Comparative methylation profiles and telomerase biology of mouse multipotent adult germline stem cells and embryonic stem cells

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ABSTRACT: Recently, several groups described the isolation of mouse spermatogonial stem cells (SSCs) and their potential to develop to embryonic stem cell (ESC)-like cells, so-called multipotent germline stem cells (mGSCs). We were the first to derive such mGSCs from SSCs isolated from adult mouse testis and, therefore, called these mGSCs multipotent adult germline stem cells (maGSCs). Here, we comparatively analyzed gene-specific and global DNA methylation profiles as well as the telomerase biology of several maGSC and male ESC lines. We show that undifferentiated maGSCs are very similar to undifferentiated male ESCs with regard to global DNA methylation, methylation of pluripotency marker gene loci, telomerase activity and telomere length. Imprinted gene methylation levels were generally lower in undifferentiated maGSCs than in undifferentiated male ESCs, but, compared with undifferentiated mGSCs derived by other groups, more similar to those of male ESCs. Differentiation of maGSCs increased the methylation of three of the four analyzed imprinted genes to almost somatic methylation patterns, but dramatically decreased global DNA methylation. Our findings further substantiate the pluripotency of maGSCs and their potential for regenerative medicine.

Key words: genome-wide methylation / imprinted genes / multipotent adult germline stem cells / pluripotency marker genes / telomerase biology

Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of the blastocyst and defined by their capacity of self-renewal as well as their broad differentiation plasticity (Evans and Kaufman, 1981; Martin, 1981). However, the therapeutic use of ESCs could be impeded by problems regarding immune rejection due to genetic differences between the patient and the donor ESCs as well as ethical issues associated with the use of embryos (Vats et al., 2005).

Spermatogonial stem cells (SSCs) are self-renewing single cells located in the periphery of the seminiferous tubules whose continuous division maintains spermatogenesis throughout the life of a male individual (Spradling et al., 2001). Recently, we and others showed that mouse SSCs can be isolated and, after culture, develop to ESC-like multipotent germline stem cells (mGSCs) (Kanatsu-Shinohara et al., 2004; Guan et al., 2006; Seandel et al., 2007; Izadyar et al., 2008). Similar to ESCs, these mGSCs could be differentiated into various somatic cell lineages, produced teratomas in immunodeficient mice and formed germline chimaeras after microinjection in blastocysts. Although Kanatsu-Shinohara et al. (2004) used the testsis of neonatal mice to isolate SSCs, we were the first to show by a genetic selection strategy in transgenic mice based on the spermatogonia-specific marker Stra8 [stimulated by retinoic acid (RA) gene 8] that even an adult tests is an appropriate source for deriving SSCs that developed to mGSCs called multipotent adult...
germline stem cells (maGSCs) (Guan et al., 2006). Remarkably, we were also able to derive maGSCs by identifying and isolating SSCs from adult testes of non-transgenic mice of the strains FVB, 129/Sv and C57BL/6 morphologically (Guan et al., 2006). The two following studies by Seandel et al. and Izadyar et al. confirmed our findings (Seandel et al., 2007; Izadyar et al., 2008). Furthermore, another recent study by Kanatsu-Shinohara et al. indicated that SSCs are not pluripotent themselves, but that each SSC is able to become, by very rare fortuity, reprogrammed to a pluripotent multipotent germ- line stem cell and, thereby, loses its spermatogenic potential (Kanatsu-Shinohara et al., 2008). The molecular processes underlying this fortuitous reprogramming event have not yet been identified.

