In the Japanese population, both haplotypes *DPA1*01:03-DPB1*04:01* and *DPA1*01:03-DPB1*04:02* showed significant associations with protection against HBV infection ($P = 1.17 \times 10^{-5}$; OR = 0.32; 95% CI, 0.18–0.56 for *DPA1*01:03-DPB1*04:01* and $P = 1.95 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55 for *DPA1*01:03-DPB1*04:02*). In the Korean subjects, a significant association of *DPA1*01:03-DPB1*04:02* was also demonstrated; however, no association was observed for *DPA1*01:03-DPB1*04:01*. Because the observed number of each haplotype was small, none of the other haplotypes showed a significant association with protection against HBV infection.

In order to identify trans-ethnic DPA1-DPB1 haplotypes associated with HBV infection, a meta-analysis was performed. A meta-analysis further revealed that the *DPA1*01:03-DPB1*02:01* haplotype was significantly associated with protection against HBV infection ($P = 1.45 \times 10^{-5}$; OR = 0.69; 95% CI, 0.58–0.82) (Fig. S1C).

Discussion

Among 2.2 billion individuals worldwide who are infected with HBV, 15% of these are chronic carriers. Of chronic carriers, 10–15% develops LC, liver failure and HCC, and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in HBsAg negative and anti-HBc positive, i.e. HBV-resolved individuals. To identify host genetic factors associated with HBV-related disease progression may lead HBV patients to discriminate individuals who need treatment.

The *HLA-DPA1* and *HLA-DPB1* genes were identified as host genetic factors significantly associated with CHB infection, mainly in Asian populations [7–12], and not in European populations [13]. In the previous association analyses of *HLA-DPB1* alleles with HBV infection, one risk allele *HLA-DPB1*05:01* (OR = 1.52; 95% CI, 1.31–1.76), and two protective alleles, *HLA-DPB1*04:01* (OR = 0.53; 95% CI, 0.34–0.80) and *HLA-DPB1*04:02* (OR = 0.47; 95% CI, 0.34–.64), were identified in the Japanese population [7]. In this study, we further identified a new risk allele *HLA-DPB1*09:01* (OR = 1.94; 95% CI, 1.45–2.62) for HBV infection and a new protective allele *HLA-DPB1*02:01* (OR = 0.71; 95% CI, 0.56–0.89) in the Japanese population, in addition to the previously reported alleles (Table S2) [7]. The discrepancy in the association of *HLA-DPB1*09:01* allele with risk for HBV infection in a previous study [7] results from the elevated frequency of *HLA-DPB1*09:01* in the controls (12.2%), which is higher than our controls (8.7%). In this study, healthy subjects were recruited as controls. In contrast, individuals that were registered in BioBank Japan as subjects with diseases other than CHB were recruited as controls in the previous study [7], which may have included patients with diseases with which *HLA-DPB1*09:01* is associated. Although no significant association of *HLA-DPB1*09:01* with risk for HBV infection was observed in the Korean subjects, *HLA-DPB1*09:01* appears to have a susceptible effect on HBV infection, as it showed the same direction of association. When the association analyses in Japanese and Korean subjects were combined in meta-analysis, the association was statistically significant ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59). Thus, *HLA-DPB1*09:01* may be a Northeast Asian-specific allele associated with risk for HBV infection.

Moreover, a significant association of *HLA-DPB1*13:01* with risk of HBV infection (OR = 2.17; 95% CI, 1.40–3.47) was identified in the Thai subjects. However, the frequency of *HLA-DPB1*13:01* in Thai healthy controls (11.5% in the present study) reportedly varies, ranging from 15.4% to 29.5%, due to the population diversity [15–17]. Therefore, a replication analysis is required to confirm the association of *HLA-DPB1*13:01* with HBV infection in the Thai subjects. There were four other marginally associated *HLA-DPB1* alleles with low allele frequencies below 5% in HBV patients and healthy controls, including *HLA-DPB1*28:01*, *-DPB1*31:01*, *-DPB1*100:01*, and *-DPB1*105:01*, in the Hong Kong and Thai subjects. Because these infrequent alleles may have resulted from false positive associations, the association needs to be validated in a large number of subjects.

*HLA-DPB1*02:01* showed a significant association with protection against HBV infection in both Japanese and Hong Kong populations (Table S2); however, the *HLA-DPB1*02:01* allele was not associated with HBV infection in the previous study [7]. Although *HLA-DPB1*02:01* showed no association in either Korean or Thai populations, a significant association of *HLA-DPB1*02:01* with protection against HBV infection among four Asian populations was detected in meta-analysis ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) (Fig. S1B). We therefore conclude that the present finding is not a false positive.

A recent report showed that *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, **05:01*, **09:01*, and **14:01* were significantly associated with response to booster HB vaccination in Taiwan neonatally vaccinated adolescents [18]. The *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, and **14:01* were significantly more frequent in recipients whose post-booster titers of antibodies against HBV surface antigen (anti-HBs) were detectable, on the other hand, *HLA-DPB1*05:01* and **09:01* were significantly more frequent in recipients who were undetectable. Moreover, the *HLA-DPB1*05:01* and **09:01* significantly increase the likelihoods of undetectable pre-booster anti-HBs titers. These results seem consistent with our findings, in which *HLA-DPB1*05:01* and **09:01* are associated with susceptibility to chronic hepatitis B infection.

We also identified a protective effect of *HLA-DPB1*02:01* allele on disease progression in Asian populations. Previous studies identified the association of HLA class II genes including *HLA-DQ* and *HLA-DR* with development of HBV related hepatocellular carcinoma in the Chinese population [6,19,20]. In this study using Japanese and Korean samples, we identified significant associations between *HLA-DPB1*02:01* and disease progression in CHB patients ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67, for Japanese and $P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75 for Korean) (Table S4). Although the association of *HLA-DPB1*02:01* with disease progression was weaker after adjustment for age and gender in Korean subjects ($P = 2.54 \times 10^{-2}$; OR = 0.55; 95% CI, 0.33–0.93), the same direction of association was observed (i.e. protective effect on disease progression) (Table 2). The protective effects of *HLA-DPB1*02:01* on disease progression showed a significant association after adjustment for age and gender in the Japanese population ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70); moreover, a significant association between *HLA-DPB1*02:01* was observed among four Asian populations, under which population was adjusted by using dummy variables in a multivariate logistic regression analysis ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) (Table 2).

The *HLA-DPA1* and *HLA-DPB1* belong to the HLA class II alpha and beta chain paralogues, which make a heterodimer consisting of an alpha and a beta chain on the surface of antigen presenting cells. This HLA class II molecule plays a central role in the immune system by presenting peptides derived from extracellular proteins. We identified two susceptible haplotypes (*DPA1*02:02-DPB1*05:01* and *DPA1*02:01-DPB1*09:01*) and three protective haplotypes (*DPA1*01:03-DPB1*04:01*, *DPA1*01:03-DPB1*04:02*, and *HLA-DPA1*01:03-DPB1*02:01*) to chronic hepatitis B infection, which may result in different binding

affinities between HLA-DP subtypes and extracellular antigens. Although functional analyses of HLA-DP subtypes to identify HBV-related peptides are not fully completed, identification of susceptible and protective haplotypes as host genetic factors would lead us to understand the pathogenesis of HBV infection including viral factors.

