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ORIGINAL ARTICLE

# Effect of donor chimerism to reduce the level of glycosaminoglycans following bone marrow transplantation in a murine model of mucopolysaccharidosis type II

**Kentaro Yokoi · Kazumasa Akiyama · Eiko Kaneshiro · Takashi Higuchi · Yohta Shimada · Hiroshi Kobayashi · Masaharu Akiyama · Makoto Otsu · Hiromitsu Nakauchi · Toya Ohashi · Hiroyuki Ida**

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**Abstract** Mucopolysaccharidosis type II (MPS II) is a lysosomal storage disorder caused by deficient activity of the iduronate-2-sulfatase. This leads to accumulation of glycosaminoglycans (GAGs) in the lysosomes of various cells. Although it has been proposed that bone marrow transplantation (BMT) may have a beneficial effect for patients with MPS II, the requirement for donor-cell chimerism to reduce GAG levels is unknown. To address this issue, we transplanted various ratios of normal and MPS II bone marrow cells in a mouse model of MPS II and analyzed GAG accumulation in various tissues. Chimerism of whole leukocytes and each lineage of BMT recipients' peripheral blood was similar to infusion ratios. GAGs were significantly reduced in the liver, spleen, and heart of recipients. The level of GAG reduction in these tissues depends on the percentage of normal-cell chimerism. In contrast to these tissues, a reduction in GAGs was not observed in the kidney and brain, even if 100 % donor chimerism was achieved. These observations suggest that a high degree of chimerism is necessary to achieve the maximum effect of BMT, and donor lymphocyte infusion or enzyme replacement therapy might be considered options in cases of low-level chimerism in MPS II patients.

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K. Yokoi (✉) · E. Kaneshiro · H. Kobayashi · M. Akiyama · T. Ohashi · H. Ida
Department of Pediatrics, The Jikei University School of Medicine, 3-25-8 Nishishimbashi, Minato-ku Tokyo 105-8461, Japan
e-mail: kenta451@jikei.ac.jp

K. Yokoi · K. Akiyama · T. Higuchi · Y. Shimada · H. Kobayashi · T. Ohashi · H. Ida
Division of Gene Therapy, Research Center for Medical Sciences, The Jikei University School of Medicine, Tokyo, Japan

M. Otsu · H. Nakauchi
Division of Stem Cell Therapy, Center for Stem Cell and Regenerative Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan

## Introduction

Mucopolysaccharidosis type II (MPS II, also known as Hunter syndrome, OMIM 309900) is an X-linked, recessive lysosomal storage disorder (LSD) caused by decreased activity of the lysosomal enzyme iduronate-2-sulfatase (IDS, EC3.1.6.13) (Scarpa et al. 2011). This enzyme, which maps to chromosome Xq28.2, initiates degradation of the sulfate group from glycosaminoglycans (GAGs), heparin sulfate, and dermatan sulfate (Muenzer et al. 2009a, b). In a nationwide survey in Japan (Tanaka et al. 2012), MPS II was found to be the most prevalent type of MPS, accounting for 60 % of documented cases, and BMT in the early stage was shown to have some effects on central nervous system (CNS) involvement of MPS II. GAG accumulation leads to progressive multi-body-system dysfunction and results in various patterns of clinical manifestations. Patients develop skeletal deformities, macrocephaly, cardiac-valve abnormalities/hypertrophy, hepatosplenomegaly, coarse facies, upper-airway obstruction, hearing loss, and CNS involvement (Scarpa et al. 2011; Muenzer et al. 2009a, b, 2011; Tanaka et al. 2012; Simonaro et al. 2008; Link et al. 2010). Enzyme-replacement therapy (ERT) for MPS II, which was approved in many developing countries in 2006, is the first choice of treatment (Muenzer et al. 2002); however, its limitations have recently been disclosed. The most commonly observed ERT-related adverse event (AE) is infusion-related antibody response, which in some cases is life threating (Burton et al. 2011). Because of poor penetration across the blood–brain barrier (Garcia et al. 2007a, b), low efficacy has been observed for ERT in brain

tissue. Moreover, weekly injections impose a physical and economic burden on patients and their families.