The mammalian epigenome, which is defined as the sum of potentially heritable DNA and histone modifications in a given cell type, undergoes dramatic changes during germ cell development and shortly after fertilization (Reik and Walter, 2001; Li, 2002; Bernstein et al., 2007). In primordial germ cells (PGCs) representing the precursors of SSCs and all other germ cells, the genome is demethylated and, in particular, the genomic imprints, i.e. the parent-specific methylation marks of imprinted genes, of the previous generation are erased from the grandparental chromosomes (with respect to the new embryo). In the mouse, this wave of genome-wide epigenetic reprogramming starts between day 10.5 post conceptionem (p.c.) before migration of PGCs into the genital ridge and is completed by day 13.5 p.c. (Hajkova et al., 2002; Yamazaki et al., 2003). In the male germline, the establishment of novel methylation marks for imprinted genes begins around day 15.5 p.c., but is finished only after birth (Davis et al., 1999, 2000; Li et al., 2004). After fertilization, a second wave of genome-wide epigenetic reprogramming takes place in which the vast majority of male and female germline-derived methylation patterns are erased again and new somatic methylation patterns for development of the new organism are established (Mayer et al., 2000a, b). To the extent of present knowledge, imprinted genes escape this second wave and maintain their germline-specific methylation and parent-specific expression patterns throughout further development (Morgan et al., 2005). Thus, imprinted genes display a differential methylation of their parental alleles and maintenance of genomic imprinting in both ESCs and somatic cells. In contrast, pluripotency marker genes such as Oct4 and Nanog switch from a transcriptionally active and demethylated state in ESCs to a transcriptionally repressed and fully methylated state in somatic cells (Okita et al., 2007; Wernig et al., 2007).

The telomere consists of DNA–protein complexes containing DNA repeat sequences and is essential for protecting chromosomes against degradation and rearrangement (Blackburn, 2001). Telomerase serves to maintain this telomere function by addition of telomeric repeats onto chromosome ends and comprises an RNA component (TERC) and a reverse transcriptase component (TERT) (Blasco, 2005). Telomerase activity is present in immortal cells such as cancer cells, germ-line cells and ESCs, but not detectable in most somatic cells. The critical role of telomerase activity in male germ cells is underlined by the gradual loss of spermatogenic cells and eventual sterility of mice lacking the telomerase RNA component (Lee et al., 1998).

The possibly germ cell-specific epigenome, in general, and germ cell-specific genomic imprinting, in particular, of mGSCs compared with ESCs was repeatedly discussed as a major obstacle to the potential application of mGSCs in cell substitution therapy (Hochedlinger and Jaenisch, 2006). To clarify this discussion and test the similarity of maGSCs and ESCs also at the epigenomic level, more systematic studies are warranted. In the present study, we therefore comparatively analyzed the methylation status of several imprinted gene differentially methylated regions (DMRs) (H19, Igf2r, Meg3 and Snrpn) and promoter regions of pluripotency-marker genes (Nanog and Oct4), the global DNA methylation, as well as the telomerase activity and telomere length of undifferentiated and differentiated mouse maGSCs and male ESCs from different genetic backgrounds. We show that the maGSCs and male ESCs are very similar with regard to telomerase activity, telomere length and methylation of pluripotency marker genes. In addition, we demonstrate that maGSCs, compared with mGSCs derived by other groups (Kanatsu-Shinohara et al., 2004; Izadyar et al., 2008), share more similarities regarding imprinted gene methylation with male ESCs, although imprinted gene methylation and global DNA methylation of maGSCs are not completely concordant with those of male ESCs.

Materials and Methods

Derivation and culture of maGSC and ESC lines

Derivation of the maGSCs was previously described (Guan et al., 2006). In brief, testis of adult mice was digested using a two-step enzymatic digestion protocol with collagenase and trypsin. In order to obtain single cells, the suspension was filtered through a 70 µm nylon mesh and cells were plated on gelatine-coated culture dishes. Culture medium consists of Dulbecco’s minimal essential medium (DMEM) (PAN, Aidenbach, Germany) supplemented with 20% fetal calf serum (FCS) (PAN), 2 mM L-glutamine (PAN), 50 µM β-mercaptoethanol (Gibco/Invitrogen, Eggenstein, Germany), 1 x non-essential amino acids (NEAA) (Gibco/Invitrogen), sodium pyruvate (Gibco/Invitrogen), penicillin/streptomycin (PAN) and gel cell line-derived neurotrophic factor (GDNF) (Chemicon, Temecula, CA, USA). After 5–10 days in culture, ESC-like colonies could be observed. These colonies were picked, separated and plated on a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts (MEFs) in the same culture medium as described above but with 1000 U/ml leukemia inhibitory factor (LIF) (Chemicon) instead of GDNF. Using this protocol, we established the four maGSC lines maGSC Stra8, maGSC 129/Sv, maGSC FVB and maGSC C57BL/6 from the testes of adult Stra8-enhanced green fluorescent protein (EGFP)/Rosa26 mice with a mixed genetic FVB/C57BL6/129Sv genetic background. After 2–3 months in culture, ESC-like colonies could be observed. To obtain single cells, the suspension was filtered through a 70 µm nylon mesh and cells were plated on gelatine-coated dishes and culture medium was supplemented with 10^{-4} M RA (Sigma-Aldrich, Steinheim, Germany) instead of LIF. Cells were cultured for 20 days before DNA was isolated.

