出し、パラフィンオイルで覆った培養液（ドロップ）で培養を行った。パラフィンオイルで覆うことにより、培養液の急速な温度変化およびCO_2濃度の変化を防ぐことができる。

（倫理面への配慮）

本研究では実験動物としてマウスを用いた研究を行った。動物実験については、国立成育医療センター動物実験指針に従い、特に、動物愛護と動物福祉の観点から、実験動物の使用は目的に合致した最小限にとどめ、またその際、麻酔などの手段動物個体への苦痛を最小限に食い止め、また、その際、麻酔等の手段により苦痛を与えない等の倫理的配慮を行った。また、各施設に当該研究所に設置されている動物委員会の審査機関に実験計画書を申請し承認を得て実験を実施した。

C．研究結果

（1）性成熟マウスにおける排卵卵子の質の検討

8週齢マウスからの卵子の採取を行ったところ、23℃に10分以上保温したマウスから採取した排卵卵子では90％以上が形態の異常を呈し、1 時間以内に断片化（フラグメンテーション）を引き起こし、残りの10％についても6時間以内に死滅してしまった。この結果はマウスの系統による差異は認められず、どの系統でも卵子の6時間以内での死滅が確認された。それに対して、コントロールとして安楽死直後に採卵した卵子では、細胞死の割合は10％以内であった。

（2）未成熟マウスにおける排卵卵子の質の検討

3週齢マウスからの卵子の採取を行ったところ、8週齢マウスと同様に、23℃に10分以上保温したマウスから採取した排卵卵子では90％以上が形態の異常を呈し、1 時間以内に断片化（フラグメンテーション）を引き起こし、残りの10％についても6時間以内に死滅してしまった。この結果はマウスの系統による差異は認められず、どの系統でも卵子の6時間以内での死滅が確認された。それに対して、コントロールとして安楽死直後に採卵した卵子では、細胞死の割合は10％以内であった。

（3）卵巣内の卵子における質の検討

卵巣内にある卵子について、卵巣を細切して取り出したところ、体外培養によって24時間培養することにより、卵子は成熟した卵子の直径（約80μm）に達し、透明帯を除くことにより精子との融合も可能であった。この結果は系統間および週齢による差異は認められなかった。

（4）CD9トランスジェニックマウス（CD9Tgマウス）を用いた細胞死の原因の検討

CD9は膜4回貫通型タンパク質でCD9のN末端に蛍光タンパク質を結合させた融合タンパク質はCD9と同等の機能をもち、卵子の細胞膜を可視化できる。こ

の卵子を用いて、前述の実験を行ったところ、顕微鏡観察により卵子の細胞膜は崩壊していることがわかった。この卵子の細胞死がアポトーシスによるものかどうかは現在検討を行っているところである。

D．考察
　卵子、特に排卵させた卵子では、マウス母体を安楽死させた後に短時間で細胞死が引き起こされることが明らかとなった。しかし、この現象が急激な温度変化によるものなのか、母体内からのストレス物質により、排卵後の卵子のみが選択的に細胞死を誘導するのかは現在は不明である。ただし、このようなストレス物質が存在すれば、排卵卵子のみが選択的に排除され、卵巣内での卵子は何ら影響を受けないことになり、短期間での不妊症を引き起こす原因の一つとなる可能性が考えられる。今後は、排卵卵子の選択的な細胞死を引き起こす分子メカニズムの解明に取り組む。

E．結論
　卵子、特に排卵させた卵子では、マウス母体を安楽死させた後に短時間で細胞死が引き起こされることが明らかとなった。

F．健康危険情報
　ある種のストレスが排卵直後の卵子の質に影響を与える可能性が考えられる。詳細は現在のところ不明である。

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H．知的財産権の出願・登録状況（予定を含む。）
　1．特許取得
　　該当なし。
　2．実用新案登録
　　該当なし。
　3.その他
　　該当なし。

Ⅲ　研究成果に関する一覧表

別紙5

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
岡部　勝	精子の成熟と受精能獲得	森沢 正昭, 星 和彦, 岡部 勝	新編　精子学	東京大学出版会	東京	2006	153-173（全489）
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IV　研究成果の刊行物・別冊

Menstrual Blood-derived Cells Confer Human Dystrophin Expression in the Murine Model of Duchenne Muscular Dystrophy via Cell Fusion and Myogenic Transdifferentiation[D]

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Duchenne muscular dystrophy (DMD), the most common lethal genetic disorder in children, is an X-linked recessive muscle disease characterized by the absence of dystrophin at the sarcolemma of muscle fibers. We examined a putative endometrial progenitor obtained from endometrial tissue samples to determine whether these cells repair muscular degeneration in a murine mdx model of DMD. Implanted cells conferred human dystrophin in degenerated muscle of immunodeficient mdx mice. We then examined menstrual blood–derived cells to determine whether primarily cultured nontransformed cells also repair dystrophied muscle. In vivo transfer of menstrual blood–derived cells into dystrophic muscles of immunodeficient mdx mice restored sarcolemmal expression of dystrophin. Labeling of implanted cells with EGFP and differential staining of human and murine nuclei suggest that human dystrophin expression is due to cell fusion between host myocytes and implanted cells. In vitro analysis revealed that endometrial progenitor cells and menstrual blood–derived cells can efficiently transdifferentiate into myoblasts/myocytes, fuse to C2C12 murine myoblasts by in vitro coculturing, and start to express dystrophin after fusion. These results demonstrate that the endometrial progenitor cells and menstrual blood–derived cells can transfer dystrophin into dystrophied myocytes at a high frequency through cell fusion and transdifferentiation in vitro and in vivo.

INTRODUCTION

Skeletal muscle consists predominantly of syncytial fibers with peripheral, postmitotic myonuclei, and its intrinsic repair potential in adulthood relies on the persistence of a resident reserve population of undifferentiated mononuclear cells, termed "satellite cells." In mature skeletal muscle, most satellite cells are quiescent and are activated in response to environmental cues, such as injury, to mediate postnatal muscle regeneration. After division, satellite cell progeny, termed myoblasts, undergo terminal differentiation and become incorporated into muscle fibers (Bischoff, 1994). Myogenesis is regulated by a family of myogenic transcription factors including MyoD, Myf5, myogenin, and MRF4 (Sabourin and Rudnicki, 2000). During embryonic development, MyoD and Myf5 are involved in the establishment of the skeletal muscle lineage (Rudnicki et al., 1993), whereas myogenin is required for terminal differentiation (Hasty et al., 1993; Nabeshima et al., 1993). During muscle repair, satellite cells recapitulate the expression program of the myogenic genes manifested during embryonic development.

