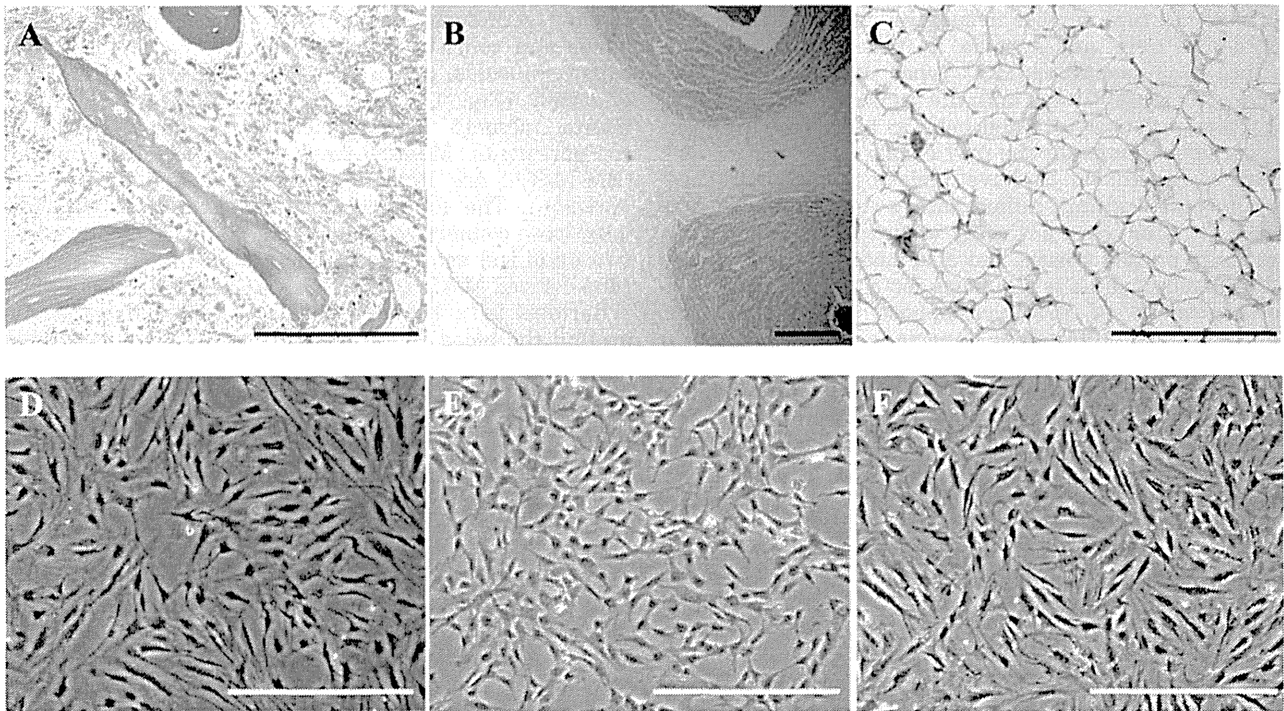


Fig. 1. Characteristic of human bone marrow, adipose tissue, and umbilical cord. (**A–C**) HE staining of human (A) bone marrow (B) umbilical cord and (C) adipose tissue. (**D–F**) Cell morphology of mesenchymal cells isolated from human (D) bone marrow (E) umbilical cord and (F) adipose tissue. Scale 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.**

| Group | Marker | 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 $7 \times 10^5$ 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 $2.1 \times 10^2$ 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).

Therefore, BM-MSCs, UC-MSCs, and AD-MSCs meet the criteria for application in regenerative medicine.

## Clinical Trials of MSCs

Generally, MSCs have an immunosuppressive effect as mentioned above. Such properties of MSCs have led them to application in the treatment of GVHD. GVHD is a complication that accompanies allogeneic tissue transplantation such as bone marrow transplantation, and is caused by immunological attack of the host cells by the transplanted immune cells. In the case of bone marrow transplantation, GVHD is induced with a probability of approximately 80% in cases of a HLA mismatch. However, even if the HLA is matched, the risk of GVHD still remains at 30 to 40%. GVHD leads to the death of recipients in the worst cases and, thus, suppression and control of GVHD is an important urgent matter for bone marrow transplantation. Steroids are generally used for the treatment of GVHD patients, but some cases are resistant. In the latter cases, MSCs are administrated to patients for potent immunosuppressive effects. Fang et al. reported a complete reversal of GVHD by administration of AD-MSCs, and almost all patients who received AD-MSC treatment survived without any side effects after a follow-up period of 40 months (Fang et al., 2007). The notable point in this clinical trial is that HLA matching between donors and recipients was not performed for the trial and, thus, suppression of GVHD was achieved in HLA-mismatched allo-transplantation. The same effect is also expected for BM-MSCs and UC-MSCs and, therefore, already operating marrow and cord banks are expected to contribute to allo-transplantation of MSCs for the treatment of GVHD.

In addition to GVHD, MSCs are used for treating other diseases. BM-MSCs were the first MSCs to be used in clinical trials and have accumulated many promising results. In 1999, Horwitz et al. first applied allogeneic BM-MSCs to children with osteogenesis imperfecta, which led to the improvement of total-body bone mineral content and subsequent osteogenesis (Horwitz et al., 1999). Other groups used autologous BM-MSCs in clinical trials for treating patients with liver cirrhosis, which resulted in increases of hepatocyte proliferation and an improvement of bilirubin as well as albumin levels, and finally led to clinical improvement of the liver cirrhosis (Terai et al., 2002, 2003; Gordon et al., 2006). BM-MSCs have also been applied to acute myocardial infarction patients, resulting in efficient cardiac function recovery for up to 1 year (Misao et al., 2006; Schachinger et al., 2009). In 2005, Park et al. reported the first trial of autologous BM-MSCs in the treatment of spinal cord injury patients, and demonstrated an improvement in functional recovery (Park et al., 2005).

Clinical trials of UC-MSCs have been mainly performed for allo-transplantation of cord blood that includes UC-MSCs. Haller et al. reported that cord blood transplantation in patients with type 1 diabetes can attenuate the symptoms (Haller et al., 2008). In the case of UC-MSCs alone, transplantation into patients with decompensated liver cirrhosis was performed, resulting in an improvement of liver function and reduction of ascites in patients (Zhang et al., 2012).

The first clinical trials with AD-MSCs were performed in 2004. Autologous AD-MSCs were used to treat a 7-year-old girl with post-traumatic calvarial defects by infusion of AD-MSCs with an autologous fibrin glue (Lendeckel et al., 2004), resulting in almost complete calvarial continuity at 3 months post-transplantation. Autologous AD-MSCs have also been applied to patients with Crohn's disease (García-Olmo et al., 2005). In such cases, 75% of transplanted sites were repaired, and 30 months of follow-up demonstrated no obvious side effects. Furthermore, autologous AD-MSCs have been used to repair tracheomediastinal fistulas caused by cancer ablation (Alvarez et al., 2008). In these cases, no side effects or complications were observed during the follow-up period, and re-epithelialization and neovascularization led to successful closure of the fistula. Recently, AD-MSCs have been used in plastic surgery such as breast reconstruction and fat supplementation for patients with facial fat atrophy (Yoshimura et al., 2008).

