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Efficacy of SSEA-3 Positive Cells Derived from Synovial Tissue in Rheumatoid Arthritis

Rie Kurose and Takashi Sawai

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Abstract

Rheumatoid arthritis (RA) is a refractory systemic autoimmune disease with chronic synovial inflammation. Sustained synovial inflammation leads to progressive destruction of bone and cartilage. Treatment to restore joints that have been destroyed irreversibly is not to be established yet even with the recent development of antirheumatic drugs and biological agents. Stage-specific embryonic antigen-3 (SSEA-3), a marker of human embryonic stem (ES) cell, acts as stem cells in the blood. SSEA-3 positive cells derived from RA synovial tissue have higher differentiating abilities than that of SSEA-3 negative cells and inhibitory effects on arthritis in collagen antibody-induced arthritis mice study. SSEA-3 positive cells derived from RA synovial tissue might have the inhibitory effect on arthritis and would be one of the cell sources for new RA treatment. The present manuscript is a brief review of mesenchymal stem cells in RA and described with the potential of RA cell therapy by SSEA-3 positive cells based on our research.

Keywords: rheumatoid arthritis, synovial tissue, SSEA-3

1. Introduction

Rheumatoid arthritis (RA) is a refractory systemic autoimmune disease with chronic synovial inflammation. Sustained synovial inflammation leads to progressive destruction of bone and cartilage. In the pathogenesis of RA, activated T cells and antigen-presenting cells such as monocytes and macrophages produce inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-2, IL-6, and interferon- γ (INF- γ). They promote release of inflammatory mediators, infiltration of inflammatory cells, production

of autoantibody, proliferation of the synovial cells, and activation of the osteoclasts, resulting in the bone and the cartilage destruction [1–3].

In the last few years, development of disease-modifying antirheumatic drugs (DMARDs) and biological agents targeting inflammatory cytokines has been major advances in the treatment of RA. Biological agents targeting inflammatory cytokines such as TNF- α , which has been shown to be a key factor in the pathology of RA, are effective for RA. These are known to improve disease activity and inhibit the progress of joint destruction. The best possible treatment goal for patients is clinical remission and consistently stopping continuing joint damage through erosions.

However, treatment to restore joints that have been destroyed irreversibly is not to be established yet. Also, DMARDs or biological agents can have serious side effects affecting the blood, liver, or kidneys rarely. Therefore, a novel RA treatment that enables restoration of destroyed joints is needed. Use of mesenchymal stem cells (MSCs) derived from bone marrow as a biological method for repairing articular cartilage defects have been investigated [4–8]. We think that the treatment by thus autologous cells can overcome these problems.

2. Mesenchymal stem cells in RA synovial tissue

2.1. Multipotency

MSCs are self-renewing, multipotent progenitor cells with multilineage potential to differentiate into various types of cells including chondrocytes, osteoblasts, and adipocytes [9–15]. While MSCs are most commonly isolated from bone marrow [13] and proliferate rapidly *in vitro*, they are also isolated from other tissues including adipose tissue [16], placenta [17], and umbilical cord blood [18]. Due to their accessibility and convenient expansion protocols, ethical dilemmas and risk of tumor formation, such as in ES cells and iPS cells, can also be avoided and therefore MSCs are easy to use in clinical application and have been recognized as promising candidates for cell therapy.

We investigated earlier the potential of chondrogenic differentiation of MSCs derived from bone marrow and synovial fluid in human osteoarthritis (OA) [19, 20]. Our study concluded that both bone marrow MSCs (BMMSCs) and synovial fluid MSCs (SFMSCs) had a potential of cell proliferation and chondrogenic differentiation. Both cells were fibroblast-like cells and had similar cell surface antigen in flow cytometry analysis, namely positive for CD13, CD44, and CD105 and negative for CD10, CD14, and CD45. However, aggrecan (AGG) mRNA expression in SFMSCs, which are traditionally associated with chondrogenic commitment, was a significant high compared to BMMSCs *in vitro*. According to other researches, SFMSCs are considered the same as synovial MSCs [21, 22]. Study of Sekiya et al. [23] reported that synovial MSCs are a candidate cell source for regenerative medicine of cartilage due to their high chondrogenic ability. They demonstrated that chondrogenic potentials of synovial MSCs between RA and OA patients were similar, as the weight of the pellet is a quantitative indicator of the ability of MSCs to produce chondrogenesis *in vitro*. Therefore, autologous synovial MSCs can be expected in cartilage regeneration for RA patients. According to previous reports [24], there was a negative relationship between chondrogenic potential of synovial MSCs and magnitude of synovitis in RA, and some properties of synovial MSCs vary dependent on the