Dystrophin is associated with a large oligomeric complex of glycoproteins that provide linkage to the extracellular membrane (Ervasti and Campbell, 1991). In Duchenne muscular dystrophy (DMD), the absence of dystrophin results in destabilization of the extracellular membrane-sarcolemma-cytoskeleton architecture, making muscle fibers susceptible to contraction-associated mechanical stress and degeneration. In the first phase of the disease, new muscle fibers are formed by satellite cells. After depletion of the satellite cell pool in childhood, skeletal muscles degenerate progressively and irreversibly and are replaced by fibrotic tissue (Cossu and Mavilio, 2000). Like DMD patients, the mdx mouse lacks dystrophin in skeletal muscle fibers (Hoffman et al., 1987; Sicinski et al., 1989). However, the mdx mouse develops only a mild dystrophic phenotype, probably because muscle regeneration by satellite cells is efficient for most of the animal's life span (Cossu and Mavilio, 2000).

Myoblasts represent the natural first choice in cellular therapeutics for skeletal muscle because of their intrinsic myogenic commitment (Grounds et al., 2002). However, myoblasts recovered from muscular biopsies are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000). An alternative source of muscle progenitor cells is therefore desirable. Cells with a myogenic potential are present in many tissues, and these cells readily

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form skeletal muscle in culture (Gerhart *et al.*, 2001). We report here that human dystrophin expression in the mdx model of DMD is attributed to cell fusion of mdx myocytes with human menstrual blood–derived stromal cells.

MATERIALS AND METHODS

Isolation of Human Endometrial Cells from Menstrual Blood

Menstrual blood samples (n = 21) were collected in DMEM with antibiotics (final concentrations: 100 U/ml penicillin/streptomycin) and 2% fetal bovine serum (FBS), and processed within 24 h. Ethical approval for tissue collection was granted by the Institutional Review Board of the National Research Institute for Child Health and Development, Japan. The centrifuged pellets containing endometrium-derived cells were resuspended in high-glucose DMEM medium (10% FBS, penicillin/streptomycin), maintained at 37°C in a humidified atmosphere containing 5% CO$_2$, and allowed to attach for 48 h. Nonadherent cells were removed by changing the medium. When the culture reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA and plated to new dishes. After 2–3 passages, the attached endometrial stromal cells were devoid of blood cells. Human EM-E6/E7/hTERT-2 cells, endometrium-derived progenitors, were obtained from surgical endometrial tissue samples and were immortalized by E6, E7, and hTERT-2 (Kyo *et al.*, 2003). C2C12 myoblast cells were supplied by RIKEN Cell Bank (The Institute of Physical and Chemical Research, Japan).

Flow Cytometric Analysis

Flow cytometric analysis was performed as previously described (Terai *et al.*, 2005). Cells were incubated with primary antibodies or isotype-matched control antibodies, followed by additional treatment with the immunofluorescent secondary antibodies. Cells were analyzed on an EPICS ALTRA analyzer (Beckman Coulter, Fullerton, CA). Antibodies against human CD13, CD14, CD29, CD31, CD34, CD44, CD45, CD50, CD54, CD55, CD59, CD73, CD90, CD105, CD117 (c-kit), CD133, HLA-ABC, and HLA-DR were purchased from Beckman Coulter, Immunotech (Marseille, France), Cytotech (Hellebæk, Denmark), and BD Biosciences PharMingen (San Diego, CA).

In Vitro Lentivirus-mediated Gene (EGFP) Transfer into EM-E6/E7/hTERT-2 Cells

Infection of EM-E6/E7/hTERT-2 cells with lentivirus having a CMV promoter and EGFP reporter resulted in high levels of EGFP expression in all cells. Cells were analyzed for EGFP expression by flow cytometry (Miyoshi *et al.*, 1997, 1998).

In Vitro Myogenesis

Menstrual blood–derived cells or EM-E6/E7/hTERT-2 cells were seeded onto collagen I-coated cell culture dishes (Biocoat, BD Biosciences, Bedford, MA) at a density of 1 × 10^4/ml in growth medium (DMEM, supplemented with 20% FBS). Forty-eight hours after seeding onto collagen I-coated dishes, cells were treated with 5-azacytidine for 24 h. Cell cultures were then washed twice with PBS and maintained in differentiation medium (DMEM, supplemented with either 2% horse serum (HS) or 1% insulin-transferrin-selenium supplement [ITS]). The differentiation medium was changed twice a week until the experiment was terminated.

RT-PCR Analysis of EM-E6/E7/hTERT-2 Cells and Menstrual Blood–derived Cells

Total RNA was prepared using Isogen (Nippon Gene, Tokyo, Japan). Human skeletal muscle RNA was purchased from TOYOBO (Osaka, Japan). RT-PCR of Myf5, MyoD, desmin, myogenin, myosin heavy chain-IIx/d (MyHC-IIx/d), and dystrophin was performed with 2 μg of total RNA. RNA for RT-PCR was converted to cDNA with a first-stand cDNA synthesis kit (Amersham Pharmacia Biotechnology, Piscataway, NJ) according to the manufacturer's recommendations. The sequences of PCR primers that amplify human but not mouse genes are listed in Supplementary Table 1. PCR was performed with TaKaRa recombinant Taq (Takara Shuzo, Kyoto, Japan) for 30 cycles, with each cycle consisting of 94°C for 30 s, 62°C or 65°C for 30 s, and 72°C for 20 s, with an additional 10-min incubation at 72°C after completion of the last cycle.

Immunohistochemical and Immunocytochemical Analysis

Immunohistochemical analysis was performed as previously described (Mori *et al.*, 2005). Briefly, the sections were incubated for 1 h at room temperature with mouse mAb against vimentin (Cone V9, DakoCytomation, Fort Collins, CO). After washing in PBS, sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin, diluted, and washed in cold PBS. Staining was developed by using a solution containing diaminobenzidine and 0.01% H$_2$O$_2$ in 0.05% M Tris-HCl buffer, pH 6.7. Slides were

counterstained with hematoxylin. In the cases of fluorescence, frozen sections fixed with 4% PFA were used. The antibodies against human dystrophin (NCL-DYS3; Novocastra, Newcastle upon Tyne, United Kingdom) or anti-human nuclei mouse mAb (clone 235-1, Chemicon, Temecula, CA) was used as a first antibody, and goat anti-mouse IgG conjugated with Alexa Fluor 488 or goat anti-mouse IgG antibody conjugated with Alexa Fluor 546 (Molecular Probes, Eugene, OR) was used as a second antibody.

Immunocytochemical analysis was performed as previously described (Mori *et al.*, 2005), with antibodies to skeletal myosin (Sigma, St. Louis, MO; product no. M 4276), MF20 (which reacts with all sarcomere myosin in striated muscles, Developmental Studies Hybridoma Bank, University of Iowa, IA), α-sarcomeric actin (Sigma, product no. A 7811), and desmin (Bio-Science Products, Bern, Switzerland; no. 010031, clone: D9) in PBS containing 1% bovine serum albumin. As a methodological control, the primary antibody was omitted. In the cases of fluorescence, slides were incubated with Alexa Fluor 546–conjugated goat anti-mouse IgG antibody.

