Global and gene-specific histone modification profiles of mouse multipotent adult germline stem cells

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Submitted on June 6, 2010; resubmitted on October 2, 2010; accepted on October 5, 2010

ABSTRACT: We previously reported the generation of multipotent adult germline stem cells (maGSCs) from spermatogonial stem cells (SSCs) isolated from adult mouse testis. In a later study, we substantiated the pluripotency of maGSCs by demonstrating their close similarity to pluripotent male embryonic stem cells (ESCs) at the epigenetic level of global and gene-specific DNA methylation. Here, we extended the comparative epigenetic analysis of maGSCs and male ESCs by investigating the second main epigenetic modification in mammals, i.e. global and gene-specific modifications of histones (H3K4 trimethylation, H3K9 acetylation, H3K9 trimethylation and H3K27 trimethylation). Using immunofluorescence staining, flow cytometry and western blot analysis, we show that maGSCs are very similar to male ESCs with regard to global levels and nuclear distribution patterns of these modifications. Chromatin immunoprecipitation real-time PCR analysis of these modifications at the gene-specific level further revealed modification patterns of the pluripotency marker genes Oct4, Sox2 and Nanog in maGSCs that are nearly identical to those of male ESCs. These genes were enriched for activating histone modifications including H3K4me3 and H3K9ac and depleted of repressive histone modifications including H3K27me3 and H3K9me3. In addition, Hoxa11, a key regulator of early embryonic development showed the ESC-typical bivalent chromatin conformation with enrichment of both the activating H3K4me3 and the repressive H3K27me3 modification also in maGSCs. Collectively, our results demonstrate that maGSCs also closely resemble ESCs with regard to their chromatin state and further evidence their pluripotent nature.

Key words: multipotent adult germline stem cells / pluripotency / histone modifications / epigenetics

Introduction

Embryonic stem cells (ESCs) derived from the inner cell mass of pre-implantation mouse blastocysts are pluripotent in nature (Evans and Kaufman, 1981; Martin, 1981) and have the ability to differentiate into all three germ layers (Chambers, 2004; O’Shea, 2004; Suda et al., 1987) as well as germ cells (Geijsen et al., 2004; Nayernia et al., 2006). For the maintenance of these functional properties, an ESC genome must be highly plastic to sustain pluripotency on one hand and to enable differentiation to any other cell type on the other hand. Numerous studies already demonstrated that epigenetic mechanisms play a key role in regulating stemness and lineage specification (Mescherer and Misteli, 2006).

The main components of the epigenetic code are DNA methylation (Weber and Schubeler, 2007) and post-translational modifications of histone proteins (Jenuwein and Allis, 2001; Margueron et al., 2005). The basic unit of chromatin structure is the nucleosome, which is composed of 146 bp of DNA wrapped around a basic core histone octamer (two copies each of histones, H2A, H2B, H3 and H4). The N-terminal tails of histones are subject to diverse post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation (Jenuwein and Allis, 2001). These histone modifications are known to control structural chromatin compaction and regulate gene transcription by establishing higher order interactions between neighboring nucleosomes, altering the positioning/spacing of nucleosomes, and modulating contacts between histones and DNA (Narlikar et al., 2002). Generally, modifications like methylation at H3K4 (lysine 4 of histone H3), H3K36 and H3K79 residues, together with acetylation at H3K9 (H3K9ac) are associated with gene activation, whereas methylation at H3K9, H3K27 and H4K20 residues are linked to gene repression (Sims et al., 2003; Martin and Zhang, 2005).

