Immunomodulatory properties of stem cells from human exfoliated deciduous teeth

Yamaza et al.
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Abstract

Introduction: Stem cells from human exfoliated deciduous teeth (SHED) have been identified as a population of postnatal stem cells capable of differentiating into osteogenic and odontogenic cells, adipogenic cells, and neural cells. Herein we have characterized mesenchymal stem cell properties of SHED in comparison to human bone marrow mesenchymal stem cells (BMMSCs).

Methods: We used in vitro stem cell analysis approaches, including flow cytometry, inductive differentiation, telomerase activity, and Western blot analysis to assess multipotent differentiation of SHED and in vivo implantation to assess tissue regeneration of SHED. In addition, we utilized systemic SHED transplantation to treat systemic lupus erythematosus (SLE)-like MRL/lpr mice.

Results: We found that SHED are capable of differentiating into osteogenic and adipogenic cells, expressing mesenchymal surface molecules (STRO-1, CD146, SSEA4, CD73, CD105, and CD166), and activating multiple signaling pathways, including TGFβ, ERK, Akt, Wnt, and PDGF. Recently, BMMSCs were shown to possess an immunomodulatory function that leads to successful therapies for immune diseases. We examined the immunomodulatory properties of SHED in comparison to BMMSCs and found that SHED had significant effects on inhibiting T helper 17 (Th17) cells in vitro. Moreover, we found that SHED transplantation is capable of effectively reversing SLE-associated disorders in MRL/lpr mice. At the cellular level, SHED transplantation elevated the ratio of regulatory T cells (Tregs) via Th17 cells.

Conclusions: These data suggest that SHED are an accessible and feasible mesenchymal stem cell source for treating immune disorders like SLE.
immunomodulatory function as seen in BMMSCs. In this study, we compare immuno-regulatory properties between SHED and BMMSCs and utilize SHED transplantation to treat SLE-like diseases in a murine model.

Materials and methods
Mice
C57BL/6J and C3MRL-Fas\(^{lpr}\) (MRL/\(^{lpr}\)) mice (female, six- to seven-week-old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Beige nude\(^{nude}\) Xid (III) mice (female, 8- to 12-week-old) were purchased from Harlan (Indianapolis, IN, USA). All animal experiments were performed under an institutionally approved protocol for the use of animal research (University of Southern California protocol #10874 and #10941).

Human tooth, bone marrow and peripheral blood samples
Human exfoliated deciduous incisors were obtained as discarded biological samples from children (six- to eight-year-old) at the Dental Clinic of the University of Southern California following the approved Institutional Review Board guidelines. Healthy bone marrow aspirates from iliac bone and peripheral blood mononuclear cells (PBMNCs) of healthy volunteers were purchased from AllCells (Berkeley, CA, USA).

Isolation and culture of SHED and BMMSCs
Mononuclear cells isolated from the remnant dental pulp tissue of the deciduous incisors were cultured as reported previously [21, 24]. BMMSCs culture was described previously [25]. The detailed protocols were described in Additional file 1.

Cell surface markers analysis
The procedure for single colored flow cytometry (FCM) was performed as described previously [26, and Additional file 1]. The samples were analyzed on a FACS\(^{Calibur}\) flow cytometer (BD Bioscience, San Jose, CA, USA). Some cells were used for immunoblotting and immunofluorescent staining.

Colony forming units-fibroblastic (CFU-F) assay
CFU-F assay was performed according to a previous study [27, and Additional file 1].

Cell proliferation assay
The proliferation of each MSC population was performed by bromodeoxyuridine (BrdU) incorporation assay as previously described [21, 27 and Additional file 1].

Telomerase activity assay
Telomerase activity was evaluated by telomeric repeat amplification protocol (TRAP) assay using real-time polymerase chain reaction (PCR) [28, and Additional file 1].

In vitro osteogenic induction assay
Osteogenic differentiation assays of SHED and BMMSCs were performed according to previous publications [21, 28]. Osteogenic markers and mineralized nodule formation were assessed as described previously [21, 28 and Additional file 1].

Adipogenic induction assay in vitro
Adipogenic assay in vitro of each stem cell population was performed as described previously [21, 28, and Additional file 1].