Western Blotting

Western blot analysis was performed as previously described (Mori *et al.*, 2005). Blots were incubated with primary antibodies (desmin, myogenin [Clone F5D, Santa Cruz Biotechnology], and dystrophin [NCL-DYSA, Novocastra]) for 1–2 h at room temperature. After washing three times in the blocking buffer, blots were incubated for 30 min with a horseradish peroxidase–conjugated secondary antibody (0.04 μg/ml) directed against the primary antibody. The blots were developed with enhanced chemiluminescence substrate according to the manufacturer's instructions.

Fusion Assay

EM-E6/E7/hTERT-2 cells (2500/cm²) or EGFP-labeled EM-E6/E7/hTERT-2 cells (2500/cm²) were cocultured with C2C12 myoblasts (2500/cm²) for 2 d in DMEM supplemented with 10% FBS and then cultured for 7 additional days in DMEM with 2% HS to promote myotube formation. The cultures were fixed in 4% paraformaldehyde and stained with a mouse anti-human nuclei IgG1 mAb and the mouse anti-human dystrophin IgG2a mAb (or anti-myosin heavy chain IgG2b mAb MF-20). The cells were visualized with appropriate Alexa-fluor–conjugated goat anti-mouse IgG1 and IgG2a (or IgG2b) secondary antibodies (Molecular Probes). Total cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole).

In Vivo Cell Implantation

Six- to 8-wk-old NOD/Shi-scid/IL-2 receptor −/− (NOG, CREA, Shizuoka, Japan) mice and 6- to 8-wk-old mdx-scid mice were implanted with EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells in seven independent experiments. The cells (2 × 10⁷) were suspended in PBS in a total volume of 100 μl and were directly injected into the right thigh muscle of NOG mice or mdx-scid mice. The mice were examined 3 wk after cell implantation, and the right thigh muscle was analyzed for human vimentin and dystrophin by immunohistochemistry. The antibodies to vimentin and dystrophin (NCL-DYS3) react with human vimentin and dystrophin-equivalent protein, but not murine protein.

RESULTS

Surface Marker Expression of Endometrium-derived Cells

We investigated myogenic differentiation of primary cells without gene introduction from menstrual blood, because menstrual blood on the first day of the period is considered to include endometrial tissue. We successfully cultured a large number of primary cells from menstrual blood. Menstrual blood–derived cells showed at least two morphologically different cell groups: small spindle-like cells and large stick-like cells, regarded as being passage day (PD) 1 or 2 (Figure 1, A and B, respectively). Surface markers of the menstrual blood–derived cells were evaluated by flow cytometric analysis. Surface markers of EM-E6/E7/hTERT-2 cells (Figure 1C) and menstrual blood–derived cells (Figure 1D) were evaluated by flow cytometric analysis (Figure 1E). In these experiments, the cells were cultured in the absence of any inductive stimuli. EM-E6/E7/hTERT-2 cells were positive for CD13, CD29 (integrin β1), CD44 (Pgp-1/ly24), CD54, CD55, CD59, CD73, and CD90 (Thy-1), implying that EM-E6/E7/hTERT-2 cells expressed mesenchymal cell–related antigens in our experimental setting. Menstrual blood–derived cells were positive for CD13, CD29, CD44, CD54, CD55, CD59, CD73, CD90, and CD105, implying that prolif-
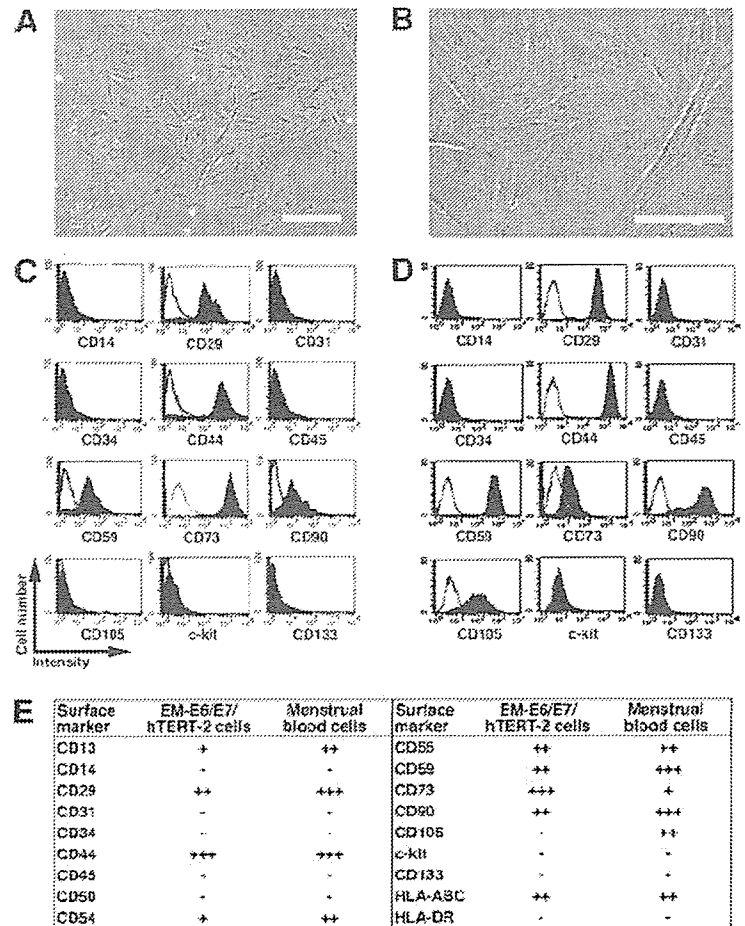
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Myogenesis by Menstrual Blood Cells

Figure 1. Surface marker expression of endometrium-derived cells. (A and B) Morphology of menstrual blood–derived cells, regarded as being PD 1 or 2. Scale bars, 200 μm (A), 100 μm (B). (C and D) Flow cytometric analysis of cell surface markers of EM-E6/E7/hTERT-2 cells (C) and menstrual blood–derived cells (D). (E) Further phenotypic analysis in EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells are summarized. Peak intensity was estimated in comparison with isotype controls. + + +, strongly positive (>100 times the isotype control); + +, moderately positive (<100 times but more than 10 times the isotype control), weakly positive (<10 times but more than twice the isotype control), −, negative (less than twice the isotype control).

Surface marker	EM-E6/E7/hTERT-2 cells	Menstrual blood cells	Surface marker	EM-E6/E7/hTERT-2 cells	Menstrual blood cells
CD13	+	++	CD55	++	++
CD14	-	-	CD59	++	+++
CD29	++	+++	CD73	+++	+
CD31	-	-	CD90	++	+++
CD34	-	-	CD105	-	++
CD44	+++	+++	c-kit	-	-
CD45	-	-	CD133	-	-
CD50	-	-	HLA-ABC	++	++
CD54	+	++	HLA-DR	-	-

erated and propagated cells express mesenchymal cell-related cell surface markers. Unlike EM-E6/E7/hTERT-2 cells, the menstrual blood–derived adherent cells were positive for CD105. EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells expressed neither hematopoietic lineage markers, such as CD34, nor monocyte-macrophage antigens such as CD14 (a marker for macrophage and dendritic cells), or CD45 (leukocyte common antigen). The lack of expression of CD14, CD34, or CD45 suggests that EM-E6/E7/hTERT-2 cells and the menstrual blood–derived cell culture in the present study is depleted of hematopoietic cells. The cells were also negative for expression of CD31 (PECAM-1), CD50, c-kit, and CD133. The cell population was positive for HLA-ABC, but not for HLA-DR. These results demonstrate that almost all cells derived from endometrium are of mesenchymal origin or stromal origin.