In summary, we identified a new risk allele *HLA-DPB1*09:01*, which was specifically observed in Northeast Asian populations, Japanese and Korean. Moreover, a new protective allele *HLA-DPB1*02:01* was identified among four Asian populations: Japanese, Korean, Hong Kong and Thai. The protective allele *HLA-DPB1*02:01* was associated with both chronic HBV infection and disease progression in chronic HBV patients. Identification of a total of five alleles, including two risk alleles (*DPB1*09:01* and *DPB1*05:01*) and three protective alleles (*DPB1*04:01*, *DPB1*04:02* and *DPB1*02:01*), would enable HBV-infected individuals to be classified into groups according to the treatment requirements. Moreover, the risk and protective alleles for HBV infection and disease progression, identified in this study by means of trans-ethnic association analyses, would be key host factors to recognize HBV-derived antigen peptides. The present results may lead to subsequent functional studies into HLA-DP molecules and viral factors in order to understand the pathogenesis of HBV infection and development of hepatocellular carcinoma.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committee of National Center for Global Health and Medicine, and by the ethics committees of all participating universities and hospitals, including The University of Tokyo, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, The University of Hong Kong, Chulalongkorn University, Yonsei University College of Medicine, Nagoya City University Graduate School of Medical Sciences, Musashino Red Cross Hospital, Tokyo Medical and Dental University, Teine Keijinkai Hospital, Hokkaido University Graduate School of Medicine, Kurume University School of Medicine, Okayama University Graduate School of Medicine, Yamaguchi University Graduate School of Medicine, Tottori University, Kyoto Prefectural University of Medicine, Osaka City University Graduate School of Medicine, Nagoya Daini Red Cross Hospital, Ehime University Graduate School of Medicine, Kanazawa University Graduate School of Medicine, National Hospital Organization Osaka National Hospital, Iwate Medical University, Kawasaki Medical College, Shinshu University School of Medicine, Saitama Medical University, Kitasato University School of Medicine, Saga Medical School, and University of Tsukuba.

Written informed consent was obtained from each patient who participated in this study and all samples were anonymized. For Japanese healthy controls, 419 individuals were de-identified with information about gender, and all were recruited after obtaining verbal informed consent in Tokyo prior to 1990. For the 419 Japanese healthy individuals, written informed consent was not obtained because the blood sampling was conducted before the "Ethical Guidelines for Human Genome and Genetic Sequencing Research" were established in Japan. Under the condition that DNA sample is permanently de-linked from the individual, this study was approved by the Research Ethics Committee of National Center for Global Health and Medicine.

Characteristics of studied subjects

All of the 3,167 genomic DNA samples were collected from individuals with HBV, HBV-resolved individuals (HBsAg-negative and anti-HBc-positive) and healthy controls at 26 multi-center hospitals throughout Japan, Korea, Hong Kong, and Thailand (Table 1). In a total of 1,291 Japanese and 586 Korean samples, 1,191 Japanese individuals and all 586 Korean individuals were included in our previous study [9]. With regard to additional Japanese individuals, we collected samples from 48 healthy controls at Kohnodai Hospital, and 52 HBV patients at Okayama University Hospital and Ehime University Hospital, including 26 individuals with LC and 26 individuals with HCC. A total of 661 Hong Kong samples and 629 Thai samples were collected at Queen Mary Hospital and Chulalongkorn University, respectively.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of liver cirrhosis. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (by at least 3 bimonthly tests). Acute exacerbation (AE) of chronic hepatitis B was defined as an elevation of ALT to more than 10 times the upper limit of normal (ULN, 58 IU/L) and bilirubin to at least three times ULN (15 μmol/L). LC was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts<100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in a de-identified manner) in this study. Some of the unrelated and anonymized Japanese healthy controls were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 μl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

Genotyping of *HLA-DPA1* and *HLA-DPB1* alleles

High resolution (4-digit) genotyping of *HLA-DPA1* and *-DPB1* alleles was performed for HBV patients, resolved individuals, and healthy controls in Japan, Korea, Hong Kong, and Thailand. LABType SSO HLA DPA1/DPB1 kit (One Lambda, CA) and a Luminex Multi-Analyte Profiling system (xMAP; Luminex, Austin, TX) were used for genotyping, in according with the manufacturer's protocol. Because of the small quantity of genomic DNA in some Korean samples, we performed whole genome amplification for a total of 486 samples using GenomiPhi v2 DNA Amplification kit (GE Healthcare Life Sciences, UK), in accordance with the manufacturer's instruction.

A total of 2,895 samples were successfully genotyped and characteristics of these samples are summarized in Table S1.

Statistical analysis

Fisher's exact test in two-by-two cross tables was used to examine the associations between *HLA-DP* allele and chronic HBV infection or disease progression in chronic HBV patients,

using statistical software R2.9. To avoid false-positive results due to multiple testing, significance levels were adjusted based on the number of observed alleles at each locus in each population. For *HLA-DPA1* alleles, the number of observed alleles was 3 in Japanese, 4 in Korean, 5 in Hong Kong, and 5 in Thai subjects. Therefore, the significant levels for α were set at $\alpha = 0.05/3$ in Japanese, $\alpha = 0.05/4$ in Korean, $\alpha = 0.05/5$ in Hong Kong, and $\alpha = 0.05/5$ in Thai subjects. In the same way, significant levels for *HLA-DPB1* alleles were $\alpha = 0.05/10$, $0.05/11$, $0.05/12$, and $0.05/16$, respectively. Multivariate logistic regression analysis adjusted for age and sex (used as independent variables) was applied to assess associations between the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) and disease progression in CHB patients. To examine the effect of *DPB1*02:01* allele on disease progression in all populations, population was further adjusted by using three dummy variables (i.e., (c1, c2, c3) = (0, 0, 0) for Japanese, (1, 0, 0) for Korean, (0, 1, 0) for Hong Kong, and (0, 0, 1) for Thai) in a multivariate logistic regression analysis. We obtained the following regression equation: logit(p) = −3.905+0.083*age+(−0.929)*sex+(−0.684)**DPB1*02:01*+1.814*c1+(−0.478)*c2+0.782*c3. Significance levels in the analysis of disease progression in CHB patients were set as $\alpha = 0.05/10$ in Japanese, $\alpha = 0.05/11$ in Korean, $\alpha = 0.05/15$ in Hong Kong, and $\alpha = 0.05/15$ in Thai subjects. The phase of each individual (i.e., a combination of two *DPA1-DPB1* haplotypes) was estimated using PHASE software [21], assuming samples are selected randomly from a general population. In comparison of the estimated *DPA1-DPB1* haplotype frequencies, significant levels were set as $\alpha = 0.05/14$ in Japanese, $\alpha = 0.05/17$ in Korean, $\alpha = 0.05/17$ in Hong Kong, and $\alpha = 0.05/18$ in Thai subjects. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) in order to calculate pooled OR and its 95% confidence interval (95% CI). We applied meta-analysis for alleles with frequency>1% in all four Asian populations. The significance levels in meta-analysis were adjusted by the total number of statistical tests; $\alpha = 0.05/20$ for *DPA1* alleles, $\alpha = 0.05/57$ for *DPB1* alleles, and $\alpha = 0.05/74$ for *DPA1-DPB1* haplotypes.

Supporting Information

Figure S1 Comparison of odds ratios in association analyses for *HLA-DP* with chronic HBV infection among four Asian populations: (A) *HLA-DPA1* alleles; (B) *HLA-DPB1* alleles; and (C) *HLA DPA1-DPB1* haplotypes. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) to calculate pooled OR and its 95% confidence interval (95% CI). Bold depicts a statistically significant association after correction of significance level.
(DOCX)

Table S1 Individuals with successfully genotyped for *HLA-DPA1* and *HLA-DPB1*.
(DOCX)

Table S2 Frequencies of HLA-DP alleles in HBV patients and healthy controls among Asian populations.
(XLSX)

Table S3 Frequencies of HLA-DP alleles in HBV patients and resolved individuals among Asian populations.
(XLSX)

Table S4 Associations of HLA-DPB1 alleles with disease progression in CHB patients among Asian populations.
(XLSX)

Table S5 Estimated frequencies of HLA DPA1-DPB1 haplotypes in HBV patients and healthy controls among Asian populations.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS MS KT M. Mizokami. Performed the experiments: NN HS KK Y. Mawatari M. Kawashima M. Minami. Analyzed the data: NN HS M. Kawashima JO. Contributed reagents/materials/analysis tools: W-KS M-FY NP YP SHA K-HH K. Matsuura YT M. Kurosaki YA NI J-HK SH TI KY IS Y. Murawaki YI AT EO YH MH SK EM KS KH ET SM MW YE NM K. Murata M. Korenaga KT M. Mizokami. Wrote the paper: NN HS JO KT M. Mizokami.