diseases patients have. Also, it was reported that chondrogenic potential in RA patients was inferior to that in OA patients. However, Jones et al. [24] reported that effective suppression of joint inflammation is necessary for the development of autologous MSC treatments aimed at cartilage regeneration in RA and synovial MSCs can be expected for RA patients with the inflammation well controlled as well as OA patients.

2.2. Immunosuppressive effect

Previous reports have suggested that synovial MSCs harvested from RA were capable of immunosuppression in vitro [24]. However, other reports have suggested that the immunomodulatory function of synovial MSCs seems to be disturbed and causes an inefficacy due to various factors within RA microenvironment and as a result of a direct contact with inflammatory cells and cytokines [25]. In RA synovial tissue, synovial MSCs appear to play an important role in controlling the inflammation and immune hemostasis.

3. Stage-specific embryonic antigen-3 (SSEA-3) positive cells in RA synovial tissue

3.1. SSEA-3

Multilineage differentiating stress enduring (Muse) cells are a novel type of pluripotent stem cells and recently reported as adult human MSCs without introducing exogenous genes. They are present in various organs such as pancreas, dermis, umbilical cord, fat, liver, trachea, bone marrow, spleen [26–31] and are contained at a proportion of several percent in cultured mesenchymal stem cells [26], 4–9% in human adipose tissue [27] and 1–2% in human skin fibroblasts [26]. Muse cells are able to differentiate into cells from all three embryonic germ layers both spontaneously and under media-specific induction. Also, Muse cells have a low tumor-forming ability compared with embryonic stem (ES) cells and a high efficiency of change to iPS cells by Yamanaka gene introduction [32]. They can migrate to damaged tissues by intravenous injection in vivo, spontaneously differentiate into cells compatible with the targeted tissue, and contribute to tissue repair. Thus, Muse cells will be expected to play an important role in regenerative therapy by further studies. SSEA-3 is a marker of human embryonic stem cell. Muse cells are able to be isolated as SSEA-3 positive cells from cultured mesenchymal cells.

SSEA-3 positive cells are autologous cells and act as stem cells in the blood and also possess immunosuppression effects [28–31]. Therefore, they could be one of novel cell sources as cell therapy in RA. We studied the possibility of SSEA-3 positive cells derived from RA synovial tissue.

3.2. SSEA-3 as cell therapy in RA

3.2.1. SSEA-3 positive cells in RA synovial tissue

We used synovial tissue harvested from 13 RA patients at the time of joint surgery in our hospital (**Table 1**) [33]. Diagnosis of RA for all patients was based on the American College of Rheumatology (ACR) criteria in 1987 [34] or the ACR/European League Against Rheumatism

Pts.	Age	M/F	Stage	Class	R/L	Surgery
1	73	M	IV	III	R	total knee arthroplasty
2	45	F	I	II	L	arthroscopic synovectomy
3	78	M	III	I	R	total knee arthroplasty
4	82	F	IV	II	R	total knee arthroplasty
5	77	M	IV	II	L	total knee arthroplasty
6	84	F	IV	II	R	total knee arthroplasty
7	69	F	III	II	R	total elbow arthroplasty
8	77	F	III	II	L	total elbow arthroplasty
9	66	F	IV	II	R	2nd, 4th PIP arthrodesis
10	69	F	IV	III	L	1st IP arthrodesis
11	61	F	IV	II	R	wrist arthroplasty
12	62	F	IV	II	R	1st IP arthrodesis
13	66	F	III	I	L	total hip arthroplasty

Pts, patients; M/F, male or female; R/L, right or left; PIP, proximal interphalangeal joint; IP, interphalangeal joint.

Table 1. Clinical data of patients with RA (n = 13) for this study.

