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Sca-1and Thy-1 Accelerate Neuron-like Differentiation in Bone Marrow Stromal Cells

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Bone marrow stromal cells taken from EGFP transgenic mice were sorted by magnetic beads with surface markers for Sca-1 and Thy-1. The cells were then co-cultured on organotypic hippocampal slice or with neuronal cell feeder in dish. On hippocampus, both Sca-1 and Thy-1 positive cells showed 4- 8 folds higher potential to show neuron-like morphology than negative cells. In dish, negative cells fewly survived but each positive cells survived and showed neuron-like differentiation. In both culture condition, retinoic acid supplement accelerate differentiation. Differentiated Sca-1 and Thy-1 positive cells were immunohistochemically GFAP- and NeuN-negative but nestin-, neurofilament- and NSE-positive. Neuron-like differentiation of bone marrow cells can be enhanced by selection using cell surface proteins.

Recent studies have examined whether bone marrow-derived cells can differentiate to neural cell types. The potential to become functioning neurons is unknown but native bone marrow-derived stem cells express many neural-specific genes at the mRNA or protein level [7]. Bone marrow stromal cells (BMSCs) cultured with epidermal growth factor and brain derived neurotrophic factor showed 0.5 –1% of cells express nestin, Neu-N and GFAP [13]. The protocol used for neuronal differentiation consists of various cocktails of growth factors, retinoic acid (RA) and supplements for induction and differentiation of embryonic stem cells or neuronal stem cells into mature neuronal cells. We have previously reported that mouse BMSCs differentiate into neuron-like cells morphologically when co-cultured with hippocampal slice culture with horse serum [1]. Although cell viability could be enhanced by RA in our previous study, only a small proportion of neural cell was generated on hippocampal slice. Among various candidates of surface marker proteins for enhancement of neuronal differentiation, we focused on Sca-1 and Thy-1 population in this study. Sca-1 is one of the surface markers in hematopoietic stem cells that generate parenchymal microglia and perivascular neovascularing cells [4]. Sca-1 is also a marker of stem cell of cardiomyocytes [10] and neural progenitor cells [15]. Thy-1(CD90) is a cell surface glycoprotein found in human neurons and glial cells but found very low frequency in normal bone marrow (less than 0.1% of nucleated cells). It is also a marker of hematopoietic stem cells and has been used to generate neural progenitor cells from adult bone marrow [6]. We also examined neuronal differentiation of sorted BMSCs on feeder cells of pure neurons and glial cells, using the same culture medium and compared neuron-like differentiation between Sca-1 and Thy-1.

MATERIALS AND METHODS

All experimental procedures were conducted according to the Guidelines for Animal Experimentation of Kobe University Graduate School of Medicine. Transgenic mice

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(C57BL16Tg14 (act-EGFP) Osb Y01) were kindly provided by Dr. Masaru Okabe (Osaka University, Japan) [12]. Bone marrow was extracted from the femur and the shinbone of mice. Bone marrow cells were plated on 10 cm plastic dishes in slice culture medium (25% heat inactivated horse serum, 25% Hanks balanced salt solution, 50% MEM w/o L-glutamine, 50 units / ml of penicillin, 50 μg /ml of streptomycin and 0.5 % of glucose). After 24 hours, BMSCs were first separated by their attachment to the plastic culture dish. Then, Sca-1 or Thy-1 positive cells were isolated from BMSCs by immunomagnetic selection using mini MACS Separation Unit (Militenyi Biotec). Briefly, BMSCs were incubated with anti-Sca-1 microbeads (1:10, Miltenyi Biotec) or anti-Thy-1 microbeads (1:10, Miltenyi Biotec) for 20 min at 4 ˚C, then washed with MACS buffer (PBS with 0.5% BSA and 2mM EDTA) and centrifuged at 1000 rpm for 10 min. After filtering through pre-separation filters to remove clumps, cells were placed in the separation column in the magnetic field. Magnetically retained positive cells were eluted and this procedure was performed twice to increase the purity. In the pilot study, the efficiency of cell sorting was evaluated using goat anti-Sca-1 mouse serum (1:100, RD), or goat anti-Thy-1 mouse serum (1:100, RD). More than 80% of sorted cells were positive against anti-Sca-1 serum or anti-Thy-1 serum.