In 1980, bone marrow transplantation (BMT) became a possible curative option for patients with severe neuropathic LSDs. such as MPS I, MPS VI, globoid-cell leukodystrophy, and metachromatic leukodystrophy (Peters et al. 2003). BMT might overcome the limitations of ERT, such as CNS efficacy and the necessity for repeated treatment. However, a major limitation of BMT is the requirement for a strong preconditioning regimen to achieve sufficient engraftment; high-intensity preconditioning is usually necessary because immune function is typically normal in these patients, and occasionally, this strong preconditioning regimen worsens the patient's condition (Tanaka et al. 2012). To overcome these problems, reduced-intensity conditioning regimens followed by BMT have been considered (Hansen et al. 2008), but this approach sometimes results in low levels of donor-cell chimerism. Although we and others reported beneficial therapeutic outcomes of small engraftment in type 1 Gaucher disease (Enquist et al. 2009), MPS VII (Soper et al. 2001), and Fabry disease (FD) (Yokoi et al. 2011), we do not know how much donor chimerism is required to obtain maximum efficacy of BMT in MPS II.

From this point of view, defining the level of chimerism necessary to achieve a therapeutic effect is crucial if less toxic conditioning followed by BMT is to be considered for MPS II. Accordingly, we produced MPS II mice with various ratios of bone marrow cells (BMCs) and analyzed the level of IDS and GAGs in some organs.

## Materials and methods

### Animals

Female C57BL/6Ly45.2 ($CD45.1^{-}CD45.2^{+}$) mice heterozygous for the X-linked allele ($IDS^{+/-}$) were generously provided by Joseph Muenzer (University of North Carolina, Chapel Hill, NC, USA) (Garcia et al. 2007a, b). The latent-carrier female mice were bred with wild-type (WT) male mice of the same genetic strain, producing C57BL/6Ly45.2 ($CD45.1^{-}CD45.2^{+}$) hemizygous IDS knockout male mice (MPS II mouse model, $IDS^{-/0}$). C57BL/6Ly45.1 male mice ($CD45.1^{+}CD45.2^{-}$) were purchased from Sankyo Labo Service (Tokyo, Japan) and mated with female C57BL/6 ($CD45.1^{-}CD45.2^{+}$) WT mice, resulting in C57BL/6 ($CD45.1^{+}CD45.2^{+}$) donor male mice. These mice were used as healthy donors. The genotype of all offspring was determined by polymerase chain reaction (PCR) analysis of DNA obtained from a tail snip. Mice were housed in groups of three to five per cage in a colony room under a 12-h light–dark cycle. Rodent diet and water were available *ad libitum*. All experiments were approved by the Animal Care Committee of the Jikei University School of Medicine, and all institutional and national guidelines for the care and use of laboratory animals were followed.

### Bone marrow transplantation

BMCs were collected from the femur and tibia of C57BL/6.Ly45.1 ($CD45.1^{+}CD45.2^{+}$) male mice and MPS-II-model mice ($CD45.1^{-}CD45.2^{+}$). Bone marrow cavities were flushed using phosphate-buffered saline (PBS) with 27-gauge needles, and cells were filtered through 100-μm nylon strainers (BD Falcon, Franklin Lakes, NJ, USA). Red blood cells were lysed using BD FACS™ lysing solution (BD Biosciences, San Jose, CA, USA). The percentage proportion of mixed BMCs from C57BL/6.Ly45.1 mice and MPS-II-model mice were 25:75, 50:50, 75:25, and 100:0, respectively. After MPS-II-model mice were exposed to myeloablative irradiation (9 Gy; 20 mA, 150 kV over 4.0 min with a 2.0-mm aluminum filter), $2.0 \times 10^{6}$ cells were injected intravenously into the mice (8 weeks of age) through the tail vein. Irradiation was performed using a Hitachi MBR1520R irradiator (Hitachi, Tokyo, Japan).

### Flow cytometry analysis

Peripheral white blood cells were harvested from transplanted mice (8 and 12 weeks after BMT) under anesthesia by superficial temporal vein puncture. Red blood cells were lysed using BD FACS™ lysing solution (BD Biosciences) based on the manufacturer's instructions. Peripheral blood cells were stained with fluorescein-isothiocyanate-conjugated anti-murine CD45.1 and allophycocyanin-conjugated anti-murine CD45.2 (eBioscience, San Diego, CA, USA). Each lineage was distinguished using the corresponding phycoerythrin-conjugated antibody: B cell–CD45R, T cell–CD3e, granulocyte–Ly6G, and macrophage–CD11b (eBioscience). Donor-derived cell engraftment was determined as the percentage of $CD45.1^{+}CD45.2^{+}$ cells. Flow cytometric analysis was performed using a MACSQuant® Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany), and results were analyzed with MACSQuantify® software (Miltenyi Biotec).

