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Original Article

In vitro MRI and Characterization of Rat Mesenchymal Stem Cells Transduced with Ferritin as MR Reporter Gene

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Purpose : This study was performed to evaluate the characteristics of rat mesenchymal stem cells (RMSCs) transduced with human ferritin gene and investigate *in vitro* MRI detectability of ferritin-transduced RMSCs.

Materials and Methods: The RMSCs expressing both myc-tagged human ferritin heavy chain subunit (myc-FTH) and green fluorescence protein (GFP) were transduced with lentiviurs. Transduced cells were sorted by GFP expression using a fluorescence-activated cell sorter. Myc-FTH and GFP expression in transduced cells were detected by immunofluorescence staining. The cell proliferative ability and viability were assessed by MTT assay. The RMSC surface markers (CD29+/CD45-) were analyzed by flow cytometry. The intracellular iron amount was measured spectrophotometically and the presence of ferritin-iron accumulation was detected by Prussian blue staining. *In vitro* magnetic resonance imaging (MRI) study of cell phantoms was done on 9.4 T MR scanner to evaluate the feasibility of imaging the ferritin-transduced RMSCs.

Results: The myc-FTH and GFP genes were stably transduced into RMSCs. No significant differences were observed in terms of biologic properties in transduced RMSCs compared with non-transduced RMSCs. Ferritin-transduced RMSCs exhibited increased iron accumulation ability and showed significantly lower T_2 relaxation time than non-transduced RMSCs.

Conclusion: Ferritin gene as MR reporter gene could be used for non-invasive tracking and visualization of therapeutic mesenchymal stem cells by MRI.

Index words : Rat mesenchymal stem cell · Reporter gene · Ferritin · Magnetic resonance imaging

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INTRODUCTION

In the preclinical research for stem cells, fluorescent proteins have been used as reporter genes for noninvasive *in vivo* imaging. However, these reporters have limited ability to provide spatial resolution and information in deeper tissue where the labeled stem cells would be located such as heart and brain. Magnetic resonance imaging (MRI) can provide high-resolution and quantitative information for biological process occurring in deep tissues with noninvasive manner, therefore the combination of fluorescent

reporter genes and MR reporter genes has been developed (1, 2). Ferritin gene is considered as a promising MR reporter gene, which augments the intracellular iron storage to produce signal changes in the surrounding environment that can be detected by MRI (3). Several reports showed that ferritin overexpression did not interfere with tumor cell growth (3–5) or stem cell differentiation and proliferation (2, 6).

For stem cell research, rats can be a most useful animal to be studied for *in vivo* MR imaging because the body size is not too small to be scanned with easily accessible clinical MR machines, and the obtained data could be integrated and interpreted with stacks of previously reported results in the field of myocardial ischemia (7), hindlimb ischemia (8, 9), and so on. However, there are yet no reports regarding the labeling of ferritin gene in rat stem cells, while murine (2) and swine (10) stem cells were reported. Mesenchymal stem cells found in various adult tissues are multipotent stromal cells that have the potential to give rise to cell of diverse lineages. Mesenchymal stem cells are widely used for stem cell therapeutic application for diverse disease (11–13)

This study were undertaken to evaluate the influence of overexpressed ferritin gene as a MR reporter gene on the biological properties of the rat mesenchymal stem cells (RMSCs), and performed the MRI study of RMSCs stably expressing ferritin [myc-tagged human ferritin heavy chain subunit (myc-hFTH)] and green fluorescent protein (GFP) on 9.4T MR scanner.

MATERIALS AND METHODS

Generation of RMSCs stably expressing myc-hFTH and GFP

RMSCs were obtained from Lonza, which was isolated from bone marrow taken from the femur and tibia of female Fischer 344 rats, cultured in Rat Mesenchymal Stem Cell Growth Medium Bullet Kit® (Lonza, Basel, Switzerland). Both Green fluorescence protein (GFP) and myc-tagged human ferritin heavy chain subunit (myc-hFTH) genes-transduced RMSCs (RMSCs-GFP/FTH) and only GFP-transduced RMSCs (RMSCs-GFP) were prepared as previously described (2).