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A New Age of Regenerative Medicine: Fusion of Tissue Engineering and Stem Cell Research

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After the biotech medicine era, symbolized by the progress of genetic engineering and the developments of physiologically active substances such as proteins and cytokines as medicine, the regenerative medicine era, when cells and tissues become medicine, is going to start and getting popular in recent years in the world. In particular, regenerative medicine is highly expected as curative-therapeutic treatments that can cure patients with obstinate diseases and physically impaired function, while the conventional symptomatic treatments are unable to do so. For supporting and enhancing the progress of regenerative medicine, remarkable advancements in regenerative medical sciences, and cell and tissue engineering are desired worldwide. Furthermore, to achieve new treatments based on regenerative medicine, multidisciplinary approaches from various fields such as molecular biology, cell biology, science and engineering, and pharmaceutical and medical sciences are essential. Regenerative medicine should be recognized as a completely new interdisciplinary academic discipline, which is unable to be obtained from the extrapolations of vertically connected and conventional academic disciplines. Therefore, for realizing regenerative medicine, not only the reconstruction of conventional medical science but also the establishment of new academic field, which integrates and assimilates scientific and engineering technologies, and biotechnology, become important. For example, technology controlling the expansion and differentiations of embryonic stem (ES) cells and induced pluripotent stem (iPS) cells is essential for obtaining the sufficient numbers of the cells allowing desired medical treatments to be realized. For separating remaining small amounts of undifferentiated ES and iPS cells from the differentiated cells with a high accuracy, an interdisciplinary research project having the horizontally integration of science, engineering, medicine should be considered. Further, the developments of technologies, which can efficiently transplant somatic cells in the body, are more important than those of cell sources, and new cooperative and integrative systems having various academic disciplines including medicine, science, engineering, and pharmacy are extremely important. For example, harvested cells, which are obtained from culture dishes by treating them with enzymes including trypsin or dispase after being cultured and expanded, are damaged in their structures and functions by enzymatically cleaving their cell-membrane proteins. Upon the direct injection of suspension containing damaged cells following the enzyme treatments to the tissue or organ, approximately 95% of the cells fail to stay in the target, and only less than several percent of the cells transplanted in the organ is speculated to be effective (Hofmann et al., 2005). The unwelcome fact that possible therapeutic effects are expected with a small number of transplanted cells should be noticed seriously. Tissue engineering is extremely important in terms of the more efficient engraftment of cell transplantation and is expected to proceed further with the integration of the technology of biomaterials contacting cell directly and bio- and medical-technologies. One of the tissue engineering approaches is biodegradable polymer scaffold-based methods (Langer and Vacanti, 1993). It is important to promote research that investigates how the shape and function of cells cultured in the scaffolds are maintained after transplantation. In this special issue, the recent progress of tissue engineering approaches using biodegradable polymer scaffolds are reviewed by Rui Reis and his colleagues on periodontal tissue, Charles Vacanti and Koji Kojima on trachea, Toshiharu Shinoka and his colleagues on vasculature, Dietmar Hutmacher and his colleagues on osteochondral tissue, and Stephan Badylak and his colleagues on skeletal muscle. For maintaining cell-dense thicker tissue viability, sufficient amounts of oxygen and nutrients should be supplied into the tissue. However, scaffold-based tissue has a heavy burden to have supplies of oxygen and nutrients from the scaffold surface by diffusion, which is hampered with increasing cell density. The developments of new technologies to break through this problem would be desired. Another approach for tissue engineering is cell sheet-based methods (Yang et al., 2007). Dense monolayered cell sheets that are harvested from temperature responsive culture dishes are fully viable and almost all of the cells are engrafted and keep their function after transplantation. In this special issue, the cutting edge research using cell sheet-based tissue engineering methods are reviewed by Teruo Okano and Kazuo Ohashi on liver and islets, Tatsuya Shimizu and Katsuhisa Matsuura on cardiac tissue, Masayuki Yamato and his colleagues on periodontal tissue, and Masato Sato and his colleagues on articular cartilage. In the cornea,

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myocardium, periodontal ligament, esophagus, and articular cartilage, the clinical studies with this cell-sheet technology have been already initiated, and the clinical efficacies of these cell-sheet transplantations have been confirmed. Remarkable research projects investigating the preparation of three-dimensional (3-D) cell-sheet tissues by layering cell sheets have been started. Especially challenging is to make capillaries in 3-D layered cell-sheet tissue and allow them to connect with living blood vessels (Sekine et al., 2013) or the micro-channels of artificial vascular beds (Sakaguchi et al., 2013). From these successful studies, the preparations of various organs such as liver, kidney, pancreas, and so forth are expected to be accelerated.

Various stem cell types are currently under investigation. Among them, the application of tissue stem cells, also called somatic stem cells, is being developed with remarkable progress (Korbling and Estrov, 2003). Some tissue stem cells have proceeded to clinical trials and even further to general medical treatment. Hematopoietic stem cells (HSCs) are representative of such cells (Weissman and Shizuru, 2008). HSCs have been successfully administered to patients with leukemia for over 50 years (Thomas, 1983). In this special issue, Atsushi Iwama and his colleagues, Yaeko Nakajima-Takagi and Mitsujiro Osawa, review HSCs, including the methods used for identifying and expanding HSCs *ex vivo*, and the niche cells that control the self-renewal, differentiation, and homing of HSCs.

One special type of tissue stem cell has attracted great attention. Mesenchymal stem cells have been intensively studied for more than a decade, with new knowledge accumulating from both basic and applied studies (Kuroda et al., 2011). Importantly, more than 450 clinical studies are currently being performed throughout the world, targeting various diseases, and there are several venture capital companies focusing on mesenchymal stem cells. Mesenchymal stem cells are indeed a hot topic and a major field in basic science, clinical medicine, and medical industry. In this special issue, Yasumasa Kuroda and Mari Dezawa provide an overview of mesenchymal stem cell studies, focusing on three representative sources, bone marrow, adipose tissue, and umbilical cord, and describe the similarities and differences among them. They also describe recent advances in clinical studies and discuss perspectives of mesenchymal stem cell use. Finally, they introduce multilineage-differentiating stress enduring (Muse) cells, which are recently discovered pluripotent stem cells that make up a large percentage of mesenchymal stem cells. Muse cells have attracted attention not only because they explain the triploblastic differentiation ability of mesenchymal stem cells, but also because they are pluripotent but non-tumorigenic. Their review discusses the great potential of Muse cells for regenerative medicine.

Neural regeneration is another hot topic in regenerative medicine (Gage, 2000). While there are some candidate stem cells that relate to neural regeneration, in this special issue James St. Johns and Jenny Ekberg review olfactory ensheathing cells. Established central nervous system tissue does not possess regenerative capacity; therefore, the only way to restore damaged brain regions and spinal cord is to supply new neural cells and reconstruct neuronal circuits. Neural stem cells have attracted great attention for quite some time, but the number of available cells from fetal or adult brain is limited. Due to their regenerative capacity and feasibility of application, olfactory ensheathing cells are considered one of the strongest candidates for the treatment of spinal cord injury, and in fact, they have been already applied to patients in some countries.