Gene-specific methylation analysis

Genomic DNA was isolated from maGSC and male ESC lines using simple isopropanol precipitation. Bisulfite treatment of genomic DNA was performed using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). The methylation status of the H19 imprinting control region (ICR), the Igf2r DMR2, the Snrpn DMR1, the Meg3 intergenic (IG) DMR and the promoters of Nanog and Oct4 was analyzed by bisulfite pyrosequencing. Bisulfite pyrosequencing was performed on a PSQ™ 96MA Pyrosequencing System (Biotage, Uppsala, Sweden) with the PyroGold SQA reagent kit (Biotage).
(Tost et al., 2003). PCR and sequencing primers for bisulfite pyrosequencing are listed in Supplementary Table S1. Pyro Q-CpG software (Biotage) was used for pyrosequencing data analysis.

Analysis of global DNA methylation
Global DNA methylation was analyzed on a PSQ™ 96MA Pyrosequencing System (Biotage) using the luminometric methylation assay (LUMA) (Karimi et al., 2006). We employed a recently described modified LUMA protocol for minimizing degradation effects on quantification by performing additional measurements of free DNA ends (Bjornsson et al., 2008). Briefly, the original dispensation order was modified by programming the machine to first carry out three dispensations of dGTP and dTTP which do not bind to the overhangs left by the used restrictions enzymes EcoRI, HpaII and MspI, but to the ends of degraded DNA. In the next three dispensations, dATP and dCTP were added which anneal to the overhangs left by the restriction enzymes, followed by five final dispensations of dGTP and dTTP.

Telomerase activity assay
Telomerase activity was measured using a telomeric repeat amplification protocol (TRAP) assay kit (Chemicon) with PBS-washed cell pellets that were stored at −80°C until use. Cell pellets were resuspended with CHAPS lysis buffer and were incubated on ice for 30 min. Cell lysates were centrifuged for 20 min at 12,000g, 4°C, and the supernatants were stored at −80°C. Two microliters of the cell extract at 750 ng/μl were added to a total volume of 50 μl PCR mix containing the TRAP reaction buffer, dNTPs, substrate oligonucleotide, telomerase primer, internal standard primer and Taq polymerase. Two microliters of telomerase positive control cell extracts were analyzed as a positive control and 2 μl of CHAPS lysis buffer as a negative control. Each sample was incubated at 30°C for 30 min for telomerase extension, followed by PCR amplification and polyacrylamide gel electrophoresis.

Telomerase length assay
Determination of the lengths of terminal restriction fragments was carried out using the TeloTAGGG Telomere length assay kit (Roche, Mannheim, Germany), according to the manufacturer’s protocol.

Results
Methylation patterns of imprinted and pluripotency marker genes
With regard to methylation of imprinted genes, the hypothesis was tested that undifferentiated maGSCs display somatic imprinting patterns (i.e. imprinting patterns typical for somatic cells opposed to those of germline cells) similar to those of undifferentiated ESCs which have already been reported in the literature (Geijsen et al., 2004). For all four imprinted gene regions, the four maGSC lines were generally less methylated showing moderate to strong hypomethylation than the four ESC lines displaying the expected somatic imprinting patterns (Fig. 1A). In detail, the two tested masculinously methylated ICRs, the Igf2r DMR2 and the Snrpn DMR1, displayed a completely androgenetic imprinting pattern with strong hypomethylation in the maGSC lines maGSC 129/Sv, maGSC FVB and maGSC C57BL/6 (between 4% and 12% methylation) and a partially androgenetic imprinting pattern with moderate hypomethylation in maGSC Stra8 (29% and 32% methylation, respectively). The four tested ESC lines, however, showed partially androgenetic or even somatic imprinting patterns (between 19% and 57% methylation) for these two regions. For the paternally methylated Meg3 IG-DMR, we detected a strong hypomethylation in maGSC FVB and maGSC C57BL/6 (6% and 8% methylation, respectively) and a rather somatic imprinting pattern with very moderate hypomethylation in maGSC Stra8 and maGSC 129/Sv (39% and 42% methylation, respectively). In comparison, the four tested ESC lines also exhibited a moderate hypomethylation (between 21% and 40% methylation) for this DMR. For the paternally methylated H19 ICR, a strong hypomethylation was only observed for maGSC 129/Sv (11% methylation), whereas the three other maGSC lines maGSC Stra8, maGSC FVB and maGSC C57BL/6 were very moderately hypomethylated, indicating a rather somatic imprinting pattern (41%, 32% and 35% methylation, respectively). In contrast, we exclusively found somatic H19 imprinting patterns (between 43% and 60% methylation) in the four ESC lines. For each cell line, we also calculated a mean imprinted gene methylation percentage by averaging the methylation percentages measured for the four imprinted genes (Fig. 1B). Strikingly, maGSC Stra8 and ESC Stra8, which are derived from the same genetic background, showed very similar mean methylation percentages and moderately hypomethylated patterns for all four tested imprinted gene regions (Fig. 1A and B). For other maGSCs and ESCs of the same genetic background, no similarities in mean methylation percentages and methylation patterns were discovered (Fig. 1A and B).