Fluorescence-activated cell sorting analysis of RMSCs-GFP and RMSCs-GFP/FTH

GFP positive cells were sorted from transduced cells using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) equipped with a 530-nm filter (bandwidth, ± 15 nm), a 585-nm filter (bandwidth, ± 21 nm). The sorted GFP-expressing cells were used for *in vitro* studies.

Immunofluorescence staining of the labeled cells

RMSC, RMSC-GFP, and RMSC-GFP/FTH were cultured on eight-well chamber slides, and rinsed in phosphate buffered saline (PBS) and followed by fixation with 2% paraformaldehyde for 30 minutes. Fixed cells were incubated with primary antibodies directed against GFP and myc in cells, and staining was visualized using secondary antibodies conjugated to Alexa 488 (green) and Alexa 594 (red) (Invitrogen, Cergy Pontoise, France). Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, blue) (Santa Cruz Biotechnology, Santa Cruz, CA). Images of stained cells were acquired with a fluorescence microscopy (Leica, Wetzlar, Germany), equipped with a CCD camera (Leica).

Flow cytometric analysis of surface markers

To determine if the surface marker expression of RMSCs is altered in transduced RMSCs, flow cytometry was performed with allophycocyanin (APC)-conjugated anti-CD 45 and anti-CD29 antibodies (E-bioscience, San Diego, CA). Data analysis was performed on FACSCalibur using the Cell Quest Pro software (BD Biosciences).

Cell proliferative ability and viability assay

To evaluate the cell proliferation activity and viability of RMSCs, RMSCs-GFP, and RMSCs-GFP/FTH, all cells were seeded at a density of 10^4 cells per well in 96-well plates, and incubated under standard conditions (37℃, 5% humidified CO_2). The viability and proliferation of cells in culture is assessed using a standard 3-, 5-diphenyltetrazolium bromide (MTT) assay. The proliferation activities were expressed as the relative ratio at day 1. The cell viabilities were evaluated after the incubation with addition of 0, 100, 250, and 500 μmol/L ferric ammonium citrate (FAC) for 3 days. The viabilities were expressed as the

relative percentage to the control of cells not treated with FAC.

Assessment of iron accumulation

To evaluate the iron storage capacity of the RMSCs, RMSCs-GFP, and RMSCs-GFP/FTH, cells were cultured in a medium supplemented with or without FAC of 500 μmol/L for 72 hours, washed three times with PBS, and incubated in 6 NHCl solution at 70℃ for 30 minutes. The cellular iron amount was determined using a total iron reagent kit (Pointe Scientific, Canton, MI). Average iron loadings were calculated by dividing the total mean values by total cell numbers. Prussian blue iron staining was also used to detect ferritin-iron accumulated within cells. Cells were grown on eight-well chamber slides in a medium with or without FAC (500 μmol/L) for 72 hours, washed thoroughly in PBS and fixed in 2% paraformaldehyde for 20 minutes at 4℃ prior to the Prussian blue staining. The eight-well chamber slides were placed in a staining solution (1% potassium ferrocyanide and 5% HCl) for 30 minutes, washed twice in PBS, counterstained with nuclear-fast red solution for 5 minutes, and mounted using an aqueous mounting medium (Biomeda Corp., Foster city, CA).

Phantom preparation

RMSCs and RMSCs-GFP/FTH cultured in medium with or without iron supplement of 300 μmol/L FAC for 3 days were harvested. For cell phantom, all cells were suspended in 300 μL of 0.7% agarose and transferred to 300 μL microtubes. To evaluate whether the MR signal change of cell phantoms is proportional to the cell number, increasing numbers of cells (1×10^3, 5×10^3, 1×10^4, 5×10^4, 1×10^5, 5×10^5, and 1×10^6) treated with FAC (300 μmol/L) were prepared. A tube filled with only 0.7% agarose was prepared for a control. The microtubes were placed into the agarose plate and styrofoam with cone-shaped cavities fitting the microtubes on an animal MR machine (9.4T) and in vitro MRI of cell phantoms was performed.