For diseases of complex tissues, such as the kidney and eye, the search for tissue stem cells has not been as simple and straightforward. Motoko Yanagida and her colleague, Koji Takaori, are pioneers in the area of kidney regeneration and stem cells. Here in this special issue, they review kidney stem cells. The retina is an organ with highly sophisticated function, such as information processing ability comparable to that of the brain. In mammals, retinal stem/progenitor cells (RPCs) are suggested to be possible retinal stem cells that are quiescent and exhibit very little activity. A number of different cellular sources of RPCs have been identified in the vertebrate retina. These include RPCs at the retinal margin; pigmented cells in the ciliary body, iris, and retinal pigment epithelium; and Müller cells within the retina. Henry Yip describes the isolation and expansion of RPCs from immature and mature eyes, and their potential application to transplantation in degenerated retinal tissue.

James Trosko, the pioneer of tissue stem cells and cancer stem cells, poses questions about the reprogramming hypothesis of iPS cells. His review proposes novel insights into the relation between iPS cells and cancer stem cells.

A survey of the stem cell world reveals that tissue stem cells are diverse and complex, just as our bodies are composed of complex sophisticated systems. Our bodies are able to maintain tissue homeostasis perhaps because of the evolutionary development of tissue stem cells to acquire a variety of functions. We hope that this special issue will stimulate development of additional tissue engineering and tissue stem cell studies.

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EDITORIAL

A New Age of Regenerative Medicine: Fusion of Tissue Engineering and Stem Cell Research

An exciting era is on our doorstep: regenerative medicine. Why? Because knowledge and technology are converging to the point where cell or tissue therapies are becoming more and more available, worldwide. Another reason is because regenerative medicine relies on interdisciplinary, team-science that brings basic scientists and clinicians together. This union, which is consistent with the Journal's emphasis of integrative biology that crosses disciplines, appears to be a paradigm for science of the future. For these reasons, *The Anatomical Record* is proud to publish this special issue on stem cells and tissue engineering.

Another reason for the Journal's pride is its substantial archive of published papers that are related to regenerative medicine. Perusing the archive reveals nearly 100 papers on stem cells and/or tissue engineering. One of the first papers characterizes spermatogonia stem-cell renewal in the mouse (Oakberg, 1971). More than a decade later, a paper describes myosatellite cell growth and regeneration in a mouse model of dystrophic muscle (Ontell et al., 1984). The number of regenerative medicine-related papers during the decade of the 1990s equals the number such papers published in the preceding two decades. The two papers focus on guided tissue regeneration related to endosseous dental implants (Listgarten, 1996) and pluripotent embryonic stem cell models of development (O'Shea, 1999).

The number of publications in *The Anatomical Record* that are related to regenerative medicine exploded in the 21st century. One paper, which reported temporary airway epithelial repopulation and rare clonal formation by mesenchymal stem cells after injury to the lung of mice (Serikov et al., 2007), provided the cover illustration for the Journal (Volume 290, 2007). Nearly 20 papers reported results for the cardiovascular system, including the heart (Eisenberg and Eisenberg, 2004; Rosen et al., 2004; Perez-Pomares et al., 2006). Also well represented is the nervous system (ca. 15 papers) (Geuna et al., 2001; Gokhan and Mehler, 2001; Zhang et al., 2010). So are bone (ca. 10 papers) (Mishra and Knothe Tate, 2003; Umehara et al., 2012) and cartilage (also ca. 10 papers) (Huang et al., 2004; Mauck et al., 2007). Other papers report results for cellular motors for manufacturing molecules (Dinu et al., 2007), matrices (Badylak, 2005), adipose tissue (Patrick, 2001), hematology (Yoder, 2004), cochlear and vestibular systems (Eshraghi et al., 2012; Fridman and Della Santina, 2012), germ lines (Oakberg, 1971), muscle (Hirschi and Majesky, 2004), skin (Casasco et al., 2001), reserve stem cells (Young et al., 2001), embryonic stem cells (Gokhan and Mehler, 2001), wound healing (Delorme et al., 2012), methods (An et al., 2001; Merzkirch et al., 2001; Liu et al., 2012), *in vivo* tracking (Cao et al., 2009), and tissue engineering (Evans, 2001; Gutowska et al., 2001; Mann and West, 2001; Walgenbach et al., 2001; Boland et al., 2003). Lastly, a number of reviews on stem cells, tissue engineering, and regenerative medicine are among the legion of papers that are published in ***The Anatomical Record*** (Carlson, 1999; Mironov and Markwald, 2001; Young and Black, 2004; Di Felice et al., 2009; Hong et al., 2010). You are invited to access and read these important papers (http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291932-8494).

A motivation for the present special issue on stem cells and tissue engineering is that injury or disease creates defects that represent challenges to surgical reconstruction or replacement to improve quality of life or life expectancy. The papers in this special issue provide contemporary assessments of the emerging fields of stem cells and tissue engineering, an important goal of which is to help develop biological substitutes that restore structure and function. Along the way, the papers consider essential principals and methods that underlie successful applications of stem cells and tissue engineering. These considerations are important because of the need to optimize techniques to acquire and expand cells and tissues, and provide scaffolds to shape tissues and organs. Equally importantly, *in vivo* testing in large animal models is necessary to determine efficacy, durability, and safety because the target is treatment of human subjects with injury or disease. We hope that the papers in this special issue will stimulate readers to continue to push the fields of stem cells and tissue engineering to expand translation to human applications of regenerative medicine (Mitrecic et al., 2009).

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Review Article

Muse cells, newly found non-tumorigenic pluripotent stem cells, reside in human mesenchymal tissues

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Mesenchymal stem cells (MSCs) have been presumed to include a subpopulation of pluripotent-like cells as they differentiate not only into the same mesodermal-lineage cells but also into ectodermal- and endodermal-lineage cells and exert tissue regenerative effects in a wide variety of tissues. A novel type of pluripotent stem cell, Multilineage-differentiating stress enduring (Muse) cells, was recently discovered in mesenchymal tissues such as the bone marrow, adipose tissue, dermis and connective tissue of organs, as well as in cultured fibroblasts and bone marrow-MSCs. Muse cells are able to differentiate into all three germ layers from a single cell and to self-renew, and yet exhibit non-tumorigenic and low telomerase activities. They can migrate to and target damaged sites *in vivo*, spontaneously differentiate into cells compatible with the targeted tissue, and contribute to tissue repair. Thus, Muse cells may account for the wide variety of differentiation abilities and tissue repair effects that have been observed in MSCs. Muse cells are unique in that they are pluripotent stem cells that belong in the living body, and are thus assumed to play an important role in 'regenerative homeostasis' *in vivo*.

Key words: cell transplantation, mesenchymal stem cells, pluripotent stem cells, regenerative medicine, telomerase

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CURRENT STATE OF MESENCHYMAL STEM CELL (MSC) RESEARCH

Nearly 400 clinical studies of mesenchymal stem cell (MSC) transplantation have been performed around the world, targeting various diseases, such as Parkinson's disease, Crohn's disease, pulmonary fibrosis, and diabetes mellitus.[1–4] Sources of MSCs vary, with the bone marrow, adipose tissue, and umbilical cord currently being the most common. These sources are easily accessible and avoid the ethical problems associated with the use of fertilized eggs and fetal tissue. Tissue banks are available for bone marrow and umbilical cord tissues. Human MSCs have high proliferative activity and therefore large numbers of harvested MSCs can be obtained for clinical use.[5,6]

The most important requirement for clinical application is safety. To date, there have been no reports of tumorigenesis related to MSCs. They are not artificially induced or manipulated, but are naturally existing stem cells, and are thus considered non-tumorigenic. Although MSCs have great advantages for clinical use, they are not superior in all aspects, and the effects of MSCs on tissue regeneration and functional recovery are controversial.

