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Members of the miR-290 cluster modulate in vitro differentiation of mouse embryonic stem cells

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ABSTRACT

We report the biological effects of miR-290 cluster via gain-of-function or loss-of-function experiments in mouse embryonic stem cells (ESCs) cultured under differentiation conditions. Under these conditions we found that overexpression of miR-290 cluster in ESCs cannot prevent downregulation of Oct-4, but inhibition results in earlier downregulation of Oct-4 compared with the negative control. In consistence with previous findings that report ectopic expression of Brachyury during gastrulation in Argonaute-2 KO mice due to impaired miRNA function, we show that miR-290 cluster regulates negatively differentiation of ESCs towards mesodermal and germ cell lineage. These results suggest that although incapable to maintain pluripotent state alone, miR-290 cluster inhibits ESC differentiation and it is involved in the pathways controlling mesoderm and primordial germ cell differentiation. Finally, we provide proofs that members of this cluster target Dkk-1 gene, a Wnt pathway inhibitor, and affect this pathway, which can partially explain why miR-290 cluster favours pluripotency against differentiation.

1. Introduction

Frequently referred to as 'micromanagers of gene expression', microRNAs (miRNAs) were first described to regulate developmental timing in *Caenorhabditis elegans* (Bartel, 2004). Since then many studies have shown that miRNAs have key roles in diverse regulatory pathways, including control of developmental timing, hematopoietic cell differentiation, apoptosis, cell proliferation, carcinogenesis and organ development (for comprehensive reviews see He and Hannon, 2004; Kim, 2005). Identification of the first mouse embryonic stem cell (ESC)-specific miRNAs by Houbaviy et al. six years ago implicated that the role of miRNAs in the regulation of gene expression may extend also to early embryonic development and pluripotency. These miRNAs, which are members of the miR-290 cluster, were described to be ESC specific, with their expression being repressed during ESC differentiation and undetectable in adult mouse organs (Houbaviy et al., 2003, 2005; Strauss et al., 2006). Recently, we have shown that expression of these miRNAs is a general characteristic of pluripotent cells (Zovoilis et al., 2008), and these miRNAs were found by another group to be expressed in early mouse embryo already after two-cell stage (Tang et al., 2007). Moreover, under differentiation conditions, high expression levels of these miRNAs observed in other types of pluripotent cells like multipotent adult germline stem cells (maGSCs) and F9 cells have been associated with persistent levels of Oct-4 and slower differentiation compared with ESCs, in which miR-290 cluster is strongly downregulated (Zovoilis et al., 2008).

Although the above-mentioned studies clearly associate miR-290 cluster with pluripotency, very little is known about the role of these miRNAs in mechanisms controlling pluripotency and differentiation. Until now, most available studies in mouse have employed a global miRNA "knock out" by targeting Dicer or Argonaute-2 (Ago-2) which are necessary for miRNA maturation and function (Kanellopoulou et al., 2005; Alisch et al., 2007). These studies revealed that Dicer and Ago-2 KO early mouse embryos are lethal. This is consistent with a key role of miR-290 cluster in early embryonic development, since the miR-290 cluster consists of the most highly expressed miRNAs in mouse ESCs and thus it is expected to contribute to the observed phenotype (Calabrese et al., 2007; Sinkkonen et al., 2008). However, in these studies it was not possible to separate the effects observed due to loss of miR-290 cluster with those effects due to loss of other miRNAs that share the same miRNA seed with miR-290 cluster and may have similar functions.

Loss of Ago-2 resulted in ectopic expression of Brachyury during gastrulation, which Alisch et al. (2007) hypothesized may be attributed to the resulted impaired miRNA function. However, the mechanism behind this phenotype was not tested further.

Thus, from the above findings, it remains unclear whether under differentiation conditions miR-290 cluster has the ability to maintain Oct-4 expression and inhibit expression of Brachyury. Aim of this study was to test these possible biological effects of miR-290 cluster during ESC differentiation via gain-of-function or loss-of-function experiments. We tested ESCs, because they

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recapitulate many of the events observed during early embryonic development and provide for this reason a good model for testing the role of these miRNAs in pluripotency and differentiation.