(EULAR) classification criteria in 2010 [35]. Approval for this study was obtained from the Ethics of Human Experiments Committee at Hirosaki University Graduate School of Medicine, Hirosaki, Japan. Informed consent was obtained from all patients.

Immunohistochemical staining was performed to investigate the localization of SSEA-3 positive cells in RA synovial tissue. Harvested synovial tissue was immediately fixed in 4% paraformaldehyde/PBS and embedded in paraffin in a usual manner. Rat monoclonal antibody specific for human SSEA-3 (Merck Millipore, Darmstadt, Germany) was used as a primary antibody. Immunoreactivity was detected by incubation with a biotinylated anti-rat IgG antibody (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA), followed by streptavidin-biotin reaction (Vectastain ABC kit). Immunohistochemical staining for SSEA-3 showed a few positive cells in RA synovial tissue (**Figure 1a–c**).

Harvested synovial tissue was minced, digested with 3 mg/mL collagenase Type V (Wako Pure Chemical Industries: Osaka, Japan) for 3 hours at 37°C, and cultured in the α MEM (Sigma-Aldrich: Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific: Waltham, MA, USA) and antibiotics (100 units/mL penicillin G and 100 μ g/mL streptomycin) (Thermo Fisher Scientific) at 37°C in a 5% CO₂ incubator. SSEA-3 positive cells were sorted by suspending 1×10^6 synovial cells at passage 2 in 100 ml FACS buffer containing 1 ml of EDTA, 5 ml of BSA, and 44 ml of FluoroBrite DMEM (Thermo Fisher Scientific, Waltham, MA, USA). Cells were collected by using antibody specific for SSEA-3, approximately 1% in cultured cells (**Figure 1d**).

SSEA-3 positive cells strongly expressed CD44, CD90, and CD105 and lacked CD34 (**Figure 2**) in flow cytometry assay. SSEA-3 negative cells were similar to positive cells in immunophenotype, but they weakly expressed CD105.