### Tissue collection and homogenized organs

At 20 weeks, tissue samples were collected from the transplanted, unmanipulated MPS II model and WT mice. The mice were killed by cardiac perfusion (20 ml PBS) under general anesthesia, and the organs (cerebrum, cerebellum, heart, liver, spleen, and kidney) were removed and homogenized with a tissue homogenizer NS-310EII (Microtecnichion, Chiba, Japan) for 10 s, three times, in water. The homogenates were centrifuged at 14,000 g for 15 min at 4 °C, and the supernatant was used for all analyses. Protein concentration

was measured using bicinchoninic acid protein assay kits (Thermo Fisher Scientific, Rockford, IL, USA) in accordance with the manufacturer's instructions, and protein concentration of each assay was adjusted with water.

### Tissue enzyme activity

Enzyme activity of IDS was assayed in homogenized tissue, as previously described, using the artificial substrate 4-methyllumbelliferyl-alpha-iduronide-2-sulfate (Moscerdam Substrates, Oegstgeest, The Netherlands) (Akiyama et al. 2014; Higuchi et al. 2012). The liberated fluorescence of 4-methylumbelliferone was measured using an RF-5300PC spectrofluorophotometer (SHIMADZU Co., Kyoto, Japan). Enzyme activity was expressed in nmol/4 h/μg protein. The average of two experiments was used.

### Total GAG analysis

Total amount of GAGs in tissue was measured at room temperature using the Wieslad® sGAG quantitative Alcian blue-binding assay kit (Euro-Diagnostica, Malmö, Sweden), as previously described (Akiyama et al. 2014; Higuchi et al. 2012). The amount of GAGs in tissues was expressed as μg/mg protein, as calculated by the standard curve of the GAG substrate chondroitin sulfate-6 (Euro-Diagnostica). The average of two experiments was used.

### Bone density examination

From nasal bone to the temporomandibular joint, skeletal scanning was performed using computed tomography (LCT-200 experimental animal CT system, Hitachi-Aloka Medical, LTD, Tokyo, Japan) 20 weeks after BMT. Radiographs of bone density of cortical bone and cancellous tissue were evaluated using Latheta software (Hitachi-Aloka Medical, LTD).

### Statistical analysis

Data were assessed using Graphpad Prism software (Graphpad Software, Inc., La Jolla, CA, USA). Student's *t* test was employed for statistical evaluation, and $p<0.05$ was considered statistically significant.

## Results

### Chimerism in peripheral blood of recipients

To define the level of chimerism of healthy donor cells and IDS activity necessary to degrade GAG storage in MPS II recipient tissues, we transplanted compounds of BMCs (total $2\times10^6$) from the C57BL/6Ly5.1 ($CD45.1^+CD45.2^+$) and MPS II mice ($CD45.1^-CD45.2^+$) at various ratios into MPS II mice that had received myeloablative irradiation. First, we analyzed the chimerism of peripheral blood leukocytes using anti-CD45.1 and anti-CD45.2 antibodies at 8 and 12 weeks following BMT. Double-positive cells for CD45.1 and CD45.2 were considered as WT donor cells. As expected, the percentage of CD45.1/45.2 double-positive leukocytes (WT cells) of transplanted MPS II mice was almost equal to that of CD45.1/45.2 double-positive leukocytes used for the graft (Fig. 1a). We then assayed the mixed chimerism of each specific cell lineage. The percentage of WT cells among B-cell, granulocyte, and monocyte lineages was almost identical to that of the percentage of WT cells used for the graft (Fig. 1b). However, the percentage of WT T cells was lower than that of the other three lineages.

### Enzyme activity

The activity of IDS in various organs was assessed using an artificial substrate (Fig. 2). In the liver and spleen, 25 % chimerism was adequate to significantly increase enzyme activity compared with tissues from unmanipulated MPS II mice (Student's *t* test $p<0.05$). Activities in the liver and spleen of mice with 25 % chimerism corresponded to 2 % and 20 % of WT levels, respectively. In the heart from mice with 75 % chimerism, IDS activity was also significantly increased (Student's *t* test $p<0.05$) compared with unmanipulated MPS II mice (~7 % of WT controls). However, even in 100 % healthy donor cells, IDS activity in the kidney, cerebrum, and cerebellum were not meaningfully increased compared with unmanipulated MPS II mice.