In vitro MRI

The prepared phantom was scanned at Agilent Preclinical 9.4T/160 mm MRI system (Agilent Technologies, Santa Clara, CA). Spin echo T2-weighted images were obtained to create T2 maps. The following parameters were used to acquire T2-weighted images: TR = 3000 msec; Flip angle = 90°; TE = 9, 18, 27, 36, 45, 50, 100, 150, 200, 250, and 300 msec; bandwidth = 521 Hz/pixel; matrix = 256×256, FOV = 50×50 mm^2 and number of excitations (NEX) = 1. The effective slice thickness was 1.5 mm, and 2 slices were imaged for each tube.

T2 relaxation times were calculated by a linear fit of the logarithmic signal amplitudes versus TE. 0.05 cm^2 circular regions of interest were placed at three areas on each T2 map image and were averaged.

Statistical analysis

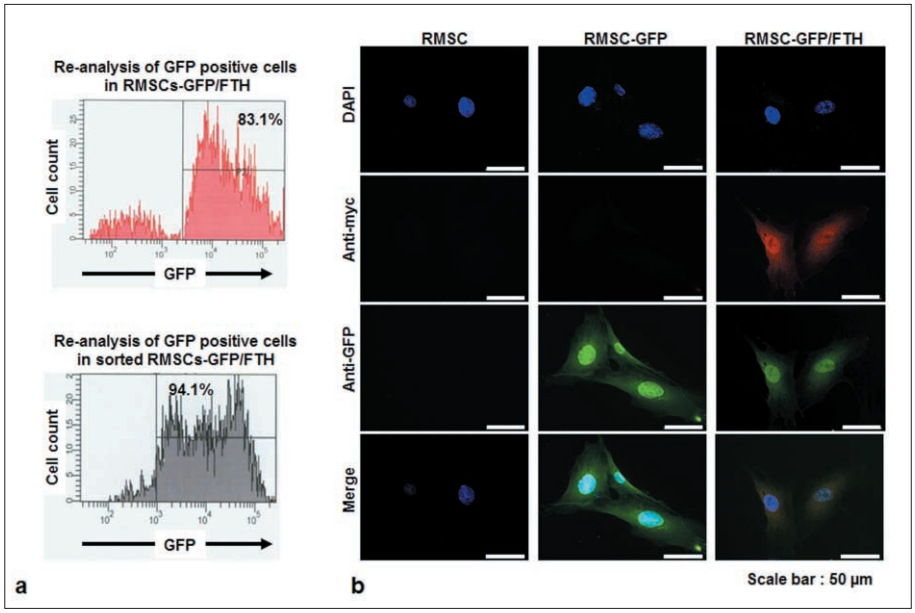


Fig. 1. Analysis of myc-tagged ferritin heavy chain subunit (myc-FTH) and green fluorescence protein (GFP) expression in rat mesenchymall stem cell (RMSC) with lentivirus.

a. The transduction efficiency of RMSC-GFP/FTH and purity of the sorted cells were approximately 83% and 94%, respectively.

b. Immunofluorescence images in fixed RMSC, RMSC-GFP, and RMSC-GFP/FTH were acquired with anti-myc and anti-GFP antibodies for myc-FTH (red) and GFP (green). Cell nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI, blue).

Data were presented as means ± standard errors. Comparisons were performed using the Student's *t*-test or ANOVA. Differences were considered significant at a *P* value of ≤ 0.05.

RESULTS

Establishment of RMSCs transduced with dual reporter genes, GFP and myc-hFTH

The transduction efficiency of RMSCs expressing myc-FTH and GFP was more than 80% using the lentivirus (Fig. 1a top panel). The purity of sorted cells was 94% (Fig. 1a bottom panel). The expressions of myc-FTH and GFP proteins in the transduced cells were confirmed using immunofluorescence staining. GFP proteins in nucleus and cytoplasm and myc-FTH proteins in cytoplasm were abundantly expressed, and co-expression of myc-FTH and GFP proteins was observed in RMSCs-GFP/FTH (Fig. 1b).

