MicroRNA signature in testes-derived male germ-line stem cells

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ABSTRACT: The testis-derived male germ-line stem (GS) cell, the in vitro counterpart of spermatogonial stem cell (SSC), can initiate donor-derived spermatogenesis in recipient testes and therefore, has been viewed as a future therapeutic modality for treatment of male infertility in azospermic patients and in cancer patients who are expecting chemotherapy. Upon extended in vitro culture, GS cells also generate a second cell type called multipotent adult germ-line stem (maGS) cell which, upon testicular transplantation, produces teratomas instead of initiating spermatogenesis. Here, we show that expressions of both Let-7a and Let-7d were consistently higher while that of miR-294 (embryonic stem cell-cycle-regulating miRNA; ESCC) was lower in GS cells than in maGS cells. Furthermore, among several putative targets of Let-7 identified by in silico bioinformatics, expressions of Igf2 and H19 mRNA targets significantly differed between GS and maGS cells. However, although the CTCF binding factor (a component of DNA methylation machinery at Igf2-H19 cluster) was also a putative target for Let-7, the difference in expressions of Igf2 and H19 between GS and maGS cells was not mediated through a change in DNA methylation. Both GS and maGS cells maintained androgenetic imprinting at the Igf2-H19 imprinting control region and Peg1 differentially methylated region. In conclusion, our study suggests that high Let-7 expression may be a unique property of GS cells and expressions of Let-7 and ESCC miRNAs may serve as miRNA signatures to distinguish them from maGS cells during clinical transplantation, to avoid the likelihood of teratoma formation due to maGS cells generated during extended in vitro culture of GS cells.

Key words: Let-7 / miR-294 / male germ-line stem cell / multipotent adult germ-line stem cell / spermatogonial stem cell

Introduction

Male infertility accounts for ~50% of total human infertility cases with ~20% as a sole cause and ~30% as a contributing cause (Swan et al., 2000; Hotaling and Walsh, 2009). It can be empirically treated with intracytoplasmic sperm injection (ICSI) as long as at least one motile sperm per oocyte is available. In cases wherein sperm are not available for ICSI or in patients who are expected to undergo chemotherapy for diseases such as cancer, spermatogonial stem cell (SSC) has been envisaged as future infertility treatment (Sadri-Ardekani et al., 2009). The usefulness of SSC in infertility treatment has been well documented in literature using a mouse model system (Ogawa et al., 2000; Fujita et al., 2005).

Germ-line stem (GS) cells are the in vitro counterpart of SSC in the testis that can self-renew in vitro in the presence of glial cell line-derived neurotrophic factor (GDNF) for more than 2 years and, when transplanted into the seminiferous tubules of an infertile male mouse, could establish donor-derived spermatogenesis to produce spermatozoa and transmit the donor haplotype to progeny (Kanatsu-Shinozuka et al., 2003a, b; 2005; de Rooij, 2006). The colony-formation ability of mouse GS cells can further be improved by the leukemia inhibitory factor (LIF) although it is not essential for maintaining their SSC characteristics (Kanatsu-Shinozuka et al., 2007). When mouse GS cells are continued in culture under these conditions, a second cell type also appears that can be expanded selectively under culture conditions used for embryonic stem (ES) cells. Unlike GS cells, these cells, called multipotent GS (mGS; from neonatal testes) or multipotent adult GS (maGS; from adult testes), are multipotent and produce teratoma upon transplantation into the seminiferous tubules of the testis (Kanatsu-Shinozuka et al., 2004; Guan et al., 2006). The mGS and maGS cells originate from the cultured GS cells themselves at a low frequency and are not some leftover of an earlier type of germ cells (Kanatsu-Shinozuka et al., 2005; de Rooij, 2006). We have also recently shown that mouse maGS cells can re-acquire GS-cell like characteristics when they were cultured in GS-like conditions (Jung et al., 2010). Thus, at any particular time point, in vitro cultured GS cells may contain some contaminating mGS or maGS cells which may produce teratoma.
instead of initiating spermatogenesis upon their transplantation into a recipient testis (Kanatsu-Shinohara et al., 2004). A molecular marker that can distinguish GS cells from mGS or maGS cells will therefore be useful for their successful application in both clinical and research settings.

