Embryonic stem cell-related miRNAs are involved in differentiation of pluripotent cells originating from the germ line

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ABSTRACT: Cells originating from the germ cell lineage retain the remarkable property under special culture conditions to give rise to cells with embryonic stem cell (ESC) properties, such as the multipotent adult germline stem cells (maGSCs) derived from adult mouse testis. To get an insight into the mechanisms that control pluripotency and differentiation in these cells, we studied how differences observed during in vitro differentiation between ESCs and maGSCs are associated with differences at the level of microRNAs (miRNAs). In this work, we provide for a first time a connection between germ cell origin of maGSCs and their specific miRNA expression profile. We found that maGSCs express higher levels of germ cell markers characteristic for primordial germ cells (PGCs) and spermatogonia compared with ESCs. Retained expression of miR-290 cluster has been previously reported in maGSCs during differentiation and it was associated with higher Oct-4 levels. Here, we show that this property is also shared by another pluripotent cell line originating from the germ line, the embryonic germ cells. In addition, we provide proof that the specific miRNA expression profile of maGSCs has an impact on their differentiation potential. Low levels of miR-302 in maGSCs during the first 10 days of leukaemia inhibitory factor deprivation are shown to be necessary for the maintenance of high levels of early germ cell markers.

Key words: embryonic stem cells / multipotent adult germ line stem cells / microRNAs, miR-290, miR-302 / differentiation / primordial germ cell marker, Dppa-3

Introduction

During recent years a number of publications reported the remarkable property of the germ cell lineage to give rise to cells with embryonic stem cell (ESC) properties after culture under special conditions (Matsui et al., 1992; Kanatsu-Shinohara et al., 2004; Guan et al., 2006). Thus, although ESCs still remain the gold standard of pluripotency, the repertoire of pluripotent stem cells in mouse has been extended to include EGCs derived from primordial germ cells (PGCs), multipotent germline stem cells (mGSCs) from neonatal testis, multipotent adult germline stem cells (maGSCs) from adult testis and gPS cells (germline-derived pluripotent stem cells) from adult testis (Matsui et al., 1992; Resnick et al., 1992; 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). Similar results have been recently obtained also for human cells (Conrad et al., 2008; Kossack et al., 2009). Despite the direct implications of these findings for regenerative medicine, the exact mechanisms that control pluripotency and differentiation in these cells are still poorly understood.

A possible regulatory mechanism includes miRNAs, a class of small non-coding RNAs that regulate gene expression at the post-transcriptional level. Regulation of gene expression by miRNAs is realized by the formation of complex regulatory networks, in which each miRNA can target many different mRNAs, and conversely, several

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different miRNAs can cooperatively control a single mRNA target (He and Hannon, 2004; Kim, 2005; Esquela-Kerscher and Slack, 2006). Surprisingly, even for the well studied ESCs, only little is known about the role of miRNAs in the transcriptional network and the mechanisms controlling pluripotency and differentiation in these cells. Recently, members of three miRNA clusters have been associated with pluripotency in ESCs: miR-290, miR-302 and miR-17-92 clusters. The first two miRNA clusters are exclusively expressed in undifferentiated pluripotent stem cells as well as during first stages of in vitro differentiation and embryonic development (Houbavty et al., 2003, 2005; Suh et al., 2004; Strauss et al. 2006; Tang et al., 2007; Chen et al., 2007; Zovoilis et al., 2008; Mendell, 2008; Foshay and Gallicano, 2009).

Until now, maGSCs is the only type of pluripotent stem cells originating from the germ line which has been tested at the miRNA level. miRNA expression analysis revealed that these cells express also miR-290 and miR-302 clusters and miRNA expression levels were similar between undifferentiated ESCs and maGSCs (Zovoilis et al., 2008). However, during in vitro differentiation expression profiles of miR-290 and miR-302 clusters were found to differ between the two cell types. miR-290 cluster expression was found to decrease much later in maGSCs compared with ESCs, in which miR-290 cluster members are strongly down-regulated within 5 days of differentiation. In addition, miR-302 cluster is strongly up-regulated in ESCs during first stages of in vitro differentiation, although it increases much more slowly in maGSCs.

