aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Animal and Veterinary Advances 7 (12): 1312-1321, 2012
ISSN 1683-9919 / DOI: 10.3923/ajava.2012.1312.1321

A Simple Method of Identification of Hematopoietic Reconstitution

Guang-Ping Ruan, Bu-Zhen Zhang, Guang-Hong Ruan, Rong-Qing Pang, Xiang Yao, Jin-Xiang Wang, Jie He, Jing Zhao, Xue-Min Cai, Yan-Bo Lv and Xing-Hua Pan
Stem Cell Engineering Laboratory of Yunnan Province, Kunming General Hospital of Chengdu Military Command, Kunming 650032, China

Corresponding Author: Xing-Hua Pan and Bu-Zhen Zhang, Stem Cell Engineering Laboratory of Yunnan Province, Kunming General Hospital of Chengdu Military Command, Kunming 650032, China

ABSTRACT

To establish a simple method of identification of hematopoietic reconstitution, Green Fluorescent Protein (GFP) transgenic male mice cells were used as the transplant cells and the percentages of peripheral GFP positive cells after transplantation at different time points were measured to test the correlations with final status of reconstitution. Peripheral white blood cells (WBCs), spleen cells and liver cells of GFP transgenic male mice were transfused into sublethally irradiated C57BL female mice. After the peripheral WBC returned to normal range which was about 35 days post transplantation, mice tails were cut to draw blood. Blood samples were hemolyzed twice with ammonium chloride, washed once with PBS. Cells were then resuspended in 400 μL fixative fluid and analyzed by flow cytometry to measure the percentages of GFP positive cells. Seventy five days post irradiation, mice were sacrificed and their bone marrows cells were subject to detection of the percentages of cells with positive Y chromosome signal by fluorescence *in situ* hybridization. The percentage of GFP positive cells in peripheral blood correlated well to the rate of cells with positive Y chromosome signal in bone marrows detected by fluorescence in situ hybridization. Thirty-five days post transplantation with GFP transgenic male mice cells; the percentage of GFP-positive cells in peripheral blood can represent the final degree of hematopoietic reconstitution. So a simple method can monitor hematopoietic reconstitution by detection of GFP positive cells in peripheral blood.

Key words: GFP transgenic mice, fluorescence in situ hybridization, Y chromosome identification, hematopoietic reconstitution

INTRODUCTION

Hematopoietic Stem Cells (HSC) could be found in several tissues of mesodermic origin. Thus some mesodermic origin tissue could be considered as an important and convenient source of cells able to support hematopoiesis (Cousin *et al*., 2003). Hematopoietic Stem Cells (HSCs) are the primitive cells that have the capability of both self-renewal and differentiation into any of the hematopoietic cell lineages. In mice, hematopoietic cells originate from mesodermal precursors that generate differentiated blood cells during embryogenesis and individual development. In adult Bone Marrow (BM), the HSC number stays relatively constant in the absence of overt injury or blood loss. Additionally, small populations of HSCs can be detected in mouse Peripheral Blood (PB) and Spleen (SL) (Huang *et al*., 2007).

In human development, transdifferentiation is appeared during embryogenesis, growth and regeneration (Thowfeequ *et al*., 2007). Understanding transdifferentiation may be useful for

designing new stem cell therapies for various diseases, such as liver, renal and heart diseases and autoimmune disease and diabetes (Eberhard and Tosh, 2008). Other stem cell therapy methods, such as embryonic stem cell (ESC) or induced pluripotent stem cell (iPS)-based therapy, may have disadvantages because of ethical controversies or oncogenic concerns. Therefore, inducing transdifferentiation of differentiated cells as an alternative therapeutic approach would be more acceptable (Slack, 2007). Fluorescent male mice peripheral WBCs, spleen cells and liver cells were transfused into sublethally irradiated C57BL female mice. Whether these cells have hematopoietic stem cells and can transdifferentiate in vivo of female mice were observed.