MicroRNA (miRNA) are a class of 20–25 nucleotide-long non-coding endogenous RNAs that post-transcriptionally modulate the gene expression through canonical base pairing between the seed sequence of the miRNA (nucleotides 2–8 at its 5′ end) and its complementary seed match sequence in the 3′UTR of target miRNAs. They are frequently present as families of redundant genes and have numerous putative targets essential for a wide array of biological processes, including control of proliferative homeostasis, differentiation or embryonic stemness (Inui et al., 2010). Lethal-7 (Let-7) family of miRNA, the first miRNA ever identified, is evolutionarily conserved from Drosophila to human and comprises twelve Let-7 genes encoding for nine distinct mammalian miRNAs (Let-7a to Let-7i) and four Caenorhabditis elegans miRNAs (Let-7, miR-48, miR-84, miR-241) that share conserved seed sequence. They regulate the cell cycle and proliferation and differentiation of ES cells, and its overexpression slows the proliferation of somatic and cancer cells (Johnson et al., 2007; Legesse-Miller et al., 2009). In mouse ES cells, ES cell-cycle-regulating (ESCC) miRNAs (miR-290-295) (Wang et al., 2008) blocks Let-7 miRNA to maintain their self-renewal (Melton et al., 2010). Though the mature Let-7 family members are down-regulated in undifferentiated ES cells, hairpin precursors and pre-Let-7 transcripts have been found to be expressed in early mouse embryo (Schulman et al., 2005). Therefore, highly mature Let-7 levels may be a marker for certain stem cells. Indeed, Hayashi et al. (2008) have recently reported that the Let-7 family was expressed at a higher level in male primordial germ (PG) cells than in female PG cells in mouse. Let-7 family members are also abundant in testes (Gillis et al., 2008) and sperm (Martins and Krawetz, 2005) thereby suggesting that Let-7 family members may be contributing to regulation of male germ-cell lineage (Hayashi et al., 2008; West et al., 2009).

In this study, using a mouse model system, we show that GS cells and maGS cells show differential expression of Let-7 and miR-294 that can be used as a miRNA signature for their identification before research or clinical application.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise specifically indicated. All animal experiments were approved and performed under the guidelines of the Konkuk University Animal Care and Experimentation Committee.

Establishment of GS and maGS cell lines

The GS and maGS cells were isolated from 4- to 6-week-old DBA/2 mouse (Orient Bio, South Korea) testes as described previously (Kanatsu-Shinohara et al., 2004; Guan et al., 2006) with partial modifications (Oh et al., 2009; Jung et al., 2010). The isolated cells were cultured on a mitomycin C-treated STO feeder cell layer in Dulbecco’s modified eagle medium supplemented with 15% (v/v) fetal bovine serum (HyClone, Logan, UT, USA), MEM non-essential amino acids (Gibco BRL, Grand Island, NY, USA), 1% penicillin-streptomycin (Gibco BRL), 50 μM β-mercaptoethanol and 10 ng/ml recombinant human GDNF (R&D Systems, Minneapolis, MN, USA) at 37°C in a humidified atmosphere of 5% CO2 in air. Additionally, 1000 unit/ml murine LIF (Chemicon, Temecula, CA, USA) was added to the initial culture medium to enhance the formation of germ cell colonies (Kanatsu-Shinohara et al., 2007). The established GS and maGS cell colonies were subsequently cultured in the absence (GS) or presence (maGS) of LIF to establish the respective cell lines (Kanatsu-Shinohara et al., 2004; Guan et al., 2006). The characteristics of established GS and maGS cell lines were verified by expression of marker genes, immunocytochemistry and DNA methylation profiling as we described previously (Oh et al., 2009; Jung et al., 2010). For in vitro differentiation studies, GS and maGS cells were induced to form embryoid bodies (EBs) by hanging drop culture for 4 days then cultured in the presence of 1.0 μM all-trans retinoic acid (EBRA) for the next 4 days as we described earlier (Oh et al., 2009; Jung et al., 2010). As control, mouse ES cells were cultured for analysis by the standard method.

