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Putative human male germ cells from bone marrow stem cells

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Abstract

Germ cells must develop along distinct male or female paths to produce spermatozoa or eggs required for sexual reproduction. Male germline stem cells maintain spermatogenesis in postnatal human testis. Here we show that a small population of bone marrow cells is able to transdifferentiate to male germ cell-like cells. We show expression of early germ cell markers Oct4, Fragilis, Stella and Vasa and male germ cell specific markers Dazl, TSPY, Piwil2 and Stra8 in these cells. Our findings provide direct evidence that human bone marrow cells can differentiate to putative male germ cells and identify bone marrow as a potential source of male germ cells that could sustain sperm production.

Introduction

Male germ cells are derived from a founder population of primordial germ cells (PGCs) that are set aside early in embryogenesis. PGCs arise from the proximal epiblast, a region of the early embryo that also contributes to the first blood lineages of the embryonic yolk sac (Lawson and Hage, 1994; Zhao and Garbers, 2002). After birth, PGCs differentiate to spermatogonial stem cells in the male which are responsible for maintaining spermatogenesis throughout life by continuous production of daughter cells that differentiate into spermatozoa (de Rooij and Grootegoed, 1998; Mc Laren, 2000). The common origin of germ cell lineage and blood lineage and the fact that bone marrow stem cells are pluripotent cells which can differentiate to other cell types, prompted us to examine the differentiation potential of human bone marrow (BM) cells to male germ cells. Accumulated evidence suggests that in addition to haematopoietic stem cells (HSCs), bone marrow also harbors endothelial stem cells (ESCs), mesenchymal stem cells (MSCs), and multipotential adult progenitor cells (MAPCs) (Jiang *et al*., 2002; Kassem, 2004; Ratajczak *et al*., 2004; Reyes *et al*., 2002). Adult bone-marrow-derived mesenchymal stem cells are capable of differentiation along several lineages. Recently, it has been discovered that bone marrow grafts to female mice, and possibly humans, can produce new follicles

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and oocytes in the recipient ovary (Johnson *et al.*, 2005). The authors have also reported that these tissues share genes typical of germ cells and proposed that bone marrow stem cells can migrate and colonize the ovaries to maintain a plentiful stock for reproduction.

In this paper, we demonstrate that human MSCs have the potential to express markers characteristic for male germ cell differentiation. This observation heralds new thinking about bone marrow stem cells as a soure for reproductive medicine.

Materials and methods

Isolation and culture of human mesenchymal stem cells

Human mesenchymal stem cells (MSCs) were prepared according to previously described methods (Pittenger *et al.*, 1999; Wulf *et al.*, 2004). Following an Internal Review Board's approval, leftover materials from disease-free bone marrow aspirates drawn for diagnostic purposes from male patients of the Haematology Service were obtained. The bone marrow aspirate was mixed with an equal amount of Dulbecco Modified Eagle medium (DMEM, Invitrogen) and centrifuged at 800 *g* for 10 minutes at 20°C. The cells were then resuspended in DMEM, layered on a Ficoll-Hypaque gradient (density = 1.077 g/cm3; Sigma, Deisenhofen, Germany), and centrifuged again. The low-density mononuclear fraction was collected, washed and resuspended in complete culture medium (DMEM with 10% fetal bovine serum [FBS, Sigma, Deisenhofen, Germany], penicillin/streptomycin [Invitrogen, Karlsruhe, Germnay], and glutamine [Invitrogen, Karlssruhe]) and plated at 2 x 107 cells/185 mm2. The cultures were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO2 and subcultured prior to confluency.

Treatment of mesenchymal stem cells

Isolated cells were cultured at 37°C and an atmosphere of 5% carbon dioxide in MesenCult Basal Medium (Cellsystems, St. Katharinen) supplemented with 10% MSC Stimulatory supplements and 50μg/ml each penicillin and streptomycin (Invitrogen, Karlsruhe). As this medium prevents differentiation, the cells were cultured during the differentiation analysis in RPMI 1640 (Invitrogen, Karlsruhe) supplemented with 10% FBS and 50μg/ml each penicillin and streptomycin. Cell passage was arbitrary, depending on the proliferation state. The cells were detached with Accutase (PAA Laboratories, Cölbe). For induction of differentiation, retinoic acid (RA, Sigma, Deisenhofen, Germnay) was added to the RPMI-medium at a final concentration of 10^{-5}M or 10^{-6}M and the cells were cultured for fifteen days in this medium.