Implanted Endometrium-derived Cells Induce De Novo Myogenesis in Immunodeficient NOG Mice

EM-E6/E7/hTERT-2 cells originate from the endometrial gland and are considered as endometrial progenitor cells or bipotential cells capable of differentiating into both glandular epithelial cells and endometrial stromal cells (Kyo *et al.*, 2003). To determine whether EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells generate complete endometrial structure in vivo, like endometriosis, the cells without any treatment or induction were injected into the right thigh

muscle of immunodeficient NOG mice. PBS without cells was injected into the left thigh muscles as a control. We failed to detect any endometrial structure in the cell-injected site. Immunohistochemical analysis using an antibody specific to human vimentin, an intermediate filament associated with a mesenchymal cell, revealed that the injected EM-E6/E7/hTERT-2 cells (Figure 2, A–F) or menstrual blood–derived cells (Figure 2, G–L) extensively migrated or infiltrated between muscular fibers (Figure 2, arrowheads). To investigate if the donor cells between muscular fibers occur as a result of cell migration, we performed a time-course analysis of implanted cells, as probed by human-specific antibody to vimentin (Supplementary Figure 1). Donor cells at 3 h after implantation are observed at the injection site, which is considered to be due to just injection of cells. Cells at 1–3 wk after implantation are detected between myocytes in the muscle bundle or muscular fascicle as well as in the interstitial tissue, implying that the donor cells between myotubes result from cell migration. Interestingly, some of the vimentin-positive implanted cells exhibited round-shaped structure (Figure 2, D, F, and J, arrows), suggesting that endometrium-derived cells are capable of differentiating into myoblasts/myotubes, and can contribute to skeletal muscle repair in patients suffering from genetic disorders such as DMD, similar to previous reports for marrow stromal cells (Dezawa *et al.*, 2005) and synovial membrane cells (De Bari *et al.*, 2003).
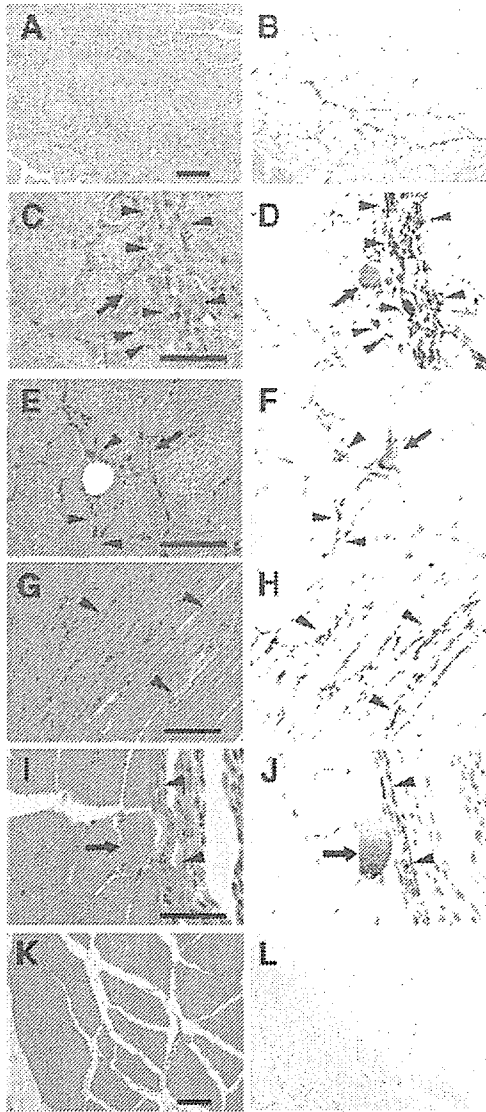
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Figure 2. Implantation of endometrium-derived cells-derived cells into the muscle of NOG mice. EM-E6/E7/hTERT-2 cells (A–F) or menstrual blood–derived cells (G–J) cultured in absence of any stimuli were directly injected into the right thigh muscle of NOG mice. Immunohistochemical analysis was performed using antibody that reacts to human vimentin but not to murine vimentin. (A, C, E, G, I, and K) hematoxylin and eosin stain (HE; B, D, F, H, J, and L) immunohistochemistry. Note that vimentin-positive EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells with a spindle morphology (C–J, arrowheads) extensively migrated into muscular bundles at 3 wk after injection, and some of the injected cells exhibited round structure (D, F, and J, arrows). Isotype mouse IgG1 served as a negative control (L). Scale bars, 100 μm (A, B, K, and L), 50 μm (C–F, I, and J), 90 μm (G and H).

Induction of Myogenic Differentiation in Endometrial Progenitor Cells In Vitro

EM-E6/E7/hTERT-2 cells at 2 wk (cultured in the DMEM supplemented with 20% FBS) after exposure to different concentrations (5, 10, and 100 μM) of 5-azacytidine were analyzed by immunostaining using anti-desmin antibody (Figure 3, A–F). The number of desmin-positive cells was

significantly higher in experimental groups with 5 or 10 μM 5-azacytidine than in untreated control groups ($p < 0.05$). To investigate whether EM-E6/E7/hTERT-2 cells are capable of differentiating into skeletal muscle cells in vitro, the cells were exposed to 5 μM 5-azacytidine for 24 h and then subsequently cultured in the DMEM supplemented with 2% HS (Figure 3, G–J) or serum-free ITS for up to 21 d (Figure 3K). Skeletal myoblastic differentiation of the cells was analyzed by evaluating expression of MyoD, Myf5, desmin, myogenin, MyHC-IIx/d, and dystrophin by RT-PCR. The MyoD, desmin, myogenin, and dystrophin genes were constitutively expressed, but MyHC-IIx/d and Myf5 genes were not. The decline of MyoD was observed in both the 2% HS (Figure 3, G and H) and the serum-free ITS (Figure 3K). The expression of MyHC, as determined by RT-PCR and immunocytochemistry, significantly increased with 2% HS (Figure 3, G–J) and serum-free ITS (Figure 3K). Immunocytochemical analysis indicated that α-sarcomeric actin (Figure 3I) and MyHC (Figure 3J) were detected in the cells incubated with 2% HS for 21 d.