With respect to methylation of pluripotency marker genes, the hypothesis was verified that undifferentiated maGSCs show a hypomethylation similar to that of undifferentiated ESCs which has already been reported in the literature (Okita et al., 2007; Wernig et al., 2007). Similar to all four analyzed ESCs, the promoters of the pluripotency marker genes Nanog and Oct4 were strongly hypomethylated in maGSC 129/Sv, maGSC FVB and maGSC C57BL/6 (between 4% and 11% methylation) (Fig. 1C). Only maGSC Stra8 displayed a slightly increased methylation leading, however, to a still markedly hypomethylated pattern (22% methylation for both genes) (Fig. 1C).

Global DNA methylation
With regard to global DNA methylation, we tested the hypothesis that undifferentiated maGSCs display genome-wide cytosine methylation levels similar to those of undifferentiated ESCs. The global DNA methylation analysis yielded slightly decreased methylation percentages between 52% and 62% (mean methylation: 57.4%) in the four maGSCs compared with methylation percentages between 57% and 68% (mean methylation: 63.8%) in the four ESCs (Fig. 1D). The MEF cell line (somatic cells) also displayed a very similar methylation percentage of 57% (Fig. 1D).

Methylation status on differentiation
To examine whether changes in methylation occur during differentiation, we induced two of the maGSC lines, maGSC Stra8 and maGSC 129/Sv, and the two ESC lines of the same genetic background, ESC Stra8 and ESC 129/Sv, to differentiate in vitro by the addition of RA and deprivation of LIF. To confirm that the cells had differentiated, the expression levels of the pluripotency marker genes Oct4 and Nanog were analyzed. As expected, both the maGSC and the ESC lines exhibited a down-regulation of the two genes during differentiation (data not shown).
Figure 1 Gene-specific and global DNA methylation levels in four undifferentiated maGSC lines, four undifferentiated ESC lines and MEFs.

(A) Methylation levels of imprinted gene DMRs (Meg3, Igf2r, H19 and Snrpn) Mean imprinted gene methylation levels calculated by averaging the methylation percentages measured for the four imprinted genes; error bars denote the standard deviation. (C) Methylation levels of promoter regions of pluripotency marker genes (Nanog and Oct4). (D) Global DNA methylation levels. The percentages of methylated CpG sites for the undifferentiated maGSC lines (blue bars), undifferentiated ESC lines (red bars) and MEFs (grey bar) are given on the Y-axis.
With respect to methylation of imprinted genes and pluripotency marker genes, we verified the hypothesis that differentiation of maGSCs results in an increase of methylation similar to that reported for Oct4, Nanog and at least some imprinted genes during differentiation of ESCs (Andollo et al., 2006; Lagarkova et al., 2006; Shovlin et al., 2008). Differentiation of the two maGSC lines and the ESC line Stra8 lead to a moderate to marked increase of methylation levels at the paternally methylated Meg3 and H19 and the maternally methylated Igf2r ICRs (Fig. 2A). The differentiated ESC line 129/Sv, however, only markedly elevated the methylation levels for Meg3.

Figure 2 Gene-specific and global DNA methylation levels in two undifferentiated maGSC and ESC lines, respectively, two differentiated maGSC and ESC lines, respectively, and MEFs.