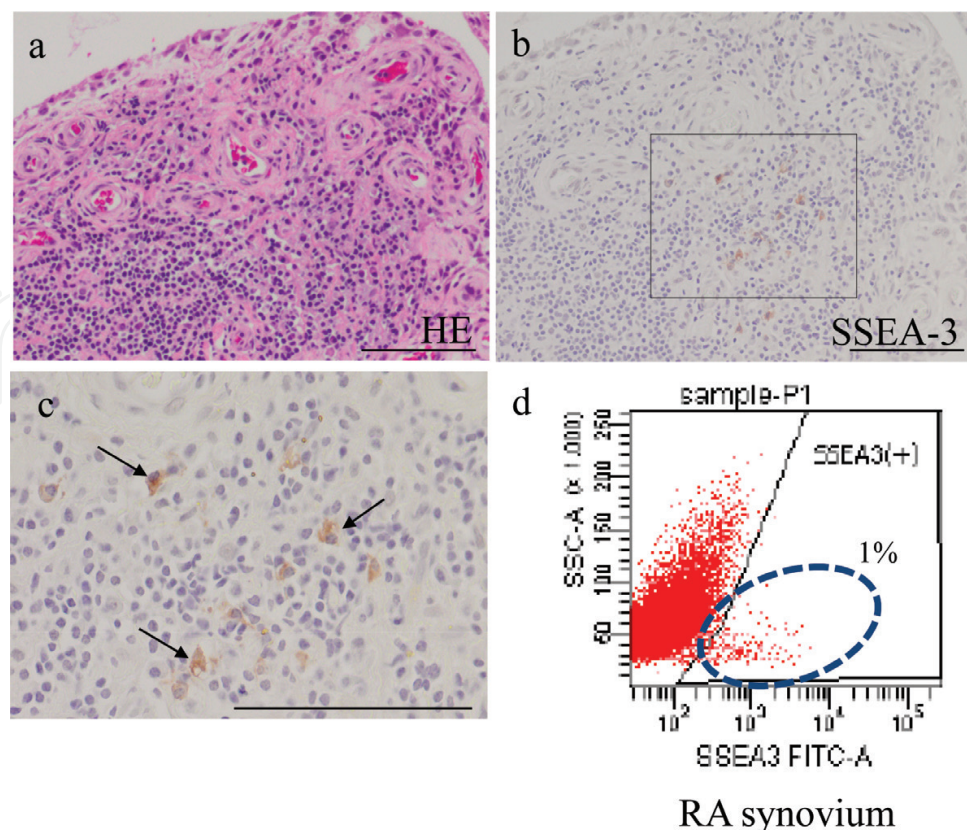


Figure 1. (a) RA synovial tissue. Hematoxylin and eosin (HE) staining. (b) Immunohistochemical staining specific for SSEA-3 in RA synovial tissue. (c) Magnified feature of (b). Bar = 100 μ m. (d) Ratio of SSEA-3 positive cells in cultured cells derived from RA synovial tissue.

Histological staining after differentiation induction culture showed differentiation into osteoblasts, adipocytes, and chondrocytes from the cultured SSEA-3 positive cells (**Figure 3**). In all mRNA expression of alkaline phosphatase (ALP) and bone morphogenetic protein 2 (BMP2) for osteogenic differentiation, peroxisome proliferator-activated receptor gamma (PPAR γ) for adipogenic differentiation and type II collagen (COL2A1), sex determining region Y (SRY)-Box 9 (SOX9) and aggrecan (AGG) for chondrogenic differentiation, SSEA-3 positive cells showed higher gene expression level than SSEA-3 negative cells although there were individual differences (**Figure 4**). These results indicate possibility of higher differentiation ability of SSEA-3 positive cells.

3.2.2. Inhibitory effect on arthritis

Collagen antibody-induced arthritis (CAIA) mice were established as the animal model for RA [36]. Induction of CAIA mice was performed on *scid/scid* mice, 7 weeks old (CLEA Japan), in which they were injected with 1.5 mg of 5-clone cocktail (arthrogen-CIA arthrogenic monoclonal antibody (mAb), Chondrex, Redmond, WA) by intraperitoneal (IP) injection at Day 0. Fifty micrograms of lipopolysaccharide (LPS) (Chondrex) was injected by IP injection at Day 3. 3×10^4 SSEA-3 positive cells labeled with cell tracker green (CTG) (Thermo Fisher Scientific) were suspended in PBS, filtered, then intravenously injected via the tail vein after the injection of LPS at Day 3. SSEA-3 negative cells labeled with CTG were used in the same procedure as control. Mice were scored for clinical arthritis; paws were assessed for signs of redness and swelling.