We hypothesized that there is a connection between the above differences in miRNA levels and the origin of maGSCs from germ cells as well as their differentiation potential. To this end, we first tested whether maGSCs express higher levels of markers representative of their germ cell origin compared with ESCs. Then, we questioned whether expressing high miR-290 cluster levels during differentiation is also a property shared by another pluripotent cell line originating from germ cells, the EGCs, and whether these levels are associated with high Oct-4 levels also in these cells. Finally, we investigated the question of whether maGSC cultures would express higher levels of germ cell markers during differentiation than ESCs due to their germ cell origin and whether differences in miRNA levels could be involved.

Materials and Methods

Cell culture

Derivation and culture of maGSC and ESC lines have been described previously (Cheng et al., 2004; Guan et al., 2006). With the exception of Fig. 1a and b, ESCs, maGSCs and EGs used in this study had the same genetic background, that of the 129/Sv mouse line, so that impact of different strain backgrounds the results could be eliminated. In brief, the ESC line (ESCs) is the well-characterized ES R1 cell line derived from the 129/Sv mouse line (Nagy et al., 1993). The maGSC line (maGSCs) is the maGSC 129SV line used and characterized previously (Zovoilis et al., 2008; Zechner et al., 2009). The EG line (EGCs) is the EG-1 line used in the work of Sharova et al. (2007), which also originates from the 129/Sv mouse line (Stewart et al., 1994). To maintain cells in undifferentiated state, cells were cultured as reported previously (Zovoilis et al., 2008). Prior to any RNA extraction and subsequent analysis, cells (two replicates per line) were cultured for at least 2 days in the absence of feeder layer (FL). For the differentiation studies cells were seeded on a 0.1% gelatine-coated 6-well culture plates (5 x 10^5 cells/well) without leukaemia inhibitory factor (LIF). Differentiation experiments were repeated twice.

miRNA transfection

For increasing miRNA levels, cells were transfected using HiPerFect transfection reagent (Qiagen, Hilden, Germany) according to manufacturer’s long-term transfection protocol. In brief, at Day 0 (beginning of culture under differentiation conditions), cells were transferred (5 x 10^5 cells/well) to 0.1% gelatine-coated 6-well plates. Subsequently, transfection complexes containing 18 μl of the transfection reagent and the miRNA precursor were added drop-wise onto the cells. Complexes were left on the cells until they reached 60–80% confluence (after 2 days), then sub-cultured again and re-transfected as described above. This procedure was repeated until Day 10 or until analysis of the cells. Pre-miR miRNA precursor molecules (Ambion, Austin, USA) for miR-302 a, b and d, were used in a final concentration of 5 nM each. The negative controls (neg) were transfected with the respective Pre-miR control, a random sequence Pre-miR molecule validated not to produce effects on known miRNA function and cells were cultured under exactly the same conditions for comparison. Pre-miR controls were used in a final concentration of 15 nM. miRNA precursor molecules used in this study are listed in detail in Supplementary, Table S1). Under these transfection conditions no cell toxicity effects were observed. miRNA transfection experiments were repeated twice.

Real-time PCR

Total RNA (including miRNAs) was isolated using the miRNeasy mini Kit (Qiagen). Conversion into cDNA and Real-time PCR detection of miRNAs were carried out using the miScript Reverse Transcription Kit and the miScript SYBR-Green PCR Kit (Qiagen), respectively, on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Optimized miRNA-specific primers for each miRNA as well as for the endogenous control RNU6B (miScript Primer Assays, Qiagen) are listed in Supplementary, Table S2. PCR specificity was checked as described previously (Zovoilis et al., 2008). For Real-time quantitative RT–PCR (qRT–PCR), the QuantiTect SYBR-Green PCR MasterMix (Qiagen) was used with gene-specific primers (listed in Supplementary, Table S3) for the following genes: Dppa-3, Stra-8, Oct-4, C-kit, Dazl and Brachyury. All quantities were further normalized to values of RNU6B and Sdha for miRNA or mRNA quantification, respectively.

Bioinformatics’ approaches and statistical analysis

For computational prediction of miRNA targets, we used the TargetScan web platform (www.targetscan.com /release 5.1), whereas for statistical analysis we used the STATISTICA software package for performing a one-way ANOVA (followed by Fischer LSD’s multiple comparison test) or t-test. Data are expressed as mean ± SD. P < 0.05 was considered statistically significant. For functional annotation of miR-302 cluster targets, we used the data mining environment provided by the DAVID bioinformatics approaches and statistical analysis platform (Huang et al., 2009). The functional annotation module was applied for gene ontology terms in PANTHER database using an EASE score of 0.001, a minimum number of two counts and a Benjamini stringency was used.