### GAG levels

GAG levels in several organs were assessed by Alcian blue staining (Fig. 3). In spleen and liver, 25 % chimerism significantly reduced GAG levels compared with unmanipulated MPS II (Student's *t* test $p<0.05$). The level of GAGs in the spleen and liver of mice with 25 % chimerism corresponded to 36 % and 28 % of unmanipulated MPS II mice, respectively. GAGs in liver and spleen were reduced in a dose-dependent manner by WT cells. Furthermore, 100 % chimerism achieved the most prominent reduction of GAGs in these tissues. Hearts of mice with 75 % chimerism showed a significant reduction in GAG level (Student's *t* test $p<0.05$). Again, GAG level reduction in the heart correlated with the percentage of WT cells. Conversely, even in mice with 100 % donor cells, GAG levels were not reduced in the kidney, cerebrum, and cerebellum. These data are relevant to the results of enzyme activity. However, in the cerebrum and cerebellum, GAG levels in WT and unmanipulated MPS II mice were not significantly

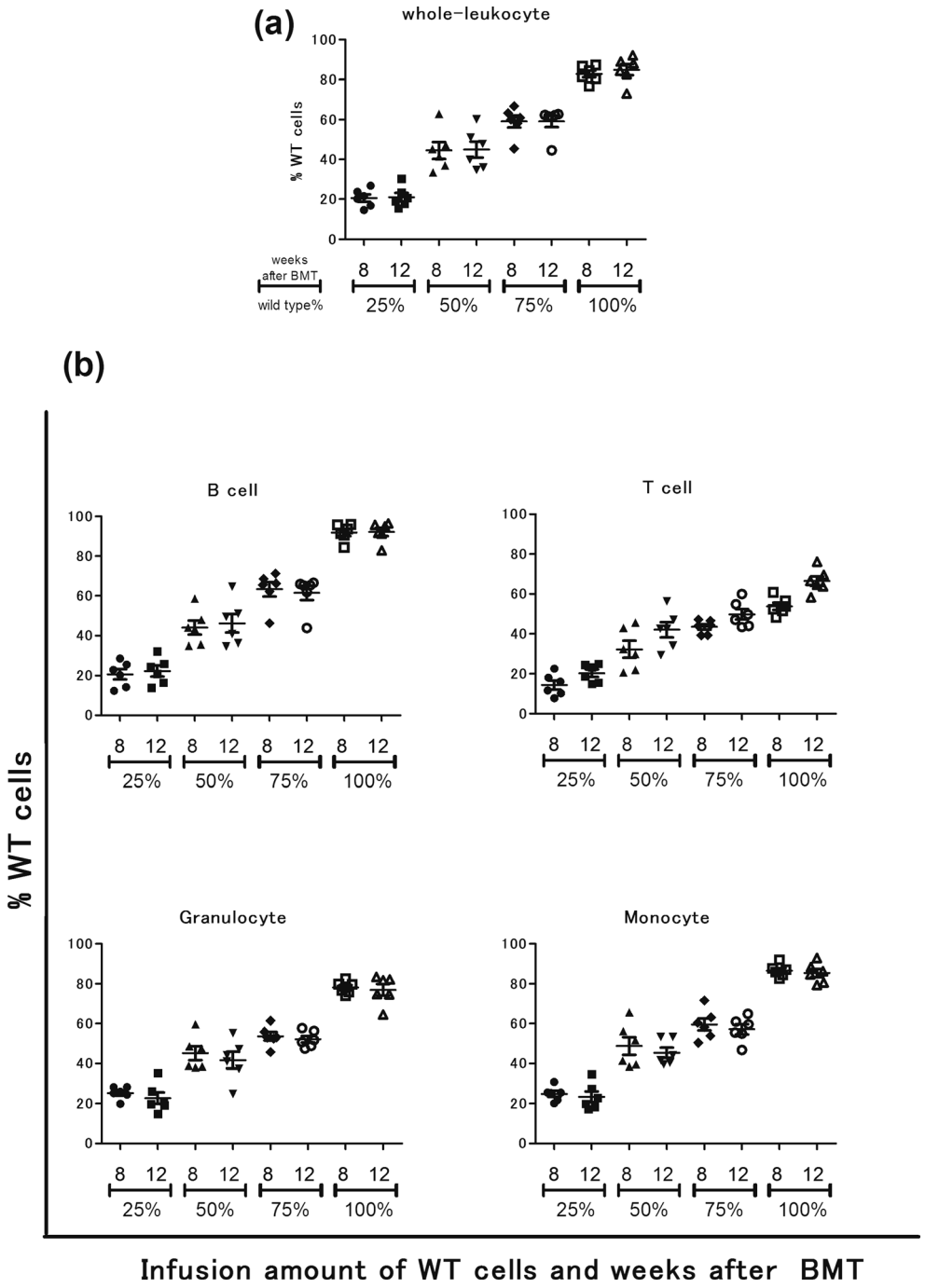


**Fig. 1** Chimerism of a recipient at 8 and 12 weeks after bone marrow transplantation (BMT). *X axis*: amount of transplanted wild-type (WT) bone marrow cells and weeks after transplantation. *Y axis*: percentage of WT cells in peripheral blood. (**a**) Whole leukocyte, (**b**) specific cell lineages (B cell–CD45R, T cell–CD3e, granulocyte–Ly6G, and macrophage–CD11b)

different ($p$=0.19 and $p$=0.10, respectively). Thus, the effect of BMT could not be evaluated in these two tissue types.

Skeletal examination

BMT does not improve bone density or cortical bone thickness (Fig. 4). However, there was no statistical difference between bone density and cortical bone thickness of MPS II and WT mice. Thus, the effect of BMT on these variables cannot be evaluated.

## Discussion

Treatment for many LSDs include ERT, cellular therapies such as BMT, and substrate reduction or chemical chaperone therapy, but the current conventional therapy for MPS II is ERT alone (Muenzer et al. 2002, 2011). However, patients with LSDs require life-long repetitive ERT administration, and the antibodies against the infused recombinant agents, could have a negative impact on ERT efficacy (Burton et al. 2011); BMT might overcome these limitations.