Analysis of biological properties of the ferritin-transduced RMSC

To evaluate the alteration in biological properties of RMSCs due to the overexpression of GFP and FTH using lentivirus, the proliferation capacity and cell surface phenotype were evaluated. No differences in

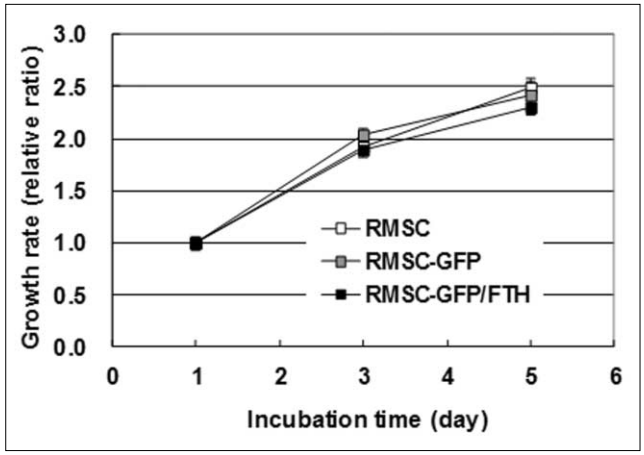


Fig. 2. Comparison of cell proliferation ability.
Proliferation of transduced and non-transduced cells was measured by a standard 3-,5-diphenyltetrazolium bromide (MTT) assay. No differences were observed between RMSC, RMSC-GFP, and RMSC-GFP/FTH.

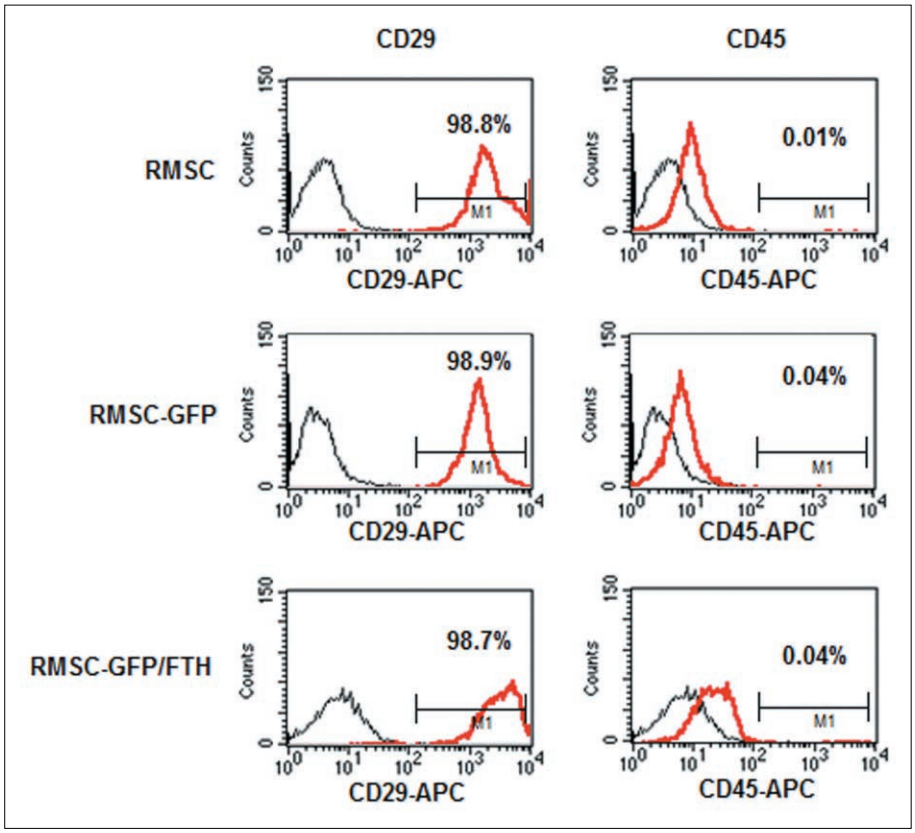


Fig. 3. Flow cytometric analysis of cell surface markers for undifferentiated rat mesenchymal stem cells.
99% of the RMSC, RMSC-GFP, and RMSC-GFP/FTH were positive for CD29 and negative for CD45. There were no significant differences in the expressions of cell surface markers between the cell groups.

