Fig. 4 – Myogenic differentiation of the EGFP-labeled 9-15c-CG cells into the quadriceps femoris muscle. EGFP-labeled 9-15c-CG cells could be recognized morphologically as the skeletal myocytes in the quadriceps femoris muscle 3 months after transplantation (A, C, E: HE staining; B, D, F: immunohistochemistry using anti-GFP antibody). The EGFP-positive donor cells exhibited skeletal myocyte-specific features such as multiple nuclei in the periphery of the cells and striation. Generation of myocytes (G) and endothelial cells (H) by the EGFP-labeled 9-15c cells. The injected donor 9-15c cells labeled with EGFP were detected by green fluorescence. (Ga, Ha) Green fluorescence of EGFP-labeled donor cells. (Gb, Hb) Immunohistochemistry for desmin (Gb, red) or CD31 (Hb, red). (Gc, Hc) The merged images of green fluorescence of injected 9-15c cells and rhodamine of desmin or CD31 clearly demonstrated that 9-15c cells differentiated into myocytes or endothelium. A–F: Longitudinal section; G, H: Cross section.

by clonal analysis [30]. In the present study using single-cell marking, we found that 9-15c cells in culture consisted of a mixture of at least three types of cells, i.e., cardiac myoblasts, cardiac progenitors and multipotent stem cells. Cardiac myoblasts are defined as cells which can differentiate into only cardiac myocytes and have low proliferative potential; cardiac progenitors have proliferative capability and the ability to become cardiomyocytes; multipotent stem cells have both proliferative capability and multipotency. The results obtained in the present study suggest that 9-15c cells are stochastically committed toward the cardiac lineage, and that following this commitment they proliferate as transient amplifying cells and differentiate into cardiac myocytes through the differentiation process, and the hierarchical model applies in the case of 9-15c multipotent cells.

In the present study, we used 5-azacytidine to induce differentiation. 5-azacytidine is a cytosine analog that causes extensive demethylation. The demethylation is attributable to covalent binding of DNA methyltransferase to 5-azacytidine in the DNA [31], with the subsequent reduction of enzyme activity in cells resulting in random loss of methylation at many sites in the genome. Previously, it has been thought that 5-azacytidine activates cardiomyogenic master genes, such as Nkx2.5/Csx, GATA4, and MEF-2C, leading to stochastic trans-

differentiation of MSCs into cardiomyocytes [32,33]. This concept is difficult to account for the existence of cardiac progenitors and multipotent stem cells we identified, and we propose two possibilities how 5-azacytidine works. First, treatment of 5-azacytidine modulates heterochromatin remodeling and leads to dedifferentiation of 9-15c cells. Second, 9-15c cells are stochastically committed toward the cardiac lineage, being independent of treatment of 5-azacytidine. At this time we cannot conclude which is feasible, but it is certain cardiomyocytes are not only transdifferentiated by treatment of 5-azacytidine.

Csx/Nkx2.5 and GATA4 are two cardiac-enriched transcription factors that are expressed in precardiac mesoderm from the very early developmental stage [34,35]. In the present study, increased frequency of cardiomyogenic differentiation of 9-15c cells was successfully achieved in vitro by forced expression of Csx/Nkx2.5 and GATA4. These results are consistent with a report showing that both Csx/Nkx2.5 and GATA4 are required for the cardiac differentiation of P19CL6 cells derived from embryonic teratocarcinoma cells [36]. Cardiomyogenic differentiation, however, could proceed only after treatment with 5-azacytidine in our experimental setting, implying that Csx/Nkx2.5 and GATA4 are required but not sufficient for cardiac differentiation. Unknown factors
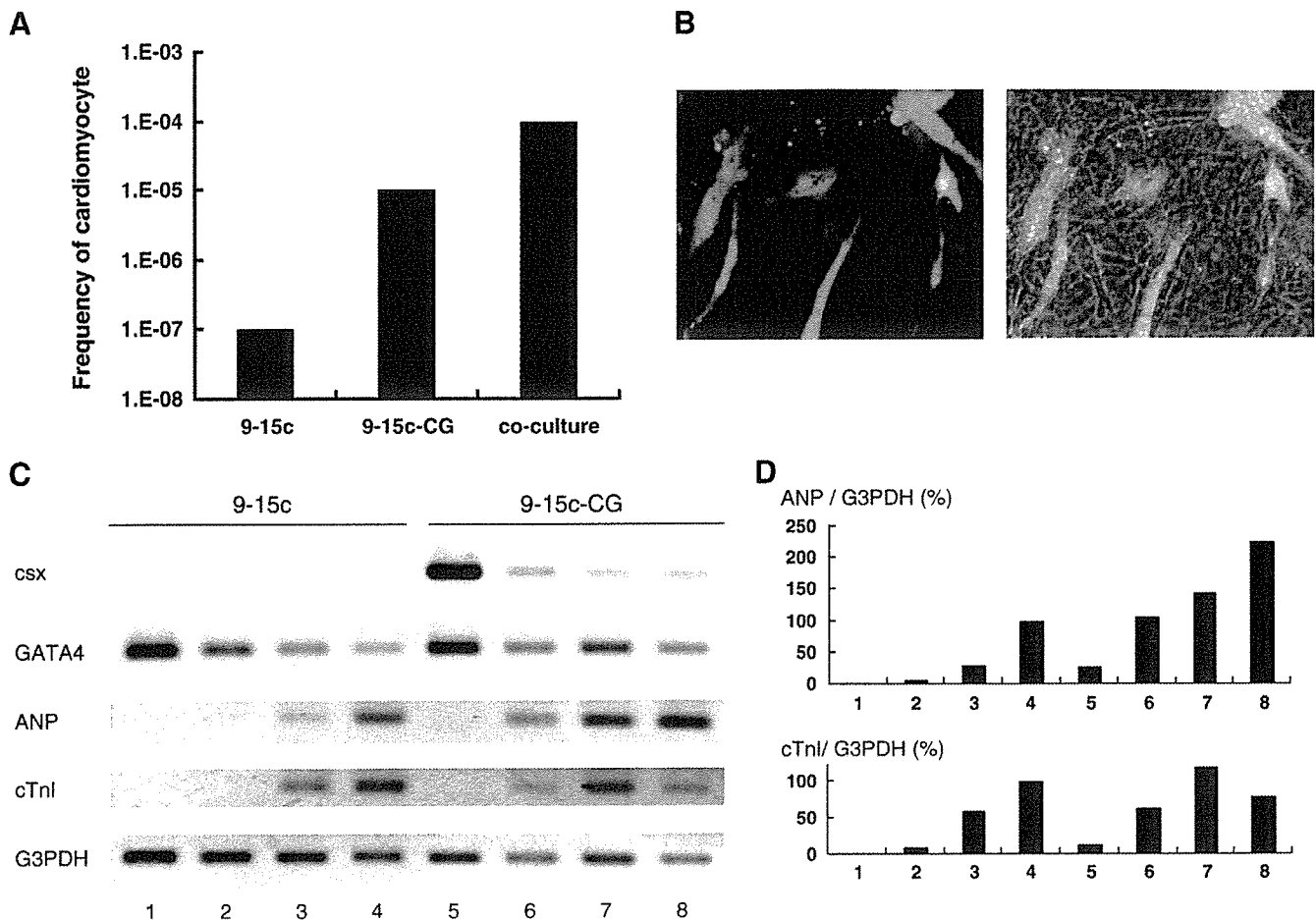
Fig. 5 – Enhancement of cardiomyogenic differentiation of 9-15c cells by co-cultivation with murine fetal cardiomyocytes. A: Frequencies of cardiomyogenic differentiation in 9-15c cells, 9-15c cells overexpressing the Csx and GATA4 genes (9-15c-CG cells), and 9-15c-CG cells co-cultured with murine fetal cardiomyocytes. B: Cardiomyogenic differentiation of EGFP-positive 9-15c-CG cells co-cultured with murine fetal cardiomyocytes. Left: Green fluorescence of EGFP-positive 9-15c-CG cells. Right: Same field visualized by phase-contrast microscopy merged with fluorescence image. C: RT-PCR analysis of the Csx, GATA4, ANP, cTnI and G3PDH genes in 9-15c cells (lanes 1–4) and 9-15c-CG cells (lanes 5–8). 9-15c cells (lane 1) and 9-15c-CG cells (lane 5) were cultured with exposure to 5-azacytidine alone (lanes 2 and 6) or 5-azacytidine and conditioned medium of cardiomyocyte cultures (lanes 3 and 7), or 5-azacytidine, conditioned medium of cardiomyocyte cultures, PDGF, retinoic acid, and fibronectin coating on a dish (lanes 4 and 8) for 4 weeks. D: Ratio mRNA expression level of ANP and cTnI to G3PDH in C. The mRNA level of 9-15c cells (lane 4) was regarded as equal to 100%.