All the cells of GFP transgenic cells glow with GFP fluorescence, so the cells from these mice can be tracked. It is unclear whether the internal organs of fluorescent mice contain hematopoietic stem cells and can reconstitute the hematopoietic function (Cousin *et al*., 2003; Ning *et al*., 2010). So a simple method was used to identify hematopoietic reconstitution. The detection of GFP positive cells in peripheral blood can identify the occurrence rate and extent of hematopoietic reconstitution which provides a simple method and platform to identify and study the roles of multipotent stem cells that can differentiate into hematopoietic stem cells in the future. The establishment of the method provides a platform to study the roles of various cells to reconstitute the hematopoietic function.

MATERIALS AND METHODS

Animal source: GFP transgenic male mice were from Professor Tinghua Wang of Kunming Medical University as gifts. C57BL female mice were purchased from Experimental Animal Center of Third Military Medical University.

Buffer preparation: Preparation of erythrocyte lysis buffer: NH_4Cl: 8 g, $NaHCO_3$: 0.84 g, Na_2EDTA: 0.37 g, distilled water added to 1000 mL.

Cells preparation: Preparation of spleen cells suspension of fluorescent male mice: sterile spleen was taken and soaked in double antibiotics and put on the 100-line screening with the syringe needle core crushing, when washed with basal medium. The cells were collected by filtration screen and hemolyzed with erythrocyte lysis buffer. After cells were counted, each mouse was injected with 5×10^6 spleen cells.

Preparation of liver cell suspension of fluorescent male mice: preparation method was the same as spleen cell suspension preparation. Each mouse was injected of 5×10^6 liver cells.

Preparation of peripheral WBCs of fluorescent male mice: eyeballs were removed to gain peripheral blood of fluorescent mice. Blood- was hemolyzed twice with erythrocyte lysis buffer. WBCs were counted and each mouse was injected with 5×10^6 peripheral WBCs.

Mice irradiation: C57BL female mice irradiation: 20 C57BL female mice in total were irradiated by the linear accelerator with dose of 600 cGy and dose rate of 50 cGy min^{-1}. The distance to box top was 98.5 cm, with 100 cm away from the mice center.

Mice group: C57BL female mice were divided into four groups, n = 5. The first group was injected with fluorescent mice peripheral WBCs, the second group were injected with fluorescent mice spleen cell suspension and the third group was injected with fluorescent mice liver cell suspension, while the fourth group was not injected with cells as negative control.

The percentages of GFP positive cells in peripheral blood were detected: Mice tails were cut and the blood was dropped into the EP tube with 200 μL anticoagulants, mixed evenly,

centrifuged and anticoagulants were aspirated. Red blood cells were hemolyzed with ammonium chloride solution and the blood samples were centrifuged at 1500 rpm for 4 min. After twice of hemolysis, cells were washed once with PBS. WBCs were resuspended in 400 μL fixative fluid for flow cytometry to detect the percentage of GFP-positive cells.

GFP-positive cells converted into CD4+ and CD8+T cells: Mice tails were cut and the blood dropped into the EP tube with anticoagulants 200 μL, mixed evenly, centrifuged and anticoagulants were aspirated. Blood was divided into two tubes and one tube was added with 2 μL CD4-PE, while another tube was added with 2 μL CD8-PE. Cells were incubated at room temperature for 30 min. Then red blood cells were hemolyzed twice and washed by PBS once. Cells were resuspended in 400 μL fixative fluid for flow cytometry analysis of GFP and PE double positive cells.

Fluorescence in-situ hybridization (FISH): Biotin-labelled mouse Y chromosome specific probe (50-100 ng) was made up to 12 mL with hybridization buffer (50% deionized formamide, 10% dextran sulphate, 2×SSC, 0.5 mol L^{-1} phosphate buffer, pH 7.3). The probe was denatured at 65°C for 10 min and preannealed by incubation at 37°C for 30-60 min. Slides were denatured by incubation in 70% formamide/2×SSC solution at 65°C for 1-2 min, quenched in ice-cold 70% ethanol and dehydrated through a 70, 90 and 100% ethanol series. The preannealed probe was applied onto slides and allowed to hybridize overnight at 37°C. Post-hybridization washes were two 5-min incubations in 50% formamide/2×SSC at 45°C, followed by two 5 min incubations in 2×SSC at 45°C. Biotin-labelled probe was visualized using one layer of Cy3-avidin (1:1000 Amersham). After detection, slides were mounted in Vectashield mounting medium with DAPI (4'6-diamidino-2-phenylindole). Images were captured using the CytoVision system (Applied Imaging) with a CCD camera mounted on a Zeiss Axioplan 2 microscope.