cell growth ability were observed between the transduced cells (RMSCs-GFP and RMSCs-GFP/FTH) and non-transduced cells (RMSCs) (Fig. 2). Flow cytometric results showed that the cultured RMSCs positive for CD29 and negative for CD45 were more than 98%. In transduced cells, the alteration in surface marker expression (CD29+/CD45-) was not observed compared to non-transduced cells (Fig. 3).

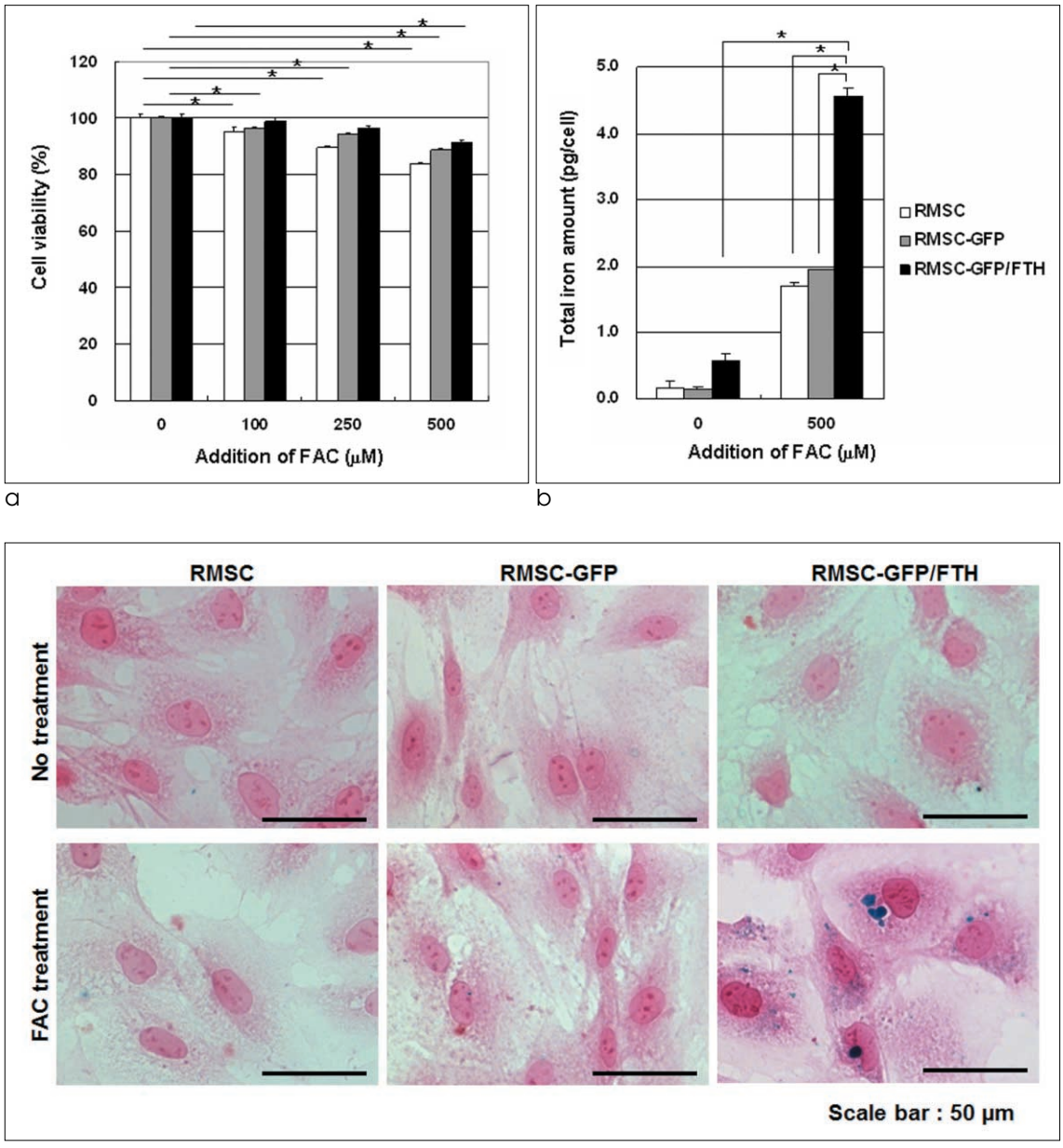


Fig. 4. Cell viability, average cellular iron amount, and Prussian blue staining.

a. Viability of cells treated with increasing concentrations of ferric ammonium citrate (FAC) for 72 hours was measured by a standard 3-, 5-diphenyltetrazolium bromide (MTT) assay. The treatment with FAC (100–250 μmol/L) did not cause cellular toxicity in RMSC-GFP/FTH, whereas the viability of RMSC and RMSC-GFP significantly decreased. Asterisks (*) point out that *P* value was statistically significant ($\leq$ 0.05).