While MSCs are referred to as 'stem cells', the rigorous methods of stem cell biology that are applied to hematopoietic and neural stem cells have not been applied in most of the studies performed using MSCs. Mesenchymal stem cells are usually collected just as adherent cells from the bone marrow and other mesenchymal tissues. While the morphology of collected adherent cells is similar to that of fibroblasts, they are not the same as fibroblasts. Some basic information about MSCs remains obscure, such as how many cells in the MSC population critically meet the criteria of stem cells, how many types of cells comprise MSCs, or the ratio of each cell type. The MSCs are a crude population and may include cells other than stem cells,

such as fibroblasts and endothelial cells, which are normally found in mesenchymal tissue.

Mesenchymal stem cells are heterogeneous, and their actions are pleiotropic. They produce humoral factors that exert trophic and anti-inflammatory effects and modulate immunologic reactions.[1,7] In addition to these humoral effects, MSCs exhibit a broad spectrum of differentiation abilities that cross the boundaries from mesodermal- to ectodermal- or to endodermal-lineage cells, suggesting that MSCs have an aspect of pluripotency.[8] Although at very low frequency, transplanted MSCs show triploblastic differentiation ability. In animal models transplanted with naive MSCs, the integration of a very small number of MSCs into damaged liver, brain, or heart, and differentiation into hepatocyte-, neural-, or cardiomyocyte-marker expressing cells in each organ were observed, suggesting the involvement of MSCs in tissue repair.[9–11] These tissue repair effects of MSCs, however, have not yet been clearly demonstrated in humans. Trophic effects are the most obvious effects of MSC transplantation, while tissue repair effects are considered to be minor and with a low frequency. Although MSCs are safe and feasible for clinical use, the low frequency of tissue repair effects limits the effectiveness of MSCs for regenerative medicine.

Nevertheless, MSCs are suggested to include a small population of stem cells that have the ability to differentiate into any cell type, much like pluripotent stem cells, and participate in tissue repair. Isolation of such stem cells from MSCs could have a critical impact in the fields of regenerative medicine and cell-based therapy. What kinds of cells might these be?

DISCOVERY OF MUSE CELLS

Pluripotent stem cells that account for one to several percent of MSCs, Muse cells, were first reported in 2010.[12] Muse cells are found in adult mesenchymal tissues such as the bone marrow, adipose tissue and dermis, but are generally distributed sparsely in organ connective tissue.[13] Muse cells can be conveniently obtained from commercially available mesenchymal cultured cells such as bone marrow- and adipose tissue-MSCs, as well as from fibroblasts, one of the most generally used cultured cells in the world, as several percentage of total MSCs. (Fig. 1)[12,14,15] Muse are pluripotent but non-tumorigenic, thus early realization of their application to regenerative medicine is highly anticipated.

The discovery of Muse cells is important in several aspects. First, the pluripotency of Muse cells and their small proportion of total MSCs are consistent with the previously reported low frequency of trans-differentiation of MSCs across triploblastic lineages. Second, the pleiotropic actions of MSCs are clarified by the division of the roles played by Muse cells and cells other than Muse cells, namely non-Muse cells. That is, Muse cells are responsible for the triploblastic differentiation and tissue repair effects, while non-Muse cells are deeply involved in trophic and immunosuppressive effects.[12,16]

Muse cells were initially identified as stress-tolerant cells. When bone marrow-MSCs (BM-MSCs) or fibroblasts are cultured for longer than overnight under stress-inducing conditions, e.g., incubated in trypsin or under low nutrition conditions, the vast majority of MSCs die and only a small number of cells, containing a high ratio of Muse cells, survive.[12] Somatic stem cells that normally reside in tissue are dormant and not usually active, but once the tissue is damaged or exposed to stress, they become activated and begin to proliferate, differentiate, and contribute to tissue restoration. In contrast to these stem cells, functioning differentiated cells tend to die after damage or stress. For example, neural stem cells that are located in the brain are normally inactive, but following stroke, these stem cells enter into the cell cycle and begin to generate neuronal and glial cells whereas mature neuronal cells tend to die.[17] Recently, Shigemoto *et al.* succeeded in efficiently collecting muscle stem cells, namely satellite cells, from adult skeletal muscle tissue by taking advantage of their stress tolerance properties.[18] In the same manner, Muse cells are stem cells that reside in mesenchymal tissues, and are tolerant to stress. In contrast to other somatic stem cells, however, such as neural and muscle stem cells, their actions are not confined to the tissue where they are located but they expand their field of activities, perhaps via the peripheral blood stream, and participate in extensive tissue repair, as described below.

CHARACTERISTICS OF MUSE CELLS

Muse cells have remarkable characteristics, including:

1 Muse cells are pluripotent stem cells that are able to differentiate into mesodermal-, ectodermal-, and endodermal-lineage cells from a single cell and can be directly collected from human tissues (Fig. 1).[12]
2 Muse cells can be obtained from easily accessed tissues, such as the bone marrow, adipose tissue, and dermis, as well as from commercially available cultured fibroblasts and BM-MSCs (Fig. 1).[12,13,15,16]
3 Muse cells have low telomerase activity and are non-turmorigenic.[14]
4 Muse cells comprise 0.03% of bone marrow mononucleated cells, and several percentage of cultured fibroblasts and BM-MSCs.[12]
5 Muse cells also comprise a part of MSCs, which are already used in clinical studies; thus, Muse cells are highly expected to be safe for clinical use.

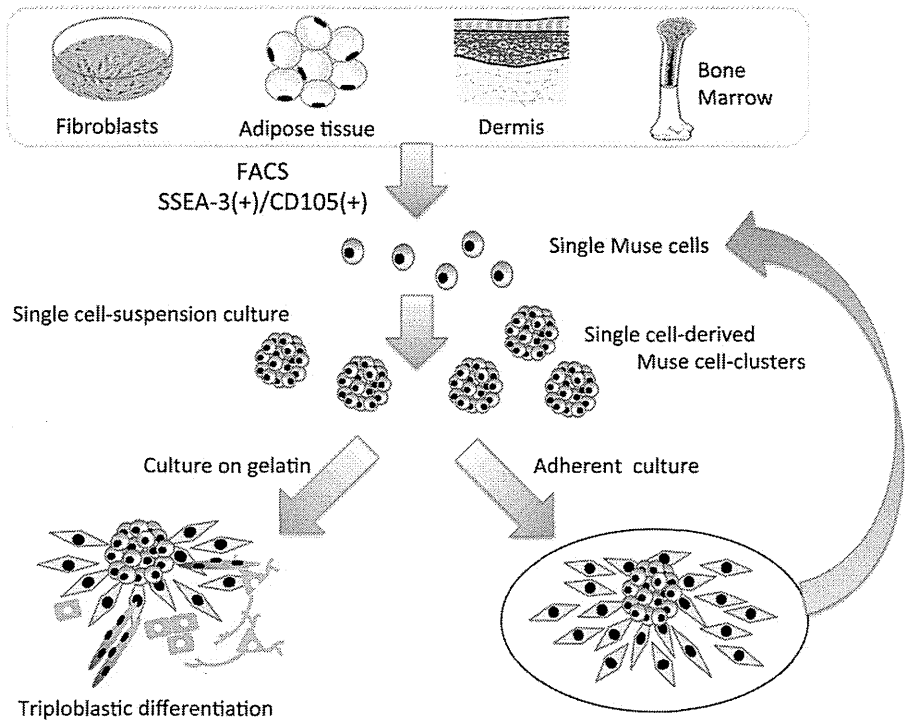

Figure 1 Pluripotency of Muse cells.
Muse cells can be collected from cultured mesenchymal cells (for example, fibroblasts and bone marrow mesenchymal stem cells (BM-MSCs)) and mesenchymal tissues (adipose tissue, dermis and bone marrow) as cells double-positive for SSEA-3 and CD105. After isolating Muse cells by fluorescence-activated cell sorting, single Muse cells cultured in suspension (single cell-suspension culture) generate characteristic clusters that are very similar to the embryoid bodies formed by human embryonic stem (ES) cells. When the cell clusters are transferred onto gelatin culture and spontaneous differentiation is induced, cells with endodermal- (i.e., hepatocytes), ectodermal- (neuronal cells), and mesodermal- (skeletal muscle cells) lineage are observed. The rest of the clusters were individually transferred to adherent culture and allowed to proliferate for 7 to 10 days, after which they underwent a second round of single cell-suspension in culture to generate second generation clusters. This experimental cycle was repeated three times, demonstrating that Muse cells maintain self-renewal, as well as triploblastic differentiation ability up to the third generation.