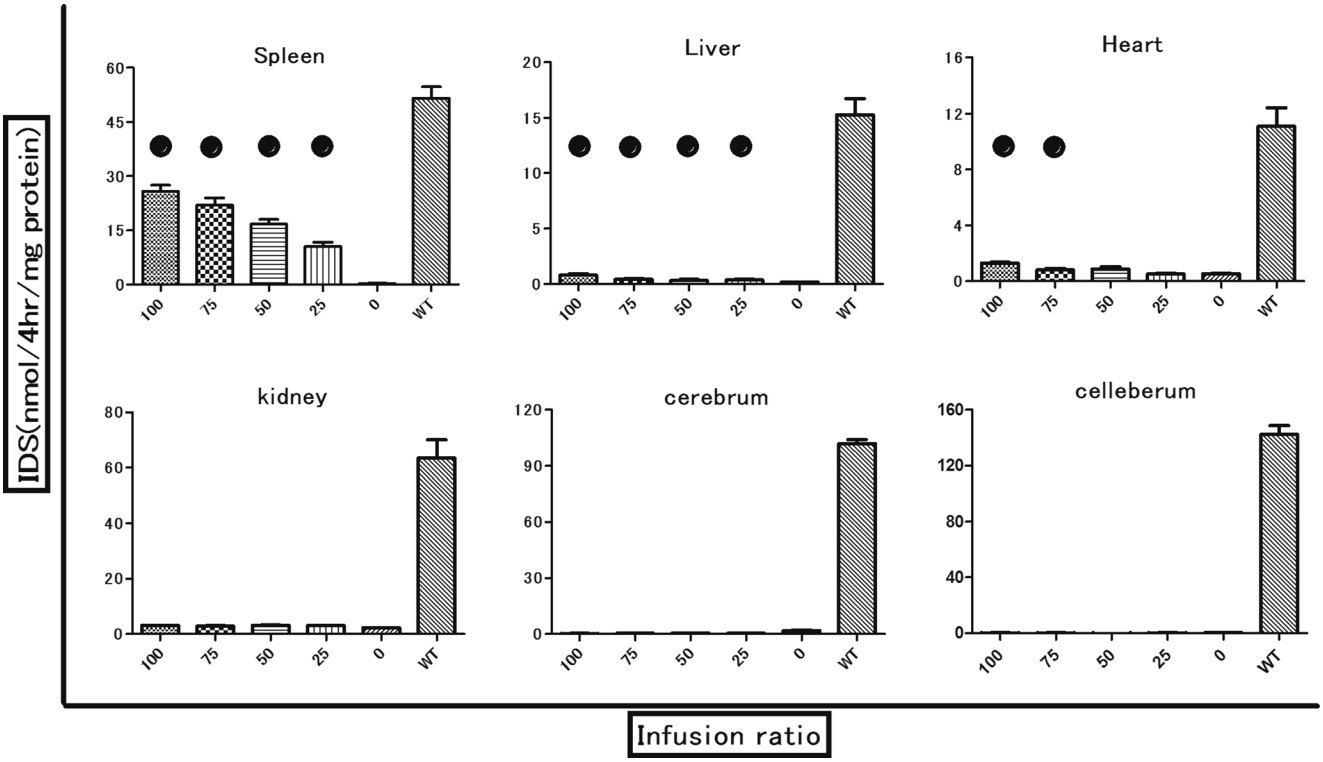


**Fig. 2** Enzyme activities of iduronate-2-sulfatase (IDS) in recipient organs 12 weeks after bone marrow transplantation (BMT): $n=6$ per group, Student's $t$ test * $p<0.05$ compared with "0" mice, which indicates unmanipulated mucopolysaccharidosis type II (MPS II) mice. *WT* wild type. *Error bar* standard error of mean

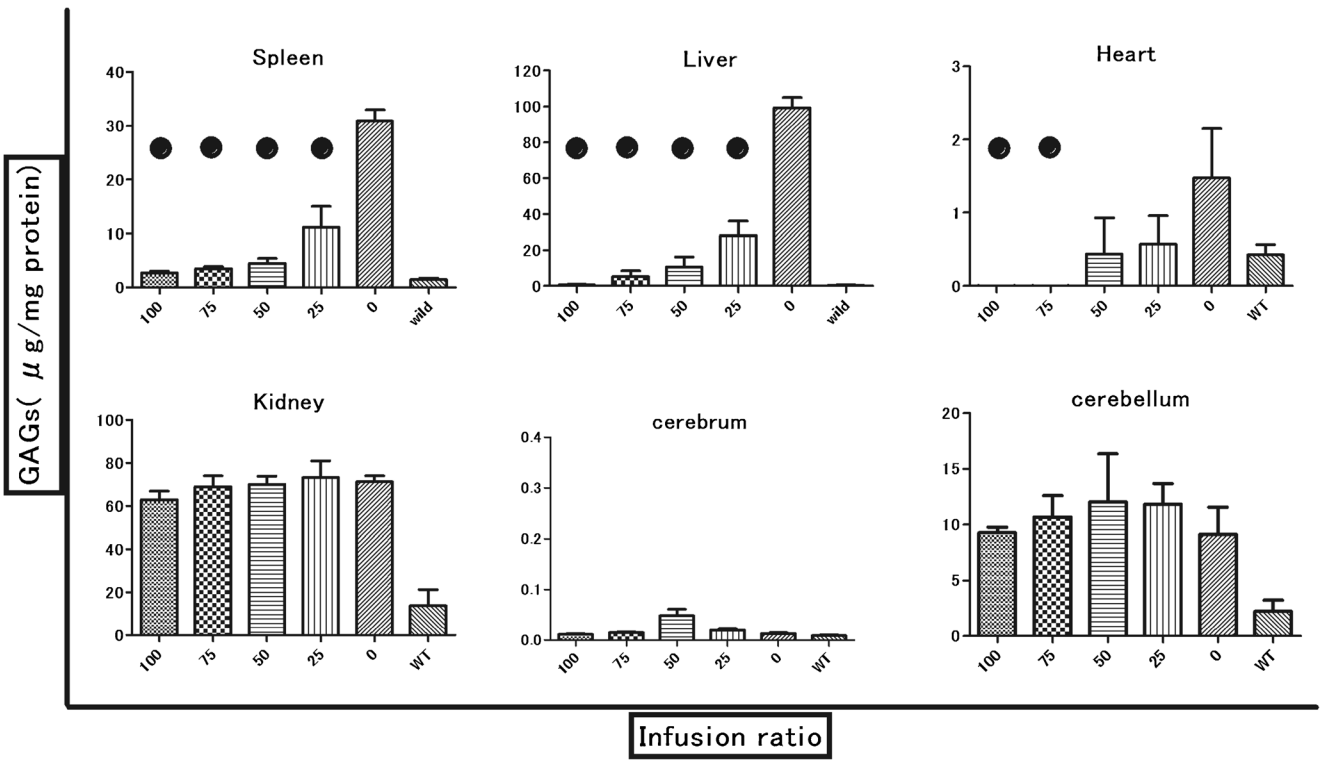


**Fig. 3** Glycosaminoglycan (GAG) storage in recipient organs 12 weeks after bone marrow transplantation (BMT) : $n=6$ per group, Student's $t$ test * $p<0.05$ compared with "0" mice, which indicates unmanipulated mucopolysaccharidosis type II (MPS II) mice. *WT* wild type. *Error bar* standard error of mean