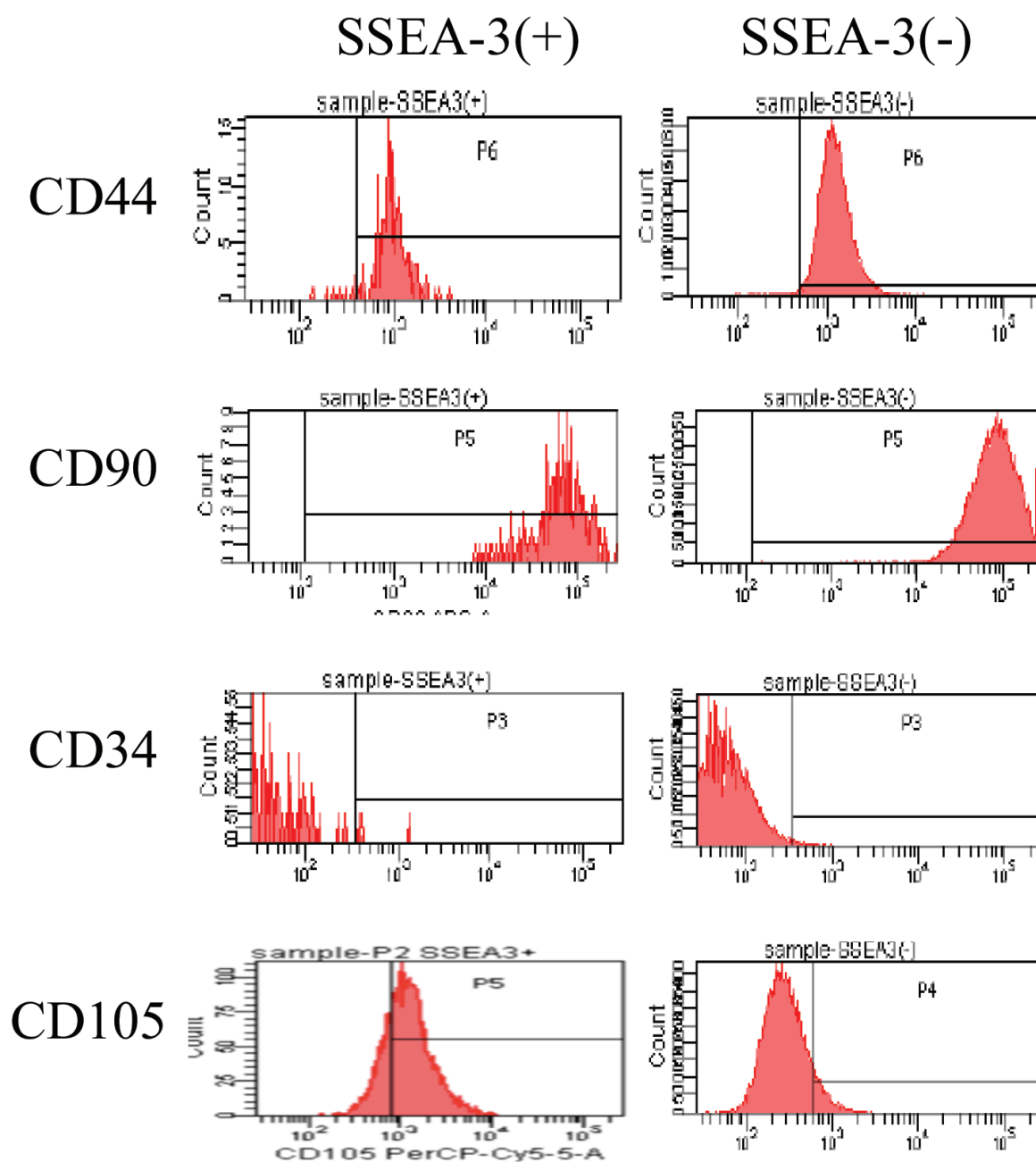


Figure 2. Representative flow cytometry analysis data.

Each paw was given a score of 0–4, giving a total maximum score of 16. (0, normal paw; 1, mild but definite redness and swelling in each joint of the digit or wrist/ankle; 2, moderate redness and swelling in two joints of the wrist/ankle with digit involvement; 3, severe redness and swelling in whole paw; 4, maximum inflammation within the wrist/ankle with many digits involved) [37]. **Figure 5a** displays the arthritis score of CAIA mice in the both transplanted groups after mAb injection. The group transplanted with SSEA-3 positive cells ($n = 3$) consisted of mice with intravenously transplanted SSEA-3 positive cells labeled with cell tracker green (CTG) seen in **Figure 5b**, while the group transplanted with SSEA-3 negative cells ($n = 3$) consisted mice with the transplanted SSEA-3 negative cells in the same procedure. Arthritis in the SSEA-3 negative cells group remained for 28 days, while arthritis score in the SSEA-3 positive cells group improved faster after peak inflammation (**Figure 5a**). There was a significant