6 Muse cells have a proliferation rate of ~1.3 day/cell division, slightly slower than that of fibroblasts in adherent culture, so a large number of Muse cells can be prepared.[14]
7 Muse cells act as repair cells *in vivo*.[12]

Muse cells have dual aspects

Muse cells belong to MSCs. Therefore, they have nearly all of the properties of MSCs. Unlike general mesenchymal cells, however, Muse cells are pluripotent. These dual aspects of Muse cells are reflected by their expression of cell surface markers; they are positive for both mesenchymal (CD105, CD90 and CD29) and pluripotency (SSEA-3) markers (Fig. 1).[12]

Muse cells are unique, not only in their surface marker expression profile, but also in their behavior and other properties. In adherent culture, they appear similar to fibroblasts, but when they are transferred to a single cell-suspension culture, they can survive and begin to proliferate to form cell clusters that resemble embryonic stem (ES) cell-derived embryoid bodies formed in suspension. Such single cell-derived Muse cell clusters are similar to ES cells in their appearance, and positive for alkaline phosphatase as well as for the pluripotency markers Nanog, Oct3/4, and Sox2. Consistent with the expression of pluripotency markers, cells derived from Muse cell clusters are able to differentiate into endodermal-, ectodermal-, and mesodermal-lineage cells when transferred to gelatin cultures, proving that single Muse cells are able to generate cells representative of all three germ layers.[12,14] Importantly, non-Muse cells in MSCs have only mesenchymal aspects; that is, they do not express pluripotency markers, nor do they survive, proliferate, or form clusters in suspension.[12,14]

Triploblastic differentiation and self-renewal abilities of Muse cells

Muse cells are pluripotent stem cells because they can generate endodermal-, mesodermal- and ectodermal-lineage cells

from a single cell and to self-renew (Fig. 1). The markers of each lineage into which Muse cells are able to differentiate are: ectodermal- (neural markers such as nestin, NeuroD, Musashi, neurofilament, microtubule associated protein-2, and markers for melanocytes such as tyrosinase, microphthalmia-associated transcription factor, gf100, tyrosinase-related protein 1, and dopachrome tautomerase[13,19]), mesodermal- (brachyury, Nkx2.5, smooth muscle actin, osteocalcin, oil red-(+) lipid droplets, and desmin[12,13]), and endodermal-lineages (GATA-6, α-fetoprotein, cytokeratin-7, and albumin[12,13]). Expression of these markers is recognized under both spontaneous differentiation on gelatin and cytokine induction systems.

With regard to ectodermal differentiation, Tsuchiyama *et al.* recently demonstrated that human dermal fibroblast-Muse cells could cross the boundary between mesodermal and ectodermal-lineages and efficiently differentiate into functional melanin-producing melanocytes by applying a cocktail of cytokines, including Wnt3a, stem cell factor, endothelin-3, and basic fibroblast growth factor, while the remainder of the fibroblasts, non-Muse cells, could not differentiate into melanocytes at all.[19] Muse cell-derived melanocytes expressed the melanocyte markers tyrosinase and microphthalmia-associated transcription factor, were positive for 3,4-dihydroxy-L-phenylalanine, an indicator of melanin production, and maintained their melanin-producing activity in the basal layer of the epidermis when transplanted into the skin. Together, these results demonstrated the absolute superiority of Muse cells over non-Muse cells in terms of pluripotency.

Although Muse cells are pluripotent, they tend to differentiate more frequently into their background lineage; they spontaneously differentiate into mesodermal-lineage cells with a higher percentage (10–15%) than into ectodermal (3–4%) or endodermal (3–4%)-lineage cells.[12]

The ratio of spontaneous differentiation of Muse cells is not very high, but an induction system with a certain combination of cytokines and trophic factors directs their differentiation more efficiently. For example, when Muse cells are treated with hepatocyte growth factor, fibroblast growth factor-4, and dexamethasone in insulin-transferrin-selenite medium, more than 90% of the cells become hepatocyte-like cells that express alpha-fetoprotein and human albumin[13] Muse cells treated with Neurobasal medium supplemented with B-27, basic fibroblast growth factor, and brain-derived neurotrophic factor differentiate into neuronal cells that are positive for MAP-2 and neurofilament.[13] In osteocyte or adipocyte induction medium, nearly 98% of Muse cells differentiate into cells positive for osteocalcin or oil-red, respectively.[13] In this manner, mesodermal-, ectodermal-, or endodermal-lineage cells can be more efficiently obtained from Muse cells, depending on the induction system. More importantly, none of the above differentiations requires the introduction of exogenous genes, and thus Muse cells produce the desired cells with lower risk.

Muse cells are self-renewable. When half of the first-generation clusters formed from Muse cells in single cell-suspension culture were transferred individually onto a gelatin culture and expanded, the expression of endodermal (alpha-fetoprotein, GATA-6), mesodermal (Nkx2.5), and ectodermal markers (MAP-2) was observed. The remaining clusters were individually transferred to an adherent culture and allowed to proliferate, after which they underwent a second round of single cell-suspension in culture to generate second generation clusters (Fig. 1). This experimental cycle was repeated up to three times and clusters from each step were analyzed. Expression of the above genes was detected in first, second, and third generation clusters, demonstrating that Muse cells maintain the gene expression profile required for self-renewal, as well as triploblastic differentiation ability.[16]

Non-tumorigenicity of Muse cells

When Muse cells are compared with tumoirgenic pluripotent stem cells such as ES and induced pluripotent stem (iPS) cells, the repertoire of the genes related to pluripotency, including Nanog, Oct3/4, and Sox2, expressed in Muse cells is similar to that of ES and iPS cells, while the expression level of those factors in Muse cells is lower compared to ES and iPS cells. Compatible with their tumorigenic activity, ES and iPS cells have high levels of telomerase activity as well as high expression levels of genes related to cell-cycle progression compared with Muse cells, which have the same low levels as naive fibroblasts.[13]

In sharp contrast with Muse cells, non-Muse cells do not originally express pluripotency genes. Expression levels of genes related to cell-cycle progression are similar between Muse and naive fibroblasts.[13]

Embryonic stem and iPS cells are known to form teratomas when transplanted *in vivo*. In fact, teratomas form when those cells are transplanted into the testes of immunodeficient mice.[12,20] In contrast, Muse cells do not develop into teratomas *in vivo*. Even after 6 months, none of the Muse cell-transplanted immunodeficient mouse testes formed teratomas (Fig. 2).[12,20] Together these results support that Muse cells are pluripotent but with non-tumorigenic and low telomerase activities. The non-tumorigenicity of Muse cells is considered to be consistent with the fact that they reside in normal adult mesenchymal tissue.