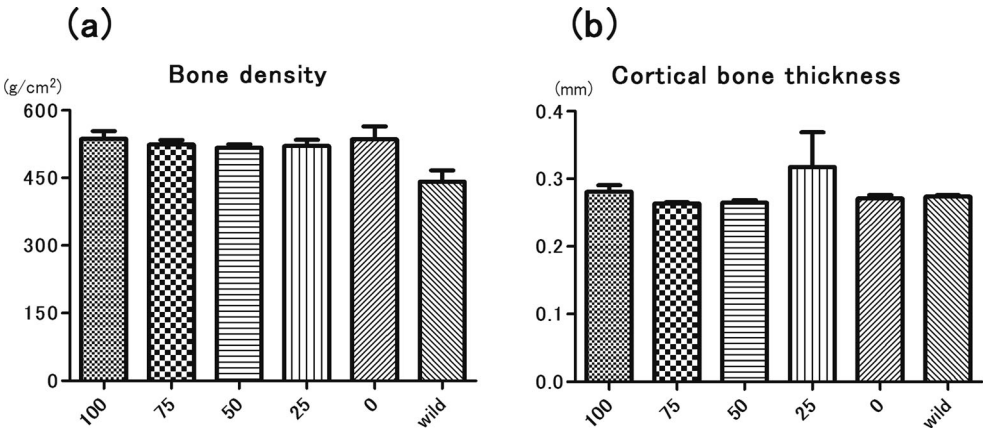


**Fig. 4** Skeletal analysis (from nasal bone to temporomandibular joint) 12 weeks after bone marrow transplantation: $n=6$ per group, Student's $t$ test * $p<0.05$ compared to "0" mice, which indicates unmanipulated mucopolysaccharidosis type II (MPS II) mice. **a** Bone density, **b** cortical bone thickness. *Error bar* standard error of mean

In the past 30 years, almost 1,000 patients with LSDs have received BMT (Tanaka et al. 2012; Prasad and Kurtzberg 2008; Mullen et al. 2000; Vellodi et al. 1999; Wynn et al. 2009; Guffon et al. 2009; Araya et al. 2009; Ito et al. 2004). The beneficial effect of BMT is principally due to the replacement of lacking enzymes by donor cells circulating in the blood and to the engraftment of donor-derived cells in many organs (Araya et al. 2009; Priller et al. 2001). For example, donor-derived macrophages can give rise to Kupffer cells in the liver, alveolar macrophages in the lung, and microglia cells in the brain.

Although therapeutic effects of BMT for several types of MPS have been indicated, especially for type I (Muenzer et al. 2009a, b; Boelens et al. 2013), BMT for MPS II patients as a curative therapy is controversial because it is clear that MPS II patients with a severe phenotype do not derive benefit if there is evidence of developmental delay at the time of BMT (Mullen et al. 2000; Vellodi et al. 1999; Wynn et al. 2009). Nevertheless, some reported improvements in clinical manifestations and somatic signs of MPS II patients have been reported (Tanaka et al. 2012; Guffon et al. 2009; Araya et al. 2009; Ito et al. 2004).

In contrast to immune-deficient or cancer patients, MPS II recipients are immunocompetent; therefore, myeloablative conditioning, including megadose chemotherapy and irradiation, is required to achieve 100 % engraftment. However, it is known that total body irradiation, which is one of the most frequent choices for the conditioning regimen and usually involves the entire brain, sometimes results in brain atrophy or dementia after a delay of many years (Tanaka et al. 2012).

To overcome these problems, efficient and less toxic BMT without poor engraftment should be considered for MPS II patients. However, less intense preconditioning regimens might cause a low level of engraftment of donor cells. Hence, we examined the degree of chimerism that is beneficial for reducing GAGs in MPS-II-model mice that underwent BMT. Some of our findings are consistent with previous reports of other murine LSD models (Enquist et al. 2009; Soper et al. 2001; Yokoi et al. 2011). At first, graft infusion ratios in our study were parallel to chimerism of engraftment, which is not only the whole leukocyte but also each lineage. This result was similar to those of other studies but with a lower degree of T-cell chimerism, perhaps because pre-existing recipient T cells may have a longer half-life.