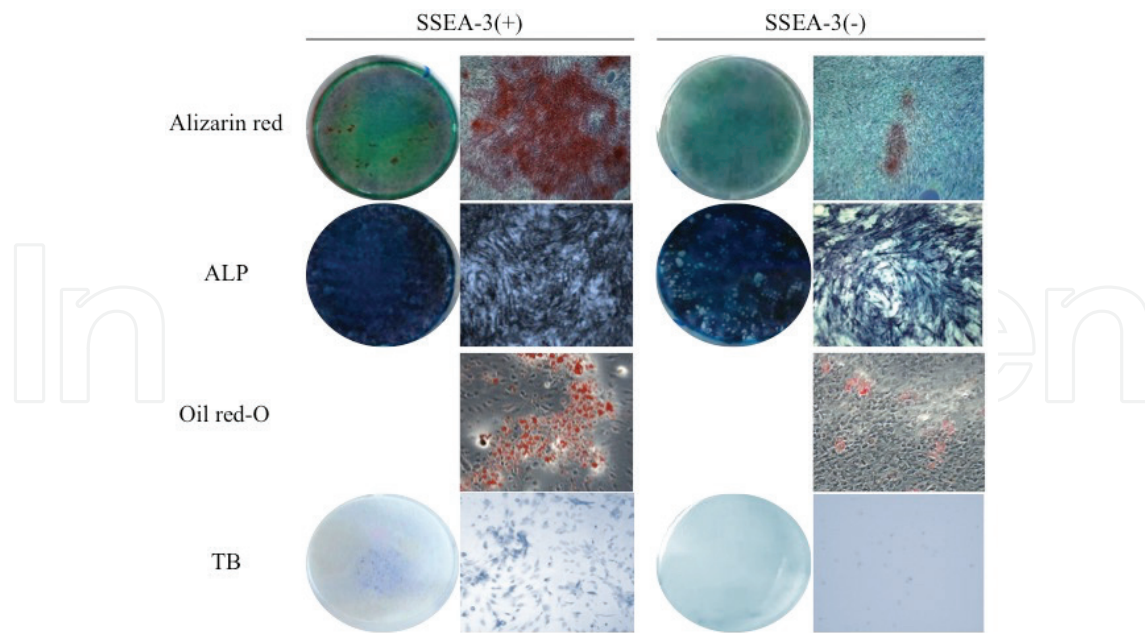


Figure 3

Figure 3. Multipotency of SSEA-3 positive cells derived from RA synovial cells. Osteogenesis was shown by alizarin red and alkaline phosphatase (ALP) staining. Adipogenesis was shown by oil red-O staining and chondrogenesis was shown by toluidine blue (TB) staining.