In comparison with differentiated cells, ESCs have highly dispersed, euchromatic nuclei, increased levels of activating histone modifications...
like H3 and H4 acetylation and diminished levels of repressive histone modifications linked to heterochromatin like H3K9 trimethylation (H3K9me3) (Meshorer and Misteli, 2006). It was also evident that the promoter regions of genes active in ESCs, i.e. pluripotency genes like Oct4, Sox2 and Nanog as well as housekeeping genes like Gapdh are marked by H3 and H4 acetylation (Hattori et al., 2004; Kimura et al., 2004; O’Neill et al., 2006) as well as solely by H3K4 trimethylation (H3K4me3; Pan et al., 2007). In addition, bivalently marked chromatin, i.e. the co-occurrence of the activating H3K4 trimethylation and the repressive H3K27 trimethylation (H3K27me3) catalyzed by Trithorax- and Polycomb-group proteins, respectively, is a hallmark of key developmental lineage-control genes not expressed in mouse and human ESCs (Ringrose and Paro, 2004; Azuara et al., 2006; Bernstein et al., 2006). These unique bivalent histone modification patterns are assumed to be essential for simultaneously silencing and priming these lineage-control genes for later activation in ESCs. Further, this bivalent conformation was shown to be resolved during the differentiation of ESCs into neural precursor cells. Promoters of genes that are markedly induced in neural precursor cells retained H3K4me, but lost H3K27me (Bernstein et al., 2006). In contrast, promoters of genes that remain silenced in neuronal precursor cells lost H3K4me, whereas they were still associated with H3K27me. Thus, in ESCs, these key developmental lineage-control genes seem to carry a unique combination of activating and repressing histone methylation marks that are normally detected exclusively in euchromatin, respectively, heterochromatin regions of differentiated somatic cells.

Spermatogonial stem cells (SSCs) of the testis are responsible for maintaining the spermatogenesis throughout male adulthood. Several studies already reported that multipotent germline stem cells (mGSCs) that are morphologically similar to ESCs and express the same group of pluripotency-related genes can be generated from SSCs of neonatal and adult mouse testis suggesting that germline stem cells retain the ability to give rise to pluripotent cells in vitro (Kanatsu-Shinohara et al., 2004; Guan et al., 2006; Seandel et al., 2007; Izadyar et al., 2008; Kanatsu-Shinohara et al., 2008; Ko et al., 2009). These mGSCs are able to differentiate into derivatives of the three germ layers in vitro and can contribute to chimeras with germ line transmission, when injected into blastocysts. mGSCs from adult mouse testis, so-called multipotent adult germ line stem cells (maGSCs) were firstly isolated and described by our group. It was shown that maGSCs and ESCs display nearly identical gene-specific patterns and nuclear distribution patterns of these modifications. We further demonstrated very strong similarities of maGSCs and ESCs regarding global levels and nuclear distribution patterns of these modifications. We further show that maGSCs and ESCs display nearly identical gene-specific patterns of these modifications at the pluripotency marker genes Oct4, Sox2 and Nanog as well as the key developmental lineage-control gene Hoxa11. Collectively, the observed similarities of maGSCs and ESCs at the chromatin level further support the pluripotent nature of maGSCs.

**Materials and Methods**

**Cell lines and cell culture**

The three mouse maGSC lines maGSC 129/Sv, maGSC C57BL/6 and maGSC Stra8 were derived from wild-type 129/Sv, wild-type C57BL/6 and transgenic Stra8-EGFP/Rosa26-LacZ-background mouse strains, respectively, without genetic selection and solely by morphological identification on the basis of formation of ESC-like colonies as previously described (Zechner et al., 2009). The corresponding male ESC lines ESC 129/Sv, ESC C57BL/6 and ESC Stra8 were isolated as described previously and used for comparison (Nagy et al., 1993; Zechner et al., 2009). The undifferentiated cell lines were maintained on Mitozym C-inactivated mouse embryonic fibroblasts (MEFs) and cultured in standard ESC culture medium consisting of DMEM (PAN, Aidenbach, Germany) supplemented with 20% defined fetal bovine serum (PAN), 1% penicillin/streptomycin, 0.1 mM non-essential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol (all the above ingredients are from Gibco BRL, Eggenstein, Germany) and 1000 U/ml leukemia inhibitory factor (Chemicon, Temecula, CA, USA). MEFs were grown in standard fibroblast culture conditions.