RNA isolation and RT-PCR analysis

RNA was extracted from cells using the RNeasy-Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. RNA from human testis was isolated using the TriReagent (Biomol, Hamburg, Germnay) according to the manufacturer's instruction.

For RT-PCR analysis, 5 μg of RNA was reverse transcribed into cDNA at 42°C for 50 min in a final volume of 20 μl containing 200 units of Superscript reverse transcriptase (Invitrogen, Karlsruhe), 0.5 μg oligo dT Primer, 10 mM DTT and 0.5 mM dNTPs. RT-PCR was carried out with 0,5 or 1 μl of cDNA, 5 to 30 pmol each of forward and reverse primers and 2 to 5 units of Platinum Taq polymerase (Invitrogen, Karlsruhe) in a final volume of 25 or 50 μl. The solution was incubated at 94°C for 4 min and then subjected to 35 cycles of amplification, each consisting of 95°C for 30s (denaturation), 57°C-61°C for 30s-45s (annealing) and 72°C for 60s (primer

extension). At the end of the temperature cycles the solution was incubated at 72°C for 10 min. The PCR products (15 ml samples) were subjected to electrophoresis on 1.5% (w/v) agarose gels containing 1 mg/ml ethidium bromide and the amplified fragments were viewed under ultraviolet light and photographed. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal control. The primers used for RT-PCR analyses are shown in Table 1.

Table 1. *Primers used for the RT-PCR analysis of human mesenchymal stem cells*

Gene	Forward/reverse	Sequence 5'-3'
c-kit	Forward	CAGACTTAATAGTCCGCGTG
	Reverse	TTTGATCATGATGCCCGCCT
CyclinA2	Forward	AGAGGCCGAAGACGAGACGGG
	Reverse	GCATAGCAGCAGTGCCCACAA
Dazl	Forward	AATCATCCTCCTCCACCACAG
	Reverse	GGGCCAGAAAGCCGCTTTAAA
Fragilis	Forward	GGGCTCTAGAGAGGAGGCCCC
	Reverse	GCAGGGGTTCATGAAGAGGGT
Gapdh	Forward	CCAGCAAGAGCACAAGAGGAAGAC
	Reverse	AGCACAGGGATACTTTATTAGATG
Oct-4	Forward	GGAGCCGGGCTGGGTTGATCC
	Reverse	GGGAGAGCCCAGAGTGGTGAC
Piwil2	Forward	ATGCTTCCATCAGGTAGAGG
	Reverse	GATCCATCAAACGCAGTGAC
Vasa	Forward	TGAGAATACAAGGACAGGAGCT
	Reverse	*TCTTCACAAGCTCCCAATCC*

Immunohistochemistry

Cells which were treated for 15 days with retinoic acid and untreated cells as controls, were washed with PBS and fixed with 4% ice cold paraformaldehyde for ten minutes. The fixed cells were incubated for 16-20 hours at 4°C with one of the following antibodies in a 1:200 dilution in PBS: Anti-Dazl, anti-Piwil2, anti-Stra8 and anti TSPY. Slides were washed three times with PBS and incubated for 1-2 hours with an anti-rabbit antibody conjugated with Cy3 at 1:500 dilution in PBS. After three further washes with PBS, slides were mounted in 4',6-diamidino,-2-phenylindole mounting solution (Vector Laboratories Inc., Burlingame, CA) and inspected under Olympus BX 60 fluorescent microscope.

For immunophenotyping of human mesenchymal progenitor cell preparations, mouse fluorochrome-conjugated isotype control antibodies, fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-coupled antibodies against the common leukocyte antigen CD45 (clone HI30, Becton Dickinson), the surface-expressed 5´-ectonucleotidase CD73 (clone 37865X, Becton Dickinson), the beta 1 integrin CD29 (clone HUTS-21, Becton Dickinson, Heidelberg, Germany) were used following the manufacturers instructions. Binding of antibodies against the stroma cell surface proteoglycan CD105 (clone 8E11, Biotrends, Cologne, Germany), and the marker for differentiated fibroblasts AS02 (clone AS02, Dianova, Hamburg, Germany) as primary antibodies was detected by anti-mouse IgG conjugate (Sigma, Deisenhofen, Germany). Saturating amounts of antibodies were added to cells for 30 minutes at 4°C, before extensive washing and measurement of 10.000 cells each on a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany), followed by data analysis with the cell quest (Becton Dickinson, Heidelberg, Germany) or WinMDI programs.