Analysis of miRNAs

To evaluate the expression of Let-7 family of miRNA, we arbitrarily chose Let-7a and Let-7d miRNAs for analysis as all members of Let-7 family share conserved seed sequence which is important for the specificity of target miRNA recognition (Lee and Dutta, 2007; Hayashi et al., 2008). Similarly, miR-294 was chosen as a representative of ESCC miRNAs (Wang et al., 2008; Melton et al., 2010). The expressions of mature miRNAs in cultured cells were quantified by real-time TaqMan® MicroRNA Assay as described previously (Melton et al., 2010). Briefly, cells were washed twice in PBS, lysed in 300 μl of lysis/binding solution and then total RNA, including miRNA, was extracted using the mirVanaR MicroRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer’s protocol. The isolated miRNAs (10 ng) were then converted to cDNA using the miRvana® MicroRNA Reverse Transcription Kit (Ambion) in 15 μl reaction mixture containing 100 mM dNTPs, 50 μM Multiscrbe® reverse transcriptase, 20 U/μl RNase inhibitor and 3 μl of miRNA-specific reverse transcription (RT) primer according to the manufacturer’s instructions. Subsequently, 1.33 μl of the RT product was PCR amplified in 20 μl of reaction mixture containing TaqMan universal PCR master mix (Ambion) and commercially available optimized miRNA-specific primers for each miRNA and for the endogenous sno202 control (Ambion) on a 7500 Fast Real-Time PCR System (Applied Biosystems) as per the manufacturer’s instructions. The amplification parameters for RT and real-time PCR were employed according to the manufacturer’s protocol (TaqMan® MicroRNA assay kit; Applied Biosystems). This method quantifies exclusively mature miRNAs but not their precursors. The expression values for each miRNAs were normalized to endogenous sno202 control and relative expression values were obtained using the 2−ΔΔCt method (Applied Biosystems user bulletin PN 4347825). All experiments were performed in triplicate.

Identification and analysis of putative Let-7 targets

Putative targets of Let-7 miRNA were predicted in silico by bioinformatic analysis using web-based TargetScan (URL: http://www.targetscan.org/mmu_50/) and MicroCosm Targets Version 5 (URL: http://www.ebi.ac.uk/entrez-srv/microcosm/htdacc/targets/v5/) softwares. Published known targets of Let-7 (URL: http://www.ncbi.nlm.nih.gov/pubmed/) were also included in the analysis. The identified putative targets were annotated by using functional annotation tools at the Database for Annotation, Visualization and Integrated Discovery 2010 (URL: http://david.abcc.ncifcrf.gov). A selected number of putative miRNA targets of Let-7 miRNA were analyzed for their expression in GS and maGS cells by RT–PCR and/or real-time quantitative RT–PCR (real-time qRT–PCR).
essentially as we described earlier (Jung et al., 2010). The primer sets used for RT–PCR and real-time qRT–PCR are listed in Supplementary data, Table S1. The housekeeping gene, Gapdh, was used as an internal standard in all experiments.

Analysis of DNA methylation at Igf2-H19 genomic cluster

Since in silico analysis identified the H19, CTCF binding protein (a controller of DNA methylation at Igf2-H19 genomic cluster) and Imp1 (an inhibitor of Igf2 gene expression) genes as putative targets of Let-7 miRNA, we analyzed the DNA methylation status at the imprinting control region (ICR) of the Igf2-H19 genomic cluster in GS and maGS cells by combined bisulfite restriction analysis (COBRA) essentially as we described earlier (Jung et al., 2010). As control, genomic DNA from sperm and somatic STO cells were also analyzed. Additionally, androgenetic DNA methylation of GS and maGS cells were analyzed by COBRA of differentially methylated region (DMR) of Pegl gene. The primer pairs used for COBRA are listed in Supplementary data, Table S2.