In Vitro Myogenic Differentiation of Menstrual Blood-derived Cells

Menstrual blood–derived cells at 3 wk (cultured in DMEM supplemented with 20% FBS) after exposure to different concentrations (5, 10, and 100 μM) of 5-azacytidine were analyzed by immunostaining using anti-desmin antibody (data not shown). The number of desmin-positive cells was significantly higher in experimental groups with 5 or 10 μM 5-azacytidine than with 100 μM 5-azacytidine; for further in vitro experiments, the menstrual blood–derived cells were exposed to 5 μM 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with low serum (2% HS) or serum-free ITS for up to 21 d (Figure 4). Myogenic potential of human menstrual blood–derived cells was analyzed by evaluating the expression of Myf5, MyoD, desmin, myogenin, MyHC-IIx/d, and dystrophin by RT-PCR. MyoD, desmin, and dystrophin genes were constitutively expressed in menstrual blood–derived cells, but MyHC-IIx/d and Myf5 were not (Figure 4A). For cells treated with 2% HS or serum-free ITS, the mRNA level of desmin and myogenin significantly increased after 3 d, and desmin steadily increased until day 21 (Figure 4, C and D). MyHC-IIx/d started to be expressed at a low level at day 21 of induction (Figure 4C). We then analyzed desmin expression by immunocytochemistry. Menstrual blood–derived cells were exposed to 5 μM 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with 20% FBS for up to 2 wk. Desmin was readily detected in colonies of the menstrual blood–derived cells (Figure 4B). Western blot analysis indicated that desmin, myogenin, and dystrophin were highly expressed in the cells incubated for 3 wk (Figure 4, E–G). These results suggest that menstrual blood–derived cells are, like the EM-E6/E7/hTERT-2 cells, able to differentiate into skeletal muscle.

Regeneration of Dystrophin by Cell Implantation in the DMD Model mdx-scid Mouse

To investigate whether human EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells can generate muscle tissue in vivo, cells without any treatment or induction were implanted directly into the right thigh muscles of mdx-scid mice (Supplementary Figure 2). The left thigh muscles were injected with PBS as an internal control. After 3 wk, myotubes in the muscle tissues injected with human EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells expressed human dystrophin as a cluster (Figure 5, A, C, and D, EM-
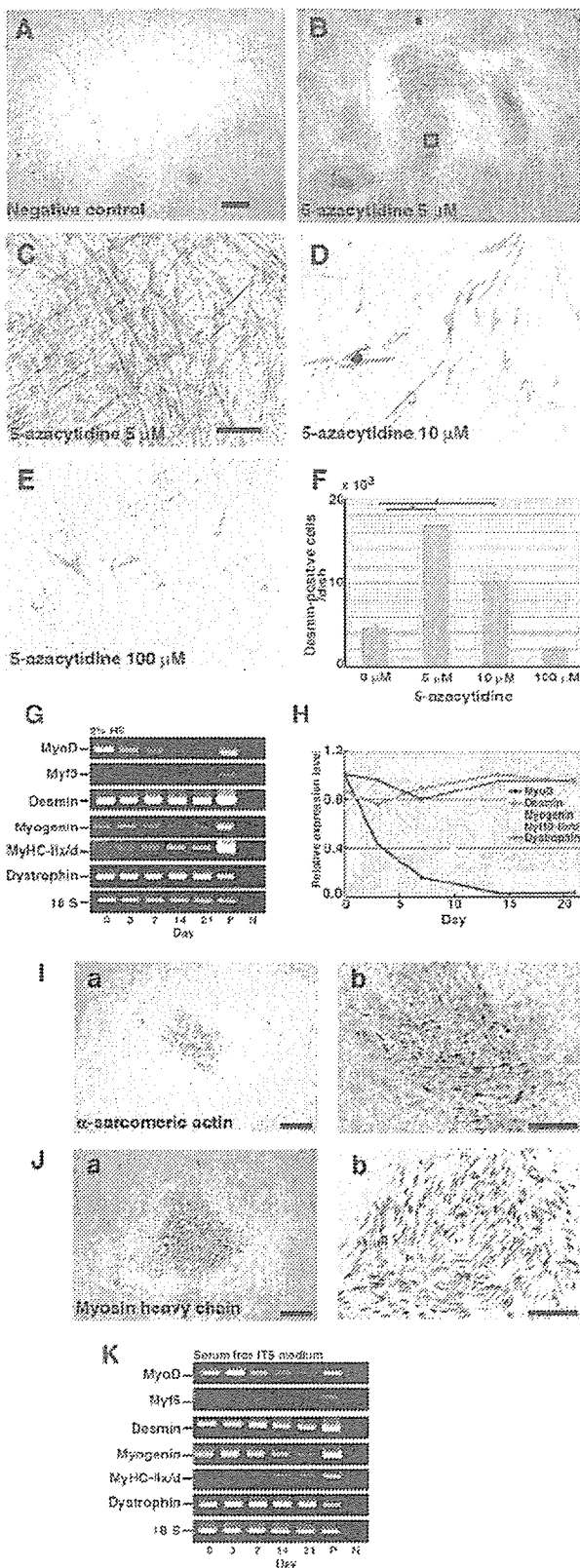
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E6/E7/hTERT-2 cells, and 5B, menstrual blood–derived cells). Quantification analysis revealed that the percentage of dystrophin-positive myofibers after implantation of menstrual blood–derived cells was high, compared with that after implantation of EM-E6/E7/hTERT-2 cells (Figure 5E). Donor cells with EGFP fluorescence participated in myogenesis 3 wk after implantation (Supplementary Figure 3). EGFP-labeled EM-E6/E7/hTERT-2 cells became positive for human dystrophin (Figure 5C). Dystrophin was not detected in the muscle of mdx-scid mice and NOG mice without cell implantation because the antibody to dystrophin used in this study is human-specific, implying that dystrophin is transcribed from dystrophin genes of human donor cells but not from reversion of dystrophied myocytes in mdx-scid mice.

To determine if dystrophin expression in the donor cells is due to transdifferentiation or fusion, immunohistochemistry with an antibody against human nuclei (Ab-HuNucl) and DAPI stain was performed. If dystrophin expression is explained by fusion, dystrophin-positive myocytes must be demonstrated to have both human and murine nuclei. We examined almost all the 7-μm-thick serial histological sections parallel to the muscular bundle (longitudinal section) of the muscular tissues by confocal microscopy and found that dystrophin-positive myocytes have nuclei derived from both human and murine cells in the longitudinal section of the myocytes (Figure 5D), implying that dystrophin expression is attributed to fusion between murine host myocytes and human donor cells, rather than myogenic differentiation of EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells per se.