Results and discussion

We established several MSC cell cultures from human bone marrow. After 3 passages *in vitro*, the MSCs showed homogenous positive surface staining for CD73, CD105 and CD29, which are typically found on mesenchymal stem cell populations, but lacked CD14, CD34, CD45 and AS02 surface expression, which denote hematopoietic or mature fibroblast differentiation, respectively (Fig. 1). To examine the potential of MSCs to differentiate to PGCs and male germ cells, we treated the cells with retinoic acid (10-5 M and 10-6 M, 15 days) and examined expression of PGC and male germ cell specific markers. By RT-PCR analysis, we found that human MSCs were positive for *Oct4*, *Fragilis*, *Stella*, *Vasa*, *c-kit*, *cyclin A2* and *Piwil2* (Fig. 2). Oct4 (also known as Oct-3) belongs to the POU (Pit-Oct-Unc) transcription factor family (Scholer *et al*., 1990). The POU family of transcription factors can activate the expression of their target genes through binding to an octameric sequence motif of an AGTCAAAT consensus sequence (Scholer, 1991). Recent evidence indicates that Oct4 is almost exclusively expressed in ES cells (Niwa, 2001). During embryonic development, *Oct4* is expressed initially in all blastomeres. Subsequently, its expression becomes restricted to the ICM and is downregulated in the TE and the primitive endoderm. At maturity, *Oct4* expression becomes confined exclusively to the developing germ cells (Pesce and Scholer, 2000). *Fragilis* and *Stella* are involved in initiating germ cell competence and specification and in the demarcation of PGCs from their somatic neighbours (Saitou *et al*., 2002). It was observed that the expression of *fragilis* first increases in the rim of the epiblast cup, suggesting that one of its roles is to keep together those cells that are predisposed to become PGCs, as they move out of the epiblast during gastrulation. Furthermore, the authors propose an elegant model in which cells at the centre of the community of precursors, which express the highest levels of *fragilis*, are the ones selected to become PGCs and to express high levels of *stella*. Expression of *fragilis* is increased in the migratory PGCs, inducing expression of other germ cell-specific genes such as *stella* (Sato *et al*., 2002) and the *VASA* homolog (Toyooka *et al*., 2000). *Vasa* encodes an ATP-dependent RNA helicase which is specific for differentiating germ cells from the late migration stage to the postmeiotic stage. PGCs express *c-Kit* at relatively high levels. In PGCs as well as in hematopoietic cells, this expression is related to a single DNaseI-hypersensitive site (HS2) which is absolutely necessary for its activity (Cairns *et al*., 2003). We found that human MSCs expressed *Oct4*, *Fragilis*, *Stella*, *Vasa*, *c-kit*, *cyclin A2* and *Piwil2*, before and after RA treatment which is an evidence that a population of MSCs shows germ cell characteristics without RA treatment (Fig. 2). Expression of some of these genes was increased after RA treatment which indicates that RA treatment promotes germ cell differentiation of human MSCs.

By immunohistochemical analysis, we found that MSCs show expression of *Stra8*, *Piwil2*, *Dazl* and *Tspy* (Fig. 3). All these genes are expressed specifically in male germ cells. During mouse embryogenesis, *Stra8* expression is restricted to the male developing gonads, and in adult mice, the expression of *Stra8* is restricted to the premeiotic germ cells (Oulad-Abdelghani *et al*., 1996). *Piwil2*, which is known in mouse as *Mili*, is expressed in premeiotic male germ cells. Spermatogenesis in the *Mili*-null mice is blocked completely at the early prophase of the first meiosis (Kuramochi-Miyagawa *et al*., 2001; Kuramochi-Miyagawa *et al*., 2004). In human, *Piwil2* is expressed specifically in testicular premeiotic germ cells (Lee *et al*., 2005). Furthermore, we showed previously that *Piwil2* modulates expression of murine spermatogonial stem cell expressed genes (Lee *et al*., 2006). DAZL proteins are germ-cell-specific RNA-binding proteins essential for gametogenesis. In humans, loss of the Y chromosomal *DAZ* genes is associated with oligozoospermia or azoospermia. The *DAZ* genes are strong candidates for the AZFc azoospermia factor, one of the most common genetic causes of male infertility (Reijo *et al*., 1995; Vogt *et al*., 1996; Ferlin *et al*., 1999). *Tspy*, the 'testis-specific protein, Y-encoded',