RESULTS

Peripheral WBCs counts of recipient mice after transplantation: Six days after transplantation, the peripheral WBC number of recipient mice was as low as about 6×10^5 mL^{-1}. Twenty six days after transplantation, peripheral WBC number of recipient mice was increased to nearly normal (~144×10^5 mL^{-1}) in all groups (Fig. 1).

Percentages of GFP-positive cells were analyzed after transplantation: Thirty five days after transplantation, GFP-positive cells in the recipient mice were detected in peripheral blood and

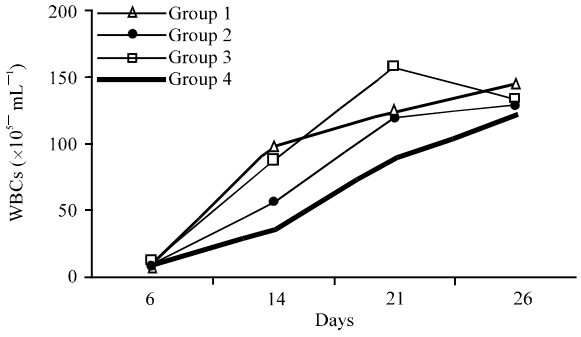

Fig. 1: Changes in white blood cells (WBCs) number of recipient mice at different days after irradiation

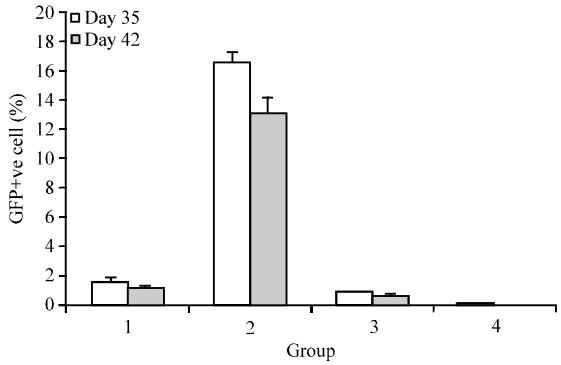

Fig. 2: GFP-positive cell analysis in peripheral blood of four groups recipient mice at different time points after transfusion (n = 5)