**Flow cytometry**

Flow cytometric experiments were performed essentially as described previously (Obier and Muller, 2010). Feeder cell-depleted single cell suspensions of ESC 129/Sv and maGSC 129/Sv (~500 000 cells per sample) were washed with phosphate-buffered saline (PBS) and resuspended in 300 μl of freshly prepared fixation buffer [21.4 mM Tris–HCl, pH 7.1, 21.4 mM NaCl, 80.25% v/v deionized formamide, 1.07% bovine serum albumin (BSA) in H2O]. The cell suspensions were then incubated for 10 min at 80°C, allowed to cool down to room temperature (RT) and centrifuged for 5 min at 960g. Supernatant was removed and the transparent cell pellets were resuspended in 200 μl of PBS. After another pelleting step, cells were washed two times in fluorescence-activated cell sorting (FACS) buffer (PBS, 0.3% BSA, 0.1% NaN3, pH 7.4), and resuspended in 100 μl of FACS buffer containing the following antibodies at 1 of 100 dilution: anti-H3 (ab 1791; abcam), anti-H3K3me3 (39159; Active Motif), anti-H3K9me3 (07-442; Millipore), anti-H3K9ac (ab 10812; Abcam) and anti-H3K27me3 (07-449; Millipore). After incubation for 1 h at RT, the cells were washed twice in 1 ml FACS buffer and incubated with anti-rabbit-phycocerythrin (eBioscience) at 1 of 100 dilution for 45 min at RT followed by two washings in FACS buffer and resuspension in 300 μl FACS buffer. The flow cytometric measurements were performed on a FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany) and analyzed with CellQuestPro software (Becton Dickinson).

**Immunofluorescence staining**

ESCs and maGSCs grown in the presence of feeder layers on round cover slips coated with gelatine were washed two times with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at RT. Cells were then incubated with 50 mM NH4Cl in PBS for 10 min and permeabilized by three incubations with 0.2% Triton X-100 in PBS for 4 min each at RT. The four different histone modifications were detected with the antibodies also used for flow cytometry (see above) at 1 of 100 dilution by incubating 1 h at RT, followed by incubation for 1 h with a secondary antibody conjugated with Cy3. Cells were also stained for OCT4 using a mouse
monoclonal antibody (MAB4419; Millipore) followed by detection using a secondary antibody conjugated with Alexa-488 (Invitrogen, Karlsruhe, Germany). The cover slips were mounted with Vectashield DAPI mounting medium (Vector Laboratories, Burlingame, CA, USA) and images were taken using a fluorescence microscope (Olympus, Hamburg, Germany).

Western blot analysis
The cells were resuspended in a buffer containing 10 mM Tris/HCl pH8, 1 mM EDTA, 2.5% SDS supplemented with protease inhibitor cocktail tablet (Roche, Mannheim, Germany) for 30 min on ice and lysed using sonication. Following centrifugation at 16 000 g for 30 min at 4 °C, the supernatant (~40 μg) was resolved on 4–12% SDS–PAGE (Invitrogen) and electro-transferred onto nitrocellulose membrane. Immunoblots were performed with the antibodies against histone H3 and the four different histone modifications also used for flow cytometry (see above except H3K9ac which was analyzed with antibody 07-352 from Millipore) at 1 of 2000 dilution. An antibody against alpha-tubulin was used as a loading control. After incubation with a horseradish peroxidase-conjugated secondary antibody, specific bands were detected using Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology, Inc., USA).

Chromatin immunoprecipitation real-time PCR assays
The cultured cells were cross-linked with 1% formaldehyde in culture medium for 10 min at RT, and cross-linking was stopped by adding glycine (to a final concentration of 125 mM). Pellets containing 2 × 10^6 cells were lysed by brief incubation in containing 10 mM Tris–HCl pH7.5, 10 mM NaCl, 3 mM MgCl2, 0.5% IGEPA and 1 mM phenylmethylsulphonyl fluoride (PMSF) followed by centrifugation and another incubation in lysis buffer supplemented with 1 mM CaCl2 and 4% IGEPA. The chromatin was then sonicated using a Branson 250 Sonifier to obtain an average DNA fragment length of ~200–500 bp. Soluble chromatin was incubated for 3 h at 4 °C with and without (negative-control) antisera (3–5 μg) against the four different histone modifications also used for flow cytometry (see above except H3K9ac which was analyzed with antibody 07-352 from Millipore) and complexed with protein-A sepharose beads overnight at 4 °C. Final linking was performed by adding elution buffer containing proteinase K followed by incubation for 1 h at 65 °C. Reverse cross-linking was performed by adding elution buffer (25 mM Tris–HCl, 10 mM EDTA, 0.5% SDS) and incubating for 1 h at 65 °C. Reverse cross-linking was performed by adding elution buffer containing proteinase K followed by incubation for 1 h at 65 °C. Finally, the DNA was extracted, purified using the Wizard SV Gel and PCR clean-up System (Promega). Quantification of precipitated DNA was carried out using SYBR green (Invitrogen)-based real-time qPCR amplification with the following primer sets: Beta-Actin-F, 5′-CCCCAACAACACCT AGCAAAT-3′ and Beta-Actin-R, 5′-CCCCAACAACACCT AGCAAAT-3′; Oct4-F, 5′-TGAGCGCGCAATCTTCAGTTC-3′ and Oct4-R, 5′-TTGAGCGCCTGCCGACAT-3′; Sox2-F, 5′-AGGTTGCTGAGTGGGA TTAG-3′ and Sox2-R, 5′-AGGTTGCTGAGTGGGA TTAG-3′; and Nanog-F, 5′-TTGAGCGCCTGCCGACAT-3′ and Nanog-R, 5′-TTGAGCGCCTGCCGACAT-3′. The qPCR data of each two biological and two independent technical replicates were calculated and expressed as percentage of input DNA.