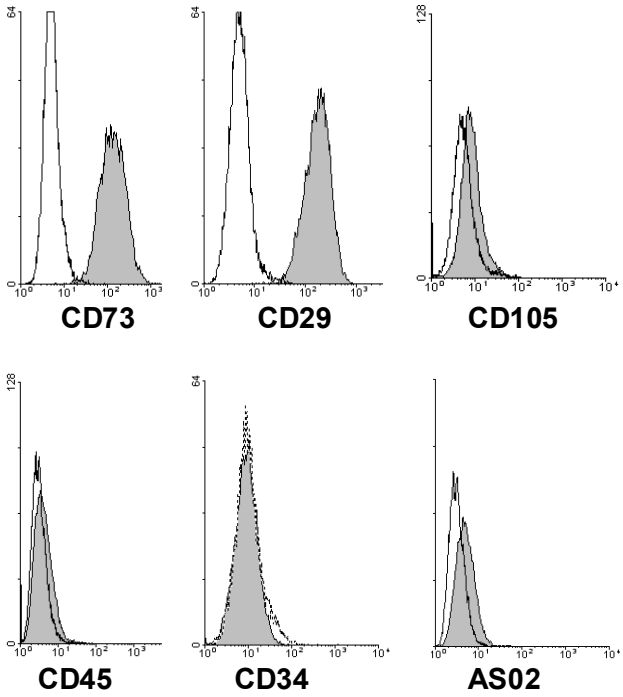

Figure 1. The immunophenotype of morphologically homogeneous populations from adherent human bone marrow mesenchymal stem cell cultures after the 3rd passage *in vitro* was analysed by immunofluorescent staining and flowcytometric documentation. Staining profiles of representative samples with 10,000 events each are shown. The markers are represented by shaded histograms, the respective isotype controls fine-lined. The MSCs stained homogeneously strong with markers for mesenchymal progenitors, such as the ectonucleotidase CD73, the ß1 integrin CD29 and weakly for the TGF-ß co-receptor endoglin (CD105). The cells were negative for the markers of hematopoetic cells (CD45, CD34), as well as a marker for differentiated fibroblasts (AS02).

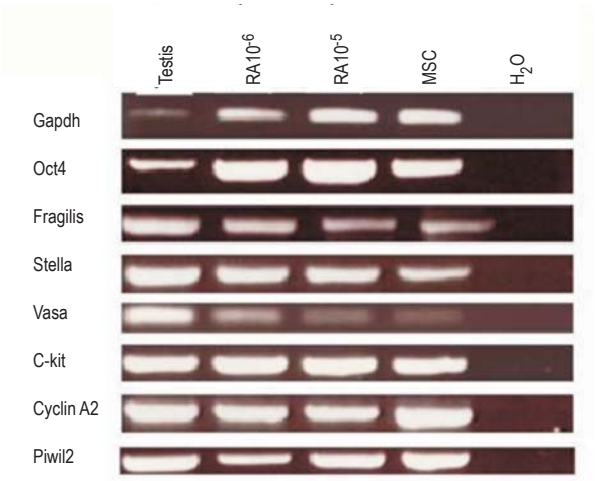

Figure 2. RT-PCR analysis of RNA isolated from human mesenchymal stem cells show expression of germ cell specific markers. RNA isolated from human testis served as positive control. H_2O, no-template negative control.