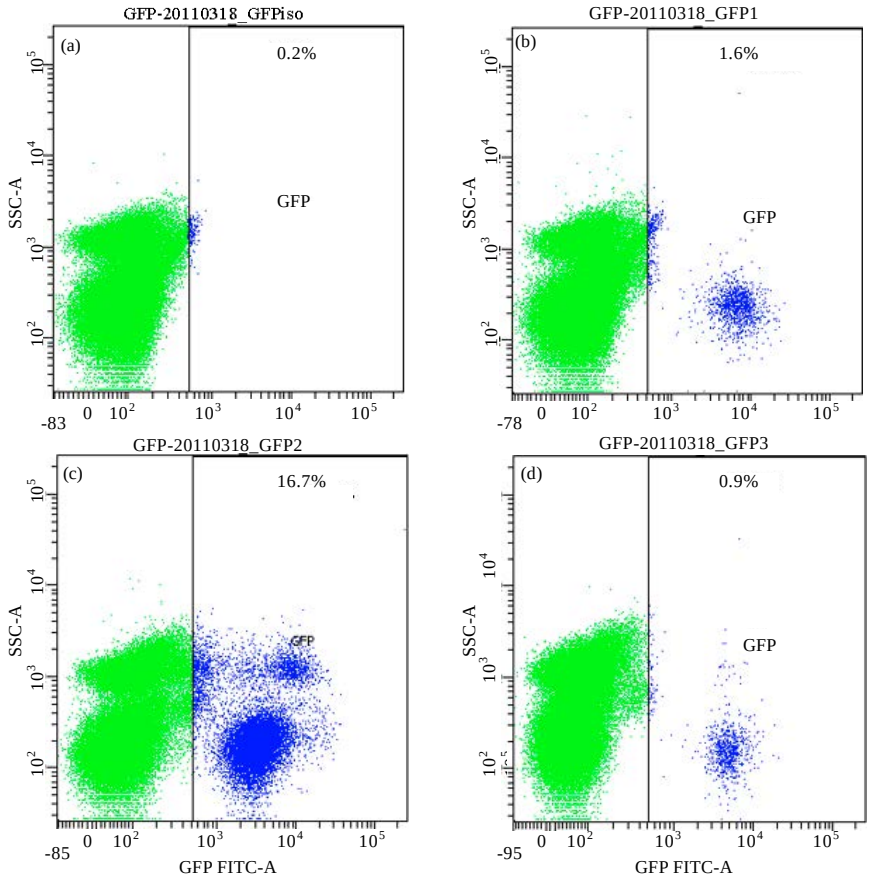

Fig. 3(a-d): GFP (a) Without transfusion of cells (0.2%) and with transfusion of (b) Peripheral WBCs (1.6%), (c) Spleen cells (16.7%) and (d) Liver cells (0.9%) analysis in peripheral blood after 35 days of transplantation

the average positive rate of four groups were as follows, respectively (Fig. 2 and 3) Mice transfused Group 2, mice transfused with spleen cell suspension exhibited 16.7% GFP-positive cells on

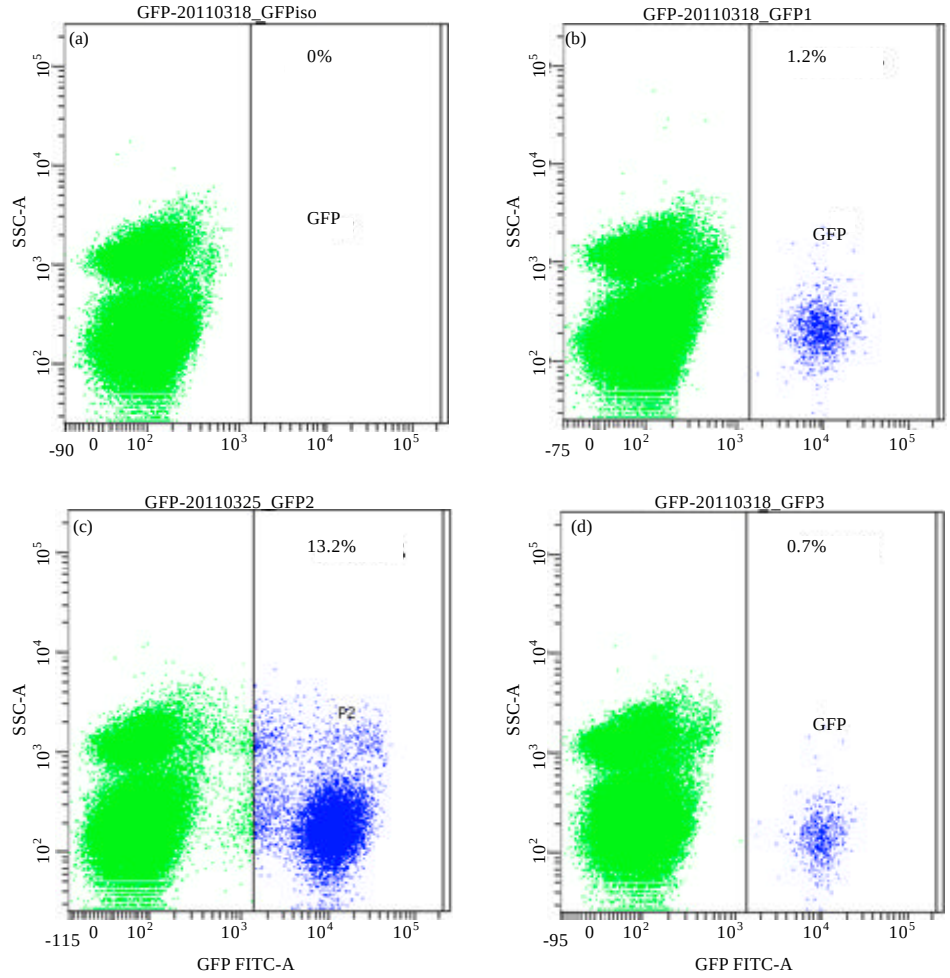

Fig. 4(a-d): GFP (a) Without transfusion of cell (0%) and with transfusion of (b) Peripheral WBCs (1.2%), (c) Spleen cells (13.2%) and (d) Liver cells (0.7 %) analysis in peripheral blood after 42 days of transplantation, Green scatter: GPF-negative cells. Blue scatter: GFP-positive cells

with peripheral WBCs showed average 1.6% GFP-positive cells in peripheral blood (Group 1). In Group 2, mice transfused with spleen cell suspension exhibited 16.7% GFP-positive cells on average. Mice transfused with liver cell suspension were detected to have average 0.9% GFP-positive cells in peripheral blood (Group 3). Group 4, in which mice were not transfused with any cells, showed only minimal amount of GFP-positive cells (average 0.2%).