Many clinical trials of patients with various diseases have been performed and no tumorigenesis has been reported thus far. This is a very important finding in terms of clinical application, because the generation of tumors has been observed after transplantation of fetal neural stem cells into a boy with ataxia telangiectasia (Amarglio et al., 2009). ES and induced pluripotent stem (iPS) cells are also expected to be used clinically, although their use in patients is limited by the fact that they are immortal cells with a serious risk of causing tumors and malignancies. The potential risks posed by the uncontrolled and unstable genomes of both ES and iPS cells have been emphasized by a recent study that demonstrated a large number of mutations acquired by the cells (Laurent et al., 2011). Because MSCs have a limited lifespan in culture, their use in patients presents a limited risk of tumorigenicity (Prockop et al., 2010). Therefore, MSCs have great advantages over fetal stem cells, as well as ES and iPS cells because of their non-tumorigenicity, ensuring that MSCs are applicable to patients.

Another issue is the effectiveness of MSCs for curing diseases in terms of tissue regeneration. As mentioned above, the regenerative effects exerted by BM-MSCs, UC-MSCs, and AD-MSCs are not always consistently successful. Many trials of MSCs have reported partial improvement, whereas some trials have shown no effect. Carrion et al. applied BM-MSCs to patients with human systemic lupus erythematosus, but no improvements were observed (Carrion et al., 2010). In this trial, no adverse effects or progression of disease activity were noted during the 14 weeks of follow-up, except one case that finally developed overt renal disease after infusion. The cause of the ineffectiveness of MSCs in such cases is still unclear. However, the accumulation of data may lead to an in-depth understanding of MSCs.

When naive MSCs are applied, a curative effect can be observed to some extent, which is attributed mainly to the trophic effect of MSCs. The cytokines and factors produced by transplanted MSCs rescue the damaged tissue. However, MSCs do not remain in the tissue for a long time unless they differentiate and integrate into the tissue. Therefore, such trophic effects do not last for many months. In addition to the trophic effect, there is evidence to support that a small number MSCs are pluripotent cells. When naive MSCs are infused into the blood stream, a very small number of them integrate into the damaged tissue and spontaneously differentiate

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

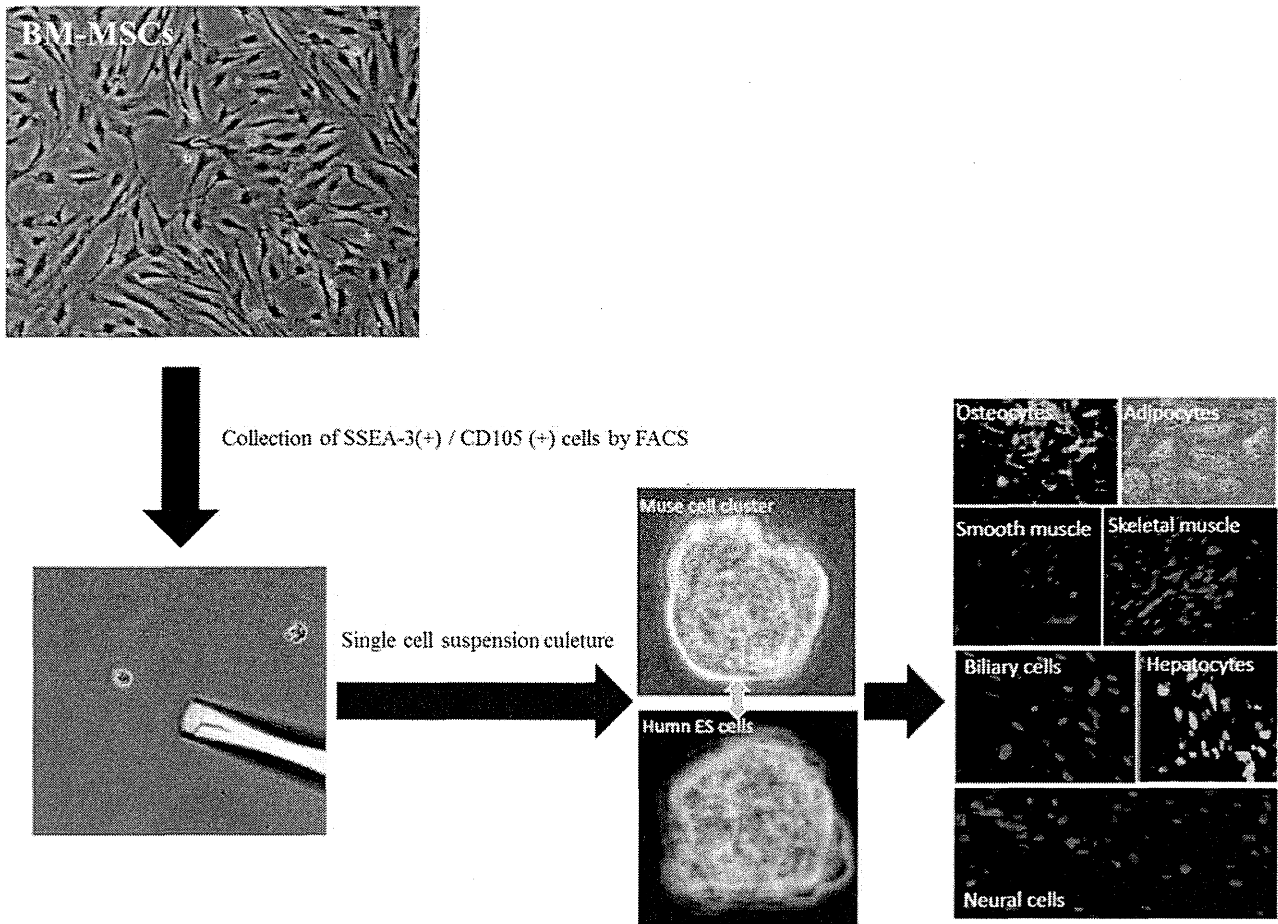


Fig. 2. Schema for isolation of Muse cells. Muse cells can be isolated from mesenchymal cells such as BM-MSCs and dermal fibroblasts as cells that are double positive for SSEA-3 and CD105. They can form clusters that are very similar to ES cell-derived embryoid bodies during maintained as a single cell-suspension 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 ($\sim$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.