induced by 5-azacytidine or microRNAs, whose key roles in stem cell biology are just emerging [37], also seem to be needed.

Adipogenic 3T3-L1 [38], osteogenic MC3T3-E1 [39], and chondrogenic ATDC5 cells [40] have been isolated from stem cells with a mesenchymal nature. In addition, cardiomyogenic precursors may be obtained from stem cells such as cardiac stem cells, embryonic stem cells, and mesenchymal stem cells. Fetal cardiomyocytes are differentiated cardiomyocytes, but not stem cells that can proliferate in vitro. Recently, cardiac stem cells capable of clonogenically self-renewing have been isolated from the adult heart [41–43]. Some cardiac stem cells also retain plasticity. The retention of plasticity, i.e., the ability to transdifferentiate into skeletal myocytes and endothelium, of 9-15c cells overexpressing Csx/Nkx2.5 and GATA4 supports the idea that these cells may be considered cardiac stem or amplifying cells in terms of differentiation and

self-renewal. On the other hand, Csx/Nkx2.5 inhibits the myogenic differentiation of C2C12 cells and promotes neuronal differentiation [44]. This unexpected effect of Csx/Nkx2.5 may be due to differential effects of the gene in different cell types, or of transient versus constitutive expression of the infected gene; dependency of the differentiated phenotypes on the gene expression period is observed for the Notch gene [45,46] and noggin gene [47].

Cell transplantation has been attempted to improve cardiac function in severe heart failure; MSCs have been transplanted to functionally restore damaged or diseased tissue in animal models, and mononuclear cells or myoblasts have been injected into ischemic hearts clinically. MSCs are capable of differentiating into many types of cells, and 'cardiomyogenic master genes' are able to enhance the commitment or determine the path to cardiomyogenic differentiation of these MSCs. The stemness of MSCs determined by single-cell

marking in this study needs to be taken into consideration when we are considering mesenchymal stem cell-based therapy: we should pay attention to the possible unexpected differentiation of donor MSCs such as osteogenesis or chondrogenesis in the implanted heart.

In conclusion, we demonstrated that cardiomyocytes were stochastically differentiated from MSCs and that forced expression of Csx/Nkx2.5 and GATA4 enhanced the commitment or determined the path to cardiogenic differentiation of these MSCs. Our findings suggest that single-cell-derived MSCs overexpressing Csx/Nkx2.5 and GATA4 behave like cardiac transient amplifying cells and that Csx/Nkx2.5 and GATA4 could be interesting target molecules for enhancing cardiogenesis of MSCs.

Acknowledgments

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Review Article

Two MSCs: Marrow stromal cells and mesenchymal stem cells

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Marrow stromal cells (MSC1) are able to generate a series of terminally-differentiated cells *in vitro*. Most experiments are performed with heterogeneous stromal cells obtained by adherence to plastic culture dishes. Since bone marrow-derived stromal cells are purified to a homogeneous population meeting the criteria for non-hematopoietic stem cells, these cells have been termed "mesenchymal stem cells" and have the capability of generating an array of cells. However, "mesenchymal stem cells" (MSC2) are also actual multi-purpose cells capable of differentiating into cells of mesoderm-origin regardless of cell sources. MSC2 can be recovered from a variety of other tissues, such as fat, muscle, menstrual blood, endometrium, placenta, umbilical cord, cord blood, skin, and eye. The terms "mesenchymal stem cell" and "marrow stromal cell" have been used interchangeably in emerging literature to describe cells that can be used in regenerative medicine, thereby introducing a degree of confusion. In this review, we re-organize the understanding of the two MSCs, describe their biology and differentiate between the two.

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Introduction

Two MSCs, i.e., marrow stromal cells (MSC1) and mesenchymal stem cells (MSC2), are attracting a great deal of attention, as they represent a valuable source of cells for use in regenerative medicine, as well as offering an excellent model of cell differentiaton in biology. However, confusion exists in the literature due to poor application or misuse of the terms and nomenclature.