DNA methylation analysis

Genomic DNA was isolated from maGSC and ESC lines using standard protocols. Bisulfite treatment of genomic DNA isolated was performed.
using the EpiTect bisulfite kit (Qiagen, Hilden, Germany). The methylation status of the promoters of Oct4, miR290 and miR302 was analysed by bisulfite pyrosequencing. Bisulfite pyrosequencing was performed on a PSQ™96MA Pyrosequencing System (Biotage, Uppsala, Sweden) with the PyroGold SQA reagent kit (Biotage). Pyro Q-CpG software (Biotage) was used for pyrosequencing data analysis. Primers that were used and genomic locations of the regions that were tested are listed in Supplementary, Table S4.

**Results**

**maGSCs express high levels of germ cell markers characteristic for PGCs and spermatogonia**

In contrast to ESCs, pluripotent cells from testis do not originate from pluripotent stem cells like those of the inner cell mass of the blastocyst but from unipotent germ cells of testis (Kanatsu-Shinohara et al., 2008; Ko et al., 2009). In this respect, they resemble EGCs that also originate from the germline, after reprogramming of PGCs to an ESC-like state. We raised the question whether maGSCs show expression of early germ cell markers after reprogramming to the ESC-like state and to what extent compared with ESCs. We tested two key germ cell markers: Dppa-3, a widely used PGC marker expressed early during germ cell development, and Stra-8, which is highly expressed in spermatogonia (Oulad-Abdelghani et al., 1996; Lacham-Kaplan, 2004; Yabuta et al., 2006; Mark et al., 2008). In addition, we used mouse testis 7 days p.p. as well as adult testis as control. maGSC cultures were consistently found to express both markers in higher levels than cultures of ESCs originating from the same mouse background (Fig. 1a and b). As expected, expression of Dppa-3, which is an early expressed germ cell marker, was very low in adult testis (Fig. 1a) in contrast to the spermatogonia marker Stra-8, which was highly expressed in adult testis. Moreover, neonatal testis, which has

**Figure 1** Expression levels of the PGC marker Dppa-3 and the spermatogonia marker Stra-8 in undifferentiated ESCs, maGSCs and EGCs. Cells were cultured under standard conditions in presence of LIF and FLs (+LIF/+FL). Expression in neonatal (7 days p.p.) and adult testis was also tested as a control. Levels have been determined by qRT–PCR and quantities were normalized to the endogenous control (Sdha). (a, b) Comparison of Dppa-3 and Stra-8 levels between ESCs and maGSCs originating from three different mouse strains. Asterisk depicts statistical significance for the difference between maGSCs of each strain and the respective ESCs from the same strain. (c, d) Comparison of Dppa-3 and Stra-8 levels between ESCs and EGCs originating from the 129/Sv strain. Asterisk depicts statistical significance for the difference in Dppa-3 levels between maGSCs and EGCs.
a high ratio of spermatogonia to more mature germ cell types (Tege-lenbosch and de Rooij, 1993), showed higher expression of Stra-8 compared with adult testis (Fig. 1b).

Next, we tested whether such high expression levels of Dppa-3 and Stra-8 in maGSC cultures are also observed in cultures of another pluripotent cell type that originates from germ cells, EGCs. EGC cultures express Stra-8 in levels similar to those of maGSCs (Fig. 1d). Consistent with their PGC origin, expression levels of Dppa-3 in EGCs were more than 8-fold higher than in maGSCs (Fig. 1c). These results suggest that even after reprogramming maGSCs and EGCs retain expression of key germ cell markers.

**Pluripotent cells originating from germ cells retain expression of pluripotency-related miRNAs and Oct-4 during differentiation**

miR-290 cluster is down-regulated in maGSCs later than in ESCs during in vitro differentiation (deprivation of FLs and LIF; Zovoilis et al., 2008). Since EGCs have similarities to maGSCs and retain a similar expression pattern of early germ cell markers after reprogramming, we tested whether maintenance of high miR-290 cluster levels during in vitro differentiation is another common trait of these cells. To this end, we used qRT–PCR to profile expression levels of members of the miR-290 cluster in EGC cultures upon deprivation of FL and LIF for 21 days, in comparison to ESCs and maGSCs (Fig. 2a). In addition, we tested representative members of another cluster, the miR-17-92 cluster, which as discussed later, has been also recently connected to ESC and PGC proliferation (Hayashi et al., 2008; Fig. 2b).