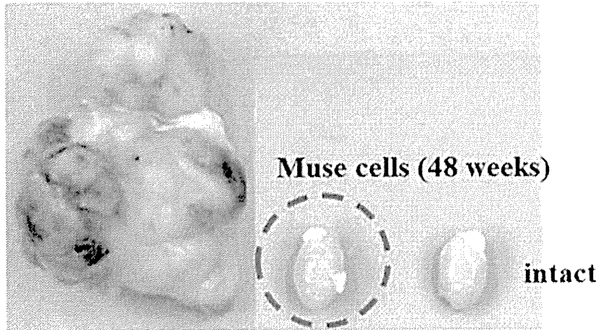


Fig. 3. Testes of immunodeficient mice injected with Muse cells. ES cells form teratomas within 8 weeks when transplanted into the testes of immunodeficient mice, whereas Muse cell-transplanted immunodeficient mouse testes do not develop teratomas even after 6 months. Pictures adapted from Kuroda et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

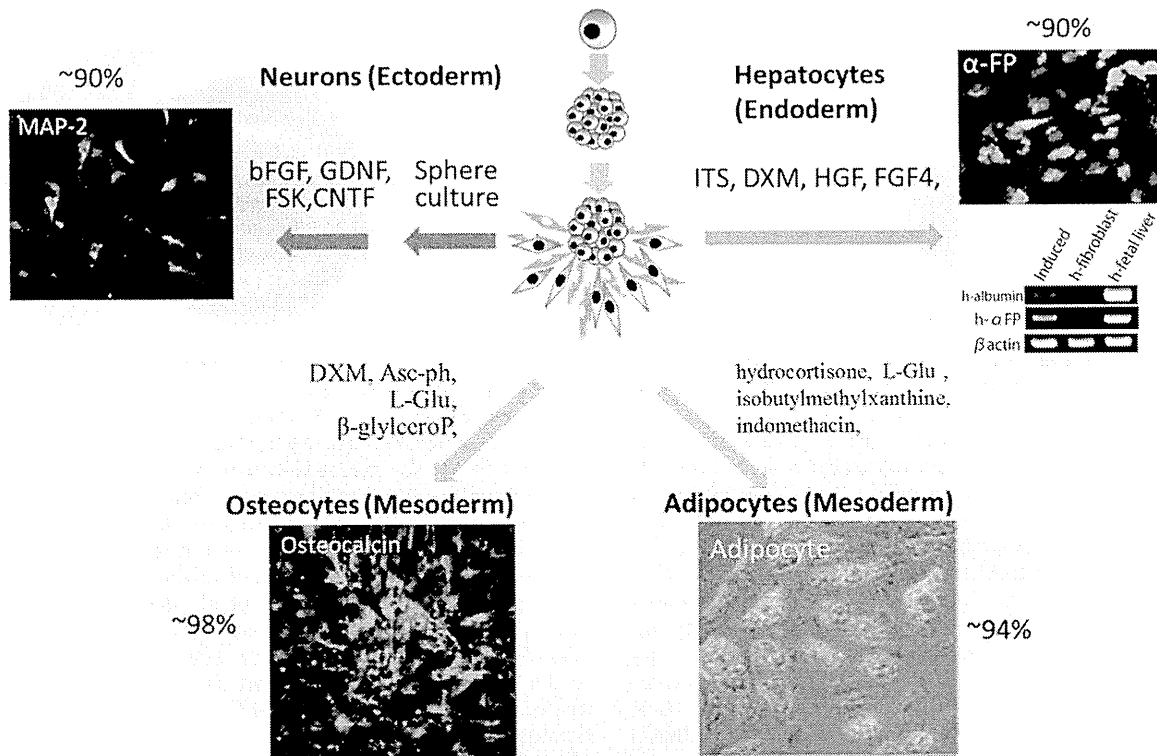


Fig. 4. Induction of Muse cells into neurons, osteocytes, adipocytes and hepatocytes. Muse cells can be directly converted into neurons, osteocytes, adipocytes and hepatocytes at very high efficiency with inductions. Pictures adapted from S. Wakao et al. (2011) with permission from The National Academy 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 $\sim$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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**ORIGINAL RESEARCH REPORT**

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

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## 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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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 $3.5 \times 10^4$ 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 $1 \times 10^6$ 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 ($1 \times 10^5$ 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, $5 \times 10^5$ 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

incubated in adipogenic differentiation medium (R&D Systems) for 14 days. Formation of new adipocytes was detected using the human MSC functional identification kit (R&D Systems) [19]. For hepatocyte induction, adipose-Muse cells (at a density of $2.0 \times 10^4$ cells/cm$^2$) were cultured on collagen-coated dishes for 14 days in DMEM supplemented with 10% FBS, insulin-transferrin-selenium (Gibco), 10 nM dexamethasone (Sigma), 100 ng/mL hepatocyte growth factor (R&D Systems), and 50 ng/mL fibroblast growth factor-4 (R&D Systems) [9]. Neuronal differentiation was performed according to the method reported by Boulland et al. [6]. Briefly, cells were induced by culture in the Neurobasal medium (Invitrogen) containing 1% FCS, 1×B27 supplement, 0.5 mM 1-methyl-3 isobutylxanthine, 1 mM dexamethasone, 0.2 mM 8CPTcAMP, 10 mM valproic acid, and 10 mM forskolin for 7 days. Comparatively, adipose-non-Muse, SSEA-3 (−) cells obtained from the same adipose tissue were used as controls in all these cell differentiation studies.

### *Quantitative PCR (q-PCR)*

Adipose-Muse cells were induced to neuronal differentiation and total RNA was extracted as described previously. Customized primers for Tuj-1 were purchased from SA Biosciences. Total RNA of BM- (Lonza) and dermal-Muse cells (Lonza) and adipose-Muse cells from LA-MSCs was collected using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using the RT2First Strand Kit (SA Biosciences). In both experiments, DNA was amplified with the Applied Biosystems 7300 real-time PCR system according to the manufacturer's instructions. Data were processed using the ΔΔCT method [22].

### *Comparative analysis of gene expression*

Total RNA of Muse cells derived from BM (Lonza), normal human dermal fibroblasts (Lonza), and LA-MSCs was extracted and purified using NucleoSpin RNA XS (TaKaRa Bio). The poly-A RNA molecules were further purified from total RNA using poly-T oligo-attached magnetic beads, and then fragmented and converted into cDNA using Illumina TruSeq RNA Sample Prep Kit (Illumina) to make libraries. The quality of libraries was determined with Agilent 2100 Bioanalyzer. The libraries were analyzed by Illumina HiSeq2000 sequencing (Illumina) according to standard procedure. Paired-end 100-bp reads were generated and subjected to data analysis with the use of the platform provided by DNAnexus.

## Results

### *Culture of adipose-MSCs*

This study utilized two sources of human adipose-MSCs; five lots of commercially available LA-MSCs that are widely used as adipose-derived MSCs, and four lots of adipose-MSCs established from human subcutaneous adipose tissue, namely, AT-MSCs. For AT-MSCs, volumes, culture duration, and total number of MSCs obtained from the four samples are shown in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/scd). On average, a culture of 15 cm$^3$ of adipose tissue for 3 weeks yielded $\sim 3 \times 10^7$ adipose-MSCs. There were no significant differences observed in morphology or doubling time between LA-MSCs and AT-MSCs (Supplementary Fig. S1).

### *Characterization of adipose-Muse cells in LA-MSCs and AT-MSCs*

We previously reported the presence of Muse cells in the adult human BM and dermis isolated by cell sorting using SSEA-3, a pluripotent stem cell marker for undifferentiated ES cells [16,17]. FACS analysis revealed the presence of SSEA-3-positive cells in LA-MSCs (3.8% ± 0.9%) and AT-MSCs (8.8 ± 1.3), which are termed as adipose-Muse cells in the following descriptions (Fig. 1A, B and Supplementary Table S1).