In general, mesenchymal stem cells are multi-potential stem cells that can differentiate into a variety of cell types (ref. http://en.wikipedia.org/wiki/Mesenchymal_stem_cell). They have been shown to differentiate, *in vitro* or *in vivo*, into osteoblasts, chondrocytes, myocytes, adipocytes and neuronal cell among others. Mesenchymal stem cells have traditionally been obtained from bone marrow, and have commonly been referred to as "marrow stromal cells"(MSC1).

While the terms"marrow stromal cell"(or"stromal cell") and "mesenchymal stem cell" have frequently been used interchangeably, they are increasingly recognized as separate entities as:

1. Stromal cells (MSC1) are a highly-heterogenous cell population, usually derived from bone marrow, consisting of multiple cell types with different potentials for proliferation and differentiation.

2. Mesenchymal stem cells (MSC2) encompass cells derived from other non-marrow tissues, such as fat, muscle, menstrual blood, endometrium, placenta, umbilical cord, cord blood, skin, and eye.

Bone marrow-derived mesenchymal stem cells or bone marrow stromal cells (MSC1) were discovered by Friedenstein in 1976, who described clonal, plastic-adherent cells from bone marrow that were capable of differentiating into osteoblasts, adipocytes, and chondrocytes. More recently, investigators have demonstrated that mesenchymal stem cells (MSC2) *per se* can be recovered from a variety of adult tissues and have the capacity to differentiate into a variety of specialist cell types. This review describes the recent advances in understanding of the two MSC cells, their biology and ongoing investigation and use.

Somatic stem cells

Somatic stem cells have been identified in hematopoietic[1], hepatic[2], epidermal[3], gastrointestinal[4], neural[5,6], muscle[6], and bone marrow[6-8] tissues. Many researchers have since demonstrated the developmental pluripotency of these cells. Bone marrow-derived stem cells can be transdifferentiated into multilineage cells, such as muscle[9] of mesoderm, lung[10] and liver[10,11] of endoderm, and brain[12-15] and skin[10] of ectoderm. Somatic stem cells are more desirable than embryonic stem (ES) cells for cell therapeutics because of ethical considerations and the possible immunologic rejection of ES cells. Mesenchymal stem cells have become the most popular somatic stem cells in medicine and biology, not least because of their high reproductive capability *in vitro*.

Bone marrow stromal cells (MSC1)

The existence of non-hematopoietic cells in bone marrow was first suggested by Cohnheim about 130 years ago[16]. Bone marrow-derived stromal cells (MSC1) can differentiate into most somatic cells, including osteoblasts, chondrocytes, myoblasts, cardiomyocytes[17-21], and adipocytes, when placed in appropriate *in vitro*[20] and *in vivo* environments[22], and thus are a useful cell source for regenerative medicine[23]. Recent studies suggest that MSC1 can also differentiate into a neuronal lineage[24], and murine bone marrow-derived adult progenitor cells can differentiate into dopaminergic neuronal cells[25,26]. Since the use of MSC1 entails no ethical or immunological problems, and bone
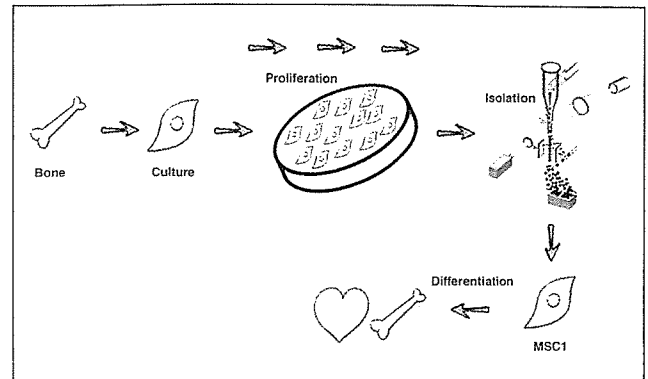

Fig.1   Development and differentiation of mesenchymal stem cells derived from bone marrow

marrow aspiration is an established routine procedure, these cells provide a useful and almost routine source of material for transplantation and tissue repair or regeneration (Fig.1).

1) Osteogenesis

KUSA-A1 cells, a murine marrow stromal cell line, are capable of generating mature bone *in vivo*[27]. They are a unique, mature osteoblast cell line and serve as a very suitable model for *in vivo* osteogenesis. Bone forms in subcutaneous tissue after subcutaneous injection of the cells into mice. The osteogenesis by KUSA-A1 is not mediated by chondrogenesis and thus is considered to be membranous ossification. Follow-up study on the fate of bone by immortalized osteoblasts shows that the ectopically-generated bone keeps its size and shape for 12 months[21]. Furthermore, the implanted cells do not metastasize like tumor cells. These unique characteristics of KUSA-A1 cells provide an opportunity to analyze the process of membranous ossification in detail.

2) Chondrogenesis

Chondrocytes differentiate from mesenchymal cells during embryonic development[28] and the phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix molecules, including type II collagen and aggrecan[29-31]. The phenotype of differentiated chondrocytes is rapidly lost since it is unstable in culture[32-35]. This process is referred to as "dedifferentiation" and is a major impediment to use of mass cell populations for therapy or tissue engineering of damaged cartilage. When isolated chondrocytes are cultured in a monolayer at low density, the typical round chondrocytes morphologically transform into flattened fibroblast-like cells, with profound changes in biochemical and genetic characteristics, including reduced synthesis of type II collagen and cartilage proteins[36]. When cultured

three-dimensionally in a scaffold such as agarose, collagen, and alginate, redifferentiated chondrocytes re-express the chondrocytic differentiation phenotype.

KUM5 mesenchymal cells, a MSC1 line, generate hyaline cartilage *in vivo* and exhibit endochondral ossification at a later stage after implantation[37]. OP9 cells, another MSC1 line, derived from macrophage colony-stimulating factor-deficient osteopetrotic mice, and also known to be niche-constituting cells for hematopoietic stem cells, express chondrocyte-specific or -associated genes, such as type II collagen $\beta 1$, Sox9, and cartilage oligomeric matrix protein at an extremely high level, as do KUM5 cells. OP9 micromasses exposed to TGF-$\beta 3$ and BMP2 form type II collagen-positive hyaline cartilage within two weeks *in vivo*. The unique characteristics of KUM5 and OP9 cells provide an opportunity to analyze the process of endochondral ossification.