Ability of Muse cells to spontaneously repair damaged tissues *in vivo*

For application of ES and iPS cells to regenerative medicine in humans, two major conditions are required: (i) the cells must be differentiated into objective cells in a cell processing

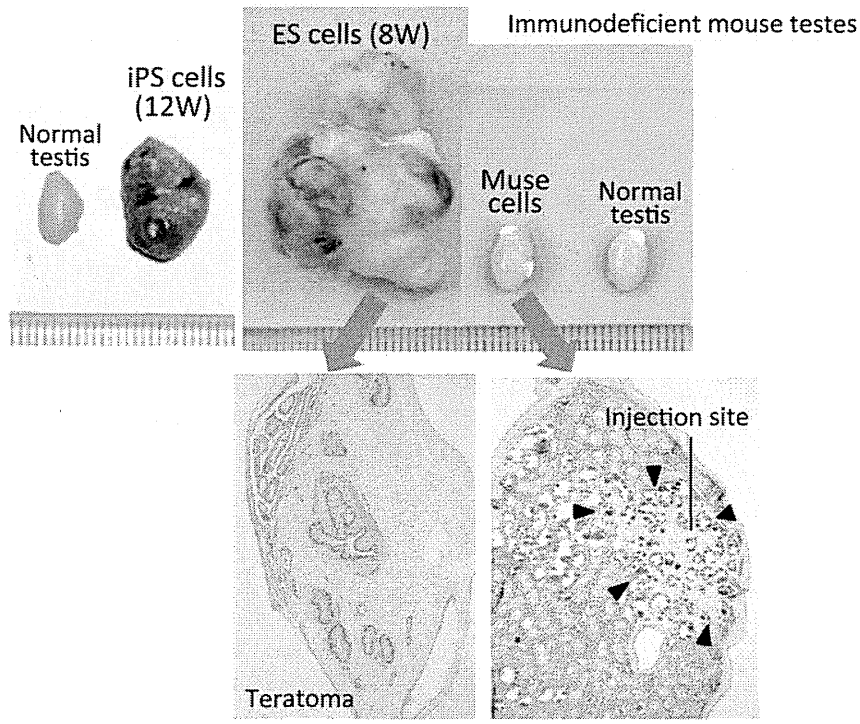

Figure 2 Muse cells are non-tumorigenic. When embryonic stem (ES) or induced pluripotent stem (iPS) cells were infused into immunodeficient mice (SCID mice) testes, they formed teratomas within 8 to 12 weeks while none of the Muse cell-transplanted testes generated teratomas and instead maintained normal tissue structure. (pictures reproduced from *Proc Natl Acad Sci USA* 2010; **107**: 8639–43 and *Proc Natl Acad Sci USA* 2011; **108**: 9875–80) (12,13).

center; and (ii) undifferentiated cells must be eliminated from the differentiated population before transplantation. These prerequisite conditions are based on the fact that undifferentiated ES and iPS cells have tumorigenic activity. As mentioned above, directly transplanted undifferentiated ES or iPS cells may form tumors *in vivo*. Furthermore, even if differentiation induction with high efficiency could be realized, some undifferentiated cells will remain.

For Muse cells, however, the above two conditions are not required. One possible scheme is that naive Muse cells can be applied directly to patients. Muse cells have the ability to migrate and integrate into the site of damage and then spontaneously differentiate into cells compatible with the tissue they target (Fig. 3). Such differentiation is observed in mesodermal, ectodermal, and endodermal tissues, and the Muse cells can act as 'repairing cells' in a wide spectrum of tissues and organs as described below.[12] Because differentiation and repair are induced spontaneously by Muse cells themselves, there is no need to control their differentiation prior to transplantation. Furthermore, as Muse cells are inherently non-tumorigenic and have low telomerase activity, it is not necessary to eliminate undifferentiated naive Muse cells. Ultimately, a cell processing center and complex systems are not necessary for Muse cell therapy.

The repairing effect of naive Muse cells is most striking in acute damage models. This was demonstrated by the infusion of green fluorescent protein-labeled naive human Muse cells into immunodeficient mouse (SCID mouse) models with fulminant hepatitis, skeletal muscle degeneration, spinal cord injury and skin injury. (Fig. 4)[12,21] Naive human Muse cells infused into the bloodstream of mouse models targeted damaged sites and differentiated into hepatocytes (positive for human albumin), skeletal muscle cells (human dystrophin), neuronal cells (neurofilament), and keratinocytes (cytokeratin 14), respectively (Fig. 4). The findings revealed that Muse cells can differentiate into ectodermal- (neuronal cell, keratinocytes), endodermal- (hepatocytes), and mesodermal-lineage cells (skeletal muscle cells) that are compatible with the targeted tissue and contribute directly to tissue repair.

While some infused Muse cells were trapped in the lung, the majority integrated into damaged tissues but not into intact tissues.[12] This suggests that disruption of blood vessels and destruction of tissues in damaged tissue are required for naive Muse cells to migrate and target, and thus Muse cells are able to perceive damage signals produced by damaged tissues. After integration, Muse cells differentiate into tissue-specific cells, but the factors that define the microenvironment of the site, which instruct the Muse cells how to differentiate correctly, remain unclear. Further elucidation of signals responsible for Muse cell migration and differentiation is needed.

DIFFERENT ROLES OF MUSE CELLS AND NON-MUSE CELLS IN MSCS

Although the action of MSCs is considered pleiotropic, recent findings of Muse cells are expected to elucidate the various

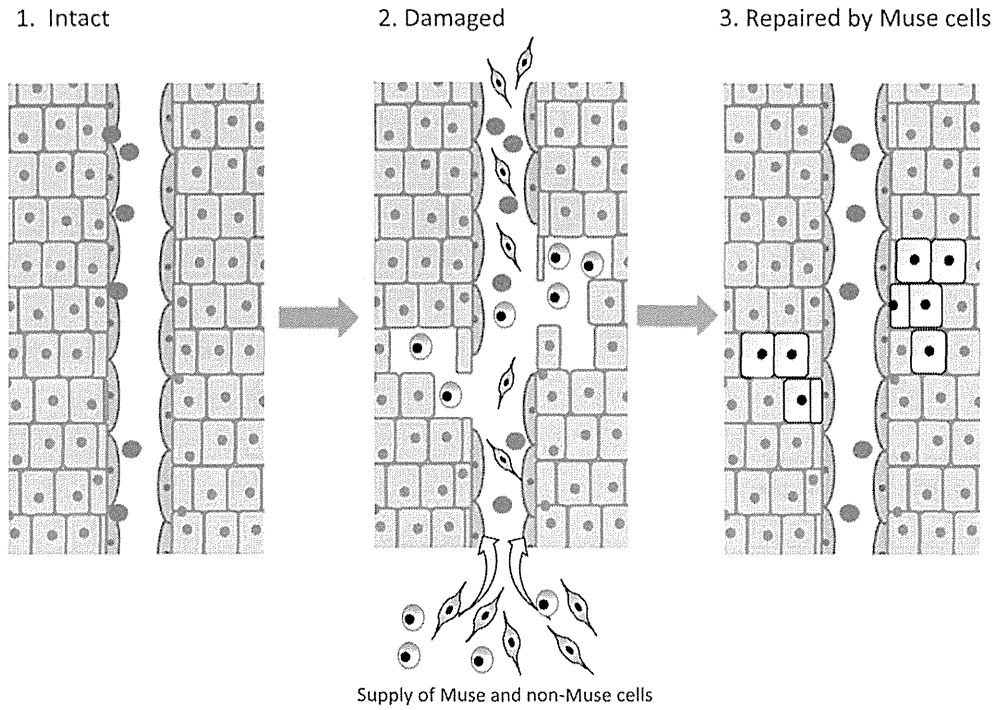

Figure 3 Tissue repair effect delivered by Muse cells. ●, red blood; ◉, Muse cells; ⌒, non-Muse cells.
When Muse and non-Muse cells were supplied to the blood stream, only Muse cells integrate into the damaged site, differentiate, and repaired the tissue, while non-Muse cells do not remain in the damaged 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*.