Surface marker expression was further analyzed in these adipose-Muse cells. They expressed general mesenchymal markers; all of SSEA-3(+)-adipose-Muse cells expressed CD105 (100%) and CD90 (100%), and in a lesser ratio (60%–70%) with CD29 (Fig. E–H). Adipose tissue is generally known to contain so-called ADSCs that express both CD34 and CD146 markers [23]. Adipose-Muse cells isolated from both LA-MSCs and AT-MSCs were, however, negative for these markers, suggesting that they are a distinct population from ADSCs (Fig. 1I, J).

When adipose-Muse cells were transferred to a single-cell suspension culture, each cell began to proliferate and form a cluster that is similar to the human-ES-cell-derived embryoid body formed in suspension culture at days 7–10 (Fig. 1C, D). On an average, adipose-Muse cells derived from LA-MSCs and AT-MSCs formed clusters in a single-cell suspension at a ratio of 31.3% ± 2.8% and 40.9% ± 6.8%, respectively (Supplementary Table S1). Importantly, none of the SSEA-3 (−) adipose-MSCs, namely, adipose-non-Muse cells, obtained from both LA-MSCs and AT-MSCS formed clusters in a single-cell suspension.

Adipose-Muse cell clusters both from LA-MSCs and AT-MSCs expressed pluripotency markers Nanog, Oct3/4, PAR4, Sox2, and TRA-1-81 and were positive for alkaline phosphatase reaction, one of the indicators of ES cells (Fig. 2). When these single-cell-derived clusters were individually transferred onto gelatin-coated dish and cultured adherently for 10–14 days, cells expanded from the cluster and proliferated. Among the expanded cell population, cells positive for α-FP (endodermal marker), SMA (mesodermal), and neurofilament (ectodermal) were recognized (Fig. 3A–D). Cells expanded from clusters of adipose-Muse cells were collected and analyzed by RT-PCR, and gene expression was detected for *NKX2-5* (mesodermal), *GATA6* (endodermal), *MAP2* (ectodermal), and *α-FP* (endodermal) (Fig. 3E). Expression of these genes further indicated that adipose-Muse cells, from either LA-MSCs or AT-MSCs, may have the ability to spontaneously generate cells representative of all three germ layers from a single cell.

To examine the potential for self-renewal, adipose-Muse cells from LA-MSCs and AT-MSCs were subjected to single-cell suspension culture in order to obtain first-generation clusters. Half of the clusters were transferred individually onto gelatin culture, maintained, and analyzed by RT-PCR for the expression of endodermal, mesodermal, and ectodermal markers, as described previously. The rest of the

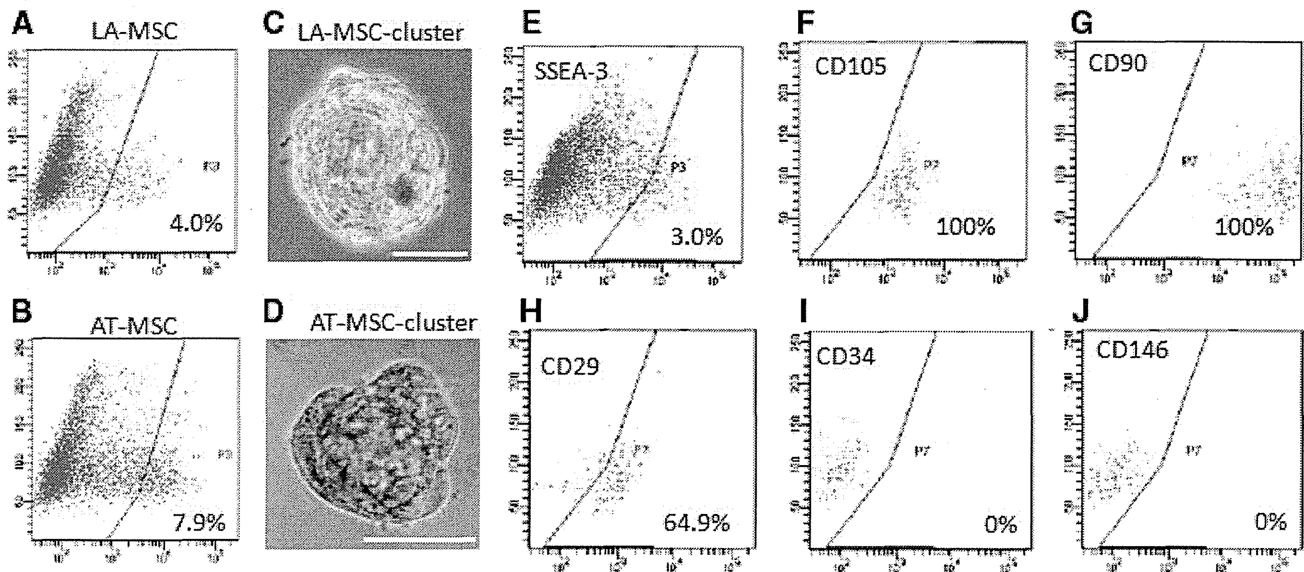


**FIG. 1.** Characterization of SSEA-3(+) cells in LA-MSCs and AT-MSCs. **(A, B)** An example of SSEA-3(+) cells in LA-MSC **(A)** and AT-MSC **(B)** FACS analysis showed the presence of SSEA-3(+) cells in both populations. **(C, D)** Clusters were formed in single-cell suspension culture from LA-MSC- **(C)** and AT-MSC-SSEA-3(+) cells **(D)**. Scale bars = 50 μm. **(E–J)** Expression of mesenchymal and ADSC markers in LA-MSC-SSEA-3(+) cells. Cells positive for SSEA-3 **(E)** were positive for CD105 **(F)**, CD90 **(G)**, and CD29 **(H)** but were negative for CD34 **(I)** and CD146 **(I)**. AT-MSCs, adipose-MSCs from subcutaneous adipose tissue; LA-MSCs, adipose-MSCs commercially purchased from Lonza. Color images available online at www.liebertpub.com/scd

clusters were individually transferred to adherent culture and allowed to proliferate for 7–10 days, after which they underwent a second round of single-cell suspension in culture to generate second-generation clusters (Fig. 4). This experimental cycle was repeated three times and clusters from each step were analyzed by RT-PCR. Again, gene expression of *MAP2*, *GATA6*, *α-FP*, and *NKX2.5* was detected in first-, second-, and third-generation clusters, demonstrating that adipose-Muse cells maintain self-renewal as well as triploblastic differentiation ability up to the third generation (Fig. 4).