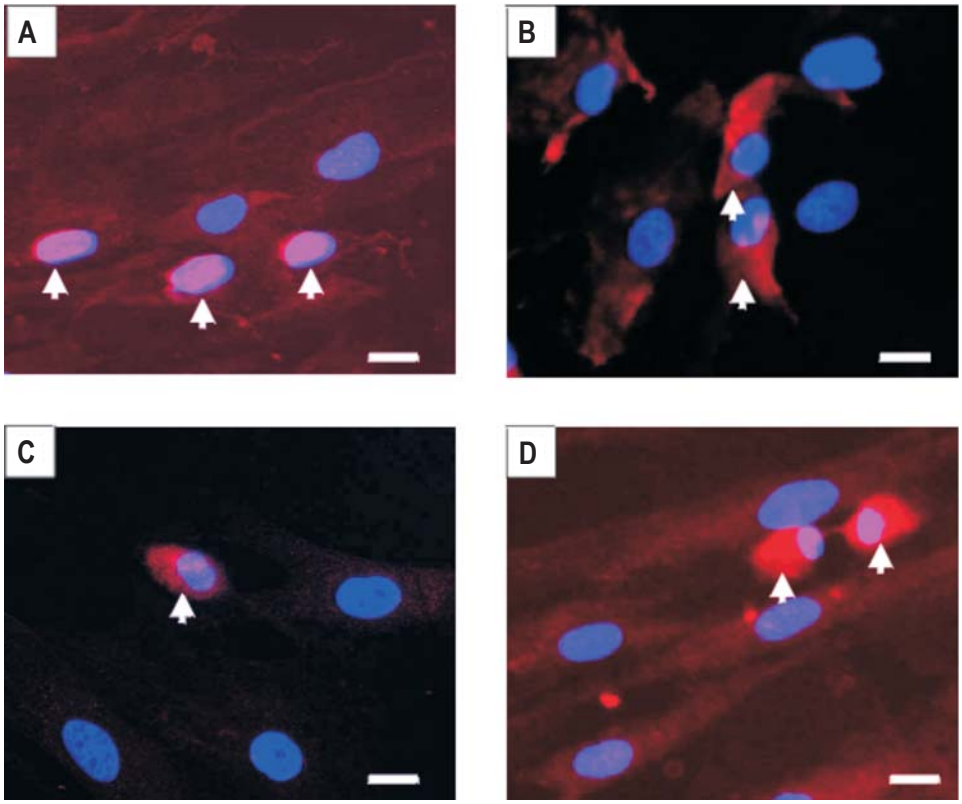

Figure 3. Immunohistochemical analysis of human mesenchymal stem cells. Specific antibodies against the male germ cell markers Dazl (A), Stra8 (B), Piwil2 (C) and Tspy (D) (all red). Nuclei are shown in blue, DAPI (4′, 6′-diamidino-2-phenylindole) staining. Bar: 10 **m**m.

is the product of a tandem gene cluster on human proximal Yp which is expressed specifically in human spermatogonia (Schnieders *et al.*, 1996). From these results it can be suggested that a population of human mesenchymal stem cells show expression of male germ cell specific markers. To examine the effect of RA on differentiation property of MSCs to germ cells, we treated the cells for 15 days with 10-5 M RA and 10-6 M RA, respectively, and performed an indirect immunostaining using antibodies against *Dazl*, *Piwil2*, *Stra8* and *Tspy*. As shown in Fig. 4, RA promotes differentiation of MSCs towards male germ cells (Fig. 4).

Our results indicate that a fraction of bone marrow cells are able to differentiate to male germ cells. Although the BM-derived human male germ cells exhibit expression of germ cell and male germ cell specific markers, we have not yet determined whether these male germ cells can undergo meiosis and form functional spermatozoa. Recently, it was demonstrated that oocytes can be generated in adult mammalian ovaries by putative germ cells in bone marrow (Johnson *et al.*, 2005). They have discovered that bone marrow grafts to female mice, and possibly humans, can produce new follicles and oocytes in the recipient ovary. This team had already discovered that these tissues share expressed genes typical of germ cells (Johnson *et al.*, 2004). They postulate that this situation is one means of replenishing ovarian follicles when numbers in the ovary decline to very few.

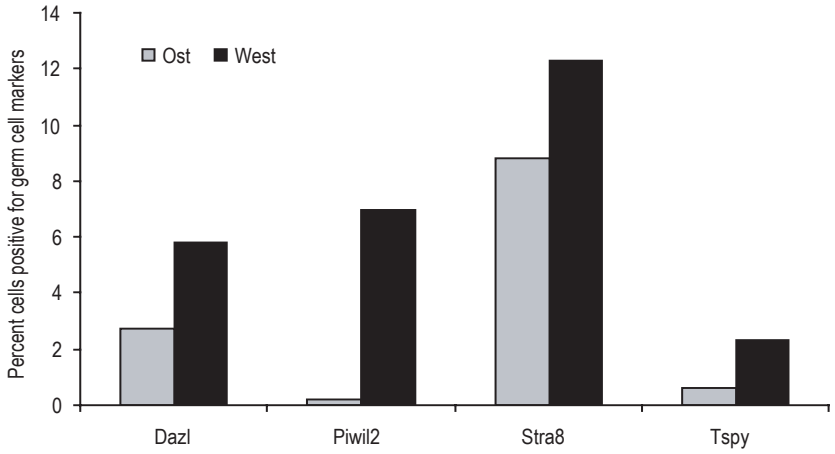

Figure 4. Effect of retinoic acid on differentiation of human MSCs to male germ cells. After retinoic acid treatment, an increase in the number of cells which expressed Dazl, Piwil2, Stra8 and Tspy (black) was detected as compared with untreated cells (white). Percentage of positive cells is shown.

These results showed for the first time that bone marrow stem cells were able in differentiate into a germ cell-like phenotype and provide a new potential source of male and female germ cells that could be used for production of oocytes and sperm.

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