Surprisingly, like maGSCs, EGCs retained high expression levels of miRNA members of both clusters upon deprivation of LIF. Although expression profiles of both cell types were definitely not the same, the similarity of their profiles compared with that of ESCs was striking: ESCs demonstrate a very early decrease in miRNA levels during the differentiation period that was tested, whereas maGSCs and EGCs do not. Importantly, these differences in miRNA expression patterns of maGSCs and EGCs compared with ESCs are observed also when other in vitro differentiation strategies are followed (Supplementary, Fig. S1).

**Figure 2** miRNA expression profiling by qRT–PCR of members of miR-290 and miR-17-92 clusters in ESCs and maGSCs during in vitro differentiation. (a, b) Intensities depict expression levels relative to those of undifferentiated cells (Day 0) and were developed using Matrix2png/version 1.0.6 as described previously (Pavlidis and Noble, 2003). Quantities were normalized to the endogenous control (RNU6B) and calibrated with levels of undifferentiated cells that depict intensity values of 1. Each column depicts expression at a different time point during differentiation and different rows represent the different miRNAs tested. (c) Schematic representation of the differentiation strategy that was followed. +LIF/+FL culture in presence of FLs and LIF, refers to undifferentiated cells, before beginning of differentiation. –LIF/–FL, culture in gelatine-coated flasks in absence of FLs and LIF that promotes differentiation.
Because high expression levels of miR-290 cluster during in vitro differentiation have been previously associated with high Oct-4 expression levels (Zovoilis et al., 2008; Zovoilis et al., 2009), we also tested Oct-4 expression profiles in EGCs during differentiation in comparison to maGSCs and ESCs (Fig. 3). Like maGSCs, and in contrast to ESCs, Oct-4 levels in EGC cultures did not decrease upon deprivation of LIF after 10 days of in vitro differentiation.

**maGSCs express high levels of germ cell markers during differentiation**

Since some properties of maGSCs associate with their germ cell origin, we hypothesized that this origin may also favour their differentiation towards germ cells upon deprivation of LIF. Indeed, cultures of maGSCs were found to express during differentiation higher levels of Stra-8 and other later expressed germ cell markers, like C-kit and Dazl (Fig. 4a) compared with ESC cultures. The in vitro differentiation environment employed here differs from that observed in vivo. However, it was reasonable to assume that increased levels of germ cell markers in spontaneously differentiating maGSC cultures may resemble the germ cell differentiation programme observed in vivo. Indeed, differentiating maGSC cultures expressed significantly higher levels of Dppa-3 than ESC cultures (Fig. 4b), which in vivo is indicative to commitment towards germ line and proceeds expression of C-kit and Dazl. It should be noted that expression levels of germ cell markers tested here refer to heterogeneous cultures undergoing spontaneous differentiation and are not indicative of expression in the single cell level.

**Low miR-302 expression levels in differentiating maGSCs correlate with increased Dppa-3 levels**

We questioned whether the specific miRNA expression profile of differentiating maGSCs can explain the observed differences in germ cell markers. Recently, over expression of members of miR-290 cluster was found to reduce Dppa-3 expression in ESCs (Zovoilis et al., 2009). However, differentiating maGSCs retain stable miR-290 levels; thus, it is rather unlikely that this could be a reason for the observed differences in Dppa-3 levels in differentiating maGSCs. Another pluripotency-related cluster, miR-302, is predicted to share same mRNA targets with some miR-290 cluster members (www.targetscan.org/Release 5.1) and could have a similar effect as miR-290 cluster. Thus, we investigated whether increased Dppa-3 levels in differentiating maGSC cultures correlate with levels of miR-302 cluster in these cells. Indeed, miR-302 cluster members were found to be expressed in lower levels in differentiating maGSCs than in differentiating ESCs (Fig. 4c).

**Increased miR-302 cluster levels result in down-regulation of Dppa-3 and Stra-8**

To confirm the association between miR-302 cluster and Dppa-3 we tested whether artificially increased levels of miR-302 cluster in differentiating maGSC cultures are able to inhibit Dppa-3 up-regulation. Figure 5a summarizes the strategy that was followed. Pre-miRNA precursor molecules (small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNAs) for miR-302 a, b, d were introduced into maGSCs cultured under differentiation conditions (−LIF/−FL) using conventional siRNA transfection methods. To monitor the efficiency of our treatment miRNA levels in treated cells were compared with those of negative control. In all cases successful treatment was confirmed by miRNA qRT–PCR (Supplementary, Fig. S2).