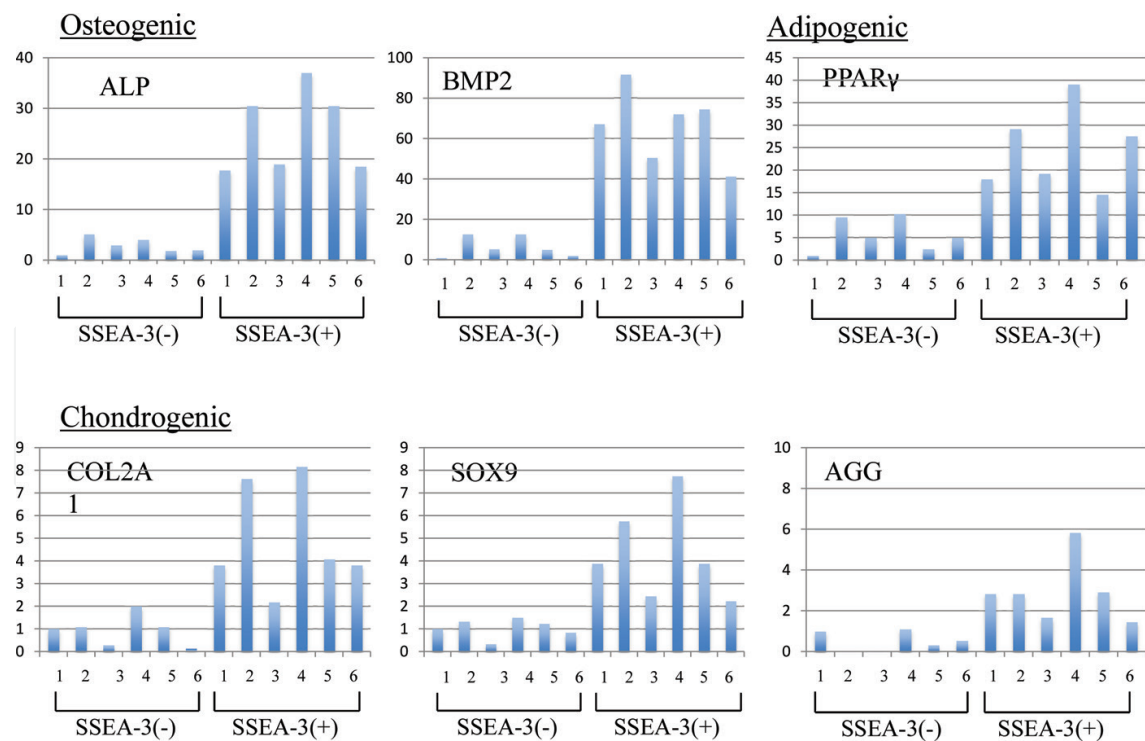


Figure 4. Real-time polymerase chain reaction (PCR) analysis. Results are reported as the mean of three independent experiments and the messenger RNA (mRNA) value of glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) was set as an internal control. Osteogenesis was shown by the expression of ALP mRNA and BMP-2 mRNA, adipogenesis was shown by the expression of PPARγ mRNA and chondrogenesis was shown by the expression of COL2A1 mRNA, SOX9 and AGG. Each data represents an average of three times. Note that the x-axis numbers represent patients in Table 1. The mRNA values of y-axis were arbitrary set.