Results
Genome-wide histone modification analysis of maGSCs
We firstly employed FACS-based detection in formamide-fixed cells to compare the global histone modification levels of maGSCs with those of pluripotent male ESCs (each derived from the 129/Sv mouse strain). To ensure the specificity of the fixation and the immunostaining method for the flow cytometric approach, we performed control stainings with antibodies against histone H3 and the four different histone modifications also used for flow cytometry (Fig. 1A and B). After immunostaining with a histone 3 (H3) specific antibody, we could observe that around 98% of both ESCs and maGSCs were stained with similar fluorescence intensities in FACS and a nuclear localization signal in fluorescence microscopy confirming the specificity of H3 staining (Fig. 1C). Then, immunostaining of both maGSCs and ESCs for the activating histone modifications H3K4me3 and H3K9ac as well as the repressive histone modifications H3K27me3 and H3K9me3 was performed and analyzed by FACS. This analysis demonstrated that ~90–98% of both cell types were positive for the four various histone modifications (Fig. 1D) with very similar high fluorescence intensities (Fig. 1E).

Fluorescence microscopy was also used to investigate the cellular localization and staining pattern of the four histone modifications in maGSCs compared with male ESCs (each derived from the 129/Sv mouse strain). Immunostainings with antibodies specific to H3K4me3, H3K27me3, H3K9me3 and H3K9ac were evaluated. An antibody specific to OCT4 was employed to distinguish the nuclei of pluripotent cells from those of MEFs. Overall, the staining patterns and intensities of the four histone modifications were very similar in both maGSCs and ESCs, but markedly differed from those detected in MEFs (Fig. 2). Very high levels of H3K4me3 staining were observed throughout the nuclei of both maGSCs and ESCs and confirming the open chromatin state of both cell types (Fig. 2A). Similarly, staining with an H3K9ac-specific antibody showed an exclusive localization in non-DAPI-dense euchromatic regions (Fig. 2B). On the other hand, repressive modifications like H3K9me3 and H3K27me3 displayed discrete spots at DAPI-dense heterochromatic regions and a diffuse pattern in the nuclear periphery, respectively, present in both cell types (Fig. 2C and D).

To validate the global histone modification levels detected by FACS analysis, we isolated total protein extracts from the ESC 129/Sv and the maGSC 129/Sv cell lines as well as from MEFs and then performed western blot analysis with antibodies against H3, H3K4me3, H3K27me3, H3K9me3 and H3K9ac (Fig. 3). This analysis revealed nearly equal levels of the analyzed histone modifications in both ESCs and maGSCs but also in non-pluripotent MEFs.

Gene-specific comparative analysis of histone modification profiles
Chromatin immunoprecipitation (ChiP) real-time PCR was used to comparatively analyze gene-specific active (H3K4me3 and H3K9ac) and repressive (H3K4me27 and H3K9me3) histone modification levels at the pluripotency marker genes Oct4, Sox2 and Nanog as well as the key developmental lineage-control gene Hoxa11 in ESC and maGSC cell lines derived from three different genetic
Histone modifications of maGSCs

Figure 1 Flow cytometric analysis of global histone modification levels in maGSCs and ESCs. Control immunostainings of ESC 129/Sv (blue) and maGSC 129/Sv (red) were performed without any antibody (A), with only secondary antibody (B) or with histone3 (H3) specific antibody (C) followed by incubation with a PE-conjugated secondary antibody. The cells were counterstained with DAPI (blue) to visualize the nuclei. Bar diagram representing the percentage of cells stained for histone H3 and the four different histone modifications (D). Fluorescence intensities of the analyzed activating (H3K4me3 and H3K9ac) and repressive (H3K27me3 and H3K9me3) histone modifications in ESC 129/Sv (blue) and maGSC 129/Sv (red) (E). All the experiments were performed in three biological replicates.