Forty two days after transplantation, the peripheral blood of the recipient mice was detected to have the presence of GFP-positive cells. The average positive rates of four groups were 1.2, 13.2, 0.7, 0%, respectively (Fig. 4). Mice transfused with spleen cell suspension exhibited the highest GFP-positive cells, followed by mice transfused with peripheral WBCs and liver cell suspension.

Sixty two days after transplantation, GFP-positive cells converted into CD4+ and CD8+ T cells: The peripheral blood of mice transplanted with peripheral WBCc had 1.1% of cells with GFP and CD8 double positive cells, indicating that cells generated by WBCs transplanted into

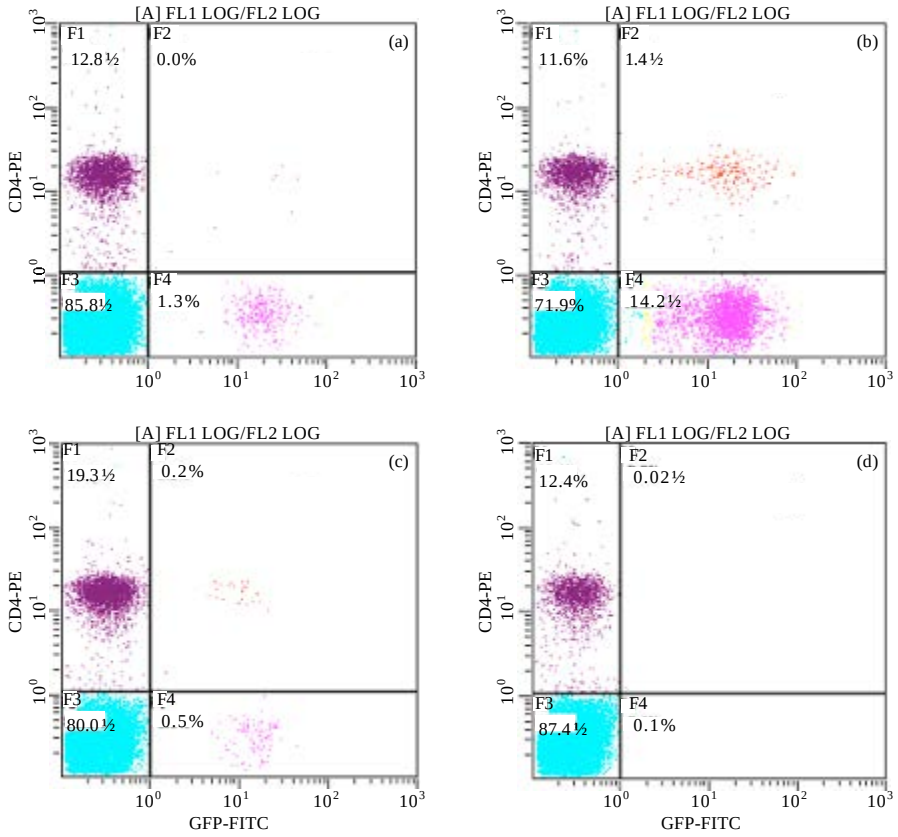

Fig. 5(a-d): CD4-PE and GFP with transfusion of (a) Peripheral WBCs (0%), (b) Spleen cells (1.4%) and (c) Liver cell (0.2%) and with (d) No transfusion of cells (0%) analysis in peripheral blood after 62 days of transplantation, Red scatter: CD4-PE and GFP-double positive cells. Blue scatter: CD4-PE and GFP-double negative cells, Purple scatter: CD4-PE positive and GFP- negative cells, Rosy scatter: CD4-PE negative and GFP- positive cells