b. Average iron amount of cells treated with FAC (500 μmol/L) for 72 hours was calculated by using a total iron reagent kit. Exposure to FAC increased the cellular iron amount of all cell groups. Average iron amount of RMSC-GFP/FTH was significantly higher than those of RMSC and RMSC-GFP. Asterisks (*) point out that *P* value was statistically significant ($\leq$0.05).

c. Prussian blue staining was performed in cells treated with FAC (500 μmol/L) for 72 hours. The iron aggregates (dark blue balls) were clearly detected within the cytoplasm of the RMSC-GFP/FTH treated with FAC.

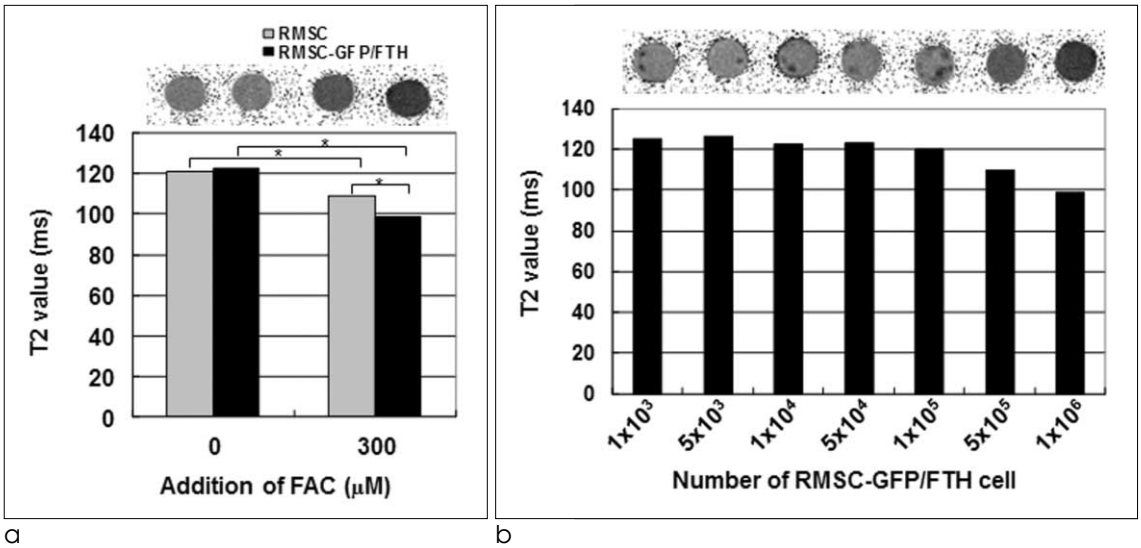
Increased intracellular iron amount in the ferritin-transduced RMSC

After treatment with increasing FAC for 72 hours, the toxicity of free soluble iron was compared in RMSCs, RMSCs-GFP, and RMSCs-GFP/FTH. The treatment with FAC (100–250 μmol/L) did not cause cellular toxicity in RMSCs-GFP/FTH, whereas the viabilities of RMSCs and RMSCs-GFP decreased. At 500 μmol/L of FAC, the viabilities of RMSCs, RMSCs-GFP, and RMSCs-GFP/FTH decreased significantly (Fig. 4a).