As shown in Fig. 5c, consistent with our hypothesis, increased levels of miR-302 cluster members prevented Dppa-3 up-regulation in maGSC cultures and resulted in decreased expression after 10 days differentiation in vitro. Using the same transfection strategy we confirmed this finding using another early germ cell marker, Stra-8 (Supplementary, Fig. S3) and also for ESC cultures. Although cultures of ESCs express already high levels of the miR-302 cluster members, further increase at their levels led to even lower Dppa-3 levels (Supplementary, Fig. S4).

We also tested the impact of high miR-302 expression levels on Oct-4 expression profile in both cell types. Increased miR-302 levels

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**Figure 3** Expression levels of the pluripotency marker Oct-4 during 10 days in vitro differentiation of ESCs, maGSCs and EGCs. Cells were cultured in absence of LIF and FLs (−LIF/−FL). Levels were determined by qRT–PCR and quantities were normalized to the endogenous control (Sdha) and calibrated with expression levels of cells before differentiation (d 0). d, day of differentiation. Asterisk depicts statistical significance at day 10 compared with undifferentiated cells (d 0).
did not affect Oct-4 expression levels in either ESCs or maGSCs (Fig. 5b).

Functional annotation of miR-302 cluster predicted targets

To get an insight into possible ways through which miR-302 cluster may control differentiation, we conducted bioinformatics analysis regarding genes predicted to be targeted by miR-302 cluster members. To this end, we employed the Targetscan platform for mouse which makes use of an algorithm identifying mRNAs with conserved complementarity to the seed (nucleotides 2–7) of the miRNA (Lewis et al., 2005). Targetscan yielded 417 conserved targets, with a total of 447 conserved sites and 133 poorly conserved sites for the miR-302 cluster seed. Genes with only poorly conserved sites were not tested further, in order to reduce the false positive rate. All genes that were included in further analysis are listed in Supplementary, Table S5. Using the data mining environment provided by the DAVID platform (Huang et al., 2009), we applied the respective functional annotation module to statistically highlight the most enriched biological annotations of miR-302 predicted targets regarding common biological processes and molecular functions. The exact parameter values used are listed in Materials and Methods section.

As shown in Fig. 6 the majority of enriched annotations include processes connected with mRNA transcription as well as regulation of transcription. Consistent with this, the most predominant molecular functions in which miR-302 targets are significantly enriched refer to transcription factor activity and DNA-protein interactions. Interestingly, enriched biological annotations included in 15% of cases developmental processes, in 5% cell cycle control and in 7% proliferation, which is in agreement with previous reports about the role of miR-302 cluster in cyclin D1 regulation (Card et al., 2008). In order to narrow our search for processes directly associated with germ cell development, we applied the functional annotation clustering module and searched for groups of annotated targets that are connected with this process. Supplementary, Fig. S5 depicts the resulting internal relationships among the clustered germ cell development terms and miR-302 predicted target genes.

miRNA promoter methylation dynamics in differentiating ESCs and maGSCs

Finally, we wanted to test whether differences between ESCs and maGSCs in expression profiles of Oct-4 as well as miR-290 and miR-302 clusters during differentiation are connected with the DNA methylation status of the promoters of these genes (Fig. 7). In general, the tested promoters showed a strong hypomethylation in the tested undifferentiated and differentiating ESCs and maGSCs, DNA methylation of Oct-4 and miR-290 promoters in maGSCs, although marginally higher than in undifferentiated ESCs, remained unchanged or even decreased during differentiation, in contrast to ESCs that increased methylation. These data suggest that, after 10 days of differentiation, ESCs have already initiated the process of promoter silencing of pluripotency-related genes like Oct-4 and miR-290 cluster. Although maGSCs depict a basal DNA methylation marginally higher than ESCs, do not seem to have initiated this process, indicating...
their need to retain miR-290 and Oct-4 expression. For the same reason, miR-302 cluster, which is up-regulated during first stages of ESC differentiation, did not show any increase of promoter DNA methylation, in contrast to miR-290 cluster and Oct-4.