(A) Methylation levels of imprinted gene DMRs (Meg3, Igf2r, H19 and Snrpn).
(B) Methylation levels of promoter regions of pluripotency marker genes (Nanog and Oct4).
(C) Global DNA methylation levels. The percentages of methylated CpG sites for the undifferentiated maGSC lines (light blue bars), differentiated maGSC lines (dark blue bars), undifferentiated ESC lines (light red bars), differentiated ESC lines (dark red bars) and MEFs (grey bar) are given on the Y-axis.
but markedly reduced the methylation levels for H19 and Igf2r (Fig. 2A). For Snrpn, only maGSC 129 showed a moderate gain in methylation upon differentiation, whereas the differentiated ESC Stra8 maintained the methylation and the differentiated maGSC Stra8 and ESC 129/Sv even decreased the methylation at this locus (Fig. 2A). Further, the two maGSCs, similar to the two ESCs, displayed dramatically elevated promoter methylation levels of the pluripotency marker genes Nanog and Oct4 upon differentiation (Fig. 2B).

Strikingly, differentiation resulted in a dramatic loss of global DNA methylation from 62% to 30% in maGSC Stra8 and from 57% to 32% in maGSC 129/Sv (Fig. 2C). In contrast, upon differentiation, the two ESC lines only moderately lost global DNA methylation from 68% to 58% (ESC Stra8) and from 68% to 62% (ESC 129/Sv), respectively (Fig. 2C).

### Telomerase activity and telomere length

The two analyzed maGSC lines maGSC 129/Sv and maGSC FVB, similar to the two analyzed ESC lines ESC 129/Sv and ESC FVB, showed high telomerase activity (Fig. 3A). Analysis of terminal restriction fragments further showed that the average telomere length in the two maGSC lines is high (>20 kb) and similar to the average telomere length measured in the two ESC lines (>20 kb) (Fig. 3B).

### Discussion

Several recent reports demonstrated that mouse SSCs being the stem cell population of spermatogenesis can be induced to become multipotent ES-like cells (mGSCs) that expressed all markers of pluripotent cells and can differentiate into the three germ layers in vitro and contribute to a variety of tissues, including germline, when injected into blastocysts (Kanatsu-Shinohara et al., 2004; Guan et al., 2006; Seandel et al., 2007; Izadyar et al., 2008). Thus, these studies provided the first derivation of pluripotent cells from a normal neonatal or adult mammal and new possibilities for exploring the molecular mechanisms governing pluripotency, establishing new genetic disease models as well as developing clinical applications in regenerative medicine.

In this context, however, questions and concerns were raised regarding the possible unbalanced genomic imprinting of these cells (Hochedlinger and Jaenisch, 2006). Indeed, a comparative methylation analysis of three paternally methylated (H19, Meg3 and Rasgrf1) and two maternally methylated (Igf2r and Peg10) ICs in SSC-derived ES-like cells (mGSCs) from neonatal mouse testis and ESCs by Kanatsu-Shinohara et al. revealed marked differences in methylation patterns with all imprinted regions being generally more methylated in ESCs than those in mGSCs (Kanatsu-Shinohara et al., 2004). Thus, in mGSCs, H19, Meg3 and Rasgrf1 were methylated to different degrees, and Igf2r and Peg10 were only rarely methylated. In contrast, a more recent comparative study of methylation imprints in ES-like cells (mGSCs) isolated from neonatal and adult mouse testis based on the expression of a Pou5f1-controlled GFP transgene and ESCs found completely androgenetic imprinting profiles in ES-like cells and only partially androgenetic imprinting profiles in ESCs (Izadyar et al., 2008). In ES-like cells, Izadyar et al. detected a strong hypermethylation of Rasgrf1 and Meg3 and a strong hypomethylation of Igf2r and Peg10, whereas, in ESCs, they found a somatic imprinting pattern for Meg3 and Igf2r, but a strong hypomethylation for Rasgrf1 and Peg10 (Izadyar et al., 2008). In our comparative study of methylation patterns in four maGSC lines derived from adult mouse testis and four male ESC lines, we included three (H19, Meg3 and Igf2r) of the six imprinted genes tested by the previous studies as well as one additional maternally methylated gene (Snrpn). Similar to the findings of Kanatsu-Shinohara et al. in mGSCs (Kanatsu-Shinohara et al.,
we detected lower imprinted gene methylation levels in maGSCs than in ESCs with the paternally imprinted genes predominantly moderately hypomethylated and the maternally imprinted genes predominantly strongly hypomethylated. Thus, we propose that maGSCs, similar to the mGSCs derived by Kanatsu-Shinohara et al. (2004), originate from germ cells which have undergone imprint erasure and already started with establishment of new paternal methylation imprints.