Results and discussion

The maGS and ES cells have been shown to have a similar miRNA profile (Zovoilis et al., 2008). However, the miRNA profile of the GS cell is not known. We found that, although GS and maGS cells did not differ with respect to the expression of stem cells (Oct4, Nanog, Cd9, Rex1), and GS cells (Stra8, Cd9) marker genes and proteins (Nanog, SSEA1 and Thy1 or CD90) (Fig. 1A and B), expressions of both Let-7a and Let-7d were consistently higher (Fig. 2A and B) while that of ESCC (miR-294) was lower (Fig. 2C) in GS cells than in maGS cells. Thus, Let-7 and ESCC miRNA could be one possible distinguishing feature of GS and maGS cells. Moreover, expression of Let-7 was high while that of ESCC was absent in sperm. The role of Let-7 in male germ-line cells is however, not known (Martins and Krawetz, 2005). Earlier studies have shown that overexpression of Let-7 reduces the proliferation of somatic and cancer cells (Johnson et al., 2007; Legesse-Miller et al., 2009). Let-7 expression was also low in rapidly proliferating ES and cancer cells (Johnson et al., 2007; Legesse-Miller et al., 2009; Melton et al.). Indeed, consistent with Let-7 expression, GS cells proliferated slower than maGS cells (~4–6 days for GS cells and ~2–3 days for maGS cells), as has also been reported earlier (Kubota et al. 2004; Jung et al., 2010). Thus, Let-7 may be a characteristic of slow-dividing GS cells. Studies in ES cells have also suggested that Let-7 may be functioning by opposing ESCC (Melton et al., 2010). Indeed, we found that expression of ESCC was lower in GS cells compared with maGS cells (Fig. 2C). Consistent with proliferation rate, expression of ESCC did not differ between maGS and ES cells (Fig. 2C).

We also evaluated whether expression of Let-7 in GS and maGS cells alters during their in vitro differentiation. As expected, stem cell (Oct4, Nanog, Cd9, Rex1), and GS cell (Stra8, Cd9) marker genes were silenced during differentiation of GS and maGS cells but were not complete in ES cells (Fig. 1C). Similarly, the expression of Let-7 miRNA was increased during differentiation of ES cells (Fig. 3). However, increase in Let-7 expression during differentiation of male germ line-derived GS and maGS cells was not as prominent as that observed with ES cells (Fig. 3). Moreover, expression of Let-7d was

Figure 1 Characterization and in vitro differentiation of male GS and maGS cells in mouse. ES cells were used as control for comparison. (A) Colony characteristic, alkaline phosphatase activity and EBs generated from GS, maGS and ES cells. (B) Expression of Nanog, SSEA-1 and Thy1 marker proteins in undifferentiated GS and maGS colonies. Colonies were immunostained with specific primary antibodies and FITC-labeled secondary antibodies and counter-stained for nuclei by DAPI. (C) Expression of stem cell and germ-cell marker genes in undifferentiated colonies, EBs and all-trans retinoic acid-treated embryoid bodies (EBRA) of GS, maGS and ES cells.
reduced during differentiation of GS cells. Thus, in male germ-line cells, Let-7 may have functions other than regulation of cellular proliferation.

In silico bioinformatic analysis showed that Let-7 has numerous putative targets (~953 for Let-7a and ~978 for Let-7d) that have disparate functions (Supplementary data, Table S3), with no means to decide a priori which one is most meaningful and thus worthy of experimental validation, as has also been reported for several other miRNAs (Inui et al., 2010). Thus, rather than querying Let-7-target pairs to predict Let-7 biological function, we asked which biological processes might be prime candidates for Let-7-mediated regulation. We narrowed down our list to cluster the putative targets into biological functions related to regulation of male germ cell and stem cell functions (Supplementary data, Table S3) and verified the expression of five targets (Fig. 4A) related to cellular proliferation (E2F5, E2F6) and spermatogenesis (Crem, Col4a5, Spata2) in undifferentiated and differentiated ES cells that differed with respect to Let-7 expression. RT–PCR analysis showed that among the tested genes, expression of only E2F5 varied inversely with the expression of Let-7 in undifferentiated and differentiated ES cells. We further found that E2F5, the most abundant member related to cell proliferation in human ES cells (Becker et al., 2007), is expressed in both maGS and ES cells but was undetectable in GS cells (Fig. 4B).