**Discussion**

In this work, we provide for a first time a connection between germ cell origin of maGSCs and their specific miRNA expression profile. Retained expression of miR-290 cluster has been previously reported in maGSCs during differentiation and it has been associated with higher Oct-4 levels. Here, we show that this property is also shared by EGCs. In addition, in this work, we provide proof that the specific miRNA expression profile of maGSCs has an impact on their differentiation potential. Low levels of miR-302 observed in cultures of maGSCs during the first 10 days of LIF deprivation were shown to be associated with high Dppa-3 and Stra-8 levels. However, it is likely that other mechanisms may also contribute to this phenotype. Of course, under the cell culture system employed here cells (ESCs, maGSCs or EGCs) do not differentiate exclusively towards a specific lineage or with the same strict hierarchical pattern that blastocyst cells do in vivo. Thus, the data presented here only indicate mRNA levels in a complex population of cells. However, this system remains invaluable for detecting general trends in differentiation potential of each of the different cell lines used here.

The ability of undifferentiated maGSCs to retain high expression of early germ cell markers after reprogramming to the ESC-like state is of particular interest. Two recent reports show that pluripotent cells originating from the germ line differ from ESCs in DNA methylation of imprinted genes (Ko et al., 2009; Zechner et al., 2009). In the context of these reports our finding suggests that the process of reprogramming towards the ESC-like state does not completely erase all properties of the cells from which they originate. This demonstrates one of the few differences observed until now between undifferentiated maGSCs and ESCs, which otherwise show similar expression patterns in global gene expression array profiling (Meyer et al., unpublished, personal communication), western blotting for key pluripotency markers (Guan et al., 2006; Zovoilis et al., 2008) as well as whole microRNAome expression profiles (Supplementary, Fig. S6).

Although ESCs and maGSCs do not differ significantly under culture conditions that inhibit differentiation, during in vitro differentiation,
Oct-4 expression is maintained for a longer time and the levels of differentiation markers increase more slowly in maGSC cultures than in those of ESCs (Zovoilis et al., 2008). These remarkable properties of maGSCs can be partially explained by their specific expression profile of miR-290 cluster during differentiation. The miR-290 cluster has been shown to maintain and induce pluripotency in multiple ways, by controlling Wnt signalling, de novo DNA methylation as well as cell cycle regulators (Sinkkonen et al., 2008; Wang et al., 2008; Zovoilis et al., 2009). Particularly, ESCs with artificially induced abnormal high levels of miR-290 cluster have been shown to resemble maGSCs’ delayed differentiation under the same differentiation conditions used in the current study (Zovoilis et al., 2009). Members of miR-17-92 cluster, although not exclusively expressed in pluripotent cells, have been also shown to control proliferation of ESCs (Foshay and Gallicano, 2009) and could also contribute to the above phenotype.

Our data are in strikingly consistent with findings of Hayashi et al. (2008) who tested miRNA expression patterns during PGC development and early spermatogenesis in vivo (Hayashi et al., 2008). In that work, miR-290 and miR-17-92 clusters as well as Oct-4 were found to be constitutively highly expressed during germ cell development. Although the differentiation environment in vitro differs significantly from that in vivo, the similarities of the findings of Hayashi et al. (2008) to our findings are striking: spontaneous in vitro differentiation of maGSC cultures is characterized by higher levels of key PGC and germ cell markers compared with ESCs and at the same time expression of the same miRNA clusters as in that work is maintained at high levels. In addition, high levels of miR-290 cluster during differentiation of maGSCs are associated with remarkably high Oct-4 levels, a gene that in vivo continues to be expressed in stem cells of adult testis (He et al., 2007). Moreover, not only maGSC cultures but also EGC cultures were found to retain high levels of miR-290 and 17/92 cluster members during first stages of in vitro differentiation in close correlation with high Oct-4 levels. Thus, in the light of findings of Hayashi et al., our data suggests that these miRNAs may play an important role in the ability of stem cells of the germ line to maintain Oct-4 expression during first stages of differentiation. This is further supported by our previous findings that maintaining artificially high levels of miR-290 cluster in ESCs during differentiation prevents their differentiation, and particularly it prevents expression of mesoderm markers (Zovoilis et al., 2009). The ability to suppress differentiation towards mesoderm is crucial for development of PGCs.
we had previously achieved it with ESCs (Zovoilis et al., 2009). Taking into account that these miRNAs consist of more than 70% of the total miRNA cell content in ESCs, these cells seem to be able to compensate for their loss during inhibition experiments at non-toxic levels. However, the importance of these miRNAs in germ cell development is supported by a recently derived miR-290 deficient mouse line, which showed a role of these miRNAs in maintaining both pluripotency and fertility. Particularly, viable miR-290 deficient mouse were found to be infertile (reviewed in Blakaj and Lin, 2008).

