Figure 3   Tissue repair effect delivered by Muse cells. ●, red blood; ◉, Muse cells; ⌒, non-Muse cells.
When Muse and non-Muse cells were supplied to the blood stream, only Muse cells integrate into the damaged site, differentiate, and repaired the tissue, while non-Muse cells do not remain in the damaged tissue nor do they participate in tissue repair.

functions of the MSC components. Although Muse cells account for only several percent of the total MSCs, they play an exclusive role in triploblastic differentiation and tissue repair, while non-Muse cells do not directly participate in these events and rather have major roles in trophic and immunosuppressive effects. There are remarkable differences between Muse and non-Muse cells. First, non-Muse cells do not form clusters in suspension like single Muse cells.[12] Assuming that non-Muse cells are just like general mesenchymal cells, such as fibroblasts, they are essentially adherent cells and thus do not inherently survive and function in suspension.

Second, pluripotency genes that are expressed in Muse cells are not expressed in non-Muse cells and thus non-Muse cells are not pluripotent. Although they have lower efficiency than Muse cells, non-Muse cells do have the ability to differentiate into osteocytes, chondrocytes, and adipocytes. They are, however, unable to differentiate into neuronal cells (ectodermal), hepatocytes (endodermal), or even into the same mesodermal lineage skeletal muscles.[16] Thus, they are not pluripotent. Consistently, as shown in melanocyte induction, Muse cells from dermal fibroblasts can differentiate into functional melanocytes that produce melanin pigment following induction with cytokine cocktails while fibroblast-derived non-Muse cells fail to differentiate.[19] Gene expression patterns in non-Muse cells during melanocyte induction are interesting to observe; they respond partially to the induction stimulation

and indeed some melanocyte markers are newly expressed in an earlier period of induction, but those markers disappear later and the gene expression pattern returns back to the original state of fibroblasts at the later stage.[19]

The partial responsiveness of non-Muse cells is also observed in iPS cell generation. Muse cells that are already pluripotent express pluripotency genes and lack tumorigenic activity, readily become iPS cells when treated with the four Yamanaka factors, and newly acquire tumorigenicity, whereas non-Muse cells do not show an increase in major pluripotency genes, including Nanog and Sox2, even after receiving the four Yamanaka factors.[13,22] Their responsiveness to the four Yamanaka factors is only partial, however, and thus non-Muse cells fail to generate iPS cells.

Third, non-Muse cells, unlike Muse cells, do not integrate nor differentiate into functional cells in damaged tissues.[12,21] Previous reports demonstrated that the large majority of MSCs do not remain in the transplanted tissue, but rather exert trophic effects that occasionally lead to some degree of functional recovery. As the majority of MSCs are non-Muse cells, the major role of non-Muse cells after transplantation might be a trophic effect.

LOCALIZATION OF MUSE CELLS *IN VIVO*

Mesenchymal tissues, such as the bone marrow, adipose tissue, and dermis, are the main reserve of Muse cells *in vivo*.
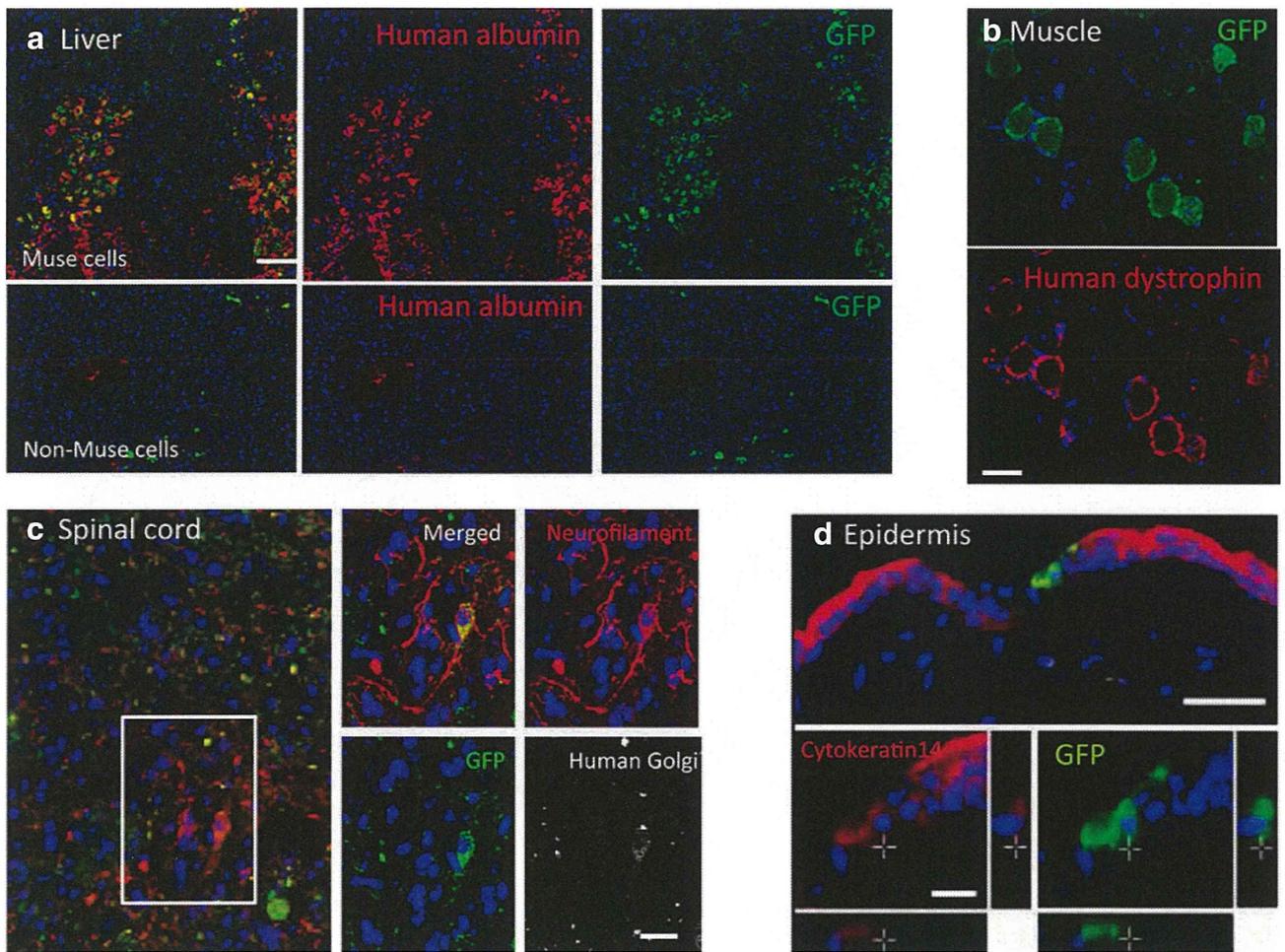
Figure 4 Tissue repair effect of Muse cells.
Green fluorescent protein (GFP)-positive human Muse cells integrated into (**a**) fulminant hepatitis, (**b**) muscle degeneration, (**c**) spinal cord injury (made by crush injury), and (**d**) skin injury models, and became (**a**) human albumin-, (**b**) human dystrophin-, (**c**) neurofilament- (cells were also positive for the human cell marker, anti-human Golgi complex, confirming that the positive cells were of human origin), and (**d**) cytokeratin 14- positive cells 4 weeks after injection. When non-Muse cells were infused into fulminant hepatitis, cells did not differentiate into albumin-positive cells. Scale bars; **a, b** = 100 μm, **c, d** = 50 μm. (Pictures reproduced from *Proc Natl Acad Sci USA* 2010; **107**: 8639–43, and *Cells* 2012; **1**: 1045–60, 2012).[12,21]

In the human dermis and adipose tissue, Muse cells detected as SSEA-3-positive cells locate sparsely in the connective tissues of the dermis and hypodermis, and do not associate with particular structures such as blood vessels or dermal papilla (Fig. 5)[13]. Similarly, they distribute in the connective tissue of many organs in the same manner as seen in the dermis and adipose tissue (unpublished data). Because tissue stem cells are generally confined to the tissue where the stem cells belong, i.e., neural stem cells in the brain, hematopoietic stem cells in the bone marrow, Muse cells are unique in that they are distributed throughout the body and are not confined to a specific organ or tissue.

Organ-derived Muse cells, however, might not be a practical source for clinical use. Rather, easily accessible mesenchymal tissues are realistic and feasible sources for obtaining Muse cells for clinical use. In the case of human bone marrow

aspirate, SSEA-3/CD105 double-positive Muse cells were identified at a ratio of 0.03%, namely, 1 in 3000 mononucleated cells.[12] The proliferation speed of Muse cells is ~1.3 day/cell division, so that 10 ml of fresh bone marrow aspirate may yield nearly 1 million Muse cells within 10 days.[12]

Commercially available cultured mesenchymal cells, such as human dermal fibroblasts and BM-MSCs, are another potential source for Muse cells. While the ratio and quality of Muse cells may be altered by handling and depend the number of subcultures, fibroblasts and BM-MSCs contain Muse cells at levels ranging from 1% to 5–6%.[13]