### Telomerase activity and in vivo transplantation of adipose-Muse cells

Being a strong indicator of tumorigenicity, telomerase activity was examined in adipose-Muse cells from LA-MSCs and AT-MSCs. High telomerase activity was observed

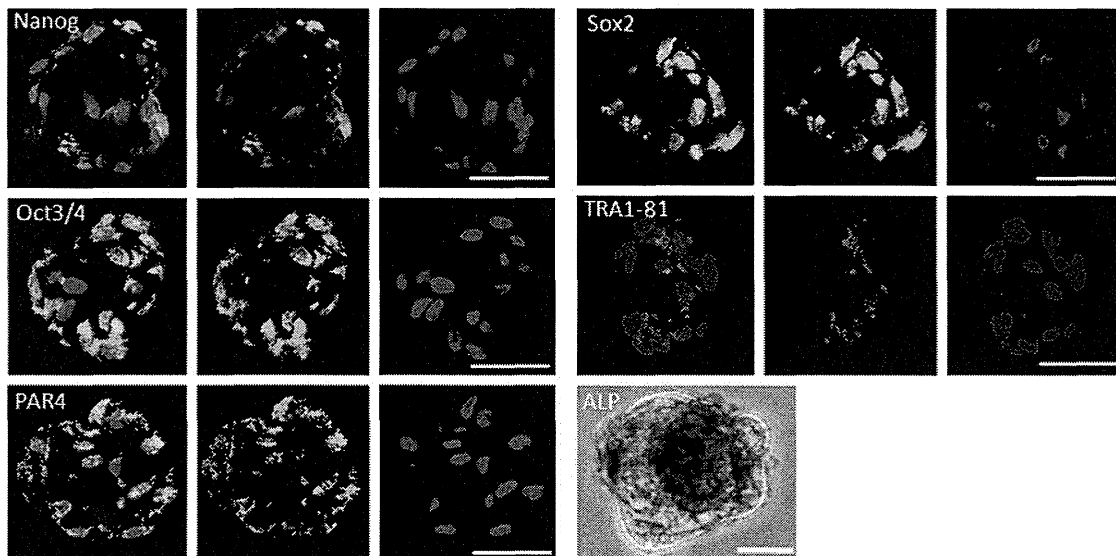


**FIG. 2.** Immunostaining of clusters formed from adipose-Muse cells in single-cell suspension culture. Clusters were positive for Nanog, Oct3/4, PAR4, Sox2, and TRA1-81, as well as for reactive alkaline phosphatase (ALP). Nanog, Oct3/4, and TRA-1-81 were from AT-MSC-SSEA-3(+) cells and PAR4, Sox2, and ALP from LA-MSC-SSEA-3(+) cells. Scale bars = 25 μm. Color images available online at www.liebertpub.com/scd

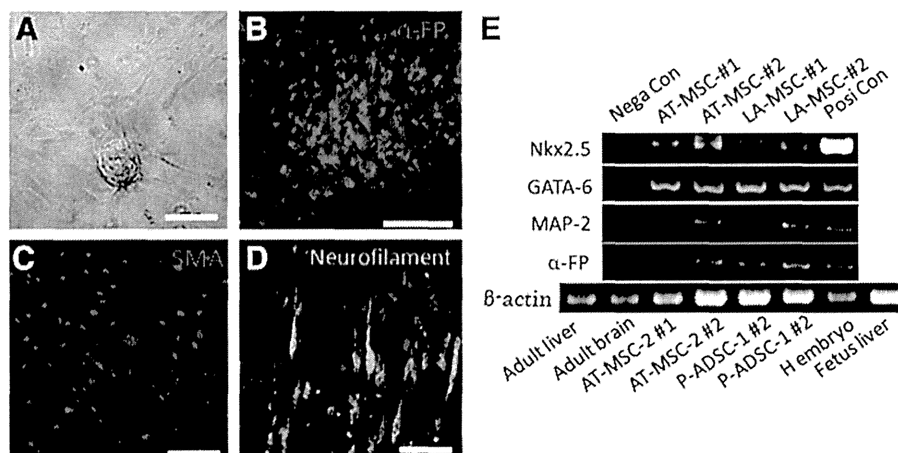


**FIG. 3.** Differentiation of single-Muse-cell-derived cluster into endodermal, mesodermal, and ectodermal lineages. Clusters formed from adipose-Muse cells of LA-MSCs in single-cell suspension culture were transferred onto gelatin-coated culture **(A)** allowing the cells to expand and differentiate spontaneously, expressing alpha-fetoprotein (α-FP: endodermal) **(B)**, smooth muscle actin (SMA: mesodermal) **(C)**, and neurofilament (ectodermal) **(D)**. Scale bars = 100 μm. RT-PCR analysis of cells expanded from single adipose-Muse cell cluster detected signals for *Nkx2.5* (mesodermal), *GATA-6* (endodermal), *MAP-2* (ectodermal), and *α-FP* (endodermal) in both AT-MSCs and LA-MSCs **(E)**. Positive controls for *Nkx2.5, MAP-2,* and *α-FP* were human whole embryo (H embryo) and for *GATA-4* was human fetus liver (Fetus liver). Negative controls for *Nkx2.5* and *MAP-2* were human adult liver (Adult liver) and for *GATA-6* and *α-FP* were human adult brain (Adult brain). RT-PCR, reverse transcription-polymerase chain reaction. Color images available online at www.liebertpub.com/scd