In vivo osteogenic differentiation
Xenogeneic transplantation was performed using immuno-compromised mice as described [21, 25, 26]. Each MSC population was subcutaneously transplanted into beige nude\(^{nude}\) Xid (III) mice using hydroxyapatite tricalcium phosphate (HA/TCP) as a carrier. Eight weeks post-transplantation, the transplants were harvested for histological analysis. Detail methods were described in the Additional file 1.

Immunoblot analysis
Ten µg total protein was loaded and analyzed by immunoblotting as previously described [21, 28, and Additional file 1].

Co-culture of human PBMNCs or T lymphocytes with SHED or BMMSCs
PBMNCs or T cells were co-cultured with or without SHED or BMMSCs under several culture conditions as described in Additional file 1. Cell death analysis and induction of Tregs and Th17 cells were described in Additional file 1.

Xenogeneic SHED or human BMMSCs into MRL/\(^{lpr}\) mice
Under general anesthesia, SHED or BMMSCs (1x10\(^5\) cells/10g body weight in 100 µl PBS) were infused into MRL/\(^{lpr}\) mice via tail vein at 16 weeks (n = 3) according to previous study [17]. MRL/\(^{lpr}\) mice (16-week-old) received physiological saline (n = 3) were used as experimentally control mice. All mice were sacrificed at 20 weeks of age, and from them were collected peripheral blood, kidney, and long bones (femur and tibiae).

FCM analysis of Treg and Th17 cells
Flow cytometric staining and analysis were performed as previously reported [29, and Additional file 1].

Measurement of biomarkers in culture supernatant, blood serum and urine
Several biomarkers, including anti-dsDNA antibody and anti-nuclear antibody ANA, complement 3 (C3), interleukin 6 (IL6), IL10, IL17, soluble receptor activator for...
nuclear factor κB ligand (sRANKL), and C-terminal telopeptides of type I collagen (CTX), creatinine, urine protein in biofluid samples (peripheral blood serum and urine) were measured by enzyme linked immunosorbent assay (ELISA) [17, and Additional file 1].

**Histological analysis of kidney and bone**

Kidneys and long bones (femurs) harvested from mice were fixed and processed to make paraffin sections. The sections were used for further experiments [Additional file 1].

**Histometry**

Histomorphometric analysis was quantified as described previously [25]. Detailed methods were described in Additional file 1.

**Statistics**

All data are expressed as the mean ± SD of, at least, triplicate determinations. Statistical difference between the values was examined by Student’s t-test. The P values less than 0.05 were considered significant.

**Antibodies and primer**

All primary antibodies used in this study were described in Additional file 1 and listed on Table S1 in Additional file 1. All primer pairs were listed in Table S2 in Additional file 1.

**Results**

**SHED possess mesenchymal stem cell properties**

Although SHED are capable of differentiating into a variety of cell types [21], their detailed mesenchymal stem cell properties remain to be elucidated. Herein, we used flow cytometry, immunoblot analysis, and immunocyto staining analysis to demonstrate that SHED at passage 3 expressed many mesenchymal surface markers, including STRO-1, SSEA4, CD73, CD105, CD146, and CD166 but were negative for CD34 and CD45 (Figures 1A-1C). In comparison to BMMSCs, SHED expressed significantly higher levels of STRO-1 and CD146, and lower levels of CD105 (Figure 1A). Additionally, SHED showed significantly high numbers of single colony clusters (colony-forming units-fibroblastic; CFU-F) and an elevated cell proliferation rate compared to BMMSCs (Figures 1D and 1E). This elevated proliferative capacity may be associated with the significantly increased telomerase activity in SHED (Figure 1F).

To compare osteogenic differentiation of SHED with BMMSCs, multiple colony-derived SHED at passage 3 were supplemented with L-ascorbate-2-phosphate, dexamethasone, and inorganic phosphate to induce mineralization in vitro as described previously [21]. After one week of induction, SHED were similar to BMMSCs, showing significantly increased alkaline phosphatase (ALP) activity (Figure 2A) and the number of ALP-positive cells by flow cytometric analysis (Figure 2B), and expression of elevated levels of ALP, Runt related transcription factor 2 (Runx2), dentin sialoprotein (DSP), and osteocalcin (OCN) by immunoblot analysis (Figure 2C). Alizarin Red-positive nodule formation in SHED and BMMSC cultures was notified after four weeks of osteogenic induction, indicating calcium accumulation in vitro (Figures 2D and 2E). However, SHED suffered remarkable impairment of adipogenic differentiation, as shown by decreased numbers of lipid-specific Oil red O-positive cells and reduced expression of adipocyte-specific molecules, peroxisome proliferator-activated receptor γ2 (PPARγ2) and lipoprotein lipase (LPL) when compared to BMMSCs (Figures 2F-2H). To validate the capacity of forming mineralized tissue in vivo by SHED, ex vivo expanded-SHED were transplanted into immunocompromised mice with hydroxyapatite/tricalcium phosphate (HA/TCP) as a carrier. SHED formed a similar amount of mineralized tissue and a reduced amount of hematopoietic marrow components when compared to BMMSC transplants (Figures 2I-2K). Next, we confirmed that SHED were similar to BMMSCs in activation of multiple signaling pathways, including TGFβ, ERK, Akt, Wnt, and PDGF (Figures 2L-2P).