female mice were mainly CD8+ T cells (~78%, Fig. 5 and 6). The peripheral blood of mice transplanted with spleen cells had 1.4% of the cells with GFP and CD4 double positive markers, 4.7% of the cells with GFP and CD8 double positive markers, indicating cells generated by spleen cells transplanted into the female mice comprised of 8% CD4+ T cells and 29% CD8+ T cells (Fig. 5, 6). The peripheral blood of mice transfused with liver cells had 0.2% of cells with GFP and CD4 double positive markers, 0.1% of the cells with GFP and CD8 double positive markers, suggesting that cells generated by the liver cells transplanted into female mice included 28% CD4+T cells and 14% CD8+T cells (Fig. 5 and 6). These results imply that GFP-positive cells in peripheral blood could be converted in to CD4+ T cells and CD8+ cells.

Seventy five days after transplantation, positive rates of Y chromosome were detected by FISH 75 days after transplantation, percentages of GFP-positive cells in peripheral blood of all 4 groups were detected to average at 1.4, 13.5, 0.8 and 0%, respectively (Fig. 7). While the FISH analysis of Y chromosome-positive rates in 4 groups, respectively, were averaging at 1, 13.3, 0.5 and 0%.

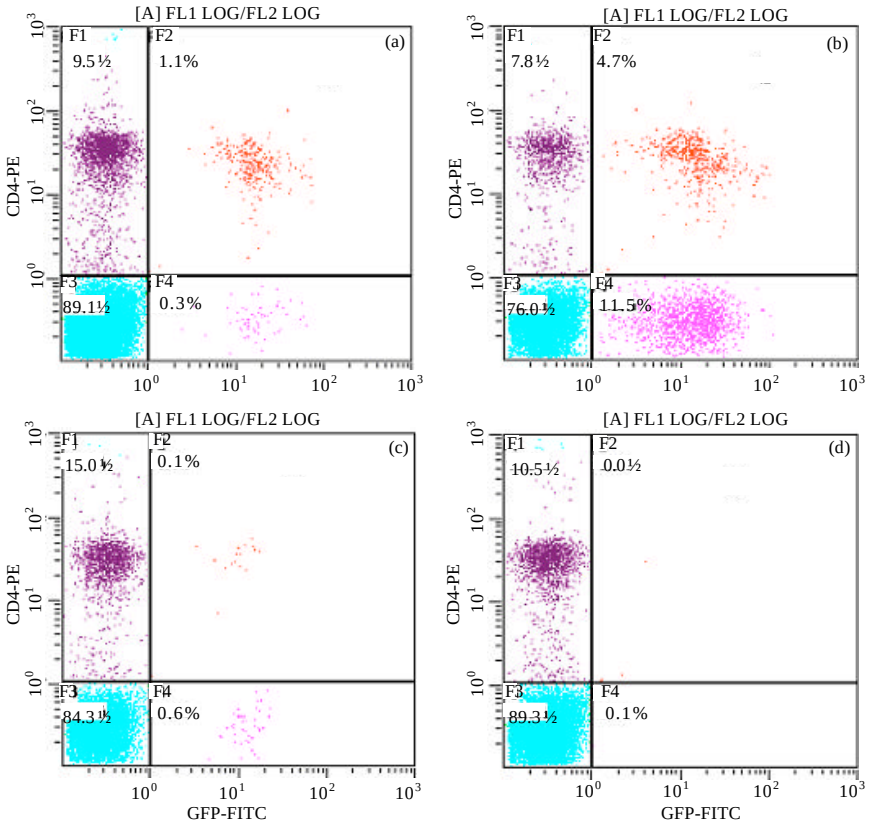

Fig. 6(a-d): GFP and CD8-PE with transfusion of (a) Peripheral WBCs (1.1%), (b) Spleen cells (4.7%) and (c) Liver cells (0.1%) and with (d) No transfusion of cells (0%) analysis in peripheral blood after 62 days of transplantation, Red scatter: CD8-PE and GFP-double positive cells, Blue scatter: CD8-PE and GFP-double negative cells, Purple scatter: CD8-PE positive and GFP-negative cells, Rosy scatter: CD8-PE negative and GFP-positive cells