In Gaucher disease models (Enquist et al. 2009), 10 % chimerism significantly reduced storage material (i.e., glucocerebroside) in the spleen and bone marrow. Moreover, it was concluded that 10 % chimerism almost normalized the level of storage material. Here as well, our findings are similar to these previously reported results: 25 % chimerism clearly reduced GAGs in the liver and spleen, but a higher degree of chimerism resulted in greater reduction in GAG accumulation. Gaucher disease is a macrophage disease, and thus mainly affects hematopoietic organs such as liver, spleen, and bone marrow. In contrast, nonhematopoietic organs, such as the heart, are usually affected in MPS disease, and it is therefore important that the percentage of chimerism required to reduce GAGs in the heart be elucidated. We found that 75 % chimerism was necessary to reduce the level of GAGs in the heart; thus, a higher degree of chimerism was necessary for BMT in MPS II.

Previous reports on MPS VII models (Soper et al. 2001) indicated that 10–15 % chimerism was sufficient to achieve a therapeutic effect in the liver, spleen, and meninges. These results were similar to ours, but comparison between the effects of 10–15 % chimerism and a higher level was not carried out in the previous study.

We formerly conducted a similar study in a murine FD model (Yokoi et al. 2011) and found that in the heart and lung, which are frequently affected in FD, 30–50 % chimerism of donor cells reduced storage levels in FD mice compared with nontransplanted mice. Although this reduction was similar to that in animals with 100 % normal cell engraftment, a higher degree of chimerism showed no additive effect. This previous observation is different from the findings of the study reported here; the discrepancy may be due to the different diseases and assay methods. Although the exact mechanism of cardiac damage in MPS

II remains unknown (Sato et al. 2013), GAGs are a normal component of cardiac-valve tissue; therefore, MPS II patients may require a higher degree of chimerism in BMT than patients with other types of LSDs in order to obtain a therapeutic effect. These results also imply that heterozygous donation of BMT for MPS II is not desirable because 50 % chimerism did not produce optimal GAG clearance in the heart. However, full engraftment of heterozygous donor is not identical to 50 % chimerism after BMT for MPS II due to skewed X-chromosome inactivation in X-linked disease. Thus, experiments using BMT in a murine MPS II model from heterozygote donor mice are underway in our laboratory.

Treatment of CNS damage in MPS II is very attractive because the brain is one of the major organs involved in this disorder. However, the degree of GAG accumulation in normal and nontransplanted MPS II brain tissue was almost the same in our assay system, and for this reason, we cannot evaluate the therapeutic effect of BMT in the brain.

In this study, we showed a direct correlation between engraftment percentage and therapeutic outcome, suggesting that full donor chimerism is highly desirable. In mice and humans, conversion of low-level chimerism to higher donor status may be obtained by donor lymphocyte infusion (DLI). In fact, an increase in chimerism was achieved by DLI in a murine model of MPS VII (Lessard et al. 2006). On the other hand, ERT was reported as an augmentation therapy to enhance the therapeutic efficacy of BMT in MPS II mice (Akiyama et al. 2014).

Limitations of this study are that the murine MPS II models were not very feasible for clinical evaluation, such as the Y-maze test, although they were suitable for biochemical evaluation of efficacy in BMT MPS II mice according to our previous reports (Akiyama et al. 2014; Higuchi et al. 2012). Also, we did not analyze a clinical endpoint, which may have affected the results.

In conclusion, a high percentage of donor-cell chimerism is necessary to obtain the maximum effect of BMT. Also, DLI or ERT might be indicated in the case of low-level donor chimerism in MPS II.

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Kentaro Yokoi, Kazumasa Akiyama, Eiko Kaneshiro, Takashi Higuchi, Yohta Shimada, Hiroshi Kobayashi, Masaharu Akiyama, Makoto Otsu, and Hiromitsu Nakauchi declare that they have no conflict of interest.

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**Contributions** K.Y., K.A. and T.O. designed and conducted research; E.K., H.H., Y.S. and H.K. performed experiments and analysed data; K.Y. wrote the paper; M.A., M.O., H.N., T.O., and H.I. discussed data and edited the paper.

**Animal Rights** All intuitional and national guidelines for the care and use of laboratory animals were followed.

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