Backgrounds (129/Sv, C57BL/6 and Stra8) as well as MEFs. Beta-Actin (Actb), a housekeeping gene enriched for activating chromatin modifications and depleted of repressive chromatin modifications, was used as a control. Quantification was performed by normalizing the results relative to the amount of input DNA and expressing them as percentage of input DNA. In maGSCs and ESCs of all three analyzed genetic backgrounds, the two active histone modifications were highly abundant at the promoter regions of the pluripotency genes Oct4, Sox2 and Nanog and the housekeeping gene Actb and represented ~20–60% of input DNA for H3K4me3 and ~30–50% of input DNA for H3K9ac (Fig. 4A–C). In contrast, the two repressive modifications were largely depleted at the promoters of the three pluripotency genes and the housekeeping gene and constituted generally below 10% and in most cases even markedly below 5% of input DNA in both maGSCs and ESCs (Fig. 4A–C). The promoter region of the
lineage-control gene *Hoxa11* was associated with the two active H3K4me3 and H3K9ac as well as the repressive H3K27me3 histone modifications which accounted for \( \approx 10-20\% \) of input DNA for H3K4me3 and H3K9ac and \( \approx 20-30\% \) of input DNA for H3K27me3 in both maGSCs and ESCs (Fig. 4A–C). However, the second repressive histone modification, H3K9me3, was not enriched at *Hoxa11* and exclusively displayed levels below 5% of input DNA in all analyzed maGSCs and ESCs. In general, the histone modification patterns detected for all five analyzed genes were very similar in maGSC and ESCs irrespective of their genetic background. With regard to the detected histone modification levels, maGSCs and ESCs of the same background tended to be slightly more similar than maGSCs and ESCs of different genetic backgrounds (Fig. 4A–C). As expected, MEFs showed a high abundance of the two active (H3K4me3 and H3K9ac), and an almost complete absence of the two repressive (H3K4me27 and H3K9me3), histone modifications at the expressed *Actb* gene (Fig. 4D). Similarly, the *Hoxa11* gene which is also known to be expressed in MEFs (Xi et al., 2007) also exhibited higher levels for the active H3K4me3 and H3K9ac modifications compared with those of the two repressive marks (Fig. 4D). The three non-expressed pluripotency marker genes, however, displayed a moderate (at *Oct4* and *Nanog*) to

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**Figure 2** Immunofluorescence analysis of histone modifications. ESC 129/Sv and maGSC 129/Sv were cultured under standard ESC culture conditions and analyzed for the staining pattern and cellular localization of H3K4me3 (A), H3K9ac (B), H3K9me3 (C) and H3K27me3 (D) (each in red). The cells were also stained with an OCT4-specific antibody (green) to distinguish the ESC and maGSC nuclei from those of feeder cells (MEFs). DAPI counterstaining (blue) was performed to visualize nuclei. DAPI-dense regions represent heterochromatin.
strong (at Sox2) enrichment for the repressive H3K4me27 modification compared with the other three histone modifications (Fig. 4D).

**Discussion**

SSCs representing the stem cell population of male germ cells have been repeatedly shown to be an appropriate source for deriving mGSCs that closely resemble ESCs with regard to their developmental potential including the ability to contribute to the germ lineage (Kanatsu-Shinohara et al., 2004; Guan et al., 2006; Seandel et al., 2007; Izadyar et al., 2008; Kanatsu-Shinohara et al., 2008; Ko et al., 2009). These findings opened new avenues to study the molecular control of stem cell pluripotency and differentiation, model human genetic disorders and generate patient-tailored regenerative therapies. To our knowledge, this is the first study to show that mGSCs also closely resemble male ESCs with regard to global and gene-specific histone modification levels and profiles.