Detection of Human Endometrial Cell Contribution to Myotubes in an In Vitro Myogenesis Model

To simulate in vivo phenomena, human endometrial cells were cocultured in vitro with murine C2C12 myoblasts for 2 d under proliferative conditions and then switched to differentiation conditions for an additional 7 d. Figure 6A

analysis of EM-E6/E7/hTERT-2 cells using an antibody to desmin. (A) Omission of only the primary antibody to desmin serves as a negative control. (C) Higher magnification of inset in B. (F) Myogenic differentiation of EM-E6/E7/hTERT-2 cells with exposure to different concentrations (B, 5 μM; C, 5 μM; D, 10 μM, E, 100 μM) of 5-azacytidine. To estimate myogenic differentiation, the number of all the desmin-positive cells was counted for each dish (n = 3). Data were analyzed for statistical significance using ANOVA. EM-E6/E7/hTERT-2 cells were cultured in the DMEM supplemented with 2% HS (G–J; horse serum), and serum-free ITS (K). (G and K) RT-PCR analysis with PCR primers allows amplification of the human MyoD, Myf5, desmin, myogenin, myosin heavy chain type IIx/d (MyHC-IIx/d), and dystrophin cDNA (from top to bottom). RNAs were isolated from EM-E6/E7/hTERT-2 cells at the indicated day after treatment with 5-azacytidine. RNAs from human muscle and H₂O served as positive (P) and negative (N) controls. Only the 18S PCR primer used as a positive control reacted with the human and murine cDNA. (H) Time course of MyoD, desmin, myogenin, MyHC-IIx/d, and dystrophin expression in the cells incubated with 2% HS for up to 21 d after 5-azacytidine treatment. Relative mRNA levels were determined using Multi Gauge Ver 2.0 (Fuji Film). The signal intensities of MyoD, desmin, and dystrophin mRNA at day 0, myogenin mRNA at day 3, and MyHC-II/d mRNA at day 21 were regarded as equal to 100%. (I and J) The cells were exposed to 5 μM 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with 2% HS for 21 d. α-Sarcomeric actin (I) and skeletal myosin heavy chain (J) was detected by immunocytochemical analysis. Scale bars, 2 mm (A and B), 300 μm (C–E), 900 μm (Ia and Ja), 425 μm (Ib and Jb).

Figure 3. Expression of myogenic-specific genes during myogenic differentiation of EM-E6/E7/hTERT-2 cells. (A–F) Immunocytochemical
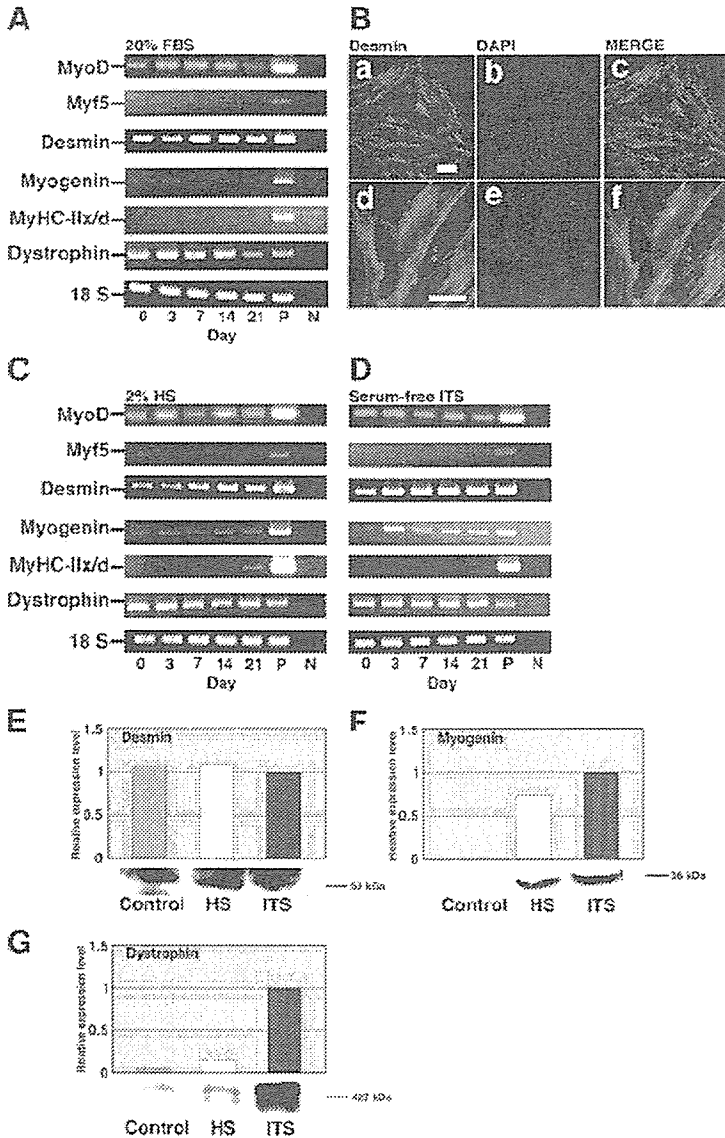
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Figure 4. Expression of myogenic-specific genes in differentiated menstrual blood–derived cells. Menstrual blood–derived cells were cultured in DMEM supplemented with 20% FBS, 2% HS, or serum-free ITS medium. (A) RT-PCR analysis with PCR primers that allows amplification of the human MyoD, Myf5, desmin, myogenin, MyHC-IIx/d, and dystrophin cDNA (from top to bottom). RNAs were isolated from menstrual blood–derived cells at the indicated day after treatment with 5 μM 5-azacytidine for 24 h. RNAs from human muscle and H_2O served as positive (P) and negative (N) controls. Only the 18S PCR primer used as a positive control reacted with the human and murine cDNA. (B) Immunocytochemical analysis using an antibody to desmin (a–f) was performed on the menstrual blood–derived cells at 2 wk after exposure to 5 μM of 5-azacytidine for 24 h. The desmin-positive cells are shown at higher magnification (d–f). Merge of a and b is shown in c, and merge of d and e is shown in f. The images were obtained with a laser scanning confocal microscope. Scale bars, 200 μm (a–c) and 75 μm (d–f). (C and D) RT-PCR analysis of menstrual blood–derived cells on DMEM supplemented with 2% HS (C) or serum-free ITS medium (D) after exposure to 5 μM 5-azacytidine for 24 h. (E–G) Western blot analysis was performed on the cells cultured in myogenic medium indicated for 21 d. The blot was stained with desmin (E), myogenin (F), and dystrophin (G) antibodies followed by an HRP-conjugated secondary antibody.

provides an example of how human and mouse nuclei in the EGFP-positive myotubes were detected. Multinucleated myotubes were revealed by the presence of specific human dystrophin (Figure 6, B and C) and myosin heavy chain (Figure 6D). Dystrophin was detected in cytoplasm in culture condition (Figure 6, B and C) despite evidence of cell surface localization in vivo. Human dystrophin and human nuclei were unequivocally identified by staining with antibodies to human dystrophin and human nuclei, whereas the numerous mouse nuclei present in this field, as shown by DAPI staining, are negative (Figure 6, B and C).