One of the four maGSCs, maGSC Stra8, displayed an overall degree of maternal and paternal imprinted gene methylation which was markedly higher than that of the other three tested maGSCs as well as that of the mGSC lines analyzed by Kanatsu-Shinohara et al. (2004). Thus, the imprinted gene methylation levels of maGSC Stra8 were very similar to those of ESC Stra8. Interestingly, both cell lines have the same mixed FVB/C57BL6/129Sv genetic background, and are doubly transgenic for ROSA26 and Stra8-EGFP (Guan et al., 2006). Hence, it may be speculated that the presence of the two transgenes and/or the mixed genetic background are beneficial to possibly reprogramme SSCs and derive maGSCs with balanced somatic imprinting patterns. Although the other three tested maGSCs are generally more similar to the mGSCs described by Kanatsu-Shinohara et al. (2004), they display slightly to markedly increased, more ESC-like methylation levels for at least one of the tested imprinted genes. The mGSCs established by Izadyar et al. (2008) displayed completely androgenetic imprinting profiles which are much more different to those of ESCs than those of the maGSCs reported here.

The already discussed differences in imprinted gene methylation patterns between maGSCs and the mGSCs established in the other two studies (Kanatsu-Shinohara et al., 2004; Izadyar et al., 2008) may be explained by the fact that different methods of stem cell isolation and culture have been used. Kanatsu-Shinohara et al. have used the testes of neonatal mice to purify SSCs and a special medium, designed for hematopoietic stem cells and including GDNF, to culture them and derive ES-like cells (Kanatsu-Shinohara et al., 2004). Izadyar et al. utilized a transgenic mouse model expressing green fluorescent protein under the control of a germ cell-specific Pou5f1 (Oct4) promoter to identify and track germine stem cells and identified a Pou5f1+/c-Ki-t+ subset of mGSCs that resembles ESCs when isolated from either neonatal or adult testis and cultured in a complex mixture of growth factors (Izadyar et al., 2008). In this study, we used testes of adult mice and no genetic selection strategy to isolate SSCs and DMEM with serum and GDNF to culture them and derive ESC-like colonies. These methodological differences may result in the enrichment of different populations of mGSCs also differing in their imprinted gene methylation.

In addition, there also seems to be no clear consistency in imprinted gene methylation patterns among the four maGSC lines as well as among the four ESC lines tested in this study. Since we used the same methodology to derive the four maGSC and four ESC lines, respectively, the observed differences in imprinted gene methylation must be rather attributed to differences in genetic background. In this context, several studies already demonstrated a modulation of imprinting patterns by genetic background. Durcova-Hills et al. (2006) reported that male germ cells from 129/Sv mice established the H19 methylation imprint more slowly than those from C57BL/6 mice. Further, Chang et al. (2009) described striking differences of H19 and Mest imprinted gene methylation patterns between C57BL/6 x DBA/2 and C57BL/6 x 129/Sv ESC lines. Overmore, parthenogenetic preimplantation development of CBA and C57BL/6 mouse embryos differs strikingly with C57BL/6 embryos forming blastocysts at a much higher frequency than CBA embryos (Penkov et al., 1996). These data argue for the presence of modifier loci possibly influencing imprint establishment and/or imprint maintenance.

Previous studies did not analyze imprinted gene methylation patterns of differentiated SSC-derived ES-like cells. We demonstrate here that differentiation of the two tested maGSC lines as well as ESC Stra8 increases the methylation of Meg3 and H19 to somatic methylation patterns and the methylation of Igf2r from strongly to moderately hypomethylated patterns. In contrast, methylation of Snrpn only increased in maGSC 129/Sv, was maintained in ESC Stra8 and even decreased in maGSC Stra8 and ESC 129/Sv. Recently, Shovlin et al. (2008) investigated imprinted gene methylation in two male and two female embryonic germ (EG) cell lines also derived from PGCs as well as one ESC line, each before and after differentiation. They found H19 methylation to be increased upon differentiation of all tested cell lines as well as Igf2r, Lit1 and Snrpn methylation to be elevated only upon differentiation of one male EG cell line. Together, these data support the hypothesis that differentiation restores the somatic methylation patterns and, thus, balanced imprinting of at least some maGSC and EG cell lines.