Hippocampus slices were prepared as reported previously [1]. The hippocampus of postnatal day 10 Sprague-Dawley rats were dissected under aseptic conditions and 350 μm slices were cut with a tissue chopper. Five slices were placed on each culture plate using a Millicell-CM 0.4 μm (Millipore, Bedford, USA) . The slices were cultured in 6-well culture clusters at 34 ˚C in 5% CO_2 with 1ml per well of the same culture medium. After 5 days in incubator, an aliquot of Sca-1 or Thy-1 positive cells was applied eventually to each culture plate at a density of 1.5×10^5 cells / millicell. A millicel plate is 314 mm^2 and the area of each hippocampal slice was about 10 mm^2. The estimated number of BMSCs was over 3,000 cells per slice. Sca-1 negative or Thy-1 negative BMSCs was cultured with hippocampal slices under the same conditions, and served as a control.

For preparation of feedercell culture, a cell suspension was made from fetus striatum of Sprague-Dawley rats (embryological day 15, Nihon Clea, Tokyo, Japan). Cells were cultured on coverslips coated by poly-D-lysine in Neurobasal (Gibco, NY, USA) with B-27 supplement for neurons or with heat-inactivated horse serum (20%) for glial cells. An aliquot of 1.0×10^5 Sca-1 or Thy-1 positive BMSCs were applied to each coverslip and co-cultured at 34 ˚C in 5% CO_2 with 30% Hanks balanced salt solution (ICN Biomedicals Inc., Ohio, USA), 60% Minimum Essential Medium without L-gultamine (GIBCO, NY, USA), 10% heat-inactivated horse serum, 50 units / ml of penicillin, 50μg / ml of streptomycin, and 0.5 % glucose. We exchanged medium every 5 days. The estimated number of BMSCs was 4.6×10^4 cells per a coverslip. We added RA (all trans; Sigma, St. Louis, USA) dissolved in DMSO to culture medium and evaluated the promotion of differentiation. RA concentration was 10^{-6} M as the most effective concentration reported previously by our laboratory [1].

Immunocytotochemistry was done after overnight fixation of the hippocampal slices or cover slips with 4% paraformaldehyde in PBS. The primary antibody was anti-nestin (mouse, Chemicon, CA, USA, 1:100), anti-neurofilament-M (rabbit, Chemicon, CA, USA, 1:100), anti-neuron specific enolase (rabbit, polyclonal, Chemicon, CA, USA, 1:100), anti-NeuN (mouse, Chemicon, CA, USA, 1:100) with overnight incubation at 4 ˚C, or anti-GFAP (rabbit, ready-to-use kit, Dako, Japan) for 30min. Secondary antibodies were biotinylated anti-mouse IgG (Leinco, 1:200) for ani-nestin, biotinylated anti-rabbit IgG (Vector, CA, USA, 1:200) for anti-GFAP, and TRITC-conjugated anti-rabbit IgG for anti-NSE and anti-NF-M. Texas red avidin (Leinco, 1:200) was used as a fluorescent marker for nestin. Each antibody was used in the slices or coverslips co-cultured for 4, 7, 10 and 14 days.

Using a confocal laser microscope and a fluorescent microscope up to 28 days, we counted the number of morphologically differentiated cells between days 4 and 28. As we reported previously, counting was done in a blinded manner using an objective 20 × lens of fluorescent microscope. Four culture plates with 20 hippocampal slices and four coverslips were used for each time point. Neuron-like differentiated cells were polygonal, angular (with pointed corners) or spindle shaped with a size ranging between 10 and 70 μm, and had 2 long axon-like processes more than 20 μm. Cells having multiple short processes shorter than 20 μm and round small cells without processes were not counted because they became debris late in co-culture. The number of neuron-like cells were expressed as mean ± S.E.M. per $50mm^2$ of hippocampal slices or coverslips. Results were evaluated with Student's *t* test. Differences between two groups were considered statistically significant when value was less than 0.01 ($p< 0.01$).

RESULTS

Sca-1 positive cells showed higher number of neuron-like cells than Sca-1 negative cells when co-cultuered with hippocampal slice and similar increase was observed with Thy-1 positive cells (Fig. 1 A and B). On culture day 7, Sca-1 positive cells reached the peak with 140 cells per a slice. This increased number was about 4-fold higher than differentiation from non-sorted BMSCs in our previous study [1]. Thy-1 positive cells had less number than Sca-1 positive cells and the highest number of neuron-like cell was obtained on culture day 4 with number of 100 cells per slice.