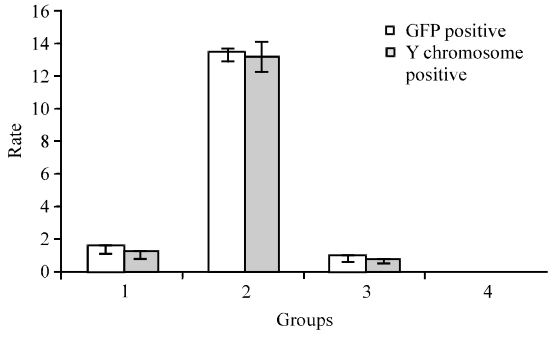

Fig. 7: Comparison of rates of GFP-positive cells in peripheral blood of recipient mice and rates of Y chromosome positive cells in bone marrow of same mice among four groups

The two positive rates manifested obvious positive correlation. Y chromosome was detected by probe in metaphase cleavage image of chromosome in bone marrow (Fig. 8).

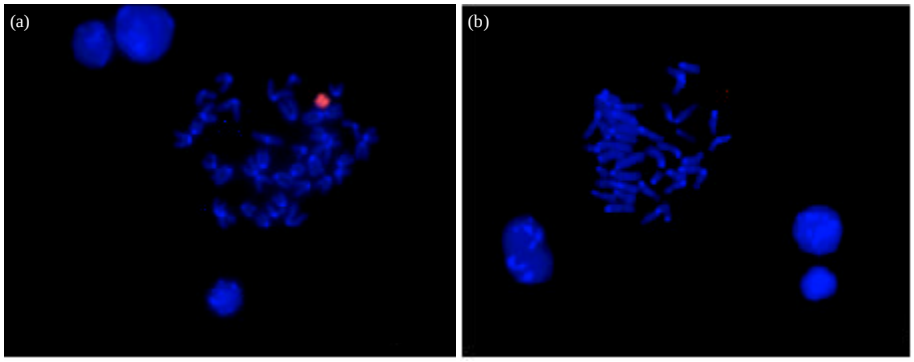

Fig. 8(a-b): Female bone marrow metaphase image (a) with Y chromosome (red) and (b) Without Y chromosome

More importantly, the trend of Y chromosome positive rates in bone marrow 75 days post transplantation is consistent with that of GFP positive cells rates in peripheral blood as early as 35 days post transplantation; strongly suggesting that the rates of GFP positive cell in peripheral blood 35 days post transplantation could represent the extent of hematopoietic reconstitution.

DISCUSSION

Transfusion of cells from fluorescent C57BL male into the C57BL female mice allows tracking the origin of the cells. Hematopoietic reconstitution of sub-lethally irradiated female mice can be monitored by a very convenient method devised in this paper. In the simple method, only 50-100 μL blood was needed through cutting mice tails and cells were analyzed by flow cytometry after erythrocyte lysis. Whether the transfused cells had rebuilt the hematopoietic function was identified. The proportion of peripheral GFP-positive cells positively correlates with the percentage of Y chromosome in the bone marrow detected by FISH. Therefore, the percentage of Y chromosome positive cells in bone marrow could be reliably predicted based on the percentage of GFP-positive cells in the peripheral blood. Since the collection of peripheral blood results into solely minimal injury to mice, the method holds the advantage of simplicity and is applicable to multiple unitizations with ease. Meanwhile, collection of peripheral blood to detect the proportion of GFP-positive cells by flow cytometry method was also very cost-effective. This low-cost approach is possible to replace the high-cost approach like FISH. The methods can preserve the intactness of the bone marrow. The percentage of GFP positive cells in peripheral blood can be detected repeatedly at different time point, while mice can survive and continue to be observed. Our study shows that peripheral WBCs, spleen cells and liver cells have the function of hematopoietic reconstitution, but the roles of these three kinds of cells are different. The spleen cells contain most of the hematopoietic stem cells among these three, the function of hematopoietic reconstitution is the largest. This can be confirmed both by the percentage of GFP-positive cells in peripheral blood and FISH results (Lapointe *et al*., 1996). Given that the peripheral WBCs and liver cells contain a limited number of hematopoietic stem cells (Aoi *et al*., 2008), only a small number of transfused cells were alive in the recipient mice and the reconstituted hematopoietic function was also limited (Spangrude *et al*., 1988). It was reported that muscle-derived cells can reconstitute hematopoietic function (Jackson *et al*., 1999; Pang, 2000). Further studies are needed to see if cells of other

lineages in mice can rebuild hematopoietic function (Haond *et al*., 2007) and our method can quickly and easily identify whether the cells transfused into the body can be converted into CD4+ or CD8+ T cells.