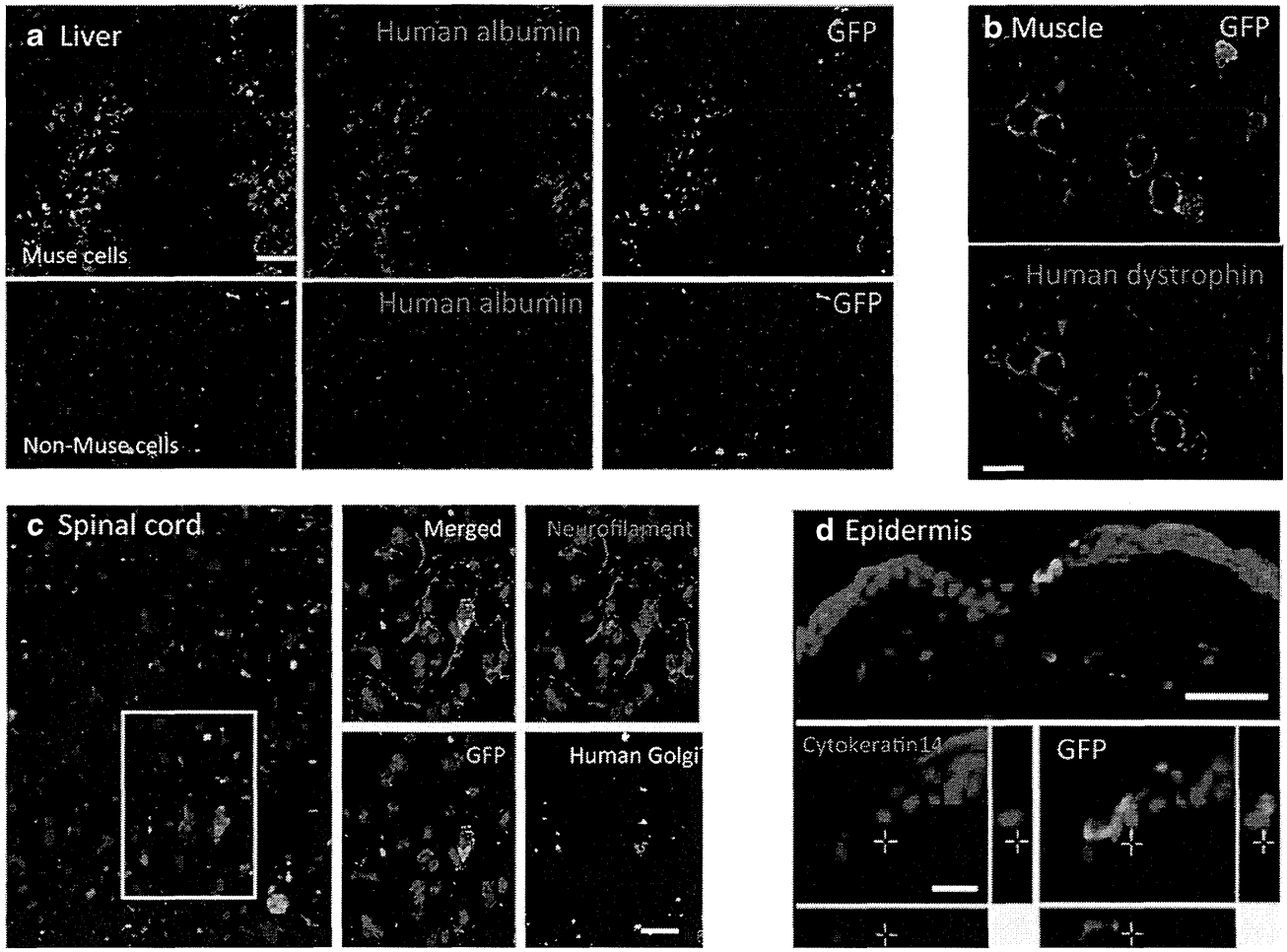

Figure 4 Tissue repair effect of Muse cells.
Green fluorescent protein (GFP)-positive human Muse cells integrated into (**a**) fulminant hepatitis, (**b**) muscle degeneration, (**c**) spinal cord injury (made by crush injury), and (**d**) skin injury models, and became (**a**) human albumin-, (**b**) 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.

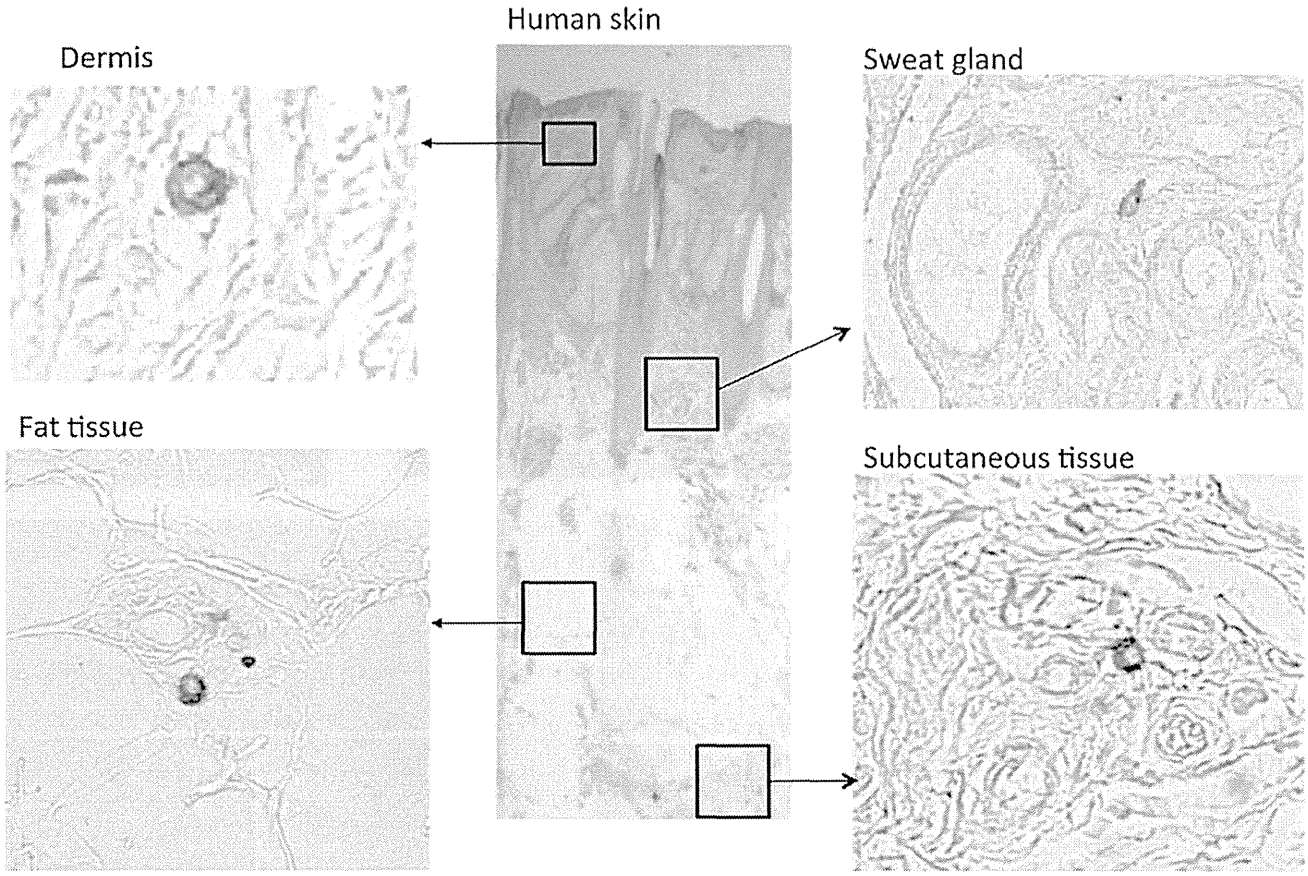

Figure 5 Muse cells sparsely locate in the adult human skin connective tissue.
Muse cells labeled by SSEA-3 are sparsely detected in the connective tissue of the dermis, sweat glands, adipose tissue and hypodermis. (pictures reproduced from *Proc Natl Acad Sci USA* 2011; **108**: 9875–80).[13]

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.

ACKNOWLEDGMENTS

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

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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, 297:98–110, 2014. © 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

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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