DISCUSSION

Skeletal muscle has a remarkable regenerative capacity in response to an extensive injury. Resident within adult skeletal muscle is a small population of myogenic precursor cells (or satellite cells) that are capable of multiple rounds of proliferation (estimated at 80–100 doublings), which are

able to reestablish a quiescent pool of myogenic progenitor cells after each discrete regenerative episode (Mauro, 1961; Schultz and McCormick, 1994; Seale and Rudnicki, 2000; Hawke and Garry, 2001). Although muscle regeneration is a highly efficient and reproducible process, it ultimately is exhausted, as observed in senescent skeletal muscle or in patients with muscular dystrophy (Gussoni et al., 1997; Cossu and Mavilio, 2000). In the present study, we investigated the myogenic potential of human endometrial tissue-derived immortalized EM-E6/E7/TERT-2 cells and primary cells derived from human menstrual blood. Human menstrual blood–derived cells proliferated over at least 25 PDs (9 passages) for more than 60 d and stopped dividing before 30 PDs. This cessation of cell division is probably due to replicative senescence or shortening of telomere length. Cell life span of menstrual blood cells is relatively short when compared with human fetal cells (Imai et al., 1994; Terai et al., 2005), and this shorter cell life span may be attributed to shorter telomere length of adult cells (i.e., endometrial stro-
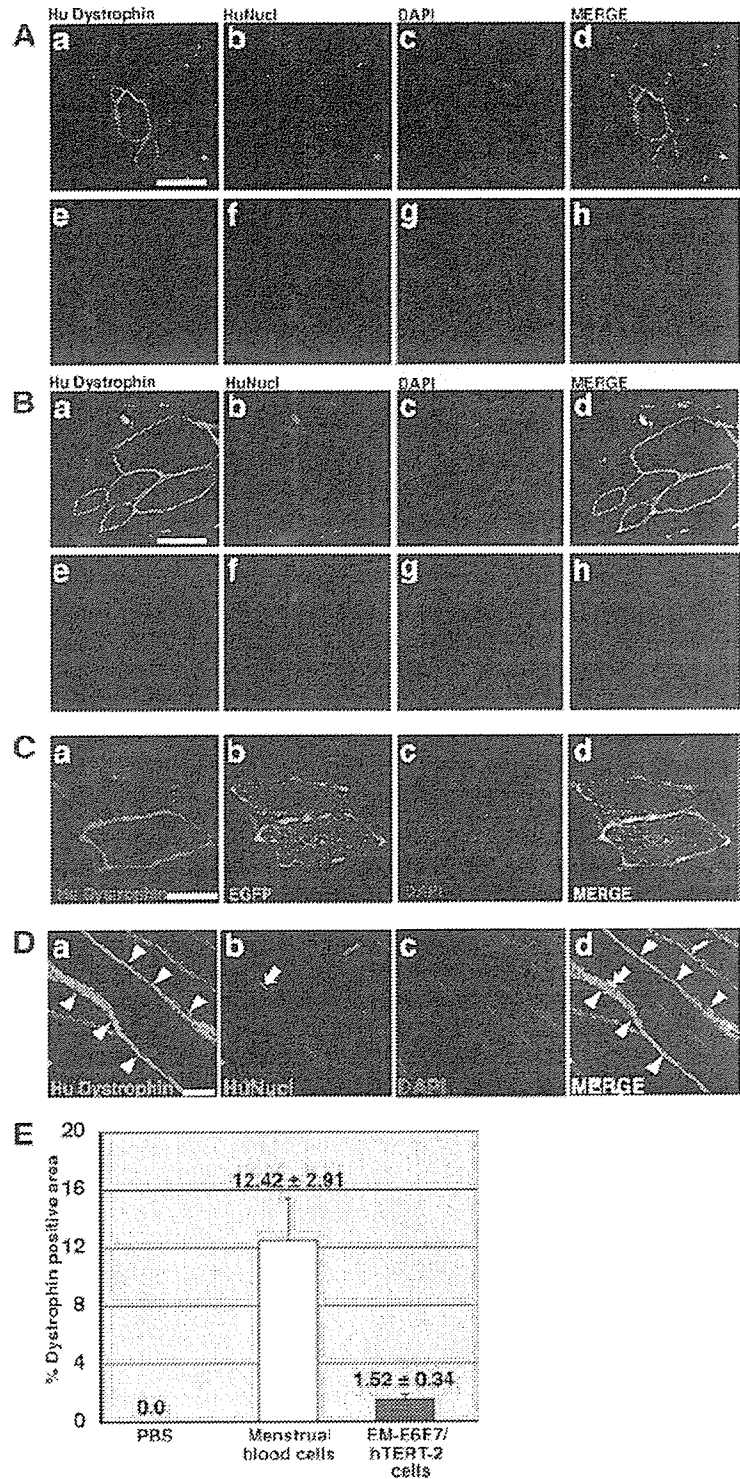
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Figure 5. Conferral of dystrophin to mdx myocytes by human endometrial cells. (A and B) Immunohistochemistry analysis using an antibody against human dystrophin molecule (green), human nuclei (HuNucl, red), and DAPI staining (blue) on thigh muscle sections of mdx-scid mice after direct injection of EM-E6/E7/hTERT-2 cells (A) or menstrual blood–derived cells (B) without any treatment or induction. (C) EGFP-labeled EM-E6/E7/hTERT-2 cells without any treatment or induction were directly injected into the thigh muscle of mdx-scid mice. Immunohistochemistry revealed the incorporation of implanted cells into newly formed EGFP-positive myofibers, which expressed human dystrophin 3 wk after implantation. (A and B) As a methodological control, the primary antibody to dystrophin was omitted (e and f). (D) Immunohistochemistry analysis using an antibody against human dystrophin molecule (green, arrowheads), human nuclei (HuNucl, red, arrow), and DAPI staining (blue) on thigh muscle sections of mdx-scid mice after direct injection of human EM-E6/E7/hTERT-2 cells without any treatment or induction. (A and B) Merge of a–c is shown in d, and merge of e–g is shown in h. (C and D) Merge of a–c is shown in d. Scale bars, 50 μm (A and B), 20 μm (C and D). (E) Quantitative analysis of human dystrophin-positive myotubes. Menstrual blood–derived cells or EM-E6/E7/hTERT-2 cells without any treatment or induction were directly injected into thigh muscle of mdx-scid mice. The percentage of human dystrophin–positive-myofiber areas was calculated 3 wk after implantation of the EM-E6/E7/hTERT-2 cells or menstrual blood–derived cells. Injection of PBS without cells into mdx-scid myofibers was used as a control.

mal cells) at the start of cell cultivation, as is the case with hematopoietic stem cells (Suda *et al.*, 1984).