In co-culture with neuron feeder, neuron-like differentiation of Sca-1 positive cells was around 44 cells per one coverslip on culture day 4, and Thy-1 positive cells showed 79 cells (Fig. 1C and D). The neuron-like cell number increased on day 7 in Sca-1 and showed 78 cells. The chronological change of the neuron-like cell counts on neuron feeder was similar to those on glial cell feeder (Fig. 1E and F). The glial cell feeder, which was expected to release various trophic factors and more favor for neuronal differentiation than neuron feeder, did not accelerate differentiation to neuron-like cell .

Neuron-like differentiation was more distinct on hippocampal slice than cell feeder, showing large cytoplasm and elongated extension of axon-like fibers which had vesicles (Fig. 2). In immunocytochemistry, few differentiated cells from Sca-1 positive or Thy-1 positive showed NF-M and NSE positive and other staining was technically difficult with hippocampal slice due to the tissue thickness (Fig. 3). With the cells on coverslips, neuron-like differentiated cells were nestin-, NF-M- and NSE-positive but GFAP- and Neu-N- negative (Fig. 4). Nestin positive neuron-like cells increased until culture day 4 in Thy-1, culture day 7 in Sca-1. NSE positive cells increased until culture day 7 in Thy-1, culture day 10 in Sca-1. The possibility of the presence of surface immunoglobulin-binding proteins that may give false-positive results with neuronal markers [14] was not detected during our confocal examination with Z-scanning, which would otherwise show a ring staining pattern.

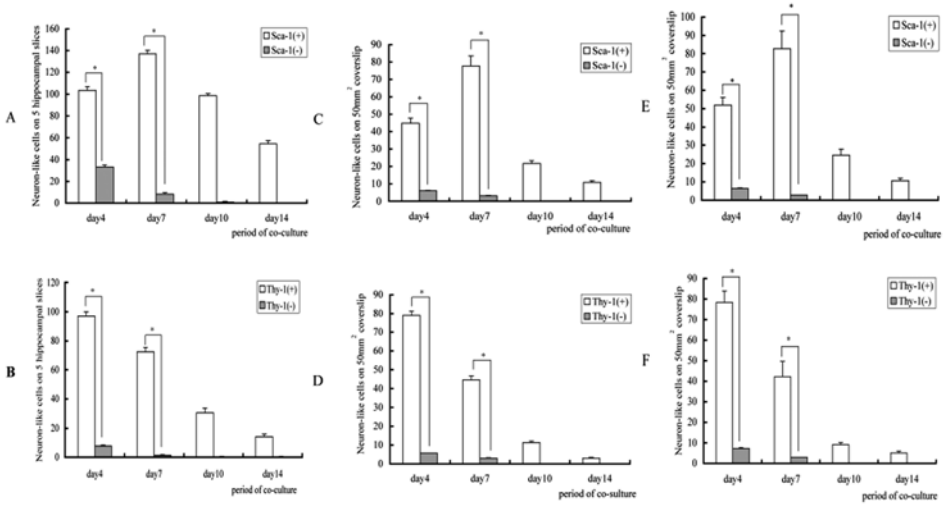


Fig. 1. Mean number ± S.E.M. of neuron-like cells per 5 hippocampal slices differentiated from Sca-1 (+) cells **(A)** and from Thy-1 (+) cells **(B)**. Mean number ± S.E.M. of neuron-like cells per 50 mm^2 coverslips covered with neural cells differentiated from Sca-1 (+) cells **(C)** and from Thy-1 (+) cells **(D)** . Neuron-like cells per 50 mm^2 coverslips covered with glial cells differentiated from Sca-1 (+) cells **(E)** and Thy-1 (+) cells **(F)**. (**P*<0.01)