3) Cardiomyogenesis

It has been generally accepted that cardiac myocytes are unable to divide once cell proliferation ceases shortly after birth in the mammalian heart, because mitotic figures have not been detected in myocytes[38]. Cardiomyocytes induce DNA synthesis *in vivo* and *in vitro*[39,40]. Adult hearts often exhibit a polypoid structure, which results from stochastic accumulation of mutations as cells pass through cell-cycle checkpoints[41]. Bone marrow-derived stromal cells (MSC1) are able to differentiate into cardiomyocytes *in vitro* and *in vivo*[19,20,42,43] and a hierarchical model has been proposed for this *in vitro* cardiomyogenic differentiation. MSC1 in culture include a mixture of at least three types of cells, i.e., cardiac myoblasts, cardiac progenitors and multi-potential stem cells, and a follow-up study of individual cells suggests that commitment of a single-cell-derived stem cell toward a cardiac lineage is stochastic[44]. Furthermore, MSC1 over-expressing well-known master transcription factors, i.e., Csx/Nkx2.5 and GATA4, unavoidably undergo cardiomyogenic fate and behave like transient amplifying cells. MSC1 also transdifferentiate into cardiomyocytes in response to humoral factors, such as demethylation of the genome, in addition to environmental factors (See the chapter "Epigenetic modifier as a differentiating inducer".

4) Neurogenesis

MSC1 can exhibit neural differentiation when exposed to demethylating agents[14]: the cells differentiating into three types of neural cells, i.e., neurons, astrocytes, and oligodendrocytes. With exposure to basic fibroblast growth factor, nerve growth factor, and brain-derived neurotrophic factor, the transdifferentiation of human stromal cells is limited to neurons[14]. The change

in gene expression during differentiation is global and drastic[45]: the differentiated cells no longer exhibit the profile of stromal cells or the biphenotypic pattern of neuronal and stromal cells. Osteoblasts capable of intra-membranous ossification are likely to differentiate into neuronal lineages, but adipocytes do not[14]. Interestingly, the cranio-facial membranous bones develop from the neural crest, which is of ectodermal origin. Development naturally progresses from neural crest cells to terminally-differentiated osteoblasts[46]. The finding of *in vitro* differentiation from mesoderm- to ectoderm-derived cells is thus the opposite of the developmental process, i.e., from ectoderm- to mesoderm-derived cells. Converting differentiated osteoblasts or MSC1 to neuronal cells, a key future task for any cell-based therapy, would thus oppose the usual direction of cell differentiation. This can now be achieved by exposing stromal cells to neurotrophic factors, at least *in vitro*.

Dopaminergic neuron-associated genes, such as nurr1 and wnt-5a, are induced at an extremely high level in the neuronally-differentiated stromal cells. Wnt5a and nurr1 are involved in the differentiation of mid-brain precursors into dopaminergic neurons[25,26]. It is quite significant that dopaminergic neurons can be generated from MSC1, since they are one of the key targets for regenerative medicine.

Epigenetic modifier as a differentiating inducer

The demethylating agent, 5-azacytidine, is a cytosine analog that has a remarkable effect on transdifferentiation of cells and has been shown to induce differentiation of stromal cells into cardiomyocytes, skeletal myocytes, adipocytes, and chondrocytes[19,42,47]. The effect of this low-molecular substance is not surprising, since it is incorporated into DNA and has been shown to cause extensive demethylation. The demethylation is attributable to covalent binding of DNA methyltransferase to 5-azacytidine in the DNA[48], with subsequent reduction of enzyme activity in cells resulting in dilution-out and random loss of methylation at many sites in the genome. This may, in turn, account for the reactivation of cardiomyogenic "master" genes, such as MEF-2C, GATA4, dHAND, and Csx/Nkx2.5, leading to stochastic transdifferentiation of MSC1 into cardiomyocytes. Use of 5-azacytidine is beneficial, but since it may have drawbacks, i.e., gene activation leading to oncogenesis and undesired differentiation, care must be exercised before using it to induce cells to differentiate into target phenotypes. Immortalized cells, including marrow stromal cells, have specific patterns of DNA methylation. The established methylation pattern of cells is maintained
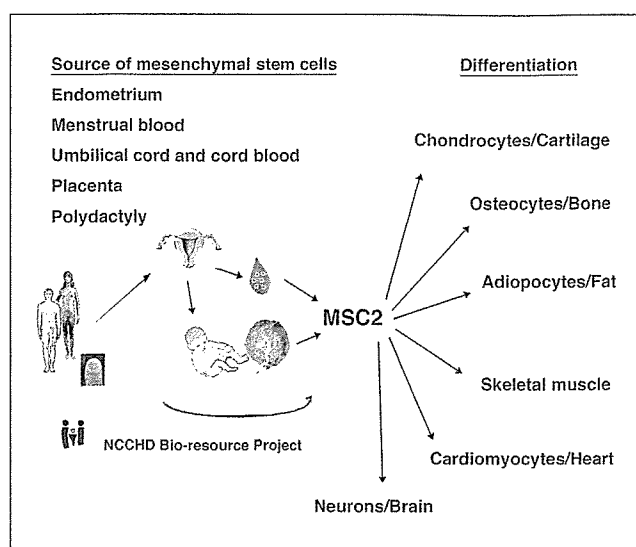
Fig.2   Sources and differentiation of mesenchymal stem cells

with considerable fidelity and silenced genes are stably inherited throughout the culture period[49-51]. The demethylating agent induces differentiation by altering the original methylated pattern and reactivating the silenced genes.

Mesenchymal stem cells (MSC2)

Tissues originating in the mesoderm include blood cells, blood vessels, heart, bone, cartilage, fat, skeletal muscle, tendon, and tissue mesenchyme. Blood cells in bone marrow are the elements that create the concept of stem cells, but bone marrow includes another cell group, i.e., mesenchymal stem cells (MSC2), which possess adherent properties. These cells have the ability to differentiate into a variety of cells and may have an organ maintenance mechanism that serves as back-up. Human mesenchymal stem cells (MSC2) are a useful source of cells for transplantation for several reasons: they have the ability to proliferate and differentiate into mesodermal tissues and they entail no ethical or immunological problems. MSC2 have been studied extensively over the past three decades and numerous independent research groups have successfully isolated them from a variety of sources, most commonly from bone marrow[19,22,52-55]. Yet, in addition to bone marrow, almost all human tissues or organs can be a source of mesenchymal stem cells, since they all have stroma or mesenchyme as well as parenchyma or epithelium.