**Interplays between SHED and T-lymphocytes**

In order to compare the immunomodulatory capacity of SHED with BMMSCs, anti-CD3/CD28 antibodies with TGFβ/IL-6 were added to the co-cultures of SHED or BMMSCs with naïve T cells, which were purified from human PBMCNs. Levels of IL17+IFNg Th17 cells and IL17 were significantly reduced in SHED and BMMSC groups compared to the naïve T cell group (Figure 3A). It appeared that SHED showed a significant inhibiting effect in reducing IL17 levels when compared to BMMSCs (Figure 3B). Our previous report indicated that activated T cells induce apoptosis of BMMSCs through the Fas/FasL pathway [28]. To determine whether activated T cells also directly impinge on SHED, as occurs in BMMSCs, SHED were co-cultured with human PBMCNs activated by anti-CD3 specific antibody treatment. We found that the activated PBMCNs were able to induce part of SHED death in the co-culture system (Figure 3C). When SHED were separated from PBMCNs using a Transwell co-culture system or treated using anti-FasL neutralizing antibody, SHED failed to show the cell death (Figure 3C), suggesting that direct cell-cell contact and the Fas/FasL pathway are required for inducing SHED death by activated splenocytes. Next, we confirmed that SHED express Fas by immunoblot analysis (Figure 3D).
dUTP-biotin nick end labeling (TUNEL) staining was used to confirm that the SHED death was due to cell apoptosis (Figure 3E).

**SHED transplantation improves SLE phenotypes in MRL/lpr mice**

Our previous study showed that systemic infusion of BMMSCs offers appropriate treatment for SLE disorders in human patients and SLE-like MRL/lpr mice [17]. Here we selected SLE-like mice at 16 weeks of age to infuse SHED for treating SLE disorders using BMMSCs as a control (Figure 4A). It is known that autoantibodies play a crucial role in SLE patients. Our previous study showed a remarkable increase in the levels of autoantibodies including anti-double strand DNA (dsDNA) IgG and IgM antibodies, and anti-nuclear antibody (ANA) in the peripheral blood [17]. As seen in BMMSC transplantation, SHED transplantation resulted in a significant reduction in serum levels of anti-dsDNA IgG and IgM, and ANA antibodies (Figures 4B-4D).

Histological analysis with hematoxylin and eosin, trichrome, and periodic acid-Schiff staining revealed that
SHED transplantation was similar to BMMSC transplantation in recovery of SLE-associated renal disorders, such as nephritis with glomerular basal membrane disorder and mesangial proliferation in MRL/lpr mice (Figure 4E). ELISA data showed that SHED and BMMSC transplantation was able to reduce the urine C3 level and elevate the serum C3 level (Figure 4F). Also, SHED transplantation significantly reduced urine protein levels compared to BMMSC transplantation (Figure 4G). Moreover, SHED and BMMSC transplantation significantly elevated creatinine levels in urine and reduced creatinine levels in serum (Figure 4H). This experimental evidence indicated that SHED transplantation is an effective approach for treating SLE disorders.
SHED transplantation regulates ratio of Tregs and Th17 cells