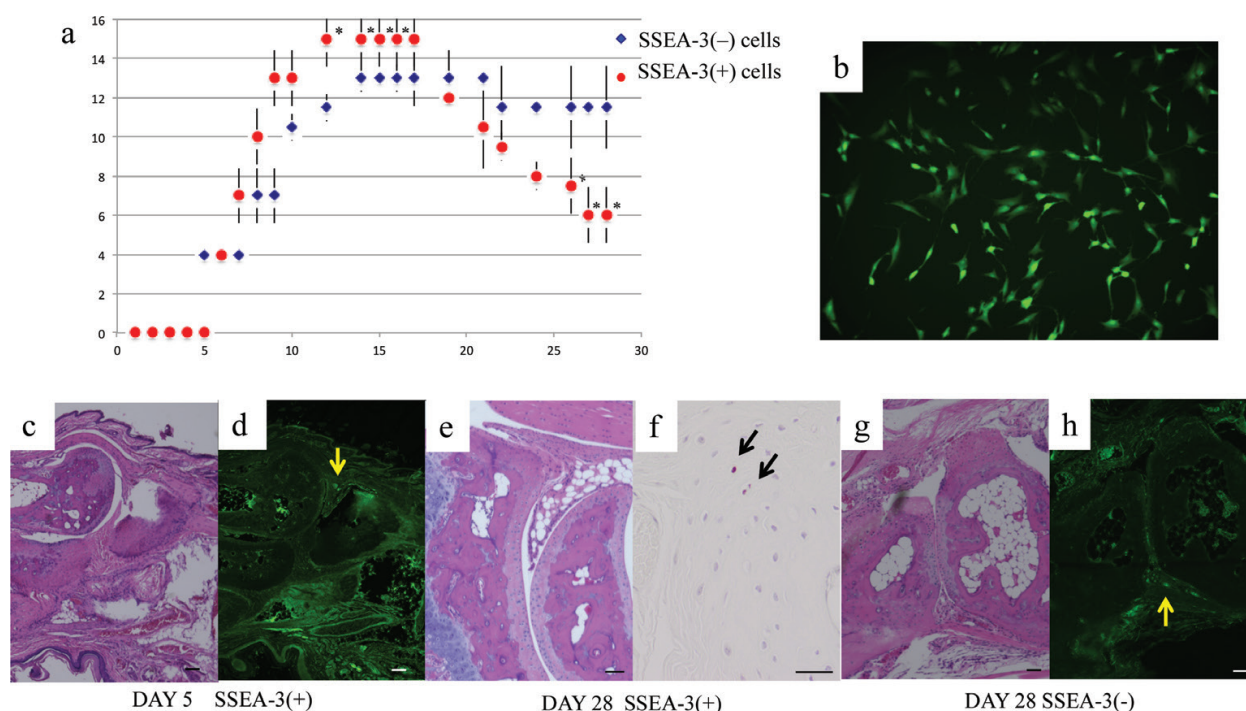


Figure 5. (a) Arthritis score of CAIA mice after mAb injection. SSEA-3 (–) cells group consisted of mice transplanted with SSEA-3 negative cells. SSEA-3 (+) cells group consisted of mice transplanted with SSEA-3 positive cells. Both groups contained three mice. Each score shows average values of three mice and * means $p < 0.05$. The X-axis means the day from day 0 when the monoclonal antibody cocktail was administered. (b) Cell tracker green (CTG)-labeled SSEA-3 positive cells (10X). (c, d and e, f) histology of joint of CAIA mouse after CTG-labeled SSEA-3 positive cells injections on day 5 and 28, respectively. Arrows shows CTG-labeled cells and positive cells for SSEA-3 in immunohistochemical staining. Bar = 100 mm. (g and h) representative histology of joint of CAIA mouse after CTG-labeled SSEA-3 negative cells injections on day 28. Bar = 100 mm.

improvement in arthritis in the SSEA-3 positive cells group. On Day 5, CTG-labeled cells were detected in the synovial tissue (**Figure 5c** and **d**) and were still present on Day 28 (**Figure 5e** and **f**) in the group transplanted with SSEA-3 positive cells. **Figure 5e** shows representative joint in the group transplanted with SSEA-3 positive cells on Day 28 and **Figure 5f** shows SSEA-3 positive cells in synovial tissue in the immunohistochemical staining of **Figure 5e**. **Figure 5g** and **h** show the progression of joint destruction in the group transplanted with SSEA-3 negative cells on Day 28. CTG-labeled cells were not detected in other healthy organs on Day 28 by fluorescent microscopy (data not shown). These results indicate that SSEA-3 positive cells have the inhibitory effect on arthritis and systemic administration of them is safety.

4. Conclusions

In our study, SSEA-3 positive cells were detected in RA synovial tissue even under pathological conditions such as RA. Although the synovial tissue we used was collected from various RA disease stages and surgical sites, SSEA-3 positive cells were detected with values of approximately 0.5–1% in all cultured SFMSCs. Collected SSEA-3 positive cells had higher gene expression level and differentiation ability in vitro compared with SSEA-3 negative cells

that were occupying most of mesenchymal stem cells. Wakao S., et al., reported that Muse and non-Muse cells had differentiation ability of osteocytes, chondrocytes, and adipocytes, while differentiation ability in non-Muse cells was lower [31]. We think that SSEA-3 positive cells in this study had a similar nature as Muse cells, considering also the results that SSEA-3 positive cells strongly expressed CD105 in FACS analysis. SSEA-3 positive cells can be systemically administered by intravenous administration like Muse cells and have possibility of differentiation into osteoblasts, adipocytes, and chondrocyte. These suggest the possibility of repairing degenerative cartilage and destroyed joints in RA. In the CAIA mice experiment, SSEA-3 positive cells that were systemically administered had an inhibitory effect on arthritis. In the transplanted group consisting of mice transplanted with SSEA-3 positive cells, arthritis score quickly decreased after the onset of arthritis compared with SSEA-3 negative cells group. There were some previous studies on immunosuppressive effect of BMMSCs [38–40] and SFMSCs [24] as mentioned earlier. Especially, SFMSCs extracted from healthy subjects are able to inhibit T-cell proliferation [25]. However, immunomodulatory function of SFMSCs and SSEA-3 positive cells may be disturbed and cause an inefficacy of SFMSCs and SSEA-3 positive cells in inflammatory environment like uncontrolled RA [41]. In RA synovial tissue, fibroblast-like synoviocytes (FLS) are key players in the perpetuation of joint inflammation and destruction. The link between FLS and SFMSCs plays an important role in controlling the inflammation and immune hemostasis in RA. In our mice study, autologous SSEA-3 positive cells proliferated in vitro might have altered the balance of immune regulation with FLS.

Our study suggests the possibility of inhibiting arthritis and joint destruction by SSEA-3 positive cells derived from synovial tissue in RA. Further study of SSEA-3 positive cells for clinical application in humans will lead to future development as a new treatment in RA.

Author details

Rie Kurose^{1*} and Takashi Sawai²

*Address all correspondence to: riekuro@hirosaki-u.ac.jp

1 Department of Orthopaedic Surgery, Hirosaki University Graduate School of Medicine, Hirosaki, Japan

2 Department of Histopathology, Tohoku University, Sendai, Japan

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