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LETTER TO THE EDITOR

Bone Marrow-Derived Stem Cells Do Not Reconstitute Spermatogenesis In Vivo

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Spermatogenesis is a self-renewing process leading to production of spermatozoa in adult testis. The existence of male germinal stem cells was demonstrated by regeneration of spermatogenesis after transplantation of total testicular germ cells in testis [1], and we have previously shown that mouse testicular cells contain a side population (SP) of stem cells (Hoechst 33342 fluorescent dye efflux) that can repopulate germ cell-depleted testis when transplanted into adult mice [2]. Several breakthroughs concerning the in vitro derivation of male and female germ cells from embryonic stem cells have challenged the field of reproductive biology and opened new perspectives for treatment of infertility [3–5]. Some reports have suggested that adult bone marrow-derived stem cells are able to regenerate various nonhematopoietic cell lineages in several organs, by transdifferentiation or cell fusion mechanisms, and that tissue-specific and pluripotent stem cells occur in the bone marrow [6]. Most strikingly, Johnson et al. recently suggested that bone marrow transplantation restores oocyte production in sterile adult mice [7], although this finding is much debated, and female bone marrow cells failed to produce haploid mature oocytes in parabiotic mouse models [8]. Concerning male germinal lineage, mouse adult bone marrow mesenchymal stem cells, grown in vitro in the presence of retinoic acid, were recently found to express germ cell markers, but they failed to undergo spermatogenesis after transplantation into testes [9]. A similar transdifferentiation process was described for adult human bone marrow cells [10]. Furthermore, another group claimed that bone marrow stem cells could differentiate in putative germ cells in mouse after transplantation in testis [11]. In a similar approach, we have tested whether male bone marrow-derived stem cells would be reprogrammed to produce germ cells that give rise to spermatogenesis when introduced into the testis or whether bone marrow might provide a reservoir of male germinal stem cells. Here we demonstrate that male bone marrow-derived stem cells do not undergo spermatogenesis when transplanted into the testis of adult mice.

To test whether bone marrow-derived stem cells might repopulate depleted testis, we first prepared total bone marrow cells from adult male donor mice and transplanted them into the seminiferous tubules of germ cell-depleted recipient C57Bl6J mice (3–5 million cells per testis) 1 month after treatment with busulfan to destroy endogenous spermatogenesis [2]. Hematopoietic stem cell potential of whole bone marrow cell suspension was confirmed by competitive repopulation assay after transplantation into the retro-orbital plexus of lethally irradiated mice. The donor mice were transgenic for the gene encoding enhanced green fluorescent protein (EGFP), which they express in all their tissues [12], thus providing a means to detect and quantify donor-derived cells by their fluorescence after testicular transplantation [2]. Recipient testes were analyzed 3 months ($n = 4$; two independent experiments), 6 months ($n = 6$; three independent experiments), and 8–9 months ($n = 11$; three independent experiments) after transplantation. We did not observe any colonization of the recipient testis when whole bone marrow cells were transplanted at any time after transplantation, in contrast to control testis transplanted with total testicular cells, which showed colonization of the seminiferous tubules (Fig. 1A, 1B). Flow cytometry analyses of single-cell suspensions from recipient testes showed that 15 of 21 transplanted testes with whole bone marrow contained only rare EGFP-positive cells (0.01%–0.1% of testicular cells), representing less than 0.06% of the number of bone marrow cells initially transplanted (Fig. 1C). During spermatogenesis, germ cells undergo meiosis, leading to the generation of haploid spermatids. We therefore analyzed the DNA content of the cells by staining with Hoechst 33342 to detect any haploid cells (Fig. 1D). No donor-derived (i.e., EGFP-positive) haploid cells were detected (Fig. 1E), demonstrating that the transplanted bone marrow cells did not reconstitute spermatogenesis in vivo in the testicular microenvironment.

The vast majority of EGFP-positive cells (91% ± 4%; $n = 5$; mean ± SEM) in recipient testis were positive for the hematopoietic cell marker CD45, and 53% ± 8% ($n = 12$) showed Hoechst dye efflux (Fig. 1E, 1F). Testis SP cells do not express the marker CD45 [2]; therefore, those EGFP-SP-CD45-positive cells that we detected in the recipient testis could not be testicular SP cells derived from donor bone marrow (BM) cells after transplantation but rather were cells that maintained their hematopoietic fate. These cells were sorted and transplanted intravenously into lethally irradiated recipients but failed to give EGFP peripheral blood chimerism, showing that those BM-derived SP cells found in recipient testis did not have any hematopoietic stem cell potential (three independent experiments with 200, 300, and 1,000 sorted cells; data not shown).