The averages of intracellular iron amount were 0.16, 0.15, and 0.59 pg/cell for RMSCs, RMSCs-GFP and RMCs-GFP/FTH without iron supplement, and increased to 1.70, 1.95, and 4.57 pg/cell with addition of FAC, respectively (Fig. 4b). The RMSCs-GFP/FTH significantly had more accumulation of intracellular iron than RMSCs and RMSCs-GFP. FAC (500 μmol/L) treatment caused 27-fold increase in intracellular iron amount of RMSCs-GFP/FTH. The presence of accumulated iron (blue dots) in RMSCs-GFP/FTH cultivated in a medium supplemented with FAC, was clearly detected by Prussian blue stain (Fig. 4c). In other five groups, there were negligible tiny blue dots.

In vitro MRI: Decreased T2 relaxation time in the ferritin-transduced RMSC

On 9.4T MR, there were no differences in T2 relaxation time between RMSCs and RMSCs-GFP/FTH at the same concentration of 1×10^6 cells in



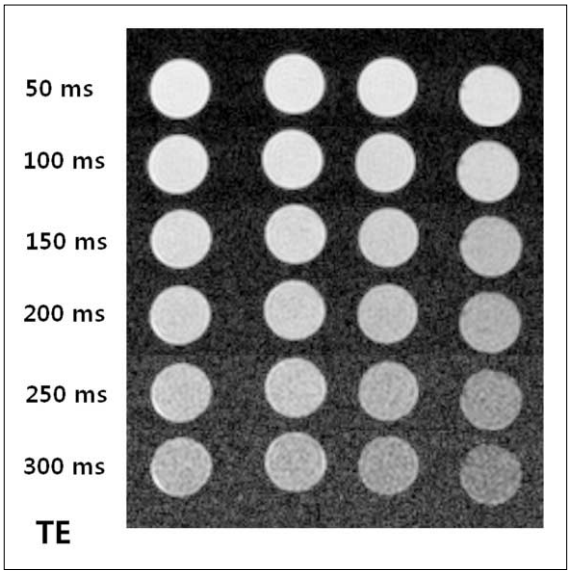


Fig. 5. *In vitro* MRI analysis of cell phantom at 9.4T.
a. T2 relaxation time significantly decreased after the addition of ferric ammonium citrate (FAC, 300 μmol/L) in RMSC-GFP/FTH and RMSC. RMSC-GFP/FTH exhibited the lower T2 relaxation time than RMSC. Asterisks (*) point out that *P* value was statistically significant ($\leq$0.05).
b. On cell phantoms containing the different cell number, RMSC-GFP/FTH treated with FAC (300 μmol/L) showed a significant decrease in T2 relaxation time when the cell number reached to 5×10^5.
c. Multiecho T2-weighted images of source data for T2 map are shown.

300μL agarose without iron supplementation. T2 shortening was easily discernible in RMSCs-GFP/FTH with iron supplementation (FAC 300 μmol/L). T2 relaxation times of RMSCs and RMSCs-GFP/FTH were 121.93 $\pm$ 0.88 and 122.48 $\pm$ 0.75 msec without iron supplement, and decreased into 108.17 $\pm$ 0.72 and 98.17 $\pm$ 0.52 msec with iron supplement, respectively (Fig. 5a and 5c). The decrease of T2 relaxation time in RMSCs-GFP/FTH was significantly greater than that in RMSCs. In MR detection sensitivity studies, the significant decrease in T2 relaxation time was observed in the phantom of RMSCs-GFP/FTH containing 5×10^5 cells (Fig. 5b).