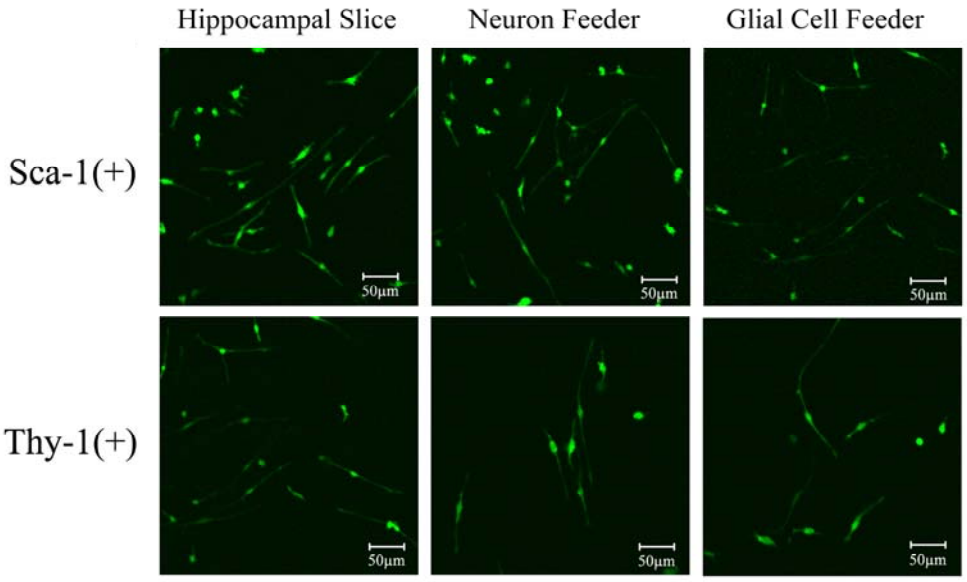


Fig. 2. Morphological differentiation of selected bone marrow stromal cells (BMSCs) into neuron-like cells after co-culture for 7 days.

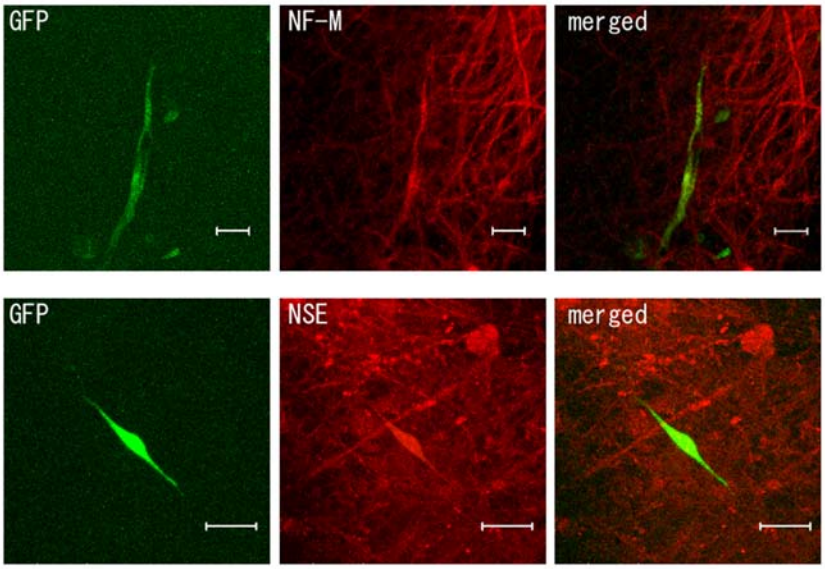


Fig. 3. Immunocytochemistry of differentiated Sca-1 (+) cells on hippocampal slice for 10 days, showing NF-M- and NSE-positive cells. Scale bars in A-F = 20 μm.