Our study shows that mice liver cells contain hematopoietic stem cells that can survive in the recipient mice and differentiate into blood cells (Vecchini *et al*., 1993). WBCs in the peripheral blood of mice also contain a small amount of hematopoietic stem cells (Matsuzaki *et al*., 2004). They also survived in peripheral blood of recipient mice and contributed to the normal function. While spleen cells contain much more hematopoietic stem cells, after the reconstitution, the peripheral blood had more fluorescent cells in recipient mice and FISH detection of Y chromosome positive cell in the bone marrow echoed this result.

CONCLUSION

A simple method to identify the roles of peripheral blood WBCs, spleen cells and liver cells was used. These cells survived in peripheral blood of recipient mice and contributed to the normal function. The method holds the advantage of simplicity and is applicable to multiple unitizations with ease.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (31172170), 973 Projects (2012CB518106) and special funding by the China Postdoctoral Science Foundation (201104748).

REFERENCES

Aoi, T., K. Yae, M. Nakagawa, T. Ichisaka and K. Okita *et al*., 2008. Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science, 321: 699-702.

Cousin, B., M. Andre, E. Arnaud, L. Penicaud and L. Casteilla, 2003. Reconstitution of lethally irradiated mice by cells isolated from adipose tissue. Biochem. Biophys. Res. Commun., 301: 1016-1022.

Eberhard, D. and D. Tosh, 2008. Transdifferentiation and metaplasia as a paradigm for understanding development and disease. Cell Mol. Life Sci., 65: 33-40.

Haond, C., F. Farace, M. Guillier, Y. Lecluse, F. Mazurier, W. Vainchenker and A.G. Turhan, 2007. Quantitative and qualitative *In vitro* analysis of the stem cell potential of hematopoietic cells purified from murine skeletal muscle. Cell Res., 17: 783-791.

Huang, X., S. Cho and G.J. Spangrude, 2007. Hematopoietic stem cells: Generation and self-renewal. Cell Death Differ., 14: 1851-1859.

Jackson, K.A., T. Mi and M.A. Goodell, 1999. Hemopoietic potential of stem cells isolated from murine skeletal muscle. Proc. Nat. Acad. Sci., 96: 14482-14486.

Lapointe, C., L. Forest, P. Lussier, L. Busque and F. Lagace *et al*., 1996. Sequential analysis of early hematopoietic reconstitution following allogeneic bone marrow transplantation with Fluorescence *In situ* Hybridization (FISH). Bone Marrow Transplantation, 17: 1143-1148.

Matsuzaki, Y., K. Kinjo, R.C. Mulligan and H. Okano, 2004. Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. Immunity, 20: 87-93.

Ning, L., E. Goossens, M. Geens, D. van Saen, I. van Riet, D. He and H. Tournaye, 2010. Mouse spermatogonial stem cells obtain morphologic and functional characteristics of hematopoietic cells *In vivo*. Hum. Reprod., 25: 3101-3109.

Pang, W., 2000. Role of muscle-derived cells in hematopoietic reconstitution of irradiated mice. Blood, 95: 1106-1108.

Slack, J.M., 2007. Metaplasia and transdifferentiation: From pure biology to the clinic. Nat. Rev. Mol. Cell Biol., 8: 369-378.

Spangrude, G.J., S. Heimfeld and I.L. Weissman, 1988. Purification and characterization of mouse hematopoietic stem cells. Science, 241: 58-62.

Thowfeequ, S., E.J. Myatt and D. Tosh, 2007. Transdifferentiation in developmental biology, disease and in therapy. Dev. Dyn., 236: 3208-3217.

Vecchini, F., K.D. Patrene and S.S. Boggs, 1993. Purified murine hematopoietic stem cells function longer on nonirradiated W41/Wv than on +/+ irradiated stroma. Blood, 81: 1489-1496.