Menstrual blood–derived cells had a high replicative ability similar to progenitors or stem cells that display a long-term self-renewal capacity and had a much higher growth rate in our experimental conditions than marrow-derived stromal cells (Mori *et al.*, 2005). In addition, the myogenic potential of menstrual blood–derived cells, i.e., a high frequency of desmin-positive cells after induction, is much greater than expected. The higher myogenic differentiation ratio can be explained just by alteration of cell characteristics from epithelial and mesenchymal bipotential cells or heter-
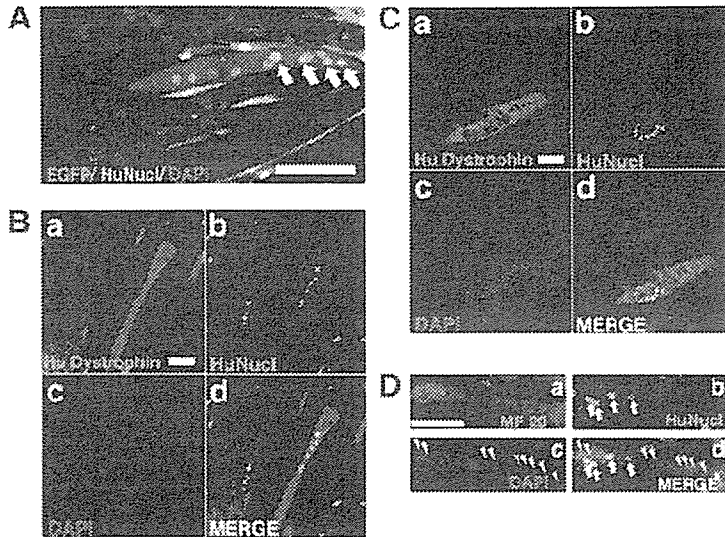
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Figure 6. Detection of human endometrial cell contribution to myotubes in an in vitro and in vivo myogenesis model. EGFP-labeled EM-E6/E7/hTERT-2 cells (A) or EM-E6/E7/hTERT-2 cells (B) or menstrual blood-derived cells (C and D) were cocultured with C2C12 myoblasts for 2 d under conditions that favored proliferation. The cultures were then changed to differentiation media for 7 d to induce myogenic fusion. (A) Myotubes were revealed by EGFP (green); human nuclei were detected by antibody specific to human nuclei (HuNucl, red, arrows). (B–D) Myotubes were revealed by specific human dystrophin mAb NCL-DYS3 (B and C, red) or anti-myosin heavy chain mAb MF-20 (D, red). (D) Human nuclei were detected by antibody specific to human nuclei (HuNucl, green, arrows). Total cell nuclei in the culture were stained with DAPI (blue, arrowheads). (B–D) Merge of a–c are shown in d. The cultures were then changed to differentiation media for 7 d to induce myogenic fusion. Scale bars, 100 µm (A–D).

ogeneous populations of cells to cells with the mesenchymal phenotype in our cultivation condition, as determined by cell surface markers (Figure 1, C–E). MyoD-positive cells are present in many fetal chick organs such as brain, lung, intestine, kidney, spleen, heart, and liver (Gerhart et al., 2001), and these cells can differentiate into skeletal muscle in culture. Constitutive expression of MyoD, desmin, and myogenin, all markers for skeletal myogenic differentiation in both immortalized EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells, implies either that most of these cells are myogenic progenitors or that these cells have myogenic potential. Expression of MyoD, one of the basic helix-loop-helix transcription factors that directly regulate myocyte cell specification and differentiation (Edmondson and Olson, 1993), occurs at the early stage of myogenic differentiation, whereas myogenin is expressed later, related to cell fusion and differentiation (Aurade et al., 1994).

Acquisition or recovery of dystrophin expression in dystrophic muscle is attributed to two different mechanisms: 1) myogenic differentiation of implanted or transplanted cells and 2) cell fusion of implanted or transplanted cells with host muscle cells. Recovery of dystrophin-positive cells is explained by muscular differentiation of implanted marrow stromal cells and adipocytes (Dezawa et al., 2005; Rodriguez et al., 2005). In contrast, implantation of normal myoblasts into dystrophin-deficient muscle can create a reservoir of normal myoblasts that are capable of fusing with dystrophic muscle fibers and restoring dystrophin (Mendell et al., 1995; Terada et al., 2002; Wang et al., 2003; Dezawa et al., 2005; Rodriguez et al., 2005). In this study using menstrual blood-derived cells, our findings—that the implantation of immortalized EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells improved the efficiency of muscle regeneration and dystrophin delivery to dystrophic muscle in mice—is explained by both possibilities or the latter possibility alone, because cells expressing human dystrophin had both murine and human nuclei, located in the center and periphery of dystrophic muscular fiber, respectively (Figures 5D, in vivo, and 6, A–D, in vitro).

DMD is a devastating X-linked muscle disease characterized by progressive muscle weakness attributable to a lack of dystrophin expression at the sarcolemma of muscle fibers (Mendell et al., 1995; Rodriguez et al., 2005), and there are no effective therapeutic approaches for muscular dystrophy at present. Human menstrual blood-derived cells are obtained by a simple, safe, and painless procedure and can be expanded efficiently in vitro. In contrast, isolation of mesenchymal stem cells/mesenchymal cells from other sources, such as bone marrow and adipose tissue, is accompanied by a painful and complicated operation. Efficient fusion systems of our immortalized human EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells with host dystrophic myocytes may contribute substantially to a major advance toward eventual cell-based therapies for muscle injury or chronic muscular disease. Finally, we would like to reemphasize that human menstrual blood-derived cells possess high self-renewal capacity, whereas biopsied myoblasts capable of differentiating into muscular cells are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000).

ACKNOWLEDGMENTS

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Hyaline Cartilage Formation and Enchondral Ossification Modeled With KUM5 and OP9 Chondroblasts

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Abstract   What is it that defines a bone marrow-derived chondrocyte? We attempted to identify marrow-derived cells with chondrogenic nature and immortality without transformation, defining "immortality" simply as indefinite cell division. KUM5 mesenchymal cells, a marrow stromal cell line, generated hyaline cartilage in vivo and exhibited enchondral ossification at a later stage after implantation. Selection of KUM5 chondroblasts based on the activity of the chondrocyte-specific cis-regulatory element of the collagen α2(XI) gene resulted in enhancement of their chondrogenic nature. Gene chip analysis revealed that OP9 cells, another marrow stromal cell line, derived from macrophage colony-stimulating factor-deficient osteopetrotic mice and also known to be niche-constituting cells for hematopoietic stem cells expressed chondrocyte-specific or -associated genes such as type II collagen α1, Sox9, and cartilage oligomeric matrix protein at an extremely high level, as did KUM5 cells. After cultured OP9 micromasses exposed to TGF-β3 and BMP2 were implanted in mice, they produced abundant metachromatic matrix with the toluidine blue stain and formed type II collagen-positive hyaline cartilage within 2 weeks in vivo. Hierarchical clustering and principal component analysis based on microarray data of the expression of cell surface markers and cell-type-specific genes resulted in grouping of KUM5 and OP9 cells into the same subcategory of "chondroblast," that is, a distinct cell type group. We here show that these two cell lines exhibit the unique characteristics of hyaline cartilage formation and enchondral ossification in vitro and in vivo. J. Cell. Biochem. 9999: 1–15, 2006.   © 2006 Wiley-Liss, Inc.

Key words: Hyaline cartilage; chondroblasts; enchondral ossification; bioinformatics; gene chip