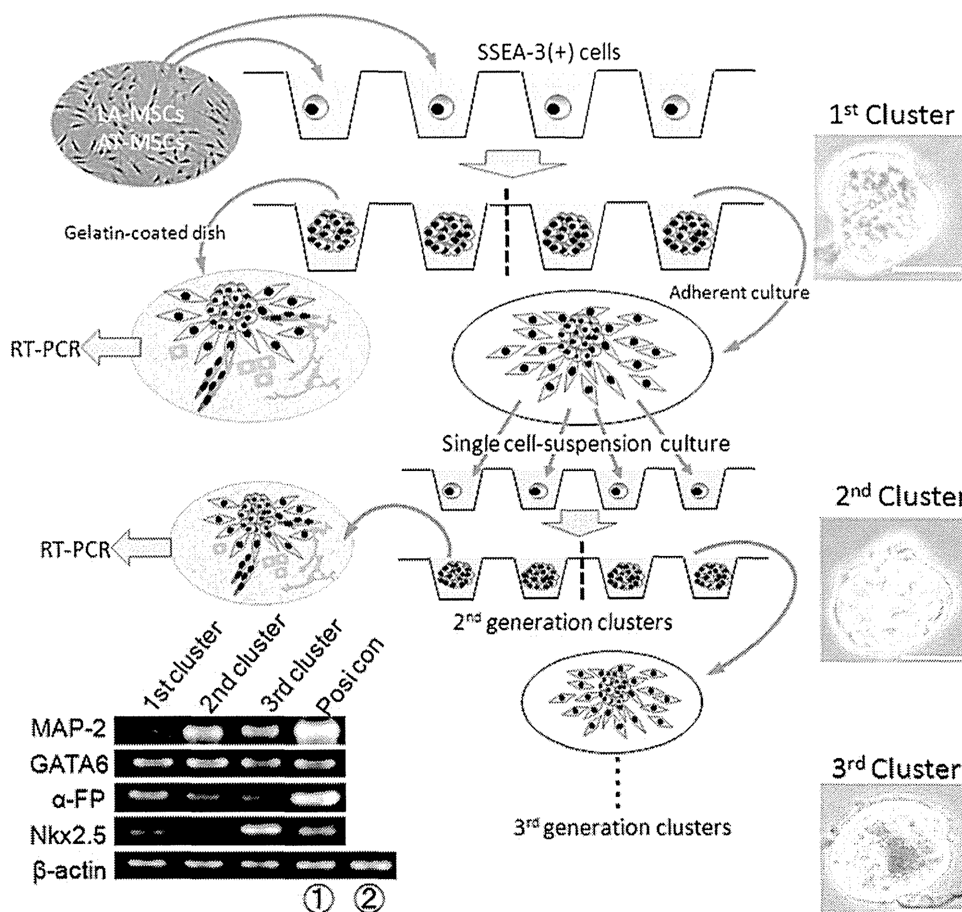


**FIG. 4.** Adipose-Muse cells demonstrate the capacity for self-renewal. Schematic diagram outlines experiments that validate self-renewal ability of adipose-Muse cells. RT-PCR data are from AT-MSC-derived adipose-Muse cells as an example. *MAP-2* (ectodermal), *GATA-6* (endodermal), *α-FP* (endodermal), and *Nkx2.5* (mesodermal) gene expression was detected in RT-PCR from cells expanded from each of clusters from first to third generations. Adipose-Muse cells from LA-MSCs showed basically same data (not shown). Positive controls for *MAP-2, α-FP,* and *Nkx2.5* were human whole embryo and for *GATA-4* was human fetus liver. 1 and 2 in *β-actin* are from human whole embryo (1) and human fetus liver (2), respectively. Scale bars = 25 μm. Color images available online at www.liebertpub.com/scd

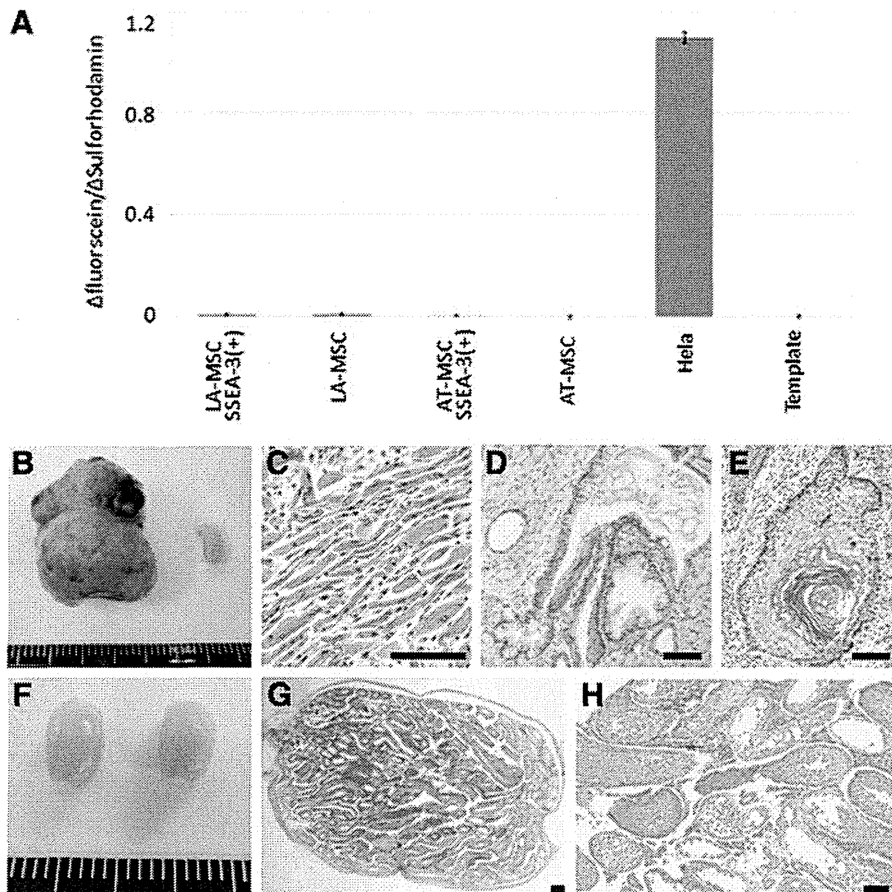


**FIG. 5.** Nontumorigenicity of adipose-Muse cells. **(A)** Telomerase activity in LA-MSCs, AT-MSCs, as well as adipose-Muse cells [SSEA-3(+)] from both populations. Hela cells (Hela) were used as positive control and template for negative control. **(B–E)** Teratoma formation in mouse testis with mouse ES cell transplantation (8 weeks after) **(B)**. Histological analysis showed that the teratoma contained muscle tissue **(C)**, intestine-like structure **(D)**, and keratinized skin formation **(E)**. **(F–H)** Transplantation of adipose-Muse cells from LA-MSCs into Nog mouse testis did not form teratoma even after 6 months **(F)** and maintained normal testis structure **(G, H)**. Scale bars = 100 μm. Color images available online at www.liebertpub.com/scd

in Hela cells, while adipose-Muse cells both from LA-MSCs and AT-MSCs were at nearly the same reduced level as cells from the original LA-MSC and AT-MSC populations (Fig. 5A).

Next, cell transplantation was performed in the testes of immune-deficient mice. When mouse ES cells were transplanted, large teratomas, consisting of mesodermal, endodermal, and ectodermal tissues, were formed by 8–10 weeks (Fig. 5B–E), while even after 6 months, the adipose-Muse cells transplanted in the mouse testes never formed teratomas. Normal testicular tubes were maintained in these testes (Fig. 5F–H).

All of the characteristics present in adipose-Muse cells, namely, expression both of pluripotency and mesenchymal markers, generation of embryoid-body-like clusters in suspension, triploblastic differentiation from a single cell, self-renewal, and nontumorigenicity, were consistent with previously reported BM- and dermal-Muse cells [16,17,21].

### *Cytokine induction into endodermal, ectodermal, and mesodermal lineages*

Adipose-Muse and -non-Muse cells were treated with cocktails of cytokines and reagents for adipocyte (mesodermal), hepatocyte (endodermal), and neuronal cell (ectodermal) differentiation. In adipocyte induction, both adipose-Muse and -non-Muse cells generated cells with lipid droplets that were positive for Oil Red-O staining. Immunolabeling of FABP-4, however, resulted in 72.4% ± 3.4% of adipose-Muse cells that display positivity, while adipose-non-Muse cells were only 34.4% ± 2.9% positive, suggesting that adipose-Muse cells have higher potential to become adipocytes than adipose-non-Muse cells (Fig. 6A–D).

Hepatocyte induction demonstrated that cells positive for hepatic stem cell marker human DLK1, and hepatocyte markers human HepPar1 and human albumin were induced from adipose-Muse cells but not from adipose-non-Muse cells. The positivity for human albumin in adipose-Muse cells was 13.7% ± 1.6% while it was undetectable in adipose-non-Muse cells (Fig. 6E–H).