In the following immunofluorescence experiments, we focused on the nuclear localization and staining pattern of the four histone modifications in maGSCs and ESCs. These experiments revealed very similar nuclear localization patterns for the four epigenetic marks in maGSCs and ESCs that are in agreement with previous findings in murine ESCs (Lehnertz et al., 2003; Meshorer et al., 2006; Luo et al., 2009). Two of these reports also showed that, as expected from its role in gene activation, methylation of H3K4, similar to acetylation of H3K9, is not colocalized with HP1α and intense DAPI staining but associated with transcriptionally active open chromatin structures in

**Figure 3** Western blot analysis of global histone modification levels in maGSCs, ESCs and MEFs. Total protein extracts from ESC 129/Sv, maGSC 129/Sv and MEFs were resolved by SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted using antibodies specific to H3, H3K4me3, H3K9ac, H3K9me3 and H3K27me3. All immunoblots were reprobed for tubulin to validate equal loading of samples. The molecular weight (Mw) in kDa of the bands is indicated on the right of each image. The utilized anti-H3K9ac antibody from Millipore has already been reported (see http://www.millipore.com/catalogue/item/07-352#) to detect H3K9ac at an Mw of 17 kDa and an additional unknown protein at an Mw of 23 kDa (indicated by the asterisk at the right of the image).
mouse ESCs (Lehnertz et al., 2003; Luo et al., 2009). In addition, the H3K9me3 and H3K27me3 nuclear localization patterns observed in our study are reminiscent of those already shown in late embryonic day 12.5–13.5 mouse primordial germ cells (PGCs), which represent the non-pluripotent embryonic precursors of SSCs and, thus, also of maGSCs (Hajkova et al., 2008) and can be transformed in vitro to pluripotent embryonic germ cells (EGCs) (Shamblott et al., 1998). Hence, these findings strengthen the view that maGSCs, ESCs, and also PGCs are closely related stem cells. Future immunofluorescence stainings of H3K9me3 and H3K27me3 in EGCs could possibly help to further

Figure 4 Analysis of histone modification levels at Oct4, Sox2, Nanog and Hoxa11 in maGSCs, ESCs and MEFs. The chromatin of ESC lines (each left) and maGSC lines (each right) from three different genetic backgrounds (129/Sv, A; C57BL/6, B; Stra 8; C) as well as that of MEFs (D) were subjected to ChIP analysis using antibodies against the four different histone modifications. The precipitated DNA was analyzed by real-time qPCR for abundance of the various modifications at the promoter regions of the housekeeping gene beta-actin; the key pluripotency genes Oct4, Sox2 and Nanog and the key developmental lineage-control gene Hoxa11. The qPCR data are presented as percentage of input DNA.
elucidate if the observed nuclear localization patterns are characteristic for pluripotent stem cells.

Similar to our genome-wide studies, our ChiP real-time PCR-based gene-specific analysis of histone modifications also revealed very similar profiles in maGSCs and ESCs. Overall, these profiles were not only similar in the maGSCs and ESCs analyzed in our study, but also closely resembled those repeatedly reported for murine ESCs. Thus, several ChiP-PCR-based studies of Oct4, Nanog and Sex2 promoters in murine ESCs also detected a hyperacetylation and hypermethylation for the two permissive marks H3K9ac and H3K4me3, respectively, and a strong hypomethylation for the two repressive marks H3K9me3 and H3K27me3 marks (Hattori et al., 2004; Kimura et al., 2004; Azuara et al., 2006; O’Neill et al., 2006). Further, Hoxa11 is one of the numerous key developmental genes that were already previously described pluripotent cell type, i.e. mouse and human induced pluripotent stem cells (Takahashi et al., 2007; Wernig et al., 2007).

Taken together, this report describes the first analysis of global and gene-specific histone modifications in maGSCs. We provide evidence for a high degree of similarities in histone modification profiles between maGSCs and ESCs. After several previous studies that reported strong similarities in DNA methylation and microRNA expression profiles between maGSCs and ESCs, these novel data further extend the characterization of maGSCs at the epigenetic level and additionally support their pluripotent nature. To obtain a more detailed and clear picture of the epigenetic landscape of maGSCs, further studies will be necessary aiming at the genome-wide analysis of DNA methylation and histone modifications at single-base-pair resolution.

**Funding**

The work was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft) in the Priority Programme SPP 1356 (EN 84/22-1 to W.E.; ZE 442/4-1 to U.Z.) and the Research Unit FOR 1041 (EN 84/23-1 to W.E.; ZE 442/5-1 to U.Z.).

**Authors’ roles**


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