Tregs prevent pathogenic autoimmunity by suppressing proliferation and production of pro-inflammatory cytokines in effector immune cells, such as helper T-lymphocytes [30]. In contrast, Th17 cells that produce IL17 are inflammatory cells responsible for the pathogenesis of autoimmune diseases [31] and bone destruction [32]. Our previous study suggested that BMMSC transplantation affects the immune balance between Tregs and Th17 cells in SLE-like disorders [17]. Here we found SHED transplantation regulated the ratio of Tregs and Th17 cells in comparison to BMMSC transplantation in MRL/lpr mice (Figures 5C and 5F). Our previous study suggested that BMMSC transplantation-mediated therapy in SLE-like mice may associate with the reconstructing trabecular bone [17]. Here we found SHED were also capable of reconstructing trabecular bone in MRL/lpr mice (Figure 6A). In contrast to BMMSC/osteoblast lineage, osteoclasts play a significant role in the maintenance of bone homeostasis by the bone resorption function. We compared SHED transplantation with BMMSC transplantation in inhibiting osteoclast activity in MRL/lpr mice and found that both SHED and BMMSC transplantation were able to reduce the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts in the distal femur epiphysis of MRL/lpr mice (Figure 6B), serum levels of runt-related NF-κB ligand (RANKL), a critical factor for osteoclastogenesis (Figure 6C), and bone resorption marker C-terminal telopeptides of type I collagen (CTXs) as compared to untreated MRL/lpr mice (Figure 6D).

**Discussion**

BMMSCs have been successfully utilized to treat a variety of human diseases, such as bone fracture [33], severe aplastic anemia [34], acute GVHD [13], and SLE [17]. SLE is a common and potentially fatal immune disease in humans...
which autoantibodies damage multiple organs, including the kidneys, cardiovascular system, nervous system, joints, and skin [35]. The pathology of SLE involves the destruction of targeted organ tissues and accumulation of auto-reactive lymphocytes and immune complexes. Although intensity and organ involvement vary significantly among SLE patients, abnormalities of T and B lymphocytes are universal [35-37]. Moreover, SLE provokes multifaceted immune modulation, including both deficiency and hyperactivity of the immune system. An understanding of the underlying pathology is crucial to developing optimal therapies for the restoration of immune homeostasis without compromising the protective immune responses to pathogens [38]. MRL/lpr mice were generated by the insertion of the early transposable element ETn in the Fas gene, which causes a striking reduction in Fas mRNA expression and is associated clinically with marked acceleration of the lupus-like disease [39]. Levels of circulating immune complexes rise enormously from about three months of age in MRL-1pr/1pr but not in MRL mice. In this study, we used MRL/lpr mice as a SLE mouse model to indicate that SHED are an appropriate population of postnatal stem cells for SLE treatment as seen in BMMSC-mediated therapy.

SHED are derived from a very accessible tissue resource and capable of providing enough cells for potential clinical application via high proliferation rate and expression of telomerase [21]. The reason that SHED provokes multifaceted immune modulation, including both deficiency and hyperactivity of the immune system. An understanding of the underlying pathology is crucial to developing optimal therapies for the restoration of immune homeostasis without compromising the protective immune responses to pathogens [38]. MRL/lpr mice were generated by the insertion of the early transposable element ETn in the Fas gene, which causes a striking

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**Figure 4.** SHED transplantation reduced levels of autoantibodies and improved renal function in MRL/lpr mice. Figure 4A shows the scheme of SHED and BMMSC transplantation procedures. (B-D) ELISA quantified that levels of anti dsDNA IgG (B), IgM (C) and nuclear (D) antibodies (ANA) (mean ± SD) were significantly reduced in the peripheral blood of SHED and BMMSC treated MRL/lpr mice (n = 6) when compared to un-treated MRL/lpr mice (n = 6). (**P < 0.001). It appeared that SHED transplantation resulted in a more significant reduction in anti IgG when compared to BMMSC transplantation (B). (E) MRL/lpr mice showed renal disorders such as nephritis with glomerular basal membrane disorder and mesangium cell over-growth. SHED and BMMSC transplantation resulted in a reduced basal membrane disorder and mesangium cell over-growth in glomerular (G) (upper panels, H&E staining; middle panels, trichrome staining; lower panels, periodic acid-schiff staining). Representative images of un-treated, SHED and BMMSC MRL/lpr mice. (F) ELISA analysis showed that SHED transplantation has the same effect as seen in BMMSC transplantation in significantly reducing C3 level in urine and elevating C3 level in serum (n = 6, *P < 0.05, **P < 0.01). (G) SHED transplantation significantly reduced urine protein levels (mean ± SD) compared to BMMSC transplanted MRL/lpr mice (n = 6). (***P < 0.001). (H) Markedly increased urine creatinine and reduced serum creatinine were observed in SHED and BMMSC transplanted MRL/lpr mice (n = 6) compared to un-treated MRL/lpr mice (n = 6, ***P < 0.001, **P < 0.01).
Figure 5. The ratio of Tregs and Th17 cells may contribute to SHED mediated treatment in MRL/lpr mice. (A-C) Flow cytometric analysis showed that the number of CD25+Foxp3+ Tregs in CD4+ T lymphocytes of MRL/lpr spleen was not significantly changed in SHED and BMMSC transplantation (A). In contrast, SHED and BMMSC transplantation were capable of significantly reduced levels of CD4+IL17+ cells in spleen as compared to un-treated MRL/lpr mice (B). SHED transplantation significantly increased the ratio of Tregs and Th17 cells when compared to BMMSC transplantation group (C) (**P <0.01, *P <0.05). Results were shown as mean ± SD from un-treated, SHED and BMMSC MRL/lpr (n = 6).