maGSC cultures have been shown in the current and a previous work (Zovoilis et al., 2008) to increase levels of differentiation markers for all three germ layers later than ESC cultures. However, here we show that this does not apply to early germ cell markers that are significantly up-regulated during differentiation in cultures of maGSCs. For example, as shown in Supplementary, Fig. S8, a flow cytometric approach for the spermatogonia marker Stra-8, which quantified how many of differentiating maGSCs actually activated Stra-8, revealed a range from 37 to 49% in all different mouse strains tested. Levels of miR-302 cluster, which share the same miRNA seed (and thus targets) with some miR-290 cluster members, could partially explain this increase in germ cell markers in differentiating maGSCs. During differentiation, miR-302 cluster is up-regulated in maGSCs later than in ESCs. Low levels of miR-302 cluster in maGSCs as compared with ESCs are necessary for high Dppa-3 and Stra-8 expression in maGSCs. Thus, this delay in up-regulation of miR-302 observed in maGSCs seems to be one of the underlying mechanisms for the high early germ cell marker levels in maGSC cultures during differentiation.

At the moment it remains unclear through which targets miR-302 induces this effect, miR-302 cluster is predicted to target more than 400 mRNAs. In addition, as noted above, miR-302 cluster shares at the same time targets with many other miRNAs, so that application of massive parallel sequencing after HITS-CLIP is needed to elucidate completely the exact regulated networks. However, through functional annotation analysis of the predicted targets it was possible to show here that miR-302 cluster acts more likely by influencing transcription through targeting of transcriptional factors. This is in agreement with a key role of miR-302 cluster during first stages of development shown in the current work, since at that point the transcriptome is characterized by a dynamic switch on and off of genes related to differentiation or pluripotency, respectively. In addition, as shown in Supplementary, Fig. S5, predicted miR-302 cluster targets include genes direct associated with germ cell development.

The most interesting conclusion that can be drawn from our findings is that pluripotent cells have two miRNA clusters (miR-290 and miR-302) that at least for some members share the same targets but are differentially expressed in different cell types during in vitro differentiation, as it is the case of ESCs and maGSCs. In this way, response of the different pluripotent cell types to different needs or decisions (for example, differentiation towards germ cell lineage or not) is co-ordinately orchestrated by the different expression patterns of miR-290 and miR-302 clusters in the different cell types. This is further supported by the different dynamics of DNA promoter methylation of these miRNA clusters that have been observed here, which confirmed that their expression is subjected to different transcriptional control in ESCs and maGSCs.

**Figure 7** DNA methylation levels of (a) the Oct-4 5′ upstream region, (b) the miR-290 cluster pri-miRNA 5′ upstream region and (c) the miR-302 cluster pri-miRNA 5′ upstream region in undifferentiated (+LIF) and differentiating (−LIF) ESCs and maGSCs.

Previous works have suggested that the destiny of PGC-competent proximal epiblast cells is initially directed towards mesoderm lineage exhibiting expression of mesoderm genes like Brachyury. These genes continue to increase in the neighbouring mesoderm somatic tissue, but become repressed along with progression of specification to PGCs (Hayashi et al., 2007). Interestingly, EGCs are already known not to preferentially differentiate in vitro towards cell types of mesoderm lineage (Sharova et al., 2007), a fact that could be now explained by the high levels of miR-290 cluster that characterizes these cells. Furthermore, as shown in Supplementary, Fig. S7, such a property applies also to maGSCs, in which Brachyury levels are significantly lower than in ESCs during differentiation, although for the ectoderm marker Nestin such a difference is much less profound.

The inhibitory role of miR-290 cluster in maGSCs’ differentiation towards different cell lineages could be ultimately confirmed by inhibiting these miRNAs in maGSCs. However, it was not possible to achieve such an effect in maGSCs and EGCs at non-toxic levels as...
Supplementary data

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

Authors’ roles


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