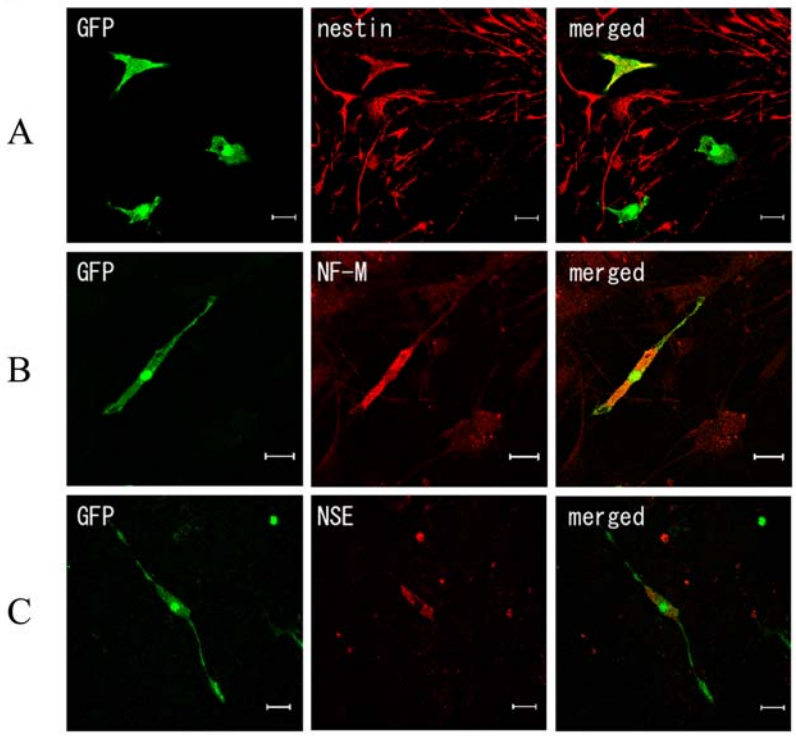


Fig. 4. Representative immunocytochemistry of differentiated cells on cell feeders. **(A)** Nestin-positive cell from Sca-1 (+) cells on neuron feeder at day 4. **(B)** NF-M-positive cell from Sca-1 (+) cells on neuron feeder at day 10. **(C)** NSE-positive cells from Thy-1 (+) cells on glial cell feeder at day 10. Scale bars in A-C = 20 μm.

DISCUSSION

It has been reported that Sca-1 and Thy-1 positive BMSCs can express some neural markers by chemical induction, RA, FGF, BDNF, EGF [8,9]. In this study, we evaluated the behavior of Sca-1 or Thy-1 positive cells in direct contact with neural or glia cells. The differentiation ratio of each Sca-1 and Thy-1 positive cells co-cultured with hippocampal slices was 0.86 % and 0.61%, respectively, and those were 2-3 fold higher than the differentiation of BMSCs in our previous study [1]. A previous study has demonstrated Thy-1- and Sca-1 positive bone marrow cells selected magnetic cell sorting resulted in a higher yield of nestin-positive spheres [8] and our present study is the first to quantify the promotion of those cells into neuron-like differentiation.

Thy-1-positive cells in adult bone marrow cells gave rise to cellular spheres which differentiated into neurons and glia [6] and express the neuronal marker genes such as NSE, beta-tubulin, GAP43 and MAP2 [2]. Those cells acquired astrocyte-like and neuron-like morphologies in specific culture condition. Transplantation of green fluorescent protein labeled bone marrow cells in the neonatal rat brain also showed extensive migration of the cells and expression of hematopoietic identity including CD90 [5].

Sca-1 is a glycosylphosphatidylinositol-linked cell surface maker protein known to enrich for somatic stem cells and found in bone marrow cells, cardiomyocytes and neuronal stem cells. The Sca-1 cell surface marker enriches for a prostate-regenerating cells and has been suggested to have immature cell properties and multilineage differentiation potential [17]. Sca-1 is used for a marker for neuronal stem cells which posses lysophosphatidic acid receptor dependent proliferation [15].

Thy-1 or Sca-1 surface marker is obviously not a specific marker for neuronal differentiation but a marker of multilineage differentiation. Our present study demonstrated those marker positive BMSCs also respond to RA treatment. This effect is likely to that of embryonic stem cells as reported previously [11, 14]. The most number of neuron-like cells was observed on culture day 7 in Sca-1, and on culture day 4 in Thy-1 positive cells. It is only a difference between Sca-1 and Thy-1 positive cells in this study. BMSCs survived until around culture day 17 on coverslips, but until around culture day 28 on hippocampal slices. This may be because of, in hippocampal slices, the preservation of neural and glial cytoarchitecture, the maintenance to some degree of normal cell-to-cell interrelationships.

As for nestin positive neuron-like cells, some groups of BMSCs can express nestin positive before morphological differentiation [3, 8, 16], so they can not mean neural differentiation. In Sca-1 or Thy-1 positive cells, the number of nestin positive cells increased until culture day 7, decreased after that, but NSE positive cells increased until culture day 10. This may indicate that some groups of neuron-like cells can gradually acquire some neural specificity. Considering the neural marker expression and differentiation ratio of Sca-1 or Thy-1 positive BMSCs, we suggest that they may be a group of immature cells.

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