(D-F) Although SHED and BMMSC transplantations failed to alter IL10 (D) and IL6 (E) levels in serum of MRL/lpr mice, IL17 levels were significantly down-regulated in SHED and BMMSC transplanted group compared to un-treated MRL/lpr mice (F). Results were shown as means ± SD from un-treated, SHED and BMMSC MRL/lpr (n = 6).

Figure 6. SHED transplantation reconstructed trabecular bone and inhibited osteoclast activity. (A) SHED transplantation showed the same effect in regenerating trabecular bone as seen in BMMSC transplanted MRL/lpr mice (n = 6) (**P <0.01). (B) TRAP staining showed that the number of TRAP positive osteoclasts was significantly reduced in SHED and BMMSC transplanted mice (n = 6, *P <0.05). (C, D) ELISA revealed that SHED and BMMSC transplantations were capable of significantly reducing the levels (mean ± SD) of soluble RANKL (sRANKL) (C) and C-terminal telopeptides of type I collagen (CTX) (D) in serum of MRL/lpr mice (n = 6) (*P <0.05, **P <0.01).
cell levels in peripheral blood. In addition, SHED transplantation, as seen in BM-MSC transplantation, is capable of recovering trabecular bone and inhibiting osteoclast activity, suggesting that SHED transplantation, as seen in BM-MSC transplantation, could lead the reconstruction of osteoelastic niche to improve SLE disorders in SLE patients and a SLE-like murine model [17]. Therefore, SHED may be an appropriate stem cell resource for treating immune disorders via improved immunomodulatory properties. Systemic infusion of SHED fails to show a significant promoting Treg level in SLE-like mice as seen in an in vitro co-culture system, which may be associated with a complex in vivo condition that hardly compares to a simple co-culture system. However, SHED infusion resulted in a significantly up-regulated level of the ratio between Tregs and Th17 cells. This is an important index indicating immunomodulatory function of SHED due to the fact that Tregs prevent autoimmunity and Th17 cells promote autoimmunity and inflammation [40].

The transition from deciduous teeth to adult permanent teeth is a unique and dynamic process in which the development and eruption of permanent teeth is coordinated with the resorption of deciduous teeth. We found that exfoliated deciduous tooth crowns contain a remnant of living pulp comprised of a normal dental pulp structure, including connective tissue, blood vessels, and odontoblasts [21]. We demonstrated that these remnants of pulp tissues in exfoliated deciduous teeth contain SHED [21]. These studies provide the first evidence that a naturally occurring exfoliated organ contains stem cells with the ability to form multiple phenotypes, and that these stem cells may offer a unique stem cell resource for potential clinical applications. SHED are very easily acquired from exfoliated teeth and can be expanded ex vivo to achieve sufficient numbers of cells for tissue regeneration such as repairing parietal defects [24].

Conclusions

SHED possess similar stem cell properties as those seen in BM-MSCs, including osteo/odontogenic and adipogenic differentiation in vitro, forming mineralized tissue in vivo, and expression of extensive mesenchymal stem cell markers. Moreover, systemic SHED transplantation is capable of offering similar, if not better, therapeutic effect on SLE murine model, suggesting that easily accessed SHED may be a feasible stem cell source for stem cell therapies.

Additional file 1. Supplementary Materials and methods and 2 supplementary tables. A PDF file containing supplementary Materials and methods and 2 supplementary tables: Table S1 displays information on antibodies; and Table S2, lists PCR primers.
Yamaza et al. Stem Cell Research & Therapy 2010, 1:5
http://stemcellres.com/content/1/1/5

Page 10 of 10

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