Interestingly, we found that Let-7 targets both DNA methylation regulatory protein and miRNAs of Igf2-H19 system. The Igf2-H19 system regulates growth and proliferation of cells and has recently been shown to be important for maintenance of SSC pluripotency (Huang et al., 2009) and for regulation of spermatogenesis through selective stimulation of spermatogonia, but not meiotic, DNA
synthesis (Soder et al., 1992). While H19 was found to be a potential target of Let-7 in our in silico analysis, Imp1, which binds specifically to the 5’ UTR of Igf2 mRNA to block its translation, is a validated target of Let-7 miRNA in several studies (Lu et al., 2007; Boyerinas et al., 2008). Additionally, our in silico analysis also revealed the CTCF binding factor, which regulates the DNA methylation machinery of the Igf2-H19 gene cluster by protecting its ICR from de novo methylation besides binding with unmethylated ICR to control gene expression, as another potential target of Let-7. Thus, we evaluated the mRNA expression and DNA methylation of the Igf2-H19 cluster in GS and maGS cells. We found that GS cells (showing high expression of Let-7) had significantly higher expression of Igf2 and lower expression of H19 than maGS cells (showing lower expression of Let-7) (Fig. 4D). However, the difference in expression of Igf2 between GS and maGS cells was possibly not mediated through change in DNA methylation as we did not find any difference between GS and maGS cells with respect to DNA methylation at ICR of the Igf2-H19 gene cluster (Fig. 4C). Both GS and maGS cells also maintained their androgenetic imprinting at DMR of Peg1 gene (Fig. 4C). It cannot be ruled out that Let-7 may be targeting the CTCF binding factor to regulate other signaling pathways such as CTCF and Smad crosstalk in the TGFβ signaling pathway (Bergstrom et al., 2010). Although further studies with gain- and loss-of-function studies are needed, the difference between GS and maGS cells for expression of Igf2 and H19 may partly be explained by Let-7 targeting of H19 and Imp1. Imp1 is a validated

**Figure 4** Expression and DNA methylation of putative targets of both Let-7a and Let-7d miRNAs. (A) Expression of putative targets in control ES cells during in vitro differentiation. Undifferentiated colonies were induced to form EBs by hanging drop method for 4 days and further treated with all-trans retinoic acid (EBRA) for another 4 days. (B) Expression of putative targets, selected from (A), in male GS and maGS stem cells. ES cells were used as control for comparison. The maGS (1) and maGS (2) indicate different expression in two different cell lines. (C) DNA methylation of Igf2-H19 ICR (Igf2-H19 ICR) and Peg1 DMR (Peg1 DMR) in GS and maGS cells. Sperm and somatic STO cells were used as control for comparison. (D) Expression of Igf2 and H19 genes in undifferentiated GS and maGS cells. Values above the bars indicate relative abundance of mRNAs normalized to the expression of respective mRNA in undifferentiated ES cells. Bars with different alphabet (a, b, c) differ significantly (P < 0.01).
target of Let-7 in number of cell types (Lu et al., 2007; Boyerinas et al., 2008) and is expressed mainly in the spermatogonia of the adult mouse testis (Hammer et al., 2005).

Together with previous work demonstrating the biallelic expression of Igf2 in the mouse male germ-line, these results suggest that the androgenic DNA methylation at ICR of the Igf2-H19 gene cluster and high expression of Let-7 miRNA in GS cells reflect the characteristics of the original germ-line cells from which they originate (Ko et al., 2008). Given that Lin-28, a negative regulator of Let-7 expression, is one of the markers of undifferentiated spermatogonia (Wang et al., 2005; Zheng et al., 2009), it is plausible that Let-7 expression may be a unique property of male germ-line cells and expression of Let-7 and ESCC miRNAs may serve as a miRNA signature to distinguish GS cells from maGS cells. Further studies should be directed at deciphering the molecular function of Let-7 in male germ-line cells in vivo. Our study suggests that analysis of Let-7 and ESCC miRNAs in GS cells may help avoid the likelihood of teratoma formation during their clinical transplantation, by distinguishing maGS cells generated from GS cells during extended culture. Putative GS/maGS colonies may be screened for the expression of Let-7 and ESCC miRNAs that Let-7 expression may be a unique property of male germ-line cells and expression of Let-7 and ESCC miRNAs may serve as an miRNA signature to distinguish GS cells from maGS cells. Further studies should be directed at deciphering the molecular function of Let-7 in male germ-line cells in vivo.

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

Authors’ roles
Y.H.J.: performed most of the experiments, analyzed the data and wrote the manuscript; M.K.G.: designed the experiment, interpreted the data and wrote the manuscript; J.Y.S.: assisted in cell culture and PCR experiments; S.J.U.: provided intellectual input in design of experiment, data interpretation and manuscript writing; H.T.L.: designed the experiment, analyzed the data, provided support and approved the manuscript.

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