Neuronal induction in adipose-Muse cells resulted in generation of cells positive for Tuj-1 with neuron-like morphology. These cells were generated from adipose-Muse cells but not from adipose-non-Muse cells. These results were also confirmed by q-PCR of Tuj-1 expression (Fig. 6I–M).

These results suggest that both adipose-Muse and -non-Muse cells are capable of differentiating into mesodermal lineage cells, such as adipocytes; however, higher efficiency is anticipated in adipose-Muse cells rather than -non-Muse cells. In contrast, differentiation from mesodermal to ectodermal or endodermal lineages was only possible in adipose-Muse cells.

### *Comparison among BM-, dermal-, and adipose-Muse cells*

Muse cells collected as SSEA-3(+) cells from human BM-MSCs (BM-Muse), dermal fibroblasts (dermal-Muse),

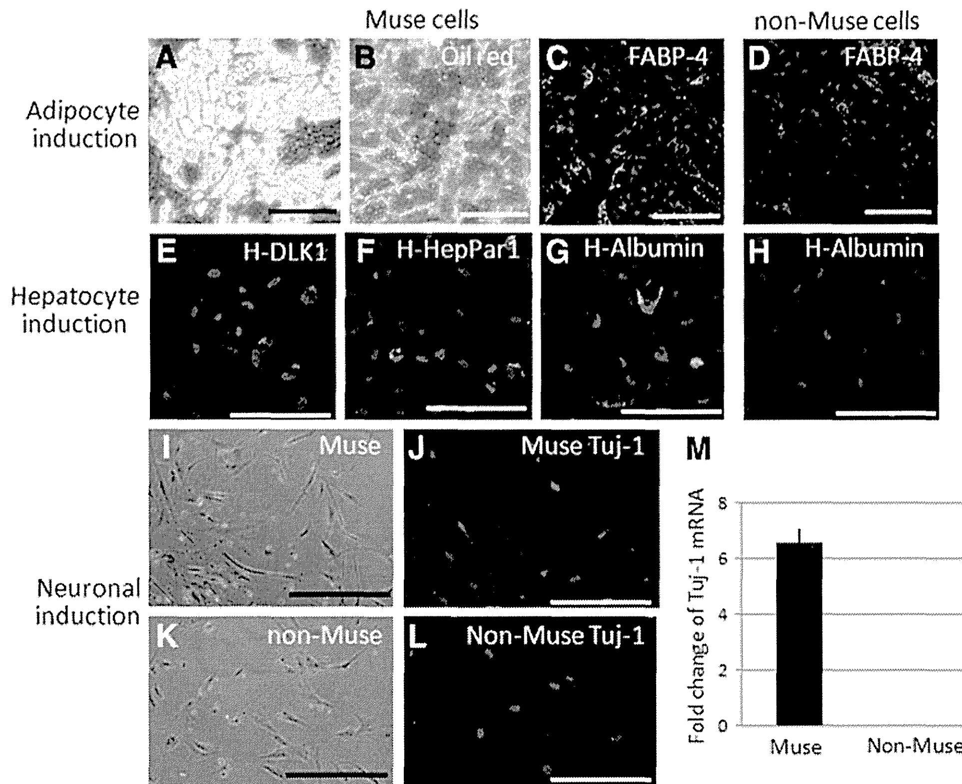


**FIG. 6.** Induced differentiation of adipose-Muse and -non-Muse cells. **(A–D)** Muse **(A–C)** and non-Muse **(D)** cells from LA-MSCs were subjected to adipocyte induction. Cells with lipid droplets **(A)** that are stained with Oil Red-O **(B)** were detected in adipose-Muse cells. Those cells were also positive for the adipocyte marker FABP-4. Adipose-non-Muse cells also contained cells positive for FABP-4 but with lower ratio **(D)**. **(E–H)** After hepatocyte induction, Muse cells were positive for liver stem cell marker human-DLK1 **(E)** and hepatocyte markers human HepPar1 **(F)** and human albumin **(G)**, while non-Muse cells lacked these expression. An example of non-Muse cells was shown in human albumin expression **(H)**. **(I–M)** After neuronal induction, Muse cells demonstrated a morphology similar to neuronal cells **(I)**, and some were also positive for Tuj-1 **(J)**. However, adipose-non-Muse cells were not like neuronal cells **(K)** and all cells were Tuj-1 negative **(L)**. Q-PCR consistently detected *Tuj-1* signal only in Muse cells and not in non-Muse cells **(M)**. Scale bars = 100 μm. Color images available online at www.liebertpub.com/scd

and LA-MSCs (adipose-Muse) were subjected to next generation sequencing to compare expression levels of genes related to endodermal, mesodermal, and ectodermal differentiation (Table 1). Analysis of mesodermal lineage expression revealed that osteogenic, adipogenic, and myogenic genes were generally higher in adipose-Muse rather than BM-Muse or dermal-Muse cells, and some of the factors, such as SP7, osteogenic factor, and Pax7 muscle stem cell marker, were only detected in adipose-Muse cells. Different from mesodermal factors, endodermal factors were more predominantly expressed in BM-Muse cells than in adipose-Muse cells. However, cholesterol 7, alpha-hydroxylase (CYP7A1), insulin gene enhancer binding protein (ISL1), and hepatocyte nuclear factor 4 alpha (HNF4A) were only expressed in adipose-Muse cells and not in BM-Muse or dermal-Muse cells (Table 1). Ectodermal genes that relate to neuronal differentiation were higher in both BM-Muse and dermal-Muse cells than in adipose-Muse cells while factors such as genes encoding for musashi RNA-binding protein 1 (MSI1), ISL1, and myelin transcription factor 1-like (MYT1L) were not expressed in BM-Muse or dermal-Muse cells, but only in adipose-Muse cells (Table 1).

## Discussion

In this study, we demonstrate that both adult human subcutaneous adipose tissue and commercially available adipose-MSCs contain a small percentage of stem cells with the capacity for triploblastic differentiation and self-renewal. These cells do not undergo tumorigenic proliferation in vitro, nor do they elicit teratomas when transplanted in vivo. These characteristics match those of previously reported Muse cells that were isolated from the BM and dermis [16,17,21], indicating that adipose tissue also contains Muse cells.

In adipose tissue, single-cell-derived cluster formation in suspension was unique to adipose-Muse cells; however, cluster formation ratio did not always reach 100% (Supplementary Table S1). This may be in part because of cellular damage caused by laser irradiation in the process of FACS isolation. Alternatively, cells might have been in an inactive dormant state, such that they did not proliferate. This property of Muse cells requires further study.