DISCUSSION

The present study suggests that the transduction of the myc-hFTH as a MR reporter gene into the RMSC did not affect the proliferative activity, cell viability, and surface maker expression.

The toxicity assay showing that the viability of RMSCs-GFP/FTH did not decrease until the FAC concentration was at least 250 μmol/L, implies that ferritin gene overexpression was protective against for free soluble iron toxicity. This result was in accord with previously published in vitro results (2, 3). Also it means that preloading of intracellular iron prior to in vivo injection can be possible, which may enable short term in vivo cell tracking before the endogenously produced ferritins were accumulated to make MR signal changed.

The result that iron amount of RMSCs-GFP/FTH was significantly larger than those of RMSCs and RMSCs-GFP without iron supplement would be due to the increased capacity for iron storage in overexpressed ferritin. In addition, iron supplement resulted in increase of iron amount in both of transduced or non-transduced cells. It can be explained by the function of native ferritin gene. Despite of increase in amount of intracellular iron by myc-FTH overexpression or iron supplementation, Prussian blue stain showed little blue dots in RMSCs and RMSCs-GFP. This result suggests the ferritin-iron particle in those groups could not be detected by Prussian blue staining.

The most important feature of the RMSCs-GFP/FTH would be the MR detectability. The change in T2 relaxation time started from 5×10^5 RMSCs-GFP/FTH treated with FAC (250 μmol/L). In the stem cell experiments regarding neovascularization after myocardial ischemia or hindlimb ischemia, cells were injected intra-muscularly, intra-venously or intra-arterially with at least the number of 5×10^5. Considering the concentration of the stem cells injected into injured site, in vivo imaging using this RMSCs-GFP/FTH would be promising.

The differentiation ability of RMSCs-GFP/FTH into multilineage cells would be further evaluated and *in vitro* and *in vivo* MRI study of RMSCs-GFP/FTH on clinical MR scanner such as 1.5T and 3T would be required.

In summary, this study demonstrated that ferritin overexpression did not exhibit the alteration in the viability and proliferations, and can be used as MRI reporter for imaging of RMSCs.

Acknowledgements:

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대한자기공명의과학회지 16:47-54(2012)

페리틴 리포터 유전자를 발현하는 백서 중간엽 줄기세포의 특성과 자기공명영상 연구

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목적: 백서 중간엽 줄기세포에 페리틴 유전자를 형질 도입시켜 생물학적 특성의 변화 유무를 평가하고, 자기공명영상에서 신호강도의 차이를 확인해보고자 하였다.

대상과 방법: 백서 중간엽 줄기세포에 렌티바이러스를 이용하여 사람유래 재조합 페리틴과 녹색형광단백질 유전자의 과발현을 유도하였다. 페리틴 유전자가 발현된 백서 중간엽 줄기세포의 증식성과 생존능을 분석하기 위해 MTT 어세이를 수행하였으며, 유세포 분석을 수행하여 중간엽 줄기세포의 표면 마커 발현을 평가하고, 세포 내 철 함량을 측정하고 프러시안 블루 염색을 시행하여 철 축적능력을 분석하였다. 세포 팬텀을 이용하여 9.4 T 자기공영영상 기기를 이용하여 검출가능성을 평가하였다.

결과: 페리틴과 녹색형광 유전자는 백서 중간엽 줄기세포에서 안정적으로 발현되었다. 페리틴 유전자의 과발현으로 인해 백서 중간엽 줄기세포의 생물학적 특성 (증식능력, 생존능, 표면마커)은 영향을 받지 않았다. 페리틴을 발현하는 중간엽 줄기세포에서 철의 축적능력이 증가된 것이 확인되었고, T2 이완 시간은 유의하게 감소하였다.

결론: 줄기세포 치료 연구에서 자기공명 리포터 유전자 페리틴은 자기공명영상법을 이용하여 중간엽 줄기세포를 비침습적으로 가시화 할 수 있고 이를 이용하여 생체추적이 가능할 것으로 기대된다.

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