Our comparative genome-wide methylation analysis using the LUMA method yielded similar methylation levels in undifferentiated maGSCs, ESCs and MEFs (somatic cells) with a very slightly decreased mean methylation in maGSCs and MEFs compared with ESCs. Another genome-wide methylation study, which was, however, limited to promoters, recently described very similar methylation patterns of undifferentiated ESCs and EG cells as well as sperm (Farthing et al., 2008). Overall, the methylation levels measured in ESCs and MEFs are well within the range of methylation levels detected in male ESCs and somatic cells by comparable methods such as nearest-neighbor analysis (Ramashoye et al., 2000; Zvetkova et al., 2005). Since Zvetkova et al. also described very similar slight variations of methylation levels among different male ESC lines as well as among different somatic cell samples, the observed slightly decreased mean methylation in maGSCs and MEFs compared with ESCs may be attributable to technical rather than biological influences. Interestingly, differentiation lead to a dramatic decrease of methylation levels only in maGSCs, but not in ESCs. We hypothesize that this decrease mainly affects non-genic repetitive elements which represent ~48% of the mouse genome and are hypermethylated (Ohgane et al., 2008). These elements have been repeatedly reported to retain high levels of methylation in PGCs at day 12.5 p.c., when methylation is already erased from imprinted and non-imprinted single copy genes, and undergo, however, incomplete demethylation, only at later, more differentiated germ cell stages (Walsh et al., 1998; Hajkova et al., 2002; Lane et al., 2003; Lees-Murdock et al., 2003). Thus, the analyzed maGSCs, similar to day 12.5 p.c. PGCs, may also represent a germ cell population which has already undergone erasure of methylation at single-copy genes, but has retained methylation at repetitive elements. It is well conceivable that RA induces maGSCs, similar to SSCs, but not ESCs to develop preferentially to differentiated germ cells which, then, undergo demethylation at repetitive elements (Dann et al., 2008).
Oct4 and Nanog play key roles in maintaining the pluripotency of cells (Nichols et al., 1998; Mitsui et al., 2003). Both genes were shown to be expressed in pluripotent cells such as ESCs and induced pluripotent stem (iPS) cells and down-regulated during their differentiation (Nichols et al., 1998; Mitsui et al., 2003; Okita et al., 2007; Wernig et al., 2007). Down-regulation of both genes during differentiation is generally associated with a transition of their promoters from a hypomethylated to a hypermethylated state (Farthing et al., 2008). Thus, our findings that Oct4 and Nanog promoters were strongly hypomethylated in undifferentiated maGSCs and ESCs expressing both genes and hypermethylated in differentiated maGSCs and ESCs down-regulating both genes agree very well with those described previously and underline the pluripotency of maGSCs (Guan et al., 2006; Seandel et al., 2007; Izadyar et al., 2008).

Similarly, telomerase activity and maintenance of telomere length are important markers for pluripotent cells such as ESCs and iPS cells allowing them to divide repeatedly and become immortal (Hyiama and Hiyama, 2007; Takahashi et al., 2007). Down-regulation of both genes during differentiation is generally associated with a transition of their promoters from a hypomethylated to a hypermethylated state (Farthing et al., 2008). Thus, our findings that Oct4 and Nanog promoters were strongly hypomethylated in undifferentiated maGSCs and ESCs expressing both genes and hypermethylated in differentiated maGSCs and ESCs down-regulating both genes agree very well with those described previously and underline the pluripotency of maGSCs (Guan et al., 2006; Seandel et al., 2007; Izadyar et al., 2008).

In conclusion, our findings demonstrate that maGSCs share additional important pluripotency features with male ESCs such as telomerase activity, telomere length and hypomethylation of pluripotency marker genes. Although maGSCs are not completely concordant with male ESCs regarding global DNA methylation and methylation of imprinted genes, imprinted gene methylation of maGSCs, compared with mGSCs derived by other groups (Kanatsu-Shinohara et al., 2004; Izadyar et al., 2008), is more similar to that of male ESCs. Thus, we could further strengthen the view that maGSCs are pluripotent cells and derivation of such cells from humans may open new avenues for clinical applications in regenerative medicine.

Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/.

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