Available mesenchymal cell lines and mesenchymal cells in culture

MSC2 have been extracted from fat, muscle, menstrual blood,

endometrium, placenta, umbilical cord, cord blood, skin, and eye (Fig.2). Moreover, the source tissues can be obtained without difficulty from resected tissues at surgery and from birth deliveries (http://www.nch.go.jp/reproduction/cellbank2.htm and http://www.nch.go.jp/reproduction/cells/primary.html); menstrual blood can be provided from volunteers. The placenta is composed of amniotic membrane, chorionic villi and decidua, each of which can be a source of different types of MSC2. Large numbers of MSC2 can be easily obtained because the placenta is usually provided for research purposes. Menstrual blood also contains a large number of MSC2, although it is usually regarded as waste material.

We have also isolated many specific cell lines from adhering cells of mouse bone marrow (http://www.nch.go.jp/reproduction/cellbank2.htm) as follows:

a. Multi-potential stem cell line: 9-15c cells (originally KUM2 cells) have multi-potential allowing differentiation into bone, fat, skeletal muscle, and myocardial cells through continued passage;

b. Oligo-potential cell lines: KUM9 cells that lose the ability to differentiate to myocardial cells but retain differentiation to bone, fat, and skeletal muscle and NRG cells that lose the capability to differentiate into myocardial cells and skeletal myocytes but retain differentiation to bone and fat;

c. Bi-potential cells: KUSA-O cells are capable of differentiating into osteoblasts and adiopocytes;

d. Precursor cells: KUSA-A1 and H-1/A are osteoblasts and preadipocytes, respectively. Adipogenic 3T3-L1[56], osteogenic MC3T3-E1[57], and chondrogenic ATDC5 cells[58] have been isolated from stem cells of a mesenchymal nature.

Focusing on human MSC2 derived from umbilical cord blood (UCBMSC) as an example, isolation, characterization, and differentiation of clonally-expanded UCBMSCs have been reported[59,60], and UCBMSCs have been found to have multi-potential[61]. Most of the surface markers are the same as those detected in their bone marrow counterparts[42], with both UCB- and bone marrow-derived cells being positive for CD29, CD44, CD55, and CD59, and negative for CD34 and CD117. Significantly, the differentiation capacity of UCB-derived cells is unaffected during establishment of a plate-adhering population of cells from UCB.

Life span of MSC1 and MSC2

Marrow stromal cells (MSC1) and mesenchymal stem cells (MSC2) are useful for cell transplantation. However, it is difficult to study and apply them because of their limited life span.

One of the reasons for this is that normal human cells undergo a limited number of cell divisions in culture and then enter a non-dividing state called "senescence" [62,63]. Human cells reach senescence after a limited number of cell replications, and the average number of population doublings (PDs) of marrow-derived mesenchymal stem cells has been found to be about 40[42], implying that it would be difficult to obtain enough cells to restore the function of a failing human organ. Large numbers of cells must be injected into damaged tissues to restore function in humans, and cells sometimes need to be injected throughout entire organs.

A system that allows human cells to escape senescence by using cell-cycle-associated molecules may be used to obtain sources of material for cell therapy[64,65]. Both inactivation of the Rb/p16INK4a pathway and activation of telomerase are required for immortalization of human epithelial cells, such as mammary epithelial cells and skin keratinocytes. Human papillomavirus E7 can inactivate pRb, and Bmi-1 can repress p16INK4a expression. Inactivation of the p53 pathway is also beneficial, even if not essential, to extension of the life span[66]. Human marrow stromal cell strains with an extended life span can be generated by transduction of combination of TERT, and Bmi-1, E6 or E7[45]. Cells with extended life span grow *in vitro* for over 80 PDs, and their differentiation potential is maintained. Transfection of TERT alone is insufficient to prolong the life span of marrow stromal cells, despite TERT having been reported to extend the life span of cells beyond senescence without affecting their differentiation ability[67]. Human stromal cells transfected with TERT and Bmi-1, E6 or E7 do not transform according to the classical pattern: they do not generate tumors in immunosuppressed mice; they do not form foci *in vitro*; and they stop dividing after confluence. The possibility that gene-transduced stromal cells might become tumorigenic in patients several decades after cell therapy therefore cannot be ruled out. Nevertheless, these gene-modified stromal cells may be used to supply defective enzymes to patients with genetic metabolic diseases, such as neuro-Gaucher disease, Fabry disease, and mucopolysaccharidosis, which have a poor prognosis and are sometimes lethal. The "risk versus benefit" balance is essential when applying these gene-modified cells clinically, and the "risk" or "drawback" in this case is transformation of implanted cells. These marrow stromal cells (MSC1) with prolonged life span also provide a novel model for further study of cancer and stem cell biology.

Differentiation of mesenchymal stem cells

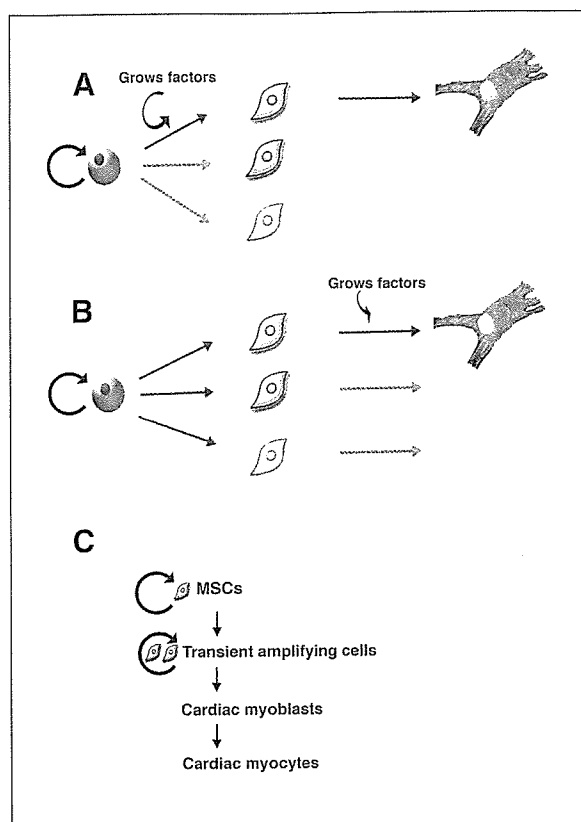Retroviral labeling of individual cells is a useful clonal assay

Fig.3 Model of stem cell differentiation
A. Deterministic model.
B. Stochastic model.
C. Differentiation model of mesenchymal stem cells.

to monitor lineage commitment at the single cell level. At present, several models have been proposed in which hematopoietic lineage determination is driven intrinsically[68], extrinsically[69], or both[70]. The issue of the mechanism and the extent of cellular differentiation that occurs when stem cells begin to differentiate is the area of furthest advanced research. Two models have been proposed: a deterministic model, in which differentiation is governed by the microenvironment (including growth factors and cytokines), and a stochastic model, in which differentiation, self-replication and the direction of differentiation emerge somewhat randomly (Fig.3A,B). The different models arise from different conceptions of mesenchymal stem cells. The mesenchymal stem cell (MSC2) line is stochastically committed toward the cardiac lineage, and following this commitment, they proliferate as transient amplifying cells and differentiate into cardiac myocytes (Fig.3C).

Considering stem cell transplant as a therapy, when mature cells arising from hematopoietic stem cells are needed, as in marrow transplant, there are no problems attending cellular dif-

ferentiation. However, in the case of cells that serve to originate cells of several different organs, as in the case of mesenchymal stem cells, there is a possibility for differentiation to cells not needed in the treatment. Ectopic tissue may therefore emerge from implanted mesenchymal stem cells, especially where the buffering system from a given site is lost and the stem cells begin to differentiate randomly into cells differing from the implanted site, thereby creating unwanted ectopic tissue.

Conclusion

Mesenchymal stem cells can be isolated from bone marrow by standardized techniques and expanded in culture through many generations, while retaining their capacity to differentiate along set pathways when exposed to appropriate conditions. This property opens up therapeutic opportunities for the treatment of lesions in mesenchymal tissues, and protocols have been devised for the treatment of defects in articular cartilage[71], bone[72], tendon[73], and meniscus[74] and for bone marrow stromal recovery[75] and osteogenesis imperfecta[76].

In this context, we prefer to use the word "stroma" rather than "mesenchymal stem cells" for accuracy and to avoid confusion. In the field of hematopoiesis, marrow stroma were originally treated as "second class citizens"[77], and represented a niche field. Today, marrow stroma are a "major player" in regenerative medicine and stem cell biology and are no longer viewed as a peripheral field of research. In addition, there is also a rapidly growing body of research into the biology and potential use of true "mesenchymal stem cells" derived from other human tissues, which are showing significant promise for future therapy, reparation or regeneration of human tissues and organs.

Clearly, this field is in its relative infancy, our understanding is at present limited but the potential benefits are great. We should perhaps, therefore, remember that the unexpected and unrivalled potential of MSCs to differentiate into a wide variety of cells represents a gift not a privilege and, with respect to the two MSCs, we should recognise and welcome their role in medicine with the words "with great power comes great responsibility".

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

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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 (Hellebaek, Denmark), and BD Biosciences PharMingen (San Diego, CA).

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

AQ: 2 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

AQ: 3 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_2O_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 AQ:4 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 ($2500/cm^2$) or EGFP-labeled EM-E6/E7/hTERT-2 cells ($2500/cm^2$) were cocultured with C2C12 myoblasts ($2500/cm^2$) 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 (2×10^7) 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 F1 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-
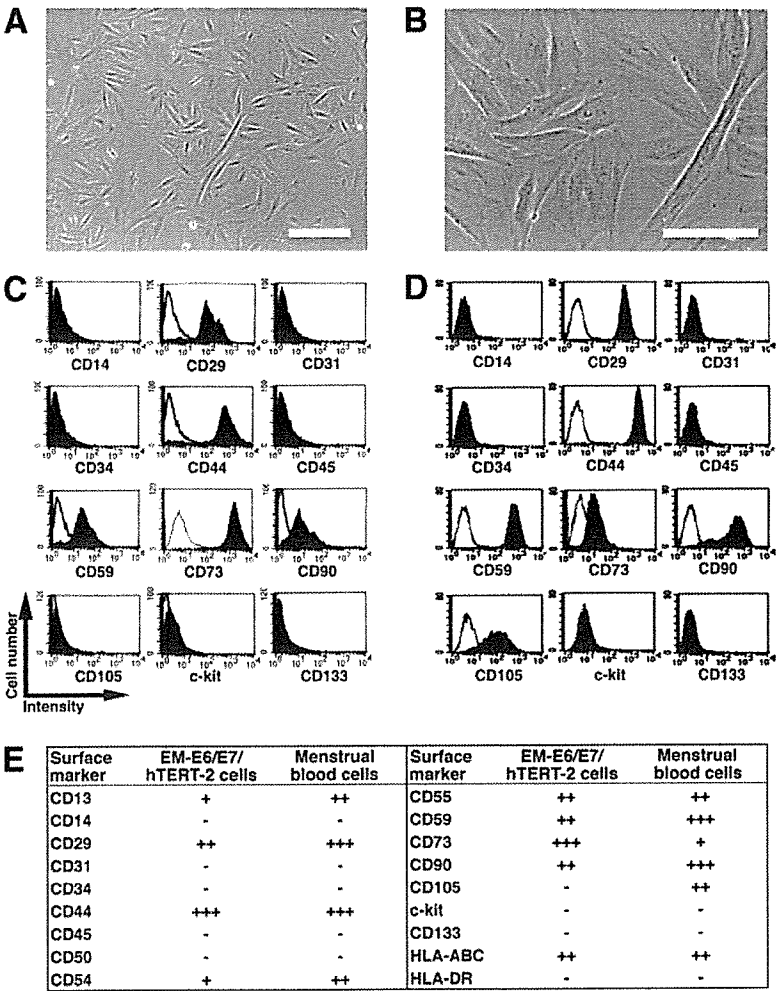
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Figure 1. Surface marker expression of endometrium-derived cells. (A and B) Morphology of menstrual blood–derived cells, regarded as being PD 1 or 2. Scale bars, 200 μm (A), 100 μm (B). (C and D) Flow cytometric analysis of cell surface markers of 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).
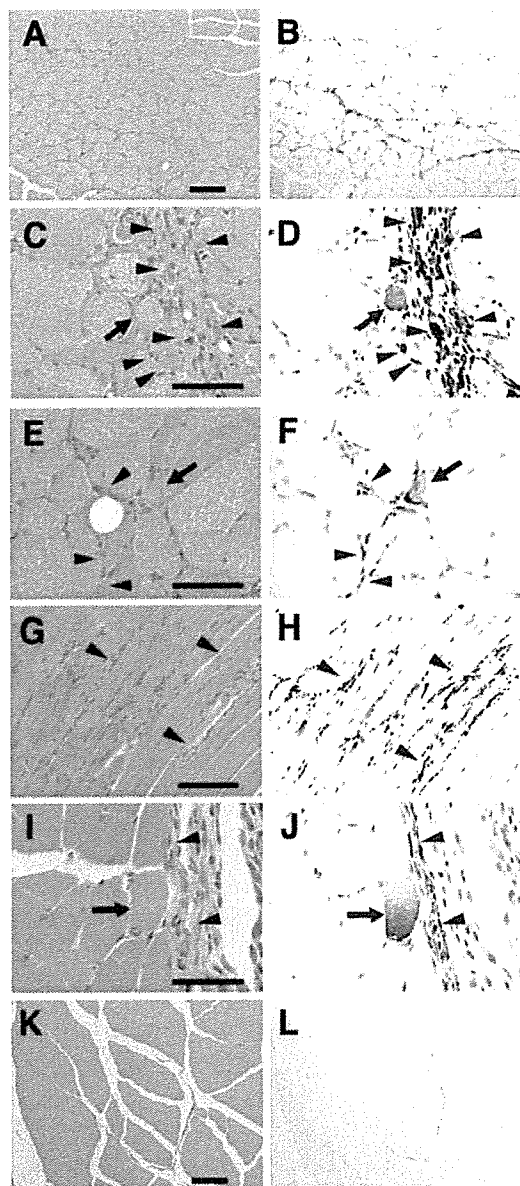
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Figure 2. Implantation of endometrium-derived cells-derived cells into the muscle of NOG mice. EM-E6/E7/hTERT-2 cells (A–F) or menstrual blood–derived cells (G–J) cultured in absence of any stimuli were directly injected into the right thigh muscle 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