2. Materials and methods

2.1. Cell culture, miRNA overexpression and inhibition

We used the well-known ESC R1 line, which was derived from the 129/Sv mouse line (Wurst and Joyner, 1993). To maintain ESCs in an undifferentiated state, the cells were cultured as reported previously (Zovoilis et al., 2008). For the differentiation studies, cells were seeded in a 0.1% gelatine-coated 6 well culture plate without leukemia inhibitory factor (LIF) for 10 days. For miRNA overexpression and inhibition during these 10 days, cells were transfected using HiPerFect transfection reagent (Qiagen, Hilden, Germany) according to manufacturer's long-term transfection protocol. In detail, at day 0 (beginning of culture under differentiation conditions), cells were trypsinized and transferred (5×10^5 cells/well) to a fresh well of a 6 well plate. Subsequently, transfection complexes containing the transfection reagent (18 μl for overexpression and 36 μl for inhibition) and the miRNA precursor/inhibitor molecules were added drop-wise onto the cells. Complexes were left on the cells until they reached 60–80% confluency (after 2 days) and then cells were passaged again and re-transfected as described above. This procedure was repeated until day 10 or until analysis of the cells. For overexpression Pre-miR miRNA precursor molecules (Ambion, Austin, USA) for miR-290_5p, miR-291_5p, miR-292_3p, miR-293, miR-294 and miR-295 were used in a final concentration of 5 nM each, while for inhibition the respective Anti-miR miRNA inhibitors (Ambion) were used in a final concentration of 20 nM each. Cells transfected with the respective Pre-miR/Anti-miR controls from Ambion were used as negative control (negative). Pre-miR and Anti-miR controls were used in a final concentration of 30 and 120 nM, respectively. miRNA precursor/inhibitor molecules used in this study are listed in detail in Supplementary Material (Tables 1 and 2). Under these transfection conditions no cell toxicity effects were observed. Transfection conditions for overexpression or inhibition of miRNAs in our cell system were optimized using a Cy3 dye-labelled Pre-miR/Anti-miR control, respectively, from Ambion. Monitoring transfection efficiency by FACS analysis ensured that our transfection–retransfection scheme could maintain transfection levels constantly between 70% and 80% of all gated cells. Experiments were performed in duplicates.

2.2. Real-time PCR

Total RNA including miRNAs was isolated from cultured cells using the miRNeasy mini Kit (Qiagen). Conversion of miRNA and mRNA into cDNA and real-time PCR detection of miRNAs was carried out according to manufacturer's protocols 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 are also commercially available (miScript Primer Assays, Qiagen) (listed in Supplementary Material—Table 3). PCR specificity was checked by melting curves, gel electrophoresis and sequencing of the PCR products after gel extraction and cloning into a pGEM-T Easy vector (Promega, Madison, USA). For real-time quantitative RT-PCR (qRT-PCR) the QuantiTect SYBR Green PCR MasterMix (Qiagen) was used with gene-specific primers, listed in Supplementary Material (Table 4), for the following genes: Oct-4, Brachyury, Eomesodermin, Fgf-8, Nestin, Dppa-3, Dkk-1, C-myc and Lefty-1. All quantities were further normalized to values of RNU6B and Sdha for miRNA and mRNA quantification, respectively.

2.3. Bioinformatic approaches and statistical analysis

For computational prediction of miRNA targets we used the TargetScan web platform for mammals (www.targetscan.com/ release 4.2), while 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 the mean $\pm$ SD and p less than 0.05 was considered statistically significant.

2.4. 3′UTR luciferase reporter assays

For the luciferase reporter assays, PCR fragments including the 3′UTRs of Smad-1, Smad-2, Dkk-1 and Lefty-1 were cloned into the multiple cloning site of pMIR-Report Luciferase vector (Ambion). The 3′UTR sequences are available in the "ensemble" entry of each gene (http://www.ensembl.org/ Dec 2008). Detailed information about the cloned fragments is available in Supplementary Material (Table 5). NIH/3T3 cells were transfected with the luciferase vector, the pMIR-Report β-Gal Control Plasmid (for use in normalization of the results) (Ambion) and the Pre-miR molecules and controls mentioned above using Attractene transfection reagent (Qiagen). In detail, one day before transfection, cells were trypsinized and transferred (3×10^4 cells/well) to a fresh well of a 24 well plate. The optimized DNA transfection amounts per well transfection for each vector were 0.4 μg (for both Luciferase and β-Gal Vector a total of 0.8 μg). For NIH/3T3 cells, the optimized miRNA precursor final concentrations per well transfection were 50 nM for each miRNA and 300 nM for the Pre-miR control. Per well 1.125 μl transfection reagent was used and in these reagent and miRNA concentrations no cell toxicity effects were observed. Transfections including only the vectors or the Pre-miR molecules and comparison with mock transfection controls were initially assayed for validation of the assay. After validation, cells were transfected in triplicate and each transfected well was assayed in triplicate after 24 h using the Luciferase Assay System (Promega) according to manufacturer's recommendations in an Orion Microplate Luminometer (Berthold Detection Systems, Pforzheim, Germany). Luciferase activity was normalized to that of the Gal Vector.

3. Results

3.1. Under differentiation conditions, overexpression of miR-290 cluster in ESCs cannot prevent downregulation of Oct-4, but inhibition results in earlier downregulation of Oct-4 compared with the negative control

Fig. 1A summarizes the strategy that was followed. For overexpression, the respective pre-miRNA precursor molecules were introduced into ESCs cultured under differentiation conditions (−LIF) using conventional siRNA transfection methods. These pre-miRNA precursors are small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNAs of miR-290 cluster. For inhibition, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous microRNAs belonging to miR-290 cluster were applied. The negative controls (neg) were transfected with the respective Pre-miR/Anti-miR controls (which are random