While MSCs are known to provide trophic and anti-inflammatory effects, these effects are temporary and do not

TABLE 1. COMPARISON OF GENE EXPRESSION RELATED TO THE DIFFERENTIATION OF ENDODERMAL, MESODERMAL, AND ECTODERMAL LINEAGES AMONG ADIPOSE-MUSE CELLS VERSUS DERMAL- AND BM-MUSE CELLS

**Mesodermal**

| | Adipose / Dermal | Adipose / BM |
|---|---|---|
| *PPARG* | 1.8939 | 3.0752 |
| *CEBPA* | 1.6335 | 0.8092 |
| *CEBPB* | 1.4336 | 1.0564 |
| *CEBPD* | 0.8261 | 0.5665 |
| *KLF15* | 4.2002 | 7.8730 |
| *LEP* | 0.8650 | 0.7210 |
| *ADIPOQ* | 2.3432 | 1.2264 |
| *AP2B1* | 0.7541 | 0.9276 |
| *FOXO1* | 1.9200 | 1.4321 |
| *SLC2A4* | 1.2509 | 1.0284 |
| *RUNX2* | 0.4624 | 0.7576 |
| *FOS* | 0.7956 | 0.3422 |
| *JUN* | 0.6616 | 0.4266 |
| *STAT1* | 0.7229 | 0.7139 |
| *SMAD1* | 1.0395 | 1.3637 |
| *SP7* | Adipose only | Adipose only |
| *ALPL* | 10.6316 | 1.5629 |
| *PAX3* | 0.0563 | Adipose only |
| *PAX7* | Adipose only | Adipose only |
| *MEF2C* | 0.4435 | 1.2908 |
| *TBX5* | 0.2465 | Adipose only |
| *KDR* | 2.8002 | Adipose only |
| *CXCR4* | ND | BM only |
| *NKX2-5* | Dermal only | ND |

**Endodermal**

| | Adipose / Dermal | Adipose / BM |
|---|---|---|
| *AFP* | 2.0655 | 0.5843 |
| *ALB* | ND | BM only |
| *CD44* | 1.0116 | 1.3584 |
| *CDH1* | 5.3242 | 1.3244 |
| *CDH2* | 5.8358 | 1.4388 |
| *CTNNA1* | 0.9106 | 0.7770 |
| *CTNNB1* | 0.8035 | 0.7119 |
| *CXCR4* | ND | BM only |
| *CYP7A1* | Adipose only | Adipose only |
| *FN1* | 0.9322 | 0.8965 |
| *HNF1A* | 1.3105 | 0.9468 |
| *HNF1B* | 1.3378 | 0.4668 |
| *HNF4A* | Adipose only | 0.3549 |
| *HTATSF1* | 0.9685 | 1.0462 |
| *ISL1* | Adipose only | 0.2334 |
| *ITGA6* | 0.1835 | 0.4860 |
| *ITGB1* | 0.7992 | 0.8514 |
| *KRT7* | 0.7876 | 0.9359 |
| *NRP2* | 1.0134 | 1.0528 |
| *OTX1* | 0.2428 | 2.8003 |
| *SYP* | 1.0983 | 0.7001 |
| *THY1* | 0.7876 | 0.9118 |
| *TTR* | Dermal only | ND |
| *GATA6* | 4.3770 | 1.3704 |

**Ectodermal**

| | Adipose / Dermal | Adipose / BM |
|---|---|---|
| *SOX2* | 0.2190 | 0.4993 |
| *NEUROG2* | ND | BM only |
| *HES1* | 1.8092 | 0.2084 |
| *HES5* | 0.3344 | 0.2169 |
| *ASCL1* | Dermal only | ND |
| *ZNF521* | 1.2807 | 13.9544 |
| *NES* | 0.3800 | 4.6525 |
| *MSI1* | Adipose only | 0.7347 |
| *OLIG2* | ND | BM only |
| *ISL1* | Adipose only | 0.2334 |
| *ISL2* | 0.1484 | 0.1060 |
| *GFAP* | 0.8829 | Adipose only |
| *POU3F2* | 1.0385 | 3.6332 |
| *MYT1L* | Adipose only | Adipose only |
| *NR4A2* | 0.5149 | 0.0536 |
| *DLX1* | 1.9342 | 0.2784 |
| *DLX2* | 6.9419 | 0.4366 |
| *MAP2* | 0.3737 | 0.5471 |
| *TP63* | 0.1497 | 0.0956 |
| *CRABP2* | 0.1967 | 1.2002 |
| *FN1* | 0.9322 | 0.8965 |
| *NOTCH1* | 0.8426 | 1.3712 |
| *NGFR* | 0.2069 | 0.3905 |
| *S100B* | Dermal only | BM only |

Expression level in adipose-Muse cells that is higher than that in dermal- or BM-Muse cells is indicated by red, whereas lower is indicated by blue colors.
BM, bone marrow.
Color images available online at www.liebertpub.com/scd

directly contribute to cell replacement or tissue regeneration [24,25]. In the true sense of functional recovery, replenishment of functional cells is essential; however, the major consensus of the primary efficacy of adipose-MSC transplantation is also attributed to trophic effects [25]. This could be explained, in part, by the small percentage of adipose-Muse cells within adipose-MSC population. However, if the ratio of adipose-Muse cells could be increased, then there may be an improvement in the curative effect of adipose-MSC transplantation. Recently, Muse cells derived from adipose tissue were reported to have been efficiently enriched from human lipoaspirated fat by long-term incubation with collagenase. Such a simple approach would be an extremely practical strategy to increase the overall yield of Muse cells for stem cell therapy [26].

BM, dermis, and adipose tissue are representative mesenchymal sources for cell-based therapy because of their easy accessibility and versatility. Even though core properties of Muse cells, namely, triploblastic differentiation, self-renewal, nontumorigenicity, and surface marker expression, are the same among those three sources, Muse cells are not the same in their gene expression that relate to endodermal-, mesodermal-, and ectodermal-lineage differentiation. Adipose-Muse cells exhibited the tendency toward expressing mesodermal lineage genes more highly than BM- and dermal-Muse cells. Conversely, genes related to endodermal and ectodermal lineages were lower in adipose-Muse cells than in those two sources. Therefore, the source for Muse cells should be selected in accordance with target tissues.

Our data show that expression of human peroxisome proliferator-activated receptor gamma (PPAR$\gamma$), a gene of mesodermal lineage, in adipose-Muse cells exceeds that of dermal- and BM-Muse cells. Considering that PPAR$\gamma$ expression is highly sensitive to the host environment, it may play a role in the unique adipose-Muse cell response to highly stressful conditions [27]. Other mesodermal genes that are elevated in adipose-Muse cells include Krüppel-like factor 15 (KLF15) and adiponectin (ADIPOQ), which encode prominent factors in adipocyte function, further supporting the preferential adipose-Muse cell differentiation to adipocytes [26].

Genes that are downregulated in adipose-Muse cells include FOS and JUN, genes that function paradoxically in both oncogenesis and tumor suppression depending on the cell type and its differentiation state and tumor stage [28]. CDH1, which encodes Cadherin-1, was expressed more highly in adipose-Muse cells than in BM- and dermal-Muse cells. Low expression of Cadherin-1 can support tumor progression, which may allude to the absence of tumorigenesis in CDH1-rich adipose-Muse cells [29]. Further, alpha-6 integrin (ITGA6) that plays a role in mammary tumorigenesis is decreased in adipose-Muse cells as compared with dermal- and BM-Muse cells [30]. Together with low telomerase activity and nontumorigenicity, this gene expression pattern may support the safety of adipose-Muse