Finally, we tested bone marrow EGFP-enriched fraction of hematopoietic stem cells, purified on the basis of both their side population phenotype and their expression of the hematopoietic stem cell marker Sca-1 [13], for their potential to produce haploid spermatids when transplanted into testis. Like total bone marrow cells, this enriched fraction of hematopoietic stem cells,

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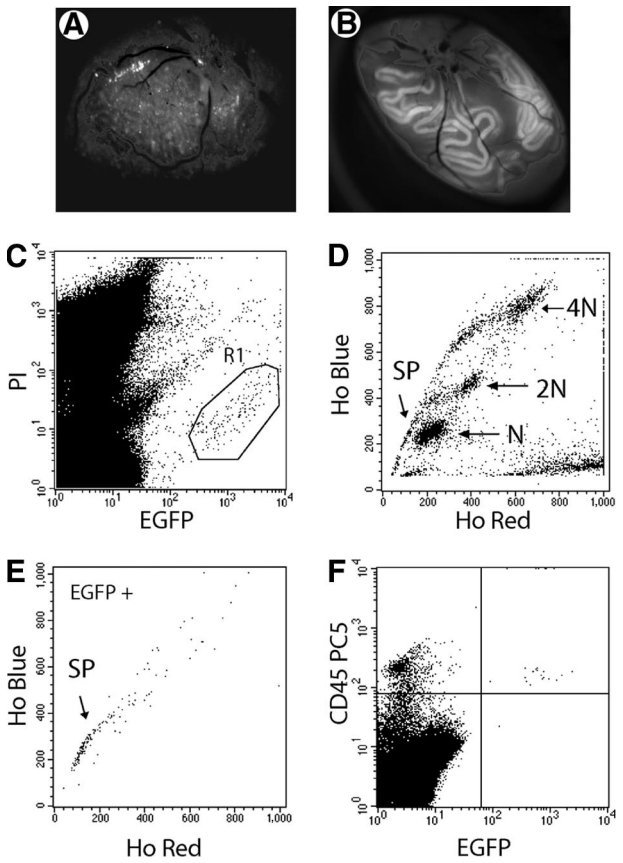

Figure 1. Bone marrow-derived stem cells do not repopulate busulfan-treated recipient testis after testicular transplantation. **(A):** EGFP whole bone marrow cells did not colonize seminiferous tubules in a recipient testis 6 months after transplantation, as determined by fluorescence microscopic analysis of whole testis. **(B):** Detection of EGFP-fluorescent seminiferous tubules in a control recipient testis transplanted with EGFP total testicular cells showing colonization of tubules 6 months after transplantation. **(C):** Flow cytometry of a testicular cell suspension from recipient testis 6 months after transplantation with whole bone marrow to detect viable donor-derived EGFP-positive cells. Donor-derived cells are found in the gate R1. PI staining was performed to exclude dead cells. **(D):** Ho fluorescence flow cytometry (red/blue Hoechst fluorescence) of a testicular cell suspension prepared from recipient testis. Residual endogenous spermatogenesis from recipient was observed 7 months after busulfan treatment. The haploid N is indicated by the arrow. **(E):** Red/blue Ho fluorescence flow cytometry of EGFP-positive cells in recipient testis gated from **(C)** (gate R1). Haploid spermatids were not detected in bone marrow donor-derived EGFP cells. **(F):** EGFP and CD45 analysis of a testicular cell suspension prepared from recipient testis transplanted with donor bone marrow cells. Donor EGFP cells expressed the hematopoietic marker CD45. Abbreviations: 2N, spermatocyte II; 4N, spermatocyte I; EGFP, enhanced green fluorescent protein; Ho, Hoechst 33342; N, spermatid population; PI, propidium iodide; SP, side population cells.

whose stem cell activity was tested by transplantation into lethally irradiated mice, did not restore spermatogenesis 4 months after testicular transplantation ($n = 14$ testis; four independent experiments; data not shown).

Our data indicate that adult male bone marrow-derived stem cells cannot transdifferentiate into germ cells to produce postmeiotic haploid cells after transplantation into testis and that male bone marrow does not appear to contain germinal stem cells. Rare bone marrow-derived, EGFP-positive cells found in transplanted testis maintain their hematopoietic fate in this environment. In addition, our study on male reproductive organs is in agreement with a recent report showing that female bone marrow cells fail to produce haploid mature oocytes in parabiotic mouse models [8], in contrast to the hypothesis that female fertility could be restored by bone marrow transplantation [7]. Future studies on the in vitro reprogramming of adult bone marrow stem cells should clearly be developed to potentially use those cells for regenerative medicine in male reproduction. Thus, our results suggest that male bone marrow in adult would not provide a simple and alternative source of stem cells for the treatment of male infertility.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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