^{F3, AQ:5}

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.

^{F4, AQ:6}

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-
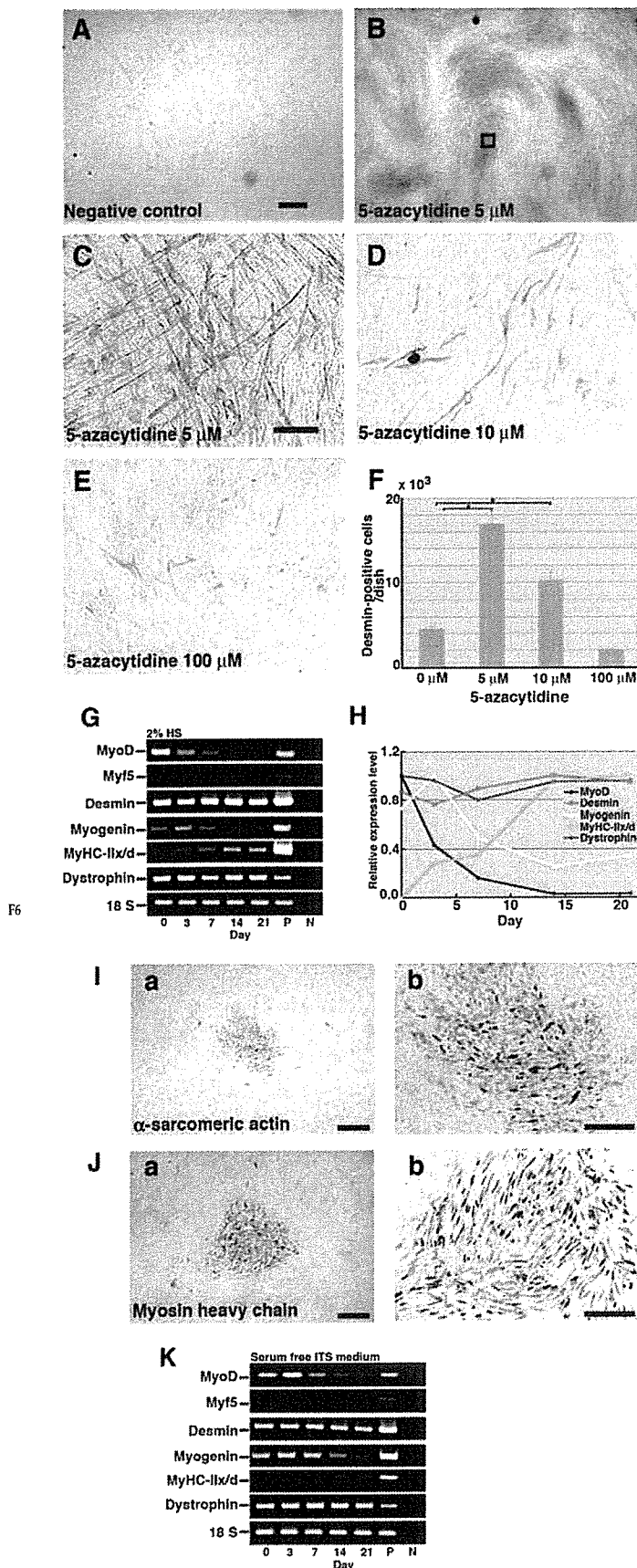
^{F5}

Figure 3. Expression of myogenic-specific genes during myogenic differentiation of EM-E6/E7/hTERT-2 cells. (A–F) Immunocytochemical

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 H$_2$O 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).
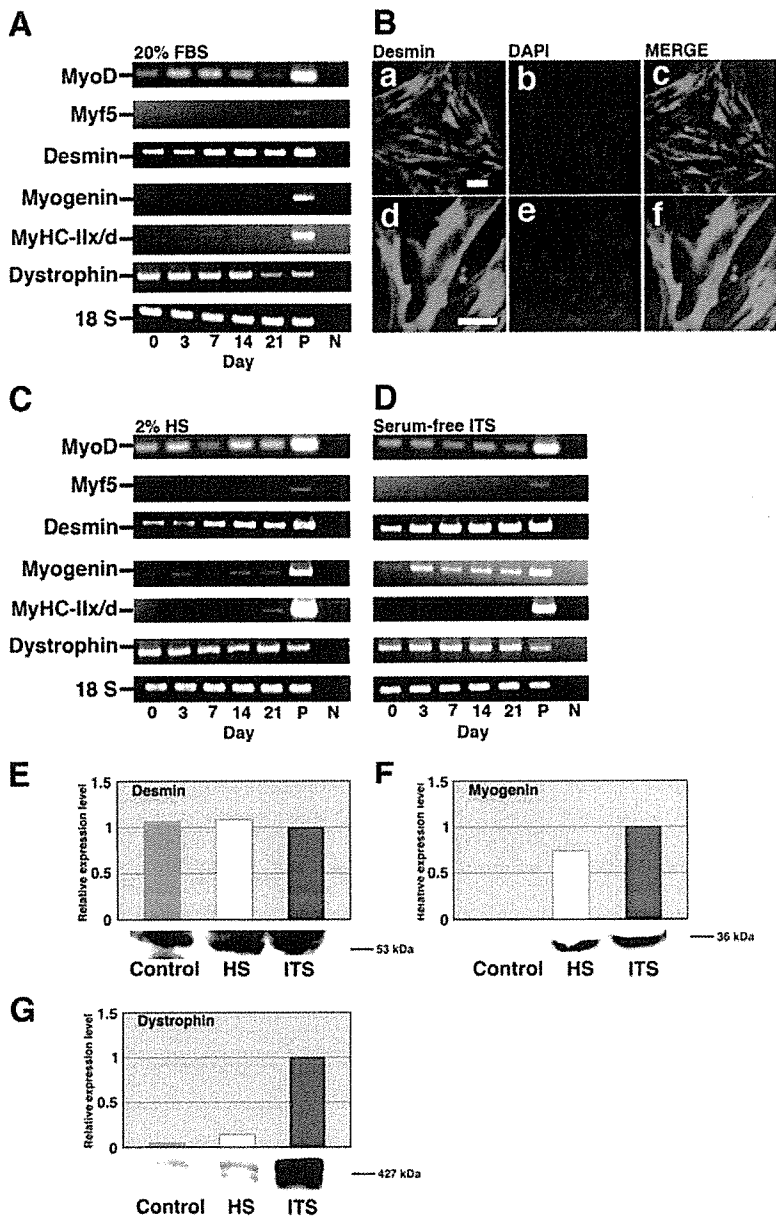
A

B

C

D

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.

E

F

G

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-
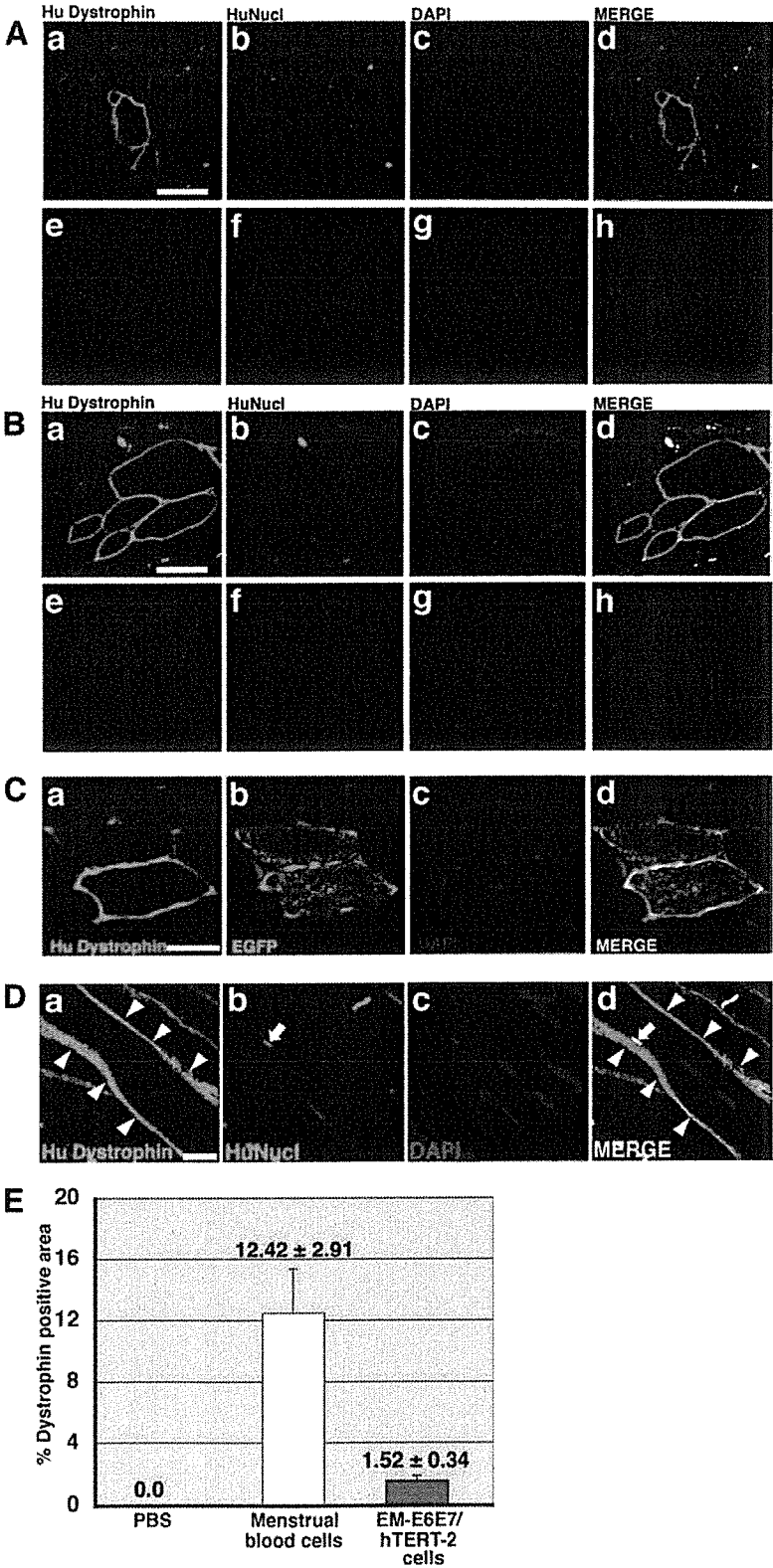
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Myogenesis by Menstrual Blood Cells

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-