MUSE CELLS AND REGENERATIVE HOMEOSTASIS

The fact that Muse cells reside in connective tissue and bone marrow suggests that they are widely distributed in the body.
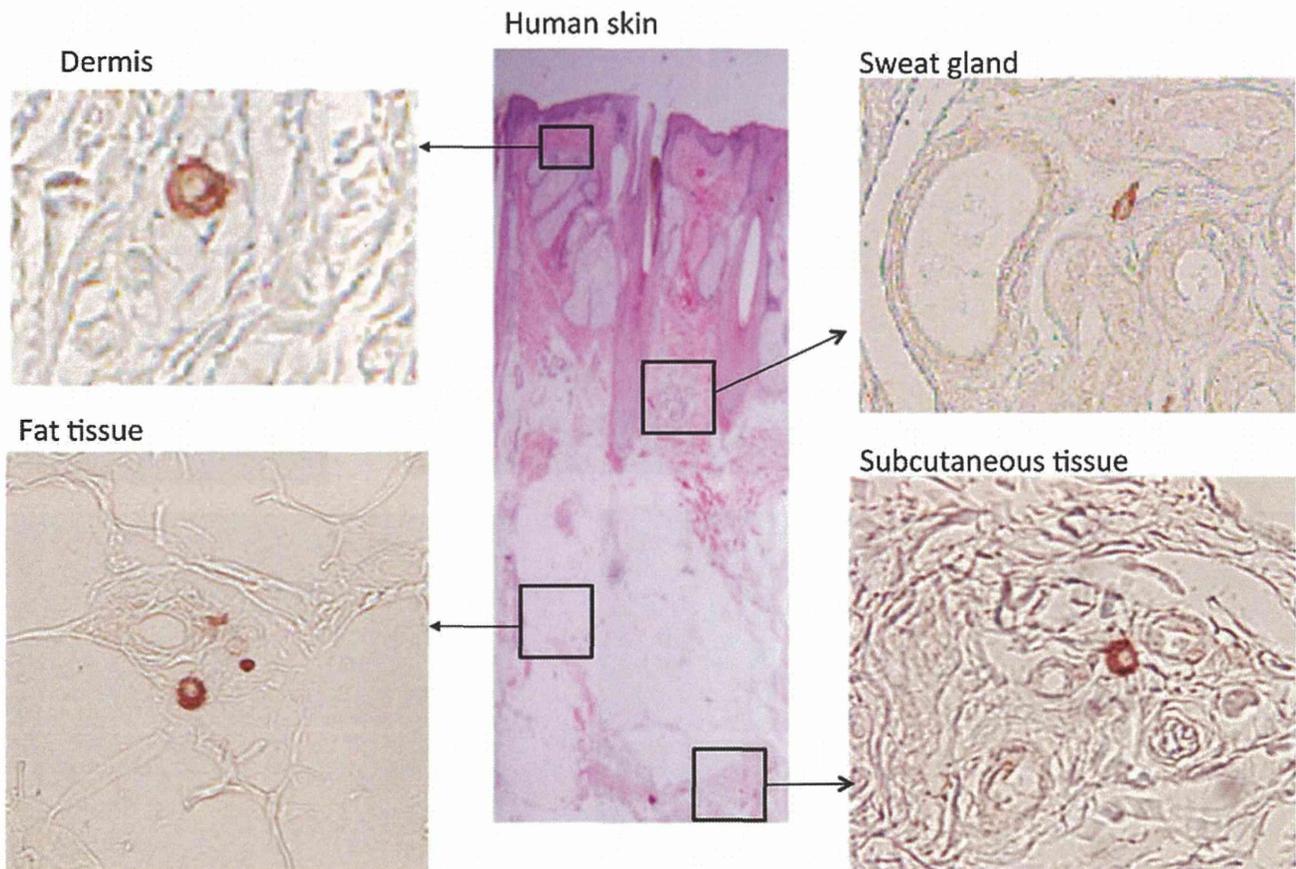
Figure 5 Muse cells sparsely locate in the adult human skin connective tissue.
Muse cells labeled by SSEA-3 are sparsely detected in the connective tissue of the dermis, sweat glands, adipose tissue and hypodermis. (pictures reproduced from *Proc Natl Acad Sci USA* 2011; **108**: 9875–80).[13]

If so, what kind of systems do Muse cells maintain *in vivo*? Because the bone marrow is directly connected to the peripheral bloodstream, the marrow is thought to be the hub of the Muse cell system in the body where the Muse cells are reserved and maintained in the normal state. Muse cells might be mobilized very slowly to the peripheral blood from the bone marrow in the normal state and distributed to the connective tissue of peripheral organs, including mesenchymal tissues such as adipose tissue and the dermis.

Comparison of the gene expression levels of Muse cells from bone marrow, adipose tissue, and dermis reveals that bone marrow-Muse express higher levels of genes related to ectodermal and endodermal-lineages than adipose- and dermal-Muse cells, suggesting that bone marrow-Muse cells have higher pluripotency than the other two types of Muse cells.[16] Bone marrow Muse cells are also unique in that they are highly dormant and more stress tolerant than adipose- and dermal-Muse cells.

Assuming that Muse cells build up a system *in vivo*, what is the function of Muse cells in the connective tissue of each organ? Because Muse cells are pluripotent, they can repair tissues that span endodermal-, mesodermal- and ectodermal-lineages. Connective tissue is very common and generally distributed in each organ, so that Muse cells residing in connective tissue can easily access small areas of damage that occur every day and replenish cells that are compatible with the tissue in the nearest parenchyma. It is conceivable that each organ is exposed to daily stress and minute damage that may cause cell degeneration. Our bodies are able to maintain function because of 'regenerative homeostasis' due to these small maintenance systems. The true mechanisms of regenerative homeostasis are still not clear, but the Muse cell system may have an important function. Further studies are needed to elucidate how Muse cells relate directly to regenerative homeostasis.

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Mesenchymal Stem Cells and Their Subpopulation, Pluripotent Muse Cells, in Basic Research and Regenerative Medicine

YASUMASA KURODA[1]* AND MARI DEZAWA[1,2]

[1]Department of Anatomy and Anthropology, Tohoku University Graduate School of Medicine, Sendai, Japan

[2]Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine, Sendai, Japan

ABSTRACT

Mesenchymal stem cells (MSCs) have gained a great deal of attention for regenerative medicine because they can be obtained from easy accessible mesenchymal tissues, such as bone marrow, adipose tissue, and the umbilical cord, and have trophic and immunosuppressive effects to protect tissues. The most outstanding property of MSCs is their potential for differentiation into cells of all three germ layers. MSCs belong to the mesodermal lineage, but they are known to cross boundaries from mesodermal to ectodermal and endodermal lineages, and differentiate into a variety of cell types both in vitro and *in vivo*. Such behavior is exceptional for tissue stem cells. As observed with hematopoietic and neural stem cells, tissue stem cells usually generate cells that belong to the tissue in which they reside, and do not show triploblastic differentiation. However, the scientific basis for the broad multipotent differentiation of MSCs still remains an enigma. This review summarizes the properties of MSCs from representative mesenchymal tissues, including bone marrow, adipose tissue, and the umbilical cord, to demonstrate their similarities and differences. Finally, we introduce a novel type of pluripotent stem cell, multilineage-differentiating stress-enduring (Muse) cells, a small subpopulation of MSCs, which can explain the broad spectrum of differentiation ability in MSCs. Anat Rec, 00:000–000, 2013. © 2013 Wiley Periodicals, Inc.

Key words: tissue engineering; stem cell; regenerative

INTRODUCTION

Our body is comprised of various kinds of tissues and cells, and all of their origins converge on a single cell, namely the zygote. The zygote undergoes cell division and develops into the blastocyst that contains the inner cell mass. Cells in the inner cell mass commit to any of the three germ layers, ectoderm (which mainly develops into the epidermis and nervous system), endoderm (including the liver, pancreas, and lung) or mesoderm (the remaining tissues including blood, bone, and bone marrow, adipose tissue, and connective tissues). Mesenchymal stem cells (MSCs), the topic of this review, belong to the mesodermal lineage and are tissue stem cells that reside in various kinds of mesenchymal tissues

i.e., bone marrow, adipose tissue, the umbilical cord, dermis, dental pulp, and synovia. In this review, we focus on three representative MSC types derived from bone marrow, adipose tissue and the umbilical cord, which have been intensely studied both in basic research and clinical applications for the past decade.

MSCs are known to have pleiotropic actions. They exert trophic and anti-inflammatory effects on damaged tissues by producing a variety of factors and cytokines that act to protect tissues, but also modulate immunological reactions, which is the basis for their application in the treatment of graft-versus-host disease (GVHD) (Fang et al., 2007). Another important property of MSCs is the broad spectrum of differentiation beyond the boundaries between germ layer lineages (Prockop, 1997; Dezawa et al., 2001; Oyagi et al., 2006). Generally, tissue stem cells generate the cell types of the tissue in which they reside, and the range of their differentiation capabilities is limited. Hematopoietic stem cells generate blood cells, and neural stem cells generate neural lineage cells (Reynolds and Weiss, 1992; Osawa et al., 1996). In this context, the differentiation potential of MSCs is exceptional and has lead to a debate over the past decade concerning whether MSCs are pluripotent or not. Pittenger et al. (1999) showed that bone marrow derived-MSCs (BM-MSCs) are multipotent and able to differentiate into some kinds of mesodermal lineage cells such as osteoblastic, chondrocytic and adipocytic cells by treatment with certain cytokines and reagents. Because MSCs belong to the mesodermal lineage, this phenomenon appears to be reasonable. However, further studies revealed that, using cytokine induction and/or gene introduction, MSCs differentiate in vitro into cells of other lineages including endodermal (hepatocytes and insulin-producing cells) and ectodermal lineages (neuronal, peripheral glial and epidermal cells) (Prockop, 1997; Dezawa et al., 2001, 2004; Oyagi et al., 2006; Wu et al., 2006; Karnieli et al., 2007). In addition, a rare subpopulation of MSCs has been reported to spontaneously differentiate into mesodermal (cardiomyocytes), ectodermal (keratinocytes) and endodermal cells (hepatocytes) in vivo according to the local microenvironment in which they integrate to ultimately contribute to tissue repair (Terai et al., 2002; Orlic et al., 2003; Tamai et al., 2011). However, the important point is that the ratios of these differentiations are generally low and, therefore, putative pluripotent cells, if they exist among MSCs, are considered to correspond to a small number of MSCs.

Besides our basic understating of MSCs, they have gained a great deal of attention in the expectation of their contribution to regenerative medicine because of several beneficial aspects. MSCs can be collected from easily accessible tissues, such as bone marrow and the umbilical cord, and can be stably expanded to a large number of cells within a reasonable time period. Unlike embryonic stem (ES) or fetal stem cells, the collection of MSCs does not involve the use of fertilized eggs or aborted fetuses, respectively, which avoids ethical concerns. Most importantly, MSCs do not have a tumorigenic proliferative activity and, thus, they are considered one of the most suitable stem cell types for cell based-therapy. In fact, some clinical trials have been diligently conducted using MSCs (Horwitz et al., 1999; Terai et al., 2003; Gordon et al., 2006).

Three mesenchymal tissues, bone marrow, adipose tissue, and the umbilical cord, have their own distinct anatomical structures. Bone marrow is located inside of bones and consists of blood vessels, specialized vessels called sinusoids, and a sponge-like network of hemopoietic cells (Fig. 1A), and has two functional compartments, namely hematopoietic and nonhematopoietic compartments. Mesenchymal cells in the bone marrow (BM-MSCs) are located in the nonhematopoietic compartment. The umbilical cord is filled with Wharton's jelly, loose connective tissue, and has three blood vessels; two umbilical arteries and one umbilical vein. Umbilical cord-derived MSCs (UC-MSCs) are mainly collected from Wharton's jelly, but can be also collected from tissues around the blood vessels as well as umbilical cord blood (Fig. 1B). Adipose tissue is comprised mainly of adipocytes, blood capillaries and small volume of connective tissues (Fig. 1C), and adipose-derived MSCs (AD-MSCs) are considered to be localized in the connective tissue around blood capillaries.

While the anatomical structures are completely distinct among these tissues, each MSC type shares common characteristics. For example, all BM-MSCs, UC-MSCs, and AD-MSCs show similar morphology (Fig. 1D–F). In addition, they are known to provide trophic immunosuppressive and anti-inflammatory effects, and a broad spectrum of differentiation, which spans from mesodermal- to ectodermal- and endodermal-lineage cells. Despite these similarities, differences among BM-MSCs, UC-MSCs and AD-MSCs exist in their cell surface marker expression, responses to cytokines and reagents, and differentiation propensity (Pittenger et al., 1999; Zuk et al., 2002; Gimble et al., 2007, Troyer and Weiss, 2008).

In this review, we focus on the similarities and differences of these representative MSC types, and introduce the recently found pluripotent stem cell type, multilineage-differentiating stress-enduring (Muse) cells, which correspond to one to several percent of MSCs, and may explain the triploblastic differentiation of MSCs (Kuroda et al., 2010; Wakao et al., 2011). Finally, the perspective of MSCs and Muse cells for regenerative medicine is discussed.

Background of MSCs

BM-MSCs collected from bone marrow have been studied for a long time. Till and McCulloch first reported cloning of bone marrow cells in 1961 (Till and McCulloch, 1961). In the 1970s, Friedenstein et al. referred to cells that are adherent, clonogenic, non-phagocytic and fibroblastic as colony-forming unit-fibroblasts (CFU-Fs), and analyzed them ex vivo. The studies conducted by Friedenstein revealed that the cells were able to differentiate into mesodermal cells including osteocytes, chondrocytes and adipocytes (Friedenstein et al., 1970; Friedenstein et al., 1974; Friedenstein et al., 1976). In subsequent studies, these bone marrow cells were given many different names that caused confusion in this area. In 1987, Owen et al. named these cells "marrow stromal stem cells", and Caplan et al. used the term "mesenchymal stem cells" in 1991, which became the generally used term in later studies (Owen et al., 1987; Caplan et al., 1991).

The general method to collect BM-MSCs from bone marrow is simple. Either bone marrow aspirates or mononucleated cells isolated by a Ficoll gradient are suspended in culture medium containing 10% bovine serum
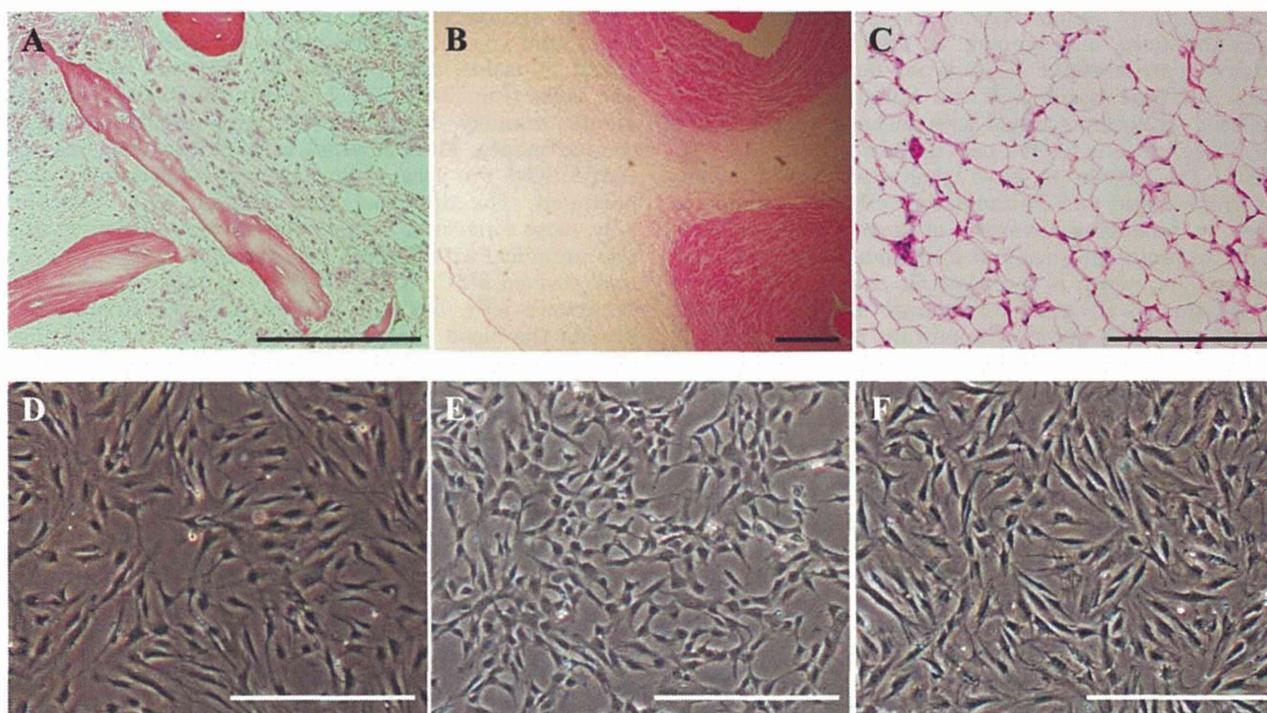
Fig. 1. Characteristic of human bone marrow, adipose tissue, and umbilical cord. (**A–C**) HE staining of human (A) bone marrow (B) umbilical cord and (C) adipose tissue. (**D–F**) Cell morphology of mesenchymal cells isolated from human (D) bone marrow (E) umbilical cord and (F) adipose tissue. Scale bar = 500 μm.

and then cultured adherently on plastic dishes. Hematopoietic stem cells, which are normally present in bone marrow aspirates, may contaminate the primary cultured cells. However, repeated washing and passaging usually eliminates hematopoietic stem cells that will not remain in adherent culture for a long period (Pittenger et al., 1999).

UC-MSCs have great advantages over other MSC types because they can be easily collected from donors during childbirth without harm, unlike bone marrow aspiration and liposuction. UC-MSCs were first isolated from umbilical cord blood (Ye et al., 1994), and in 2002, Hoerstrup et al. successfully established MSCs from Wharton's jelly (Hoerstrup et al., 2002). Since then, MSCs isolated from Wharton's jelly have been generally studied as UC-MSCs. Unlike MSCs from other tissues, UC-MSCs can be isolated from samples after 48 hr from collection at a high efficiency. Thus, the umbilical cord is very useful as a cell source for collection of MSCs. When collecting MSCs from umbilical cord blood, cells that adhere to a plastic dish are considered as UC-MSCs, as is the case with BM-MSCs. To isolate UC-MSCs from Wharton's jelly, minced tissues are placed on a plastic dish, and the cells that migrate from the tissues are considered as UC-MSCs (Troyer and Weiss, 2008).

Even though research has only begun recently, adipose tissue is the most notable tissue as a source of MSCs because adipose tissue is obtained easily. In the United States, liposuction surgery is performed more than 400,000 times per year, and adipose tissue is easily collectable from the 100 ml to 3 l per treatment (Katz et al., 1999), which is usually discarded. Collecting MSCs from these tissues was first reported by Zuk et al.

They isolated fibroblast-like cells by treating liposuction aspirates or finely minced adipose tissue with collagenase, and then the isolated cells were shown to differentiate into adipocytes, osteocytes, chondrocytes, and myocytes *in vivo* (Zuk et al., 2001). After this report, these cells were named "adipose-derived stem cells" (Zuk et al., 2002), and the widely used method to collect AD-MSCs is based on the original method by Zuk et al.

Surface Marker Expression

Essentially, the formation of MSCs, collection efficiency and ratio of colony formation do not differ largely among BM-MSCs, UC-MSCs and AD-MSCs (Izadpanah et al., 2006; Kern et al., 2006). In terms of surface markers, MSCs from each tissue commonly express CD29, CD73, CD90 and CD105, and are negative for CD45 and CD56 (Table 1) (Pittenger et al., 1999; Zuk et al., 2002; Gimble et al., 2007, Troyer and Weiss, 2008). However, there are some differences among them. Whereas both BM-MSCs and UC-MSCs are positive for CD106, AD-MSCs are negative for this marker (Zuk et al., 2002; Kern et al., 2006). In contrast, AD-MSCs express CD34, whereas both BM-MSC and UC-MSCs are negative (Pittenger et al., 1999; Gimble et al., 2007; Troyer et al., 2008). The differences in surface marker expression can be explained by several factors. For example, slight differences in the collection method, quality of serum, methods of maintaining the cultured cells, and donors may cause differences in the composition and characters of MSC populations. In addition, the species may be a factor that causes differences in surface marker expression. In fact, human and rat BM-MSCs

TABLE 1. Comparison of the protein expression profile in human mesenchymal stem cells derived from bone marrow, umbilical cord, and adipose tissue.

		BM-MSCs	UC-MSCs	AD-MSCs
Common	CD10	O	O	O
	CD13	O	O	O
	CD29	O	O	O
	CD44	O	O	O
	CD49	O	O	O
	CD73	O	O	O
	CD90	O	O	O
	CD105	O	O	O
	MHC Class I	O	O	O
	SSEA-4	O	O	O
	CD14	×	×	×
	CD31	×	×	×
	CD45	×	×	×
	CD56	×	×	×
	CD144	×	×	×
	MHC Class II	×	×	×
Uncommon	CD9			O
	CD22	O		
	CD34			O
	CD51	O		
	CD54	O		O
	CD55			O
	CD59			O
	CD64a	O		
	CD71	O		O
	CD106	O	O	
	CD133	O		
	CD140b	O		
	CD146	O		O
	CD166	O		O
	CD271	O		
	CD340	O		
	CD349	O		
	ESG1		O	
	GD2 Syntase	O	O	
	LIFr		O	
	SCF		O	
	SSEA-1	O		
	Stro-1	O		
	Telomerase		O	
	Tra-1-60		O	

are negative for CD34, whereas mouse MSCs are positive. Human UC-MSCs express CD49e and CD105 at a high level in early passages, but this expression level decreases in later passages (Weiss et al., 2006). Therefore, the surface expression pattern of MSCs differs according to the species, origin, and various factors involving their collection, maintenance, and culture period. Furthermore, the expression pattern of markers analyzed *in vitro* does not always reflect the characteristics *in vivo*.

Trophic Effect

BM-MSCs, UC-MSCs, and AD-MSCs have been reported to show a profound effect on wound healing. One of the mechanisms of this effect is the secretion of various cytokines and trophic factors such as fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), brain derived-neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF), stromal cell derived factor (SDF)-1, interleukin (IL)-6, IL-8, and IL-11. However, the ability to secrete these factors is not equal among MSC types. For example, in contrast to BM-MSCs, UC-MSCs, and AD-MSCs can produce granulocyte-macrophage colony stimulating factor and granulocyte colony stimulating factor. In addition, the expression level of some factors, such as HGF, IL-6 and IL-8, in BM-MSCs is less than that in other MSC types (Crigler et al., 2006; Yoshihara et al., 2007; Fan et al., 2011). Several groups have examined whether undifferentiated or "naive" MSCs can promote wound healing. Transplantation of autologous BM-MSCs promotes wound healing in skin-incised mice (Falanga, 2007). MSCs also show a therapeutic effect in the functional recovery of central nervous system (CNS) damage such as cerebral infarction and spinal cord injury by secretion of neuroprotective tropic factors (Yoshihara et al., 2007; Kawabori et al., 2012). AD-MSCs combined with an atelocollagen matrix enhance the healing of rat skin damage induced by mitomycin C (Nambu et al., 2007). Moreover, AD-MSCs avoid skin flap necrosis caused by inadequate blood supply and improve the cell viability (Lu et al., 2008), as well as promote periodontal tissue regeneration (Tobita et al., 2008). Similar effects have been observed by the transplantation of AD-MSCs into a rat model of hindlimb ischemia (Miranville et al., 2004; Cao et al., 2005), a mouse model of myocardial infarction (Fraser et al., 2006), and skin ulcers in a diabetic mouse model (Nambu et al., 2009).

UC-MSCs are also reported to have a therapeutic trophic effect. For example, UC-MSCs derived from umbilical cord blood or Wharton's jelly are able to promote cutaneous wound healing (Luo et al., 2010; Zhang et al., 2012). Transplantation of umbilical cord blood cells that include UC-MSCs can improve neurological and motor deficits resulting from hypoxic brain ischemia (Rosenkranz et al., 2013). All of the above effects are attributable mainly to the trophic effects of transplanted MSCs.

Anti-Inflammatory and Immunosuppressive Effects

Recent reports have revealed that each MSC type shows not only promotion of wound healing but also anti-inflammatory effects by cytokine secretion. For example, BM-MSCs decrease inflammation by secretion of soluble tumor necrosis factor receptor 1 (sTNFR1) when they were transplanted into lipopolysaccharide-induced endotoxemic model rats (Yagi et al., 2010). Similarly, transplanted BM-MSCs suppress inflammatory reactions in ischemic cardiac muscle by the expression of TNF-α stimulated gene/protein 6 (TSG-6) (Wisniewski et al., 2004). Moreover, BM-MSCs can rescue damaged cells from apoptosis by secretion of stanniocalcin-1 (Block et al., 2009).

MSCs are also known to have immunosuppressive effects by secretion of anti-inflammatory cytokines such as IL-10. Proliferation of T cells in mixed lymphocyte culture has been shown to be suppressed by co-culture

TABLE 2. Differentiation potential of human mesenchymal stem cells derived from bone marrow, umbilical cord, and adipose tissue.

		BM-MSCs	UC-MSCs	AD-MSCs
Mesodermal cells	Osteoblasts	O	O	O
	Adipocytes	O	O	O
	Chondrocytes	O	O	O
	Endothelial cells	O	O	O
	Skeletal muscle cells	O	O	O
	Cardiac muscle cells	O	O	O
	Smooth muscle cells	O	O	O
	Epithelial cells	O	O	O
	Tenocytes	O		
Ectodermal cells	Neuronal cells	O	O	O
	Peripheral glia cells	O	O	O
Endodermal cells	Pancreatic cells	O	O	O
	Hepatocytes	O		O

with MSCs, even though they were not HLA- matched (Dao et al., 2011).

Taken together, the trophic, anti-inflammatory, and immunosuppressive effects by cytokine secretion give MSCs their remarkable indirect healing effects.

Differentiation Ability

In addition to cytokine effects, all BM-MSCs, UC-MSCs, and AD-MSCs can differentiate into various types of cells, and there are no obvious differences among their differentiation potentials (Izadpanah et al., 2006; Kern et al., 2006) (Table 2). Well-known differentiated cell types from the above types of MSCs are osteocytes, adipocytes and chondrocytes. Adipocytes are differentiated by treatment with 1-methyl-3-isobutylxanthine, dexamethasone, insulin and indomethacin, osteoblasts by dexamethasone, b-glycerol phosphate and ascorbate, and chondrocytes by TGF-β stimulation plus mechanical stimulation (Prockop, 1997; Kuznetsov et al., 1997; Bruder et al., 1997; Pittenger et al., 1997; Mackay et al., 1998; Izadpanah et al., 2006; Kern et al., 2006). Differentiation of these three cell types of the mesodermal lineage demonstrates the multipotency of MSCs. In addition, BM-MSCs can differentiate into other mesodermal cell types such as skeletal muscle cells by treatment with a combination of three kinds of cytokines followed by gene transfection with Notch intracellular domain (NICD) (Dezawa et al., 2005), smooth muscle cells by TGF-β treatment (Yan et al., 2011), endothelial cells by stimulation with VEGF, and cardiac muscle cells by 5-azacytidine treatment (Makino et al., 1999). BM-MSCs can also be induced into other germ layer cell types such as those of the ectodermal lineage. For example, neuronal cells are induced by gene transduction of NICD, and then treatment with three kinds of cytokines (Dezawa et al., 2004). Peripheral glial cells are induced by treatment with β-mercaptoethanol followed by retinoic acid, forskolin, basic (b) FGF, platelet-derived growth factor and heregulin (Dezawa et al., 2001). BM-MSCs can also differentiate into endodermal lineage cells as observed by hepatocyte differentiation using HGF (Oyagi et al., 2006) and pancreatic cell differentiation using platelet lysate, retinoic acid, activin, glucagon-like peptide I, and cytokines (Zanini et al., 2011).

AD-MSCs can differentiate into skeletal muscle cells by treatment with dexamethasone and hydrocortisone (Zuk et al., 2001), as well as smooth muscle cells (Rodriguez et al., 2006) and endothelial cells (Cao et al., 2005) using other combinations of cytokines. In addition, AD-MSCs can differentiate into epithelial cells using all-trans retinoic acid (Brzoska et al., 2005), neuronal cells using valproic acid, butylated hydroxyanisole, insulin and hydrocortisone (Safford et al., 2002), peripheral glial cells by factors basically identical to a cocktail reported by Dezawa et al. (Dezawa et al., 2001; Xu et al., 2008), hepatocytes using HGF, OSM and DMSO (Seo et al., 2005), and pancreatic cells using glucose, nicotinamide, activin, exendin and HGF (Timper et al., 2006).

UC-MSCs can also differentiate into neuronal cells and peripheral glial cells (Zhang et al., 2009) by cytokine treatment (Liang et al., 2012), hepatocytes using HGF and FGF-4 (Zhang et al., 2009), and pancreatic cells by transient transfection of *Pdx1* (Fedyunina et al., 2011).

Thus, the potential for triploblastic differentiation exists in BM-MSCs, UC-MSCs, and AD-MSCs.

Accessibility of MSCs

Gimble et al. suggested that stem cells for regenerative medicinal applications should ideally meet the following criteria (Gimble, 2003, 2007):

1. Can be found in abundant quantities (millions to billions of cells)
2. Can be harvested by a minimally invasive procedure
3. Can be differentiated along multiple cell lineage pathways in a regulated and reproducible manner
4. Can be safely and effectively transplanted into either an autologous or allogeneic host
5. Can be manufactured in accordance with current Good Manufacturing Practice guidelines

Previous studies indicate that MSCs meet these criteria. In the case of BM-MSCs, 10 ml of bone marrow aspirate contains approximately 7×10^5 cells expressing CD105 that is a general marker of MSCs including BM-MSCs (this quality corresponds to criterion 1). The collection method meets the safety requirements and bone marrow aspirates can be collected with comparative ease, so that BM-MSCs are easily accessible cells with abundant quantities within a reasonable time period (corresponding to criterion 2). BM-MSCs can differentiate into cells of all three germ layers as described above (corresponds to criterion 3), and they can be safely and effectively transplanted into autologous/allogeneic hosts (corresponds to criterion 4) (Wang et al., 2012).

For UC-MSCs, 80 ml of cord blood contains approximately 2.1×10^2 MSCs (corresponds to criterion 1), and the umbilical cord can be obtained during childbirth without harm, so that it is the most easily accessible source to obtain MSCs (corresponds to criterion 1).

AD-MSCs are yielded at only $\sim 5 \times 10^3$ cells from 1 g of adipose tissue (corresponds to criterion 1), but it is possible to obtain a sufficient amount of AD-MSCs in a single liposuction procedure that obtains 100 ml to 3 l of adipose tissue. Adipose tissue is easily collected by cosmetic surgery and liposuction, and these surgeries are performed more than 400,000 per year.

Both UC-MSCs and AD-MSCs can differentiate into various kinds of cells, as observed with BM-MSCs (corresponds to criterion 3), and can be transplanted into autologous/allogeneic hosts (corresponds to criterion 4).

into tissue-specific cells. If such cells are identified, efficient regenerative treatment can be expected.

Muse Cells, Intrinsic Pluripotent Stem Cells that Reside Among MSCs

Besides trophic, immunosuppressive and anti-inflammatory effects, the scientific basis for the broad spectrum of differentiation by MSCs, which crosses the oligolineage boundaries between mesoderm and ectoderm or endoderm, is not yet clarified.

Several reports have claimed to discover pluripotent stem cells among bone marrow cells. Jiang et al. reported that MSCs derived from adult bone marrow, which they named multipotent adult progenitor cells (MAPCs), are pluripotent stem cells that show triploblastic differentiation (Jiang et al., 2002). However, the isolation of MAPCs has not been reproduced by other independent laboratories and, thus, their existence is now considered doubtful. Kucia et al. reported that they found pluripotent stem cells expressing some ES cell markers, stage specific embryonic antigen (SSEA)-1, Oct4, Nanog and Rex-1, in both adult mouse bone marrow and human cord blood, which they named very small embryonic-like (VSEL) stem cells (Kucia et al., 2006; Wojakowski et al., 2011). However, a recent report by Danova-Alt et al. demonstrated that the transcriptional profile of VSEL cells derived from human cord blood is clearly distinct from those of well-defined populations of pluripotent and adult stem cells, and mostly show an aneuploid karyotype, which questions the existence of pluripotent stem cells in umbilical cord blood (Danova-Alt et al., 2012). In both reports, there was no description of objective markers to isolate such cells and the methods that identified the cells were not specific enough to independently reproduce their data.

Even though the existence of MAPCs and VSEL stem cells is equivocal, these reports instigated the exploration of putative pluripotent stem cells that reside among MSCs. Many attempts have been made to identify pluripotent stem cells, but there are major problems to be overcome in MSC research.

First, the two main properties of pluripotent stem cells, namely triploblastic differentiation and self-renewal, need to be shown at a single cell level. Because MSCs are generally collected as adherent cells from bone marrow, adipose tissue, or the umbilical cord, they are crude heterogenous cell populations comprised of several kinds of cells. There are several reports showing the differentiation of crude bulk MSC populations into ectodermal, endodermal and mesodermal lineage cells. Because the cells were in crude populations, triploblastic differentiation from a single kind of cell could not be proven in the strict sense. Thus, from the viewpoint of basic science, there is a strong need to prove triploblastic differentiation and self-renewal at a single cell level.

Secondly, several reports have demonstrated that not all MSCs have a wide differentiation ability. For example, 10 to 25% of BM-MSCs can differentiate into alkaline phosphatase (ALP)-positive osteocytes (Birmingham et al., 2012), and differentiation across germ layers without gene transfer is much lower. Approximately 3% of MSCs differentiate into microtubule-associated protein 2 (MAP2)-positive neurons by co-culture with neurons (Hokari et al., 2008), and ~5% of MSCs differentiate

into insulin-positive β cells by stimulation with cytokines such as bFGF and epidermal growth factor (EGF) under a high glucose condition (Gabr et al., 2013). These results suggest that not all MSCs participate in triploblastic differentiation and, therefore, only a small subpopulation of MSCs are suggested to possess pluripotency.

Another possibility is that MSC populations contain several kinds of unipotent/bipotent stem cells each responsible for ectodermal, mesodermal, and endodermal lineage differentiation. In this case, individual MSCs are not pluripotent, but whole MSC populations resemble a pluripotent population of cells.

While there are many debates regarding the pluripotency of MSCs, Kuroda et al. recently demonstrated that BM-MSCs and another type of mesenchymal cell, dermal fibroblasts, include pluripotent stem cells that can self-renew and individually differentiate into cells representative of all three germ layers. These cells were found by their stress resistance, and were named "multilineage-differentiating stress-enduring (Muse)" cells (Kuroda et al., 2010).

Unique Properties of Muse Cells

Muse cells were recently identified in adult human mesenchymal tissues such as bone marrow and the dermis, and also among cultured mesenchymal cells such as BM-MSCs and dermal fibroblasts (Kuroda et al., 2010; Wakao et al., 2011). Muse cells are legitimate mesenchymal cells, which exhibit a morphology identical to those of typical mesenchymal cells such as fibroblasts, and express common mesenchymal markers CD105, CD90, and CD29. However, Muse cells are unique because they also show pluripotent stem cell properties such as the expression of pluripotency markers, self-renewal and triploblastic differentiation, indicating that Muse cells are both pluripotent and mesenchymal cell-like. These properties can be demonstrated by their marker expression. Muse cells express both pluripotency and mesenchymal stem cell markers. They can be isolated from tissues or cultured cells as cells that are double positive for SSEA-3, a well-known marker of the undifferentiated state of human ES cells, and CD105, a mesenchymal stem cell marker (Kuroda et al., 2010) (Fig. 2).

Muse cells as Nontumorigenic Pluripotent Stem Cells

When Muse are isolated and maintained as a single cell-suspension culture, which is often used for tissue stem cell culture, they proliferate and form cell clusters that are very similar to ES cell-derived embryoid bodies. The clusters express pluripotency markers, Nanog, Oct3/4 and Sox2, and are positive for ALP. Importantly, a single cell-derived cluster differentiates into endodermal (α-fetoprotein and cytokeratin 7), ectodermal (neurofilament) and mesodermal (smooth muscle actin, and desmin) marker-positive cells when cultured on gelatin, demonstrating that the original single cell possesses a triploblastic differentiation ability. Furthermore, when single Muse cell-derived clusters are expanded, SSEA-3/CD105 double positive cells can be collected again and have been shown to differentiate into endodermal, ectodermal and mesodermal cells from single cells. This cycle can be repeated, demonstrating that Muse cells
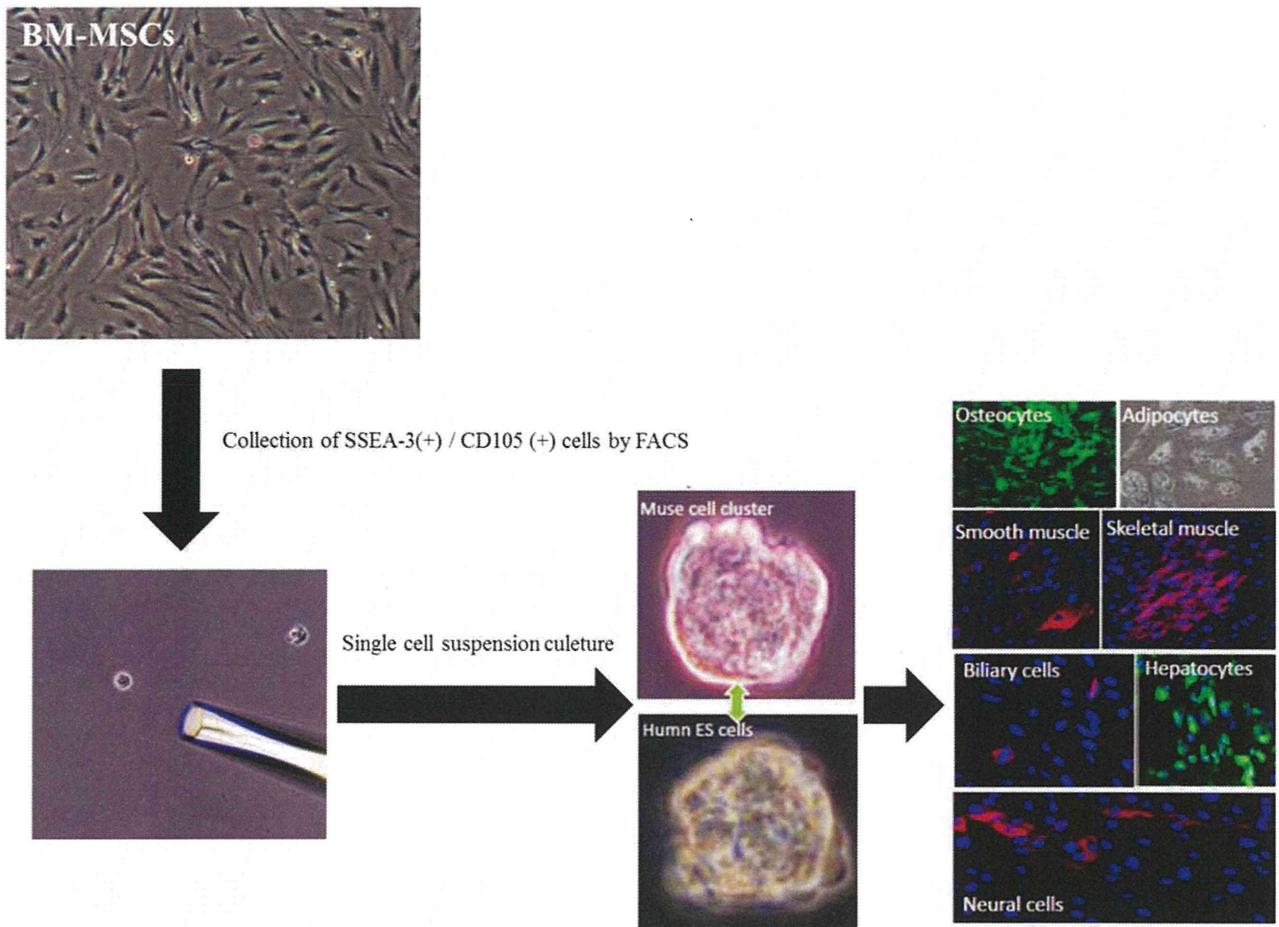
Fig. 2. Schema for isolation of Muse cells. Muse cells can be isolated from mesenchymal cells such as BM-MSCs and dermal fibroblasts as cells that are double positive for SSEA-3 and CD105. They can form clusters that are very similar to ES cell-derived embryoid bodies during maintained as a single cell-suspension culture, and they can differentiate into cells representative of three germ layers. Pictures adapted from Kuroda et al. (2010) with permission from The National Academy of Science, and adapted from Wakao et al. (2011) with permission from The National Academy of Science.

have a self-renewal ability (Kuroda et al., 2010). Thus, Muse cells are considered to correspond to putative pluripotent stem cells among BM-MSCs.

One of the great advantages of Muse cells is that they have no tumorigenicity when transplanted *in vivo*. ES and iPS cells form teratomas within 8 to 12 weeks when transplanted into the testes of immunodeficient mice, whereas Muse cell-transplanted immunodeficient mouse testes do not develop teratomas even after 6 months (Kuroda et al., 2010) (Fig. 3). Molecular analyses revealed that Muse cells have low levels of telomerase activity and gene expression related to cell-cycle progression compared with those in ES and iPS cells, and such activity and gene expression levels are at the same level as those in somatic cells such as fibroblasts (Wakao et al., 2011). Unlike popular pluripotent stem cells, namely ES and iPS cells, Muse cells are innate adult stem cells in our bodies, which is consistent with the fact that they are nontumorigenic. In addition, Muse cells have already been applied to leukemia patients as a subpopulation (~0.03%) of bone marrow mononucleated cells in bone marrow transplantation (Kuroda et al., 2010). Muse cells are pluripotent, but are non-tumorigenic, which are of great practical use for clinical application.

Stem cells are usually identified and isolated according to their surface antigens. However, Muse cells were initially found as stress-tolerant cells. Tissue stem cells are generally in a dormant state, but they are activated to repair tissues upon exposure to stress. For example, neural stem cells are usually dormant but are activated by stress such as ischemia, and enter into the cell cycle to generate neuronal cells (Ye et al., 2007). Based on this fact, pluripotent stem cells speculated to reside among BM-MSCs at a low frequency are expected to remain under a strong stress condition. In fact, when BM-MSCs are incubated with trypsin for 16 hr, the majority of cells do not survive and only a small number of cells remain alive. When these surviving cells are cultured in a single cell-suspension culture, they form clusters that are positive for pluripotency markers and are able to generate cells of all three germ layers. Because Muse cells are stress-enduring cells with a broad spectrum of differentiation, they were named "multilineage-differentiating stress-enduring" cells.

mouse ES cells (8 weeks)
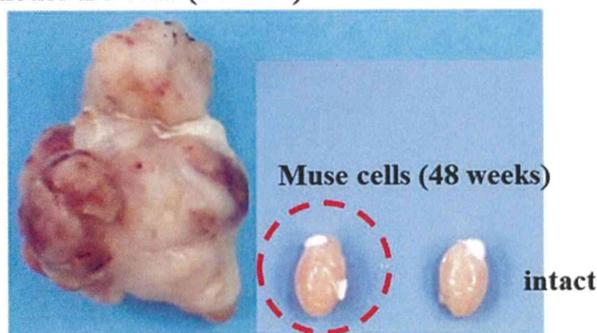
Fig. 3. Testes of immunodeficient mice injected with Muse cells. ES cells form teratomas within 8 weeks when transplanted into the testes of immunodeficient mice, whereas Muse cell-transplanted immunodeficient mouse testes do not develop teratomas even after 6 months. Pictures adapted from Kuroda et al. (2010) with permission from The National Academy of Science.

Muse cells can be directly isolated from cultured mesenchymal cells such as BM-MSCs and human dermal fibroblasts. Among human BM-MSCs, the percentage of Muse cells is around 1%, and the percentage of Muse cells among human dermal fibroblasts is several percent.

In bone marrow aspirates, as mentioned earlier, SSEA-3/CD105 double positive Muse cells are present at a ratio of 0.03% (1 in 3,000 mononucleated cells derived from bone marrow), indicating that nearly 10,000 Muse cells can be collected from 50 ml of bone marrow aspirate. Considering the doubling time of Muse cells, 1.3 days for one cell division, 10,000 freshly isolated Muse cells proliferate to more than 1 million in 8 days. Therefore, Muse cells are easily accessible pluripotent stem cells that can be obtained in large numbers for clinical applications.

Besides their broad potential for differentiation, the spontaneous differentiation rate of Muse cells *in vitro* on a gelatin-coated dish is not very high. For example, only 10 to 15% of Muse cells differentiate spontaneously into mesodermal lineage cells, and 3 to 4% of them are able to cross the boundaries between mesodermal and ectodermal or endodermal lineages to become liver or neuronal marker-positive cells. However, Muse cells show a very high differentiation rate when they are stimulated by certain combinations of cytokines and trophic factors (Fig. 4). For osteocyte or adipocyte differentiation, around 94 to 98% of Muse cells differentiate into these cells under proper induction systems. When Muse cells are treated with insulin-transferrin-selenium medium containing HGF, FGF-4 and dexamethasone, ~90% of the cells become positive for hepatocyte markers α-fetoprotein and human albumin. Similarly, approximately 90% of Muse cells differentiate into MAP-2- or
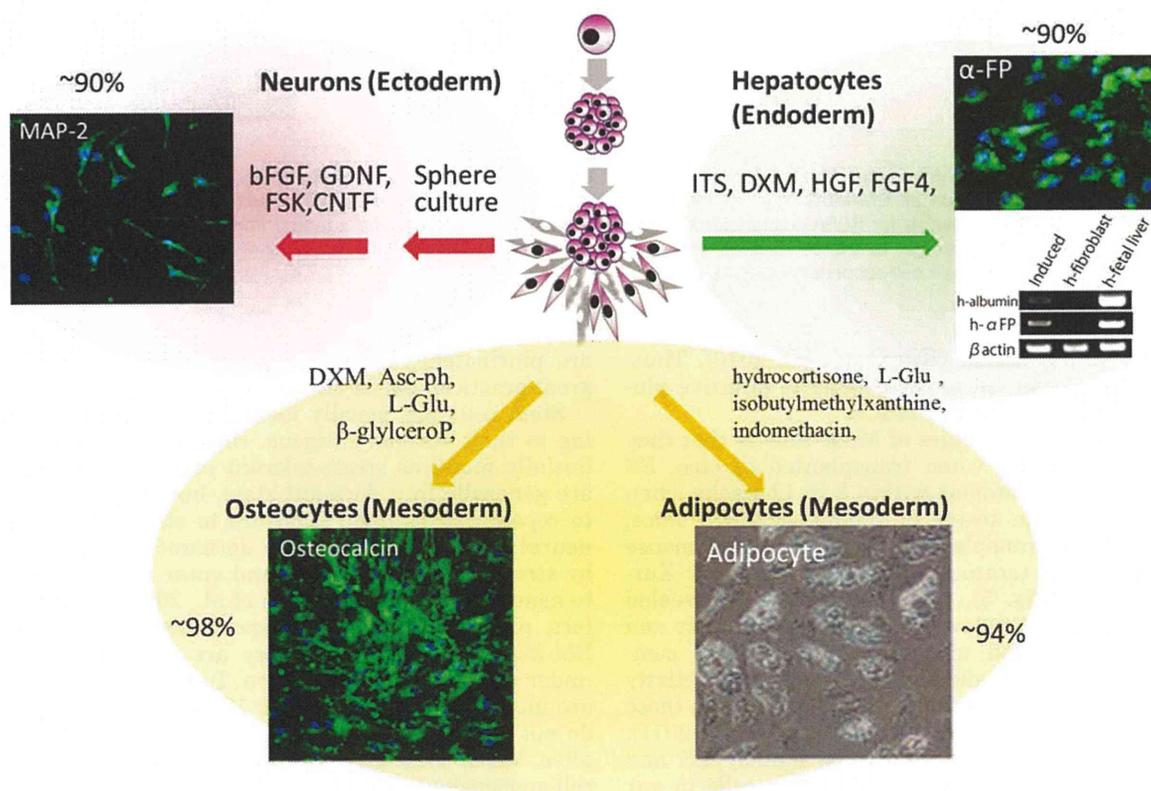
Fig. 4. Induction of Muse cells into neurons, osteocytes, adipocytes and hepatocytes. Muse cells can be directly converted into neurons, osteocytes, adipocytes and hepatocytes at very high efficiency with inductions. Pictures adapted from S. Wakao et al. (2011) with permission from The National Academy of Science.

neurofilament-positive neuronal cells when cultured in Neurobasal medium with B-27 supplement, followed by treatment with bFGF and EGF. Importantly, these inductions do not require any gene transduction, and are solely driven by cytokines and trophic factors.

Tissue Repair by Muse Cells *In Vivo*

Another attractive property of Muse cells is the ability to repair various tissues and organs that span all three germ layer lineages. When Muse cells from BM-MSCs or dermal fibroblasts are injected systemically, they recognize sites of damage, migrate, integrate into the tissue, and differentiate into cells that constitute the tissue to replace the lost cells. Such a repair effect has been shown in fulminant hepatitis (endodermal organ), muscle degeneration (mesodermal) and skin injury (ectodermal) in immunodeficient SCID mice by administration of human Muse cells. Muse cells were traced by lentivirus-GFP and human anti-golgi complex immunoreactivity, and demonstrated expression of human albumin and anti-trypsin in the integrated liver of the fulminant hepatitis model, human dystrophin in degenerated muscle, and cytokeratin 14 in injured skin. In the previous studies, a small number of Muse cells were trapped in the lung and spleen, but were rarely detected in the intact organs and tissues, suggesting that Muse cells recognize damage signals to repair the injured tissue.

Importantly, when non-Muse cells, i.e. the remaining MSC population after elimination of Muse cells, are injected systemically, they do not integrate or differentiate into functional cells in any of the above injury models. These results demonstrate that only Muse cells among MSCs can directly contribute to tissue repair, while non-Muse cells do not participate in this process.

In contrast to Muse cells, the role of non-Muse cells is complicated and multifaceted. As mentioned above, MSCs as a whole have various capabilities for cytokine secretion and immunosuppressive effects. While non-Muse cells do not have the triploblastic differentiation ability of Muse cells, they have trophic, anti-inflammatory and immunosuppressive effects that support the repair effect of Muse cells. However, such effects of non-Muse cells would be temporary because they do not remain in the tissue for a long period. Considering the differences in the actions and effects of Muse and non-Muse cells, both cell populations might be necessary for efficient tissue regeneration and, thus, there may be an optimal ratio of Muse to non-Muse cells for cell-based therapy.

Muse cells were initially identified among BM-MSCs and fibroblasts, as well as in bone marrow, suggesting that Muse cells potentially exist in other mesenchymal tissues such as adipose tissue and the umbilical cord. If so, their feasibility would be greatly extended for clinical and industrial uses.

Perspectives

Recently, MSCs have shown therapeutic effects in various kinds of tissues and organs, including promising results in clinical trials. However, the complex properties of MSCs remain unclear. Muse cells appear to explain the triploblastic differentiation and therapeutic effects of MSCs observed in various studies. Moreover, Muse cells

can be isolated from various tissues including adipose tissue and the umbilical cord, but it is unclear whether Muse cells derived from each tissue are identical or not.

MSCs derived from bone marrow, adipose tissue and the umbilical cord are useful for cell-based therapy in humans because of their low risk of tumorigenesis and easy accessibility. Furthermore, MSCs that have long been debated to have pluripotency, because they show spontaneous differentiation into mesodermal, ectodermal and endodermal cells at a very low frequency, are known to home to sites of damage and contribute to tissue repair. Recently, we found pluripotent stem cells, namely Muse cells, which comprise ~1% of cultured MSCs and 0.03% of human bone marrow mononucleated cells, and show self-renewal, triploblastic differentiation and a tissue repair effect. Importantly, Muse cells do not form tumors when transplanted, and are expected to have a greater benefit in clinical applications.

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Human Adipose Tissue Possesses a Unique Population of Pluripotent Stem Cells with Nontumorigenic and Low Telomerase Activities: Potential Implications in Regenerative Medicine

Fumitaka Ogura,[1] Shohei Wakao,[1] Yasumasa Kuroda,[2] Kenichiro Tsuchiyama,[1,3] Mozhdeh Bagheri,[1] Saleh Heneidi,[4] Gregorio Chazenbalk,[5] Setsuya Aiba,[3] and Mari Dezawa[1,2]

Abstract

In this study, we demonstrate that a small population of pluripotent stem cells, termed adipose multilineage-differentiating stress-enduring (adipose-Muse) cells, exist in adult human adipose tissue and adipose-derived mesenchymal stem cells (adipose-MSCs). They can be identified as cells positive for both MSC markers (CD105 and CD90) and human pluripotent stem cell marker SSEA-3. They intrinsically retain lineage plasticity and the ability to self-renew. They spontaneously generate cells representative of all three germ layers from a single cell and successfully differentiate into targeted cells by cytokine induction. Cells other than adipose-Muse cells exist in adipose-MSCs, however, do not exhibit these properties and are unable to cross the boundaries from mesodermal to ectodermal or endodermal lineages even under cytokine inductions. Importantly, adipose-Muse cells demonstrate low telomerase activity and transplants do not promote teratogenesis in vivo. When compared with bone marrow (BM)– and dermal-Muse cells, adipose-Muse cells have the tendency to exhibit higher expression in mesodermal lineage markers, while BM- and dermal-Muse cells were generally higher in those of ectodermal and endodermal lineages. Adipose-Muse cells distinguish themselves as both easily obtainable and versatile in their capacity for differentiation, while low telomerase activity and lack of teratoma formation make these cells a practical cell source for potential stem cell therapies. Further, they will promote the effectiveness of currently performed adipose-MSC transplantation, particularly for ectodermal and endodermal tissues where transplanted cells need to differentiate across the lineage from mesodermal to ectodermal or endodermal in order to replenish lost cells for tissue repair.

Introduction

MESENCHYMAL STEM CELLS (MSCs) derived from adipose tissue are multipotent stromal cells that can differentiate into adipocytes, chondrocytes, osteoblasts, and myoblasts in vitro and undergo differentiation in vivo [1]. MSCs are currently being applied in a number of clinical studies that target numerous diseases because of their accessibility, nontumorigenicity, and powerful trophic effects [2,3].

MSCs derived from adipose tissue (adipose-MSCs) provide an abundant and minimally invasive source of cells [4].

Adipose-MSCs can be maintained in culture for extended periods of time and can be induced in vitro to differentiate into all mesenchymal cell lineages [1,4]. Moreover, adipose-MSCs can be safely and efficiently transplanted to autologous hosts, and they are currently being used successfully for a variety of regenerative therapies [2,3].

Although not in high ratio, adipose-MSCs also have the capacity to differentiate into neuronal cells [5,6], Schwann cells [7], beta cells [8], and hepatocytes [9,10] in the presence of specific cell differentiation media. Thus adipose-MSCs may cross the oligolineage boundaries from mesodermal to ectodermal or endodermal lineages. Adipose-MSCs exhibit

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Departments of [1]Stem Cell Biology and Histology, [2]Anatomy and Anthropology, and [3]Dermatology, Tohoku University Graduate School of Medicine, Sendai, Japan.
[4]Medical College of Georgia, Georgia Regents University, Augusta, Georgia.
[5]Department of Obstetrics and Gynecology, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California.

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a wide variety of triploblastic differentiation not only in vitro, but also in vivo. At a low ratio, they may spontaneously differentiate into hepatocyte-marker-positive cells in the damaged liver [11,12], neuronal- and glial-marker-positive cells in ischemic brain injury [13,14], and cardiomyocytes in acute myocardial infarction [15] after homing to damaged sites. The low rate of adipose-MSC differentiation into ectodermal and endodermal cell lineages could be explained in part by the presence of a small population of stem cells within the adipose-MSC population that have the ability to differentiate to any type of cells, much like pluripotent stem cells. Isolation of such stem cells could have a critical impact in regenerative medicine and cell therapy.

Recently, a novel population of stem cells with pluripotent characteristics has been isolated from mesenchymal tissues, such as human skin fibroblasts and bone marrow (BM). These cells, termed multilineage-differentiating stress-enduring (Muse) cells, are of mesenchymal origin, comprise several percentages of human dermal fibroblasts and BM-MSCs, and are highly resistant to cellular stress. They are double positive for CD105, an MSC marker, and the stage-specific embryonic antigen-3 marker (SSEA-3), well known for the characterization of undifferentiated human embryonic stem (ES) cells. Muse cells can differentiate into cells of ectodermal, endodermal, and mesodermal lineages both in vitro and in vivo, and have the ability to self-renew [16]. Advantageously, Muse cells do not produce teratomas in vivo, nor do they induce immunorejection in the host upon autologous transplantation [16,17]. In addition, Muse cells are shown to home into the damaged site in vivo and spontaneously differentiate into tissue-specific cells according to the microenvironment to contribute to tissue regeneration when infused into the blood stream [16].

In the present study, we isolated Muse cells derived from human adipose tissue (adipose-Muse cells) using SSEA-3-cell-sorting techniques. Further characterization indicates that SSEA-3(+) adipose-Muse cells express general mesenchymal markers CD105, CD90, and CD29 [18,19]. They express the pluripotent stem cell markers Nanog, Oct3/4, PAR4, Sox2, and TRA-1-81 and can spontaneously differentiate into cells representative of all three germ layers from a single cell. Conversely, alternate cells in adipose-MSCs, SSEA-3 (−) adipose-MSCs (adipose-non-Muse cells), can only differentiate into mesenchymal but not into ectodermal and endodermal cell lineages even under the presence of cytokine induction. Further, adipose-Muse cells are negative for CD34 and CD146, known as classical adipose-derived stem cell (ADSC) markers [4]. While core properties of Muse cells among BM, dermis, and adipose tissues, namely, triploblastic differentiation, self-renewal, and non-tumorigenicity, are the same, BM- and dermal-Muse cells show higher expression of ectodermal and endodermal lineage markers while adipose-Muse cells show a tendency for higher expression of mesodermal markers, and preferentially differentiate into mesodermal cell lineages, suggesting that the propensity for differentiation is in accordance with the source of tissue from which Muse cells are derived.

In contrast to ES and induced pluripotent stem (iPS) cells, adipose-Muse cells have low telomerase activity and do not produce teratomas in vivo, which may alleviate one of the primary concerns with the use of pluripotent stem cells in the clinical arena. Adipose-Muse cells could be an ideal source of pluripotent stem cells with the potential to have a critical impact on regenerative medicine.

Materials and Methods

Cell source

Two different sources of adipose-MSCs were used in this study: adipose-MSCs commercially purchased from Lonza (LA-MSCs) and freshly isolated adipose-MSCs from subcutaneous adipose tissue (AT-MSCs). Cells were maintained at 37°C in Dulbecco's modified Eagle's medium high-glucose (DMEM; Gibco) containing 15% fetal bovine serum (FBS) and 0.1 mg/mL kanamycin sulfate (Gibco) in an atmosphere containing 5% CO_2.

The use of human subcutaneous adipose tissue was approved by the Ethics Committee for Animal Experiments at the Tohoku University Graduate School of Medicine. Subcutaneous adipose tissue was provided by Department of Dermatology, Tohoku University Graduate School of Medicine with informed consent. Isolation of AT-MSCs from adipose tissue was done according to the method previously reported by Estes et al. with minor modification [20]. In brief, adipose tissue was minced into small pieces and incubated in equal volume of phosphate-buffered saline (PBS, without calcium chloride and magnesium chloride) containing 1 mg/mL Collagenase Type I (Worthington Biochemical) and 1% bovine serum albumin (Nacalai) at 37°C for 1 h with mild shaking. Digested material was then centrifuged at 300 g for 5 min to obtain a cell pellet. The pellet was resuspended and filtered through a 100-μm nylon mesh filter (Becton Dickinson) and centrifuged again at 300 g for 5 min. The pellet was resuspended in DMEM containing 15% FBS and 0.1 mg/mL kanamycin sulfate and cultured. Cells were plated in adherent dishes at density of 3.5×10^4 cells/cm^2 and cultured after reaching ~90% confluence, exhibiting a fibroblast-like shape. The doubling time of the cells was 0.9–1.3 days/cell division.

Mouse ES cells (TT2 cells) were cultured at 37°C in DMEM containing 15% FBS, 0.1 mg/mL kanamycin sulfate, 0.1 mM MEM non-essential amino acid solution (NEAA; Gibco), 1 mM sodium pyruvate solution (SP; Gibco), 1000 U/mL leukemia inhibitory factor (Merk), and 100 μM 2β-mercaptoethanol on mitomycin-C-treated mouse embryonic fibroblast feeder cells established from 12.5-day embryos of C57BL/6 mice.

Fluorescence-activated cell sorting

Confluent adipose-MSCs (two to eight passages) were used for cell sorting. Cells were collected by trypsin-EDTA (0.25%) treatment, centrifuged, and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS containing 0.5% BSA and 2 mM EDTA) [21] at a concentration of 1×10^6 cells/100 μL. Cells were incubated in FACS buffer containing 15% human serum for 20 min. After two successive washes by FACS buffer, cells were incubated with anti-SSEA-3 antibody (1:50; Millipore) for 1 h at 4°C. Cells were then washed three times with FACS buffer, followed by FITC-conjugated anti-rat IgM (1:100; Jackson ImmunoResearch) for 1 h at 4°C. After three consecutive washes in FACS buffer, cells were sorted for SSEA-3(+) and SSEA-3(−) cells (adipose-Muse and -non-Muse cells)

by Special Order Research Products FACSAriaII (Becton Dickinson) using a low stream speed. This ensured a high level of cell survival and the highest purity of the sorted cells, via the four-way purity sorting mode, as previously described [21]. SSEA-3(+)-adipose-Muse cells (labeled with FITC) were analyzed by flow cytometry for the expression of cell surface antigens CD29 [labeled with phycoerythrin (PE)], CD90 (PE), CD105 (Pacific Blue), CD34 (PE), and CD146 (PE) (Becton Dickinson).

Single-cell suspension culture

Adipose-Muse cells were cultured as floating cells using poly-HEMA-coated dishes as previously described [21]. Each single cell was plated in an individual well on 96-well plates after limiting dilution with alpha-MEM medium containing 15% FBS. The actual number of cells deposited in each well was determined by visual inspection using a phase-contrast microscope, and empty wells or wells with more than one cell were excluded from analysis.

Spontaneous differentiation of clusters in vitro

After 7–10 days of single-cell suspension culture, single clusters of adipose-Muse cells were picked up with a glass micropipette and transferred onto a gelatin-coated culture dish or cover glass. After another 7–10 days of incubation, clusters were subjected to immunocytochemistry and reverse-transcription polymerase chain reaction (RT-PCR).

Immunocytochemistry

Immunocytochemistry was performed as previously described [21]. Clusters of adipose-Muse cells were fixed with 4% paraformaldehyde in 0.01 M PBS, embedded in OCT compound, and then cut into 8-μm-thick cryosections. Differentiated cells derived from adipose-Muse cell cluster were grown in gelatin-coated dishes. Cells were fixed using the same fixative described earlier. Antibodies used in this study included Nanog (1:100; Millipore), Oct3/4 (1:100; Santa Cruz Biotechnology), Sox2 (1:1000; Millipore), PAR4 (1:100; Santa Cruz Biotechnology), TRA-1-81 (1:100; Santa Cruz Biotechnology), smooth muscle actin (SMA, 1:100; Lab Vision, Thermo Fisher Scientific), neurofilament-M (1:100; Millipore), cytokeratin 7 (CK7, 1:100; Millipore), alpha-fetoprotein (α-FP, 1:100; DAKO), fatty acid-binding protein 4 (FABP-4, 1:100; R&D Systems), human hepatocyte paraffin-1 (HepPar1, 1:200; Dako), delta-like protein-1 (DLK1, 1:100; Santa Cruz), human albumin (1:100; Bethyl Laboratories), and neuronal class III β-tubulin (Tuj-1, 1:1000; Covance). All primary antibodies were diluted 1:200 in PBS/0.1% BSA solution and incubated overnight at 4°C. Following treatment with primary antibodies, cells were washed three times with PBS and incubated for 1 h at R/T with PBS/0.1% BSA containing secondary immunofluorescent antibodies. These antibodies included FITC-, Alexa-488-, or Alexa-568-labeled conjugated anti-rabbit IgG, anti-mouse IgG, anti-mouse IgM, or anti-rat IgM (1:100; Jackson ImmunoResearch). Nuclei were identified by 4′,6-diamidino-2-phenylindole (DAPI) staining (1:1000; Sigma). Cells were then washed three times with PBS. Images were acquired with a confocal laser scanning microscope (CS-1; Nikon).

RT-PCR

Total RNA was extracted from cells and purified using NucleoSpin RNA XS (Macherey-Nagel). First-strand cDNA was generated using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. The PCRs were performed using Ex Taq DNA polymerase using standard temperature cycling conditions (TaKaRa Bio). The primers used were (1) β-actin sense 5′-AGGCGGACTATGACTTAGTTGCGTTACACC-3′ and antisense 5′-AAGTCCTCGGCCACATTGTGAACTTTG-3′, (2) Nkx2.5 sense 5′-GGGACTTGAATGCGGTTCAG-3′ and antisense 5′-CTCCACAGTTGGGTTCATCTGTAA-3′, (3) α-FP sense 5′-CCACTTGTTGCCAACTCAGTGA-3′ and antisense 5′-TGCAGGAGGGACATATGTTTCA-3′; (4) microtubule associated protein-2 (MAP-2) sense 5′-ACTACC AGTTTCACACCCCCTTT-3′ and antisense 5′-AAGGGT GCAGGAGACACAGATAC-3′, and (5) GATA6 sense 5′-CCTGCGGGCTCTACAGCAAGATGAAC-3′ and antisense 5′-CGCCCCTGAGGCTGTAGGTTGTGTT-3′.

Evaluation for cell self-renewal

Cell self-renewal of adipose-Muse cell clusters was performed as previously described [21]. Briefly, adipose-Muse cells isolated by FACS were grown in single-cell suspension after limiting dilution to generate the first-generation cluster. After 7–10 days of single-cell suspension culture, first-generation clusters were transferred onto an adherent culture for expansion. After another 7 days of incubation of first-generation clusters in adherent culture, expanded cells were collected by trypsinization and returned to single-cell suspension culture after limiting dilution to form second-generation cluster. This cycle was repeated up to third-generation clusters. In each generation step, samples were subjected to RT-PCR.

Test for teratoma formation in immune-deficient mice testes

Adipose-Muse cells (1×10^5 cells) were suspended in PBS and injected using glass micropipette into the testes of 8-week-old CB17/Icr-Prkdc scid/CrlCrlj(SCID) mice ($n=6$). Mice were sacrificed for analysis 6 months after injection. For negative control, testes were injected with PBS ($n=2$) and, for positive control, 5×10^5 mouse ES cells ($n=4$) were injected, and were sacrificed 8 weeks after injection. Tissues were fixed with 4% paraformaldehyde in 0.01 M PBS and 3-μm-thick paraffin sections and analyzed by HE staining.

Telomerase activity

Telomerase activity was detected using TRAPEZE XL telomerase detection kit (Millipore) and Ex Taq polymerase (TaKaRa Bio). Fluorescence intensity was measured with a microplate reader (infinite M1000; Tecan) as described by Wakao et al. [17].

In vitro differentiation into adipocytes, hepatocytes, and neuronal cells

Experiments were repeated three times for each differentiation. Adipose